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Differentiation of human telencephalic progenitor cells into MSNs by inducible expression of Gsx2 and Ebf1

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ABSTRACT

Medium spiny neurons (MSNs) are a key population in the basal ganglia network, and their degeneration causes a severe neurodegenerative condition, Huntington’s disease. Understanding how ventral neuroepithelial progenitors differentiate into MSNs is critical for regenerative medicine to develop specific differentiation protocols using human pluripotent stem cells. Studies performed in murine models have identified some transcriptional determinants, including Gsx2 and Ebf1. Here, we have generated hES cell lines inducible for these transcription factors, with the aims of (i) studying their biological role in human neural progenitors, and (ii) incorporating TFs conditional expression in a developmental-based protocol for generating MSNs from hES cells. Using this approach, we found that Gsx2 delays cell cycle exit and reduces Pax6 expression, while Ebf1 promotes neuronal differentiation. Moreover, we found that Gsx2 and Ebf1 combined over-expression in hES cells achieves high yields of MSNs, expressing Darpp32 and Ctip2, in vitro as well in vivo after transplantation.
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INTRODUCTION

The striatum is the largest component of the basal ganglia, it is the hub of converging excitatory connections from cortex and thalamus, and it originates the direct and indirect pathways, which are distinct basal ganglia circuits involved in motor control. In humans, the degeneration of the principal striatal neuronal population, the MSNs, causes a severe neurodegenerative condition, Huntington’s disease (HD). A main goal in the field is the study of the mechanisms underlying neuronal specification and degeneration. A large number of studies performed in model organisms such as the mouse have provided fundamental insights into brain development, shedding lights on genes, signaling pathways, and general rules of brain formation. It is not incidental to point out that obvious species-specific differences exist in many aspects between mice and humans (gestation, morphology, and gene expression regulation in time and space). Thus, additional model systems are needed to uncover specific functions of a gene in human development\textsuperscript{1,2}. This task is also driven by the need to investigate neurological diseases, such as HD, in a model that more closely resembles human biology.

Here, we decided to take advantage of hES cells in order to develop a model to study the roles of selected transcription factors (TFs) in human striatal development and as a strategy to increase recovery of authentic MSNs for transplantation purposes. During brain development, a set of TFs are expressed in different regions and times and cooperate in order to establish a dorsal-ventral and medial-lateral positional identity in progenitor cells and to specify neuronal terminal differentiation. In particular, in the developing telencephalon, two TFs play a key role in contributing to the formation of the striatum: the GS Homeobox 2 (Gsx2) and Early B-cell factor 1 (Ebf1).

Gsx2 is expressed in the ventral ventricular zone (VZ) of the telencephalon where it is involved in maintaining the identity of early striatal progenitors, and it is required for
promoting a striatal fate$^{3-8}$. Recently, two studies have reported about the role of Gsx2 in mouse neural stem cells showing that Gsx2 regulates progenitors proliferation and differentiation$^{9, 10}$. Nonetheless, these studies focused on Gsx2 function in mouse neurospheres and in adult neural stem cells, models that could bear different signatures with respect to human embryonic ventral progenitors. Ebf1 is a helix-loop-helix transcription factor that has been shown to control cell differentiation in the murine embryonic striatum$^{11-13}$, but it has never been studied in a human model system of striatal development.

We have previously demonstrated that human ventral telencephalic progenitors can be generated from hES cells by using a Shh-treatment coupled with Wnt-inhibition$^{14, 15}$. These progenitors eventually differentiate into mature, electro-physiologically active neurons. However, the protocol yielded cultures containing Darpp32$^+$-Ctip2$^+$ cells never exceeding 10-15%. We therefore wished to establish a hES cell-based inducible gain-of-function (iGOF) model system whereby transcription factors expressed in the developing striatum can be harnessed to improve MSNs differentiation and to study human striatal development. We uncovered novel roles for Gsx2 and Ebf1 during human striatal specification and differentiation, in particular in cell cycle regulation. Moreover, we report that a specific temporal window of Gsx2 and Ebf1 over-expression in hES cells achieves high yields of MSNs, expressing Darpp32 and Ctip2, in vitro as well in vivo after transplantation. To our knowledge, this is the first manipulation of telencephalic determinants in hES-derived neural progenitors.
RESULTS

Generation of inducible hES cell lines.

In order to shed lights on the transcriptional program that drives human striatal differentiation, we decided to develop an inducible over-expression system in hES H9 cells. To this goal, we modified a commercially available TetON system (Clontech) by moving the TetON cassette into a chicken beta-actin promoter with CMV enhancer-based plasmid (pCAG) (see Methods), in order to avoid silencing effects\(^{16}\). This construct was introduced by nucleofection (Fig. 1a) in hES H9 cells (p40-p50) along with a linear construct carrying a gene encoding for puromycin resistance. After selection, several stable hES cell clones were picked, amplified, and tested for inducibility by using a pTRE-Luciferase construct: we selected four clones that showed no basal Luciferase activity and high induction after 48hrs of doxycycline treatment. We amplified and characterized the clones C4 and B6 that showed the highest Luciferase expression after transient transfection (Fig. 1a, chart). They expressed Oct4 and Nanog in pluripotency medium, and responded promptly to differentiation stimuli similarly to the original H9 cells (Supplementary Fig. 1a-d). Next, we constructed three conditional vectors with the pTRE promoter regulating Gsx2 (pTRE-Gsx2), Gsx2 alongside with Ebf1 by means of an IRES2 sequence (pTRE-Gsx2-Ebf1) or Ebf1 alone (pTRE-Ebf1). Using these three constructs, we carried out nucleofections in the hES inducible clones (Fig. 1a'). After selection, several stable hES cell clones were picked, amplified, and tested for Gsx2, Gsx2-Ebf1, and Ebf1 expression. Four Gsx2, one Gsx2-Ebf1, and two Ebf1 over-expressing clones were chosen for the next experiments. We quantified Gsx2, Gsx2-Ebf1, and Ebf1 overexpression in the inducible hES cell clones after 72 hours of doxycycline treatment (Fig. 1b-j). Quantification of Gsx2\(^{+}\) cells after 72hrs of doxycycline induction in Gsx2 iGOF showed 55±3% expression (Fig. 1d). Quantification of Gsx2\(^{+}\) and Ebf1\(^{+}\) cells in Gsx2-Ebf1 iGOF showed 51±19% and 48±22% expression, respectively (Fig.1 g), with virtually all the
cells co-expressing Gsx2 and Ebf1 (insets in Fig. 1f', f''). Finally, quantification of Ebf1+ cells in Ebf1 iGOF showed 60±8% expression. We next used Western Blot analysis to perform a second quantification experiment during neuronal differentiation, at day30, after 10 days of doxycycline treatment (Supplementary Fig. 1e) in the three iGOF lines. Western Blot quantification (Supplementary Fig. 1f) showed up-regulation of Gsx2 (12 fold in Gsx2-Ebf1 and 124 fold in Gsx2 iOGF) and Ebf1 (243 fold in Gsx2-Ebf1 and 267 in Ebf1 iOGF) in the three lines compared with basal culture conditions (no doxycycline).

**Gsx2 and Ebf1 regulation of patterning genes.**

The patterning activity of Gsx2 during ventral telencephalic development has been extensively studied in mouse models. However, no information is available about its roles during human development. To determine the effects of Gsx2 and Ebf1 over-expression in human neural progenitors, we used a specific protocol that we previously showed to have the potential to generate first ventral telencephalic progenitors and then mature MSNs after 80 days in vitro. However, the protocol yields cultures containing Darpp32+/Ctip2+ cells never exceeding 10-15%. We therefore wished to implement this protocol by establishing a hES cell-based inducible gain-of-function (iGOF) system whereby transcription factors expressed in the developing striatum can be utilized to increase MSNs yield. Thus, we decided to over-express Gsx2, Gsx2-Ebf1, and Ebf1 in different temporal windows during hES neural differentiation: day10-day15, day15-day20, and day20-day30. To test this TFs-mediated specification, we first analyzed regional patterning in the hES-derived neural progenitors. We found that both Gsx2, Gsx2-Ebf1, and Ebf1 iGOF down-regulated Pax6, a dorsal cortical marker, at day30 (Fig. 2a-b, e-f, i-j and quantification in m) and at day15 (Supplementary Fig. 2a-d), at the end of the doxycycline treatments. Since Pax6 is also an important early neuroectodermal marker in humans, we sought to determine if Gsx2 over-expression could compromise the process
of neural induction in hES cells. To test this possibility, Gsx2 expression was induced during the day10-day15 time window, the earliest period used in this study. Importantly, Gsx2 activation did not down-regulate Otx2 and N-Cadherin (two early neural plate markers) expression at day15, the end of the doxycycline treatment (Supplementary Fig. 2e-h), suggesting that the cells correctly went through neural induction.

Next, we performed immunostaining for Nkx2.1, a marker expressed in proliferative cells of the MGE, in striatal interneurons, and in Ctip2+ cells of the mature striatum (and in the Hypothalamus). Since at this time point (day30) most cells are still proliferating and we do not usually detect Ctip2 expression, the down-regulation of Nkx2.1 that we found in the double Gsx2-Ebf1 iGOF line (Fig 2c-d, g-h, k-l and quantification in m) suggests a suppression of a MGE fate.

Next, in order to validate these data using a different system, we took advantage of a modified mRNA (mmRNA) for Gsx2. We transfected this mmRNA into H9 hES-derived neural progenitor cells from day20 to day25 of differentiation using the same protocol utilized for the iGOF lines. As shown in Fig. 2n-p, Gsx2 over-expressing cells reduced Pax6 expression similarly to what found in the Gsx2 iGOF line (three-fold decrease in both over-expressing systems).

Together, the data indicate that in hES cells that are undergoing neuronal conversion Gsx2 and Ebf1 over-expression suppresses the dorsal marker Pax6 and the MGE marker Nkx2.1 while maintaining typical neuroepithelial markers (Otx2, N-Cadherin).

Given the in vivo expression of Gsx2 in progenitor cells and Ebf1 in early post-mitotic neurons, we next investigated if and how Gsx2 and Ebf1 over-expression modified cell proliferation.

**Gsx2 and Ebf1 regulate cell cycle kinetics**
Regulation of cell proliferation in the developing telencephalon is a tightly regulated process and it is essential in order to produce the correct number of mitotic neurons. In order to examine the effects of Gsx2 and Ebf1 over-expression in human progenitor cells, we first performed a cumulative BrdU analysis in the hES cell lines inducible for Gsx2 and Gsx2-Ebf1. After treating the cells with doxycycline for four days we administered BrdU for 30', 4, 8, and 20 hours. We found that the Gsx2 iGOF line showed a reduced BrdU incorporation compared with the untreated cells (Fig. 3a and quantification in b). In contrast, the Gsx2-Ebf1 double inducible line showed a similar BrdU incorporation propensity when compared to control line, suggesting that cell cycle alteration by Gsx2 was rescued by Ebf1. Finally, we performed the same analysis also in the Ebf1 iGOF line, finding that at 20 hours there was a significant increase in BrdU incorporation compared with control line, the opposite phenotype found in Gsx2 iGOF cells. These data suggested an involvement of Gsx2 and Ebf1 in cell cycle regulation.

In order to test this hypothesis also in hES-derived neural progenitor cells (the biological context that more closely resembles the developing embryonic human brain), we administered doxycycline from day20 to day30 of the neuronal differentiation protocol, and analyzed cell cycle kinetics by a BrdU/IddU double labeling paradigm\textsuperscript{17, 18} (see material and methods for details and experimental design in Fig. 3k) at day30. We first tested this method in our hES cell lines, in basal conditions (no doxycycline), with culture conditions permitting pluripotency, finding a cell cycle time (Tc) of 19.4±4.37 hours (data not shown), comparable to previous published data\textsuperscript{19}. Next, we analyzed the Tc of day30 hES-derived neural progenitor cells (Fig. 3g-l) and we found results in agreement with the BrdU cumulative analysis performed in Fig. 3a-e. Estimation of control cells (no doxycycline) Tc was 12±1 hours, whereas the Gsx2 over-expressing cells showed a Tc of 24±4 hours. Next, we analyzed the contribution of Ebf1 by measuring Tc in Gsx2-Ebf1 iGOF cells,
finding a value of 7±1 hours, suggesting that EBf1 could override Gsx2-mediated increase of cell cycle length.

In order to rule out the possibility that Gsx2 iGOF cells were undergoing differentiation (and thus incorporating less BrdU), we analyzed Map2 expression at day30, the same time point used for the previous analysis. Gsx2 iGOF showed a marked reduction of Map2+ cells (Supplementary Fig. 3a-d), in agreement with the previous cell cycle analysis data and further suggesting that Gsx2+ cells could not exit cell cycle. Moreover, we investigated this Gsx2-mediated cell cycle regulation also in other two hES inducible clones (G18 and G17, Supplementary Fig. 3e-k), finding similar results.

Together, these results suggest that Gsx2 regulates cell cycle progression in human neural progenitor cells.

**Gsx2 constitutive over-expression modifies proliferative characteristics and differentiation potential of hES-derived neuroepithelial stem cells.**

In order to test if this Gsx2-driven cell cycle regulation is telencephalic-dependent or represents a general role, we decided to test its over-expression in LT-NES cells. LT-NES cells (long-term self-renewing neuroepithelial stem cells) represent an excellent model for studying human neuroepithelial cells biology20. They are hES cells-derived neural progenitors with an anterior midbrain identity. Here, we decided to take advantage of this cell population and its regional identity in order to gain insights into the cell cycle regulation by Gsx2.

We generated an LT-NES cell line over-expressing Gsx2 by nucleofection of a pCAG-Gsx2-IRES-Puromycin vector and isolation of stable, positive clones. We characterized different clones, finding identical phenotypes across the different lines. A control cell line was also generated by using a pCAG-EGFP-IRES-Puromycin vector, and we found
identical self-renewal capacity and differentiation potential compared to the unmodified cell line.

First, we decided to analyze the effects of Gsx2 over-expression during proliferation of LT-NES cells, by means of BrdU studies. We first performed a BrdU pulse of 2 hours, and we found a decrease in BrdU incorporation in LT-NES cells over-expressing Gsx2 (LT-NES-Gsx2), compared to the control cell line (form 36.33±5.06% to 26.76±5.94, p<0.005, Fig. 4a-c). A similar proliferative defect was found after BrdU pulses of 4 and 24hrs (Fig. 4c).

We reasoned that this decrease in BrdU incorporation could be linked to an increase in cell differentiation or to an increase in cell cycle length, which leads to a reduction in the number of time the cells pass through the S phase, thus reducing BrdU incorporation. Thus, we performed differentiation experiments and cell cycle length studies in order to distinguish between these two possibilities.

First, we differentiated the cells for 10 days, and we analyzed the expression of the early neuronal marker βIII-Tubulin. We found that the number of newly formed neurons was decreased in LT-NES-Gsx2 compared to the control cell line (form 28.9±7.8% to 15.1±1.8%, p<0.05, Fig. 1d-f), in agreement with the results previously found in the Gsx2-iGOF hES line (Supplementary Fig. 3a-d). A similar result was found when studying the expression of a more mature neuronal marker as Map2 (Supplementary Fig. 4a,b). Moreover, even when it was possible to detect βIII-Tubulin expression in LT-NES-Gsx2 cells (at early passages), more mature and lineage-specific markers, as GABA, were absent (Supplementary Fig. 4c,d), further suggesting that Gsx2 over-expression impairs neuronal differentiation and maturation. Next, we asked if the decrease in BrdU incorporation was caused by a cell cycle dis-regulation. To this goal, we performed an analysis of cell cycle characteristics using the BrdU/IddU double labeling paradigm\textsuperscript{17,18} to estimate the cell cycle length in the two cell populations, the LT-NES-Gsx2 and LT-NES-EGFP cells. We found that Gsx2 over-expression caused a significant increase in total cell
cycle length ($T_c$) compared to the control cell line (Fig. 4g-i). This increase was even more important after a few passages (Fig. 4i, see increment between p5 and p10, from $26.88 \pm 1.84$ hours to $135.30 \pm 33.79$ hours, $p<0.005$), suggesting that Gsx2 over-expression has a cumulative effect during time. The control cell line, during the same time period, did not show a statistically significant increase in $T_c$ (from $6.92 \pm 0.11$ to $9.53 \pm 2.96$, $p>0.1$, Fig. 4i).

In conclusion, taking also into consideration that no significant programmed cell death was found in LT-NES-Gsx2 compared to the control cell line (data not shown), these data demonstrated that a constitutive Gsx2 over-expression was detrimental for proper neuronal differentiation and maturation, even in a non-telencephalic compartment, corroborating the results obtained in the hES inducible lines. Thus, to summarize the results shown in Fig. 3 and Fig. 4, Gsx2 has a major role in regulating proliferation, by lengthening the cell cycle in a context-independent manner.

**Ebf1 promotes neuronal differentiation and maturation**

The foregoing data demonstrate that Gsx2 has important roles in regulating cell cycle progression, while Ebf1 expression probably enhances differentiation. To investigate the specific role of Ebf1 in human neural progenitor cells, we first studied the Ebf1 iGOF line. After over-expressing Ebf1 (by doxycycline treatment) in the day20-day30 temporal window of hES neuronal differentiation, we compared βIII-Tubulin expression with control (no doxycycline) cells. Ebf1 over-expression resulted in a significant increase in the number of βIII-Tubulin$^+$ cells (Fig. 5a-b, quantification in c). Next, to further test the Ebf1 role in increasing neuronal differentiation, we transfected a mmRNA for Ebf1 (Miltenyi Biotec) in unmodified H9 hES-derived neural progenitor cells (exposed to the same protocol used for the iGOF lines). First, we tested transfection efficiency by staining for Ebf1 after 2 consecutive days of mmRNA delivery, finding a transfection efficiency of
32±5% (Fig. 5d-f). Next, we investigated βIII-Tubulin expression after five consecutive days of transfections (day30). We found 17±3% of cells expressing βIII-Tubulin compared with 11±1% of untreated cells (Fig. 5g-h, quantification in i, p<0.01, n=3, unpaired t-test). We then investigated if Ebf1 over-expression had an effect on neurites length or complexity. Interestingly, by using NeurphologyJ analysis, we found an increase of attachment points (Fig. 5j-l) on neuronal soma (from 3.5±0.1% to 4.7±0.7% in transfected cells, normalized over total soma number, p<0.05, n=3, unpaired t-test). These data strongly suggest that Ebf1 has a role as a neuronal differentiation player during hES differentiation.

**Gsx2 and Ebf1 over-expression differentially regulates early neuronal differentiation**

Taking into account the different proliferative responses of hES-derived neural progenitors to Gsx2 and Ebf1 over-expression, and the increased neurogenesis after Ebf1 over-expression, we decided to investigate the tendency of Ebf1 and Gsx2-Ebf1 iGOF lines towards differentiation. First, we monitored neuronal differentiation during the differentiation process, finding better neuronal morphology in the two lines after doxycycline treatment (Fig. 6a,b). In order to quantify this differentiation propensity, we performed cell cycle exit studies, by administering for two hours BrdU at day25 of neuronal differentiation in a day20-day30 temporal window of doxycycline treatment. The cells were then fixed at day30 and analyzed for BrdU and Ki67 expression (see schema in Fig. 6i). Cell-cycle exit index was calculated by dividing the total number of BrdU+ Ki67− cells by the total number of BrdU+ cells. As shown in Fig. 5c-h and quantified in Fig. 5j, the three lines showed different phenotypes. Gsx2 over-expressing cells were more likely to remain in cell cycle (2.9±2.3-fold induction over no-doxycycline cells; no doxycycline levels arbitrarily set to 1; p<0.005, n=3, Fig. 6c,d, chart in j), in agreement with the data presented in Supplementary
Fig. 3a-d. Ebf1 incorporation caused increased differentiation output in Gsx2-Ebf1 iGOF line (1.5±0.28-fold induction over no-doxycycline cells; no doxycycline levels arbitrarily set to 1; p<0.05, n=3; Fig. 6e,f, chart in j). Finally, Ebf1 single iGOF over-expressing cells were 1.13±0.07 more likely to exit cell cycle (no doxycycline levels arbitrarily set to 1; p<0.05, n=3, Fig. 6g,h, chart in j).

Again, these results were in line with the hypothesis of Gsx2 retaining neural progenitor cells in an undifferentiated state and Ebf1 controlling cell cycle exit and progenitor maturation.

**Gsx2-Ebf1 over-expression induces MSNs differentiation from hES cells.**

To determine the striatal differentiation potential of hES cells over-expressing Gsx2-Ebf1 in the day20-30 developmental window, we conducted long-term differentiation experiments, and analyzed the cells at day 60 and day 80.

First, we evaluated the number of cells expressing the striatal neuronal markers Isl1 and Ctip2 at day 60 of differentiation. Isl1+ cells increased from 4.4±0.9% in control cells (no doxycycline) to 25±5% in Gsx2-Ebf1 over-expressing cells (p<0.00005, n=3, Supplementary Fig. 5c,d). Ctip2+ cells increased from 8.5±2.3% in control cells (no doxycycline) to 20±3.9% in Gsx2-Ebf1 over-expressing cells (p<0.0005, n=3, Supplementary Fig. 5e,f). To further validate this findings using a different model, we also performed transfection experiments in RC17 hES cell line using mmRNAs for Gsx2 and Ebf1. Following the experimental strategy shown in Supplementary Fig 5b, Isl1+ cells increased from 6.2±2.2% in non-transfected cells to 17.22±3.2% in cells transfected sequentially with Gsx2 and Ebf1. At day 60 of differentiation Ctip2+ cells increased from 20.10±7.4% in non-transfected cells to 42.88±7.5% in n cells transfected sequentially with Gsx2 and Ebf1.
Next, we analyzed the neuronal population at day 80 of differentiation by studying Darpp32 and Ctip2 expression. Initially, we quantified the generated striatal neurons by expressing the density of Ctip2+/Darpp32+ area per arbitrary surface area (Fig. 7a-b), finding a higher efficiency of Darpp32+/Ctip2+ neurons generation in the iGOF line compared to the control line (from 3.78±3.08% to 38.76±13.70%). Then we focused on the number of Darpp32+ cells by performing automating soma cell counting (by using the NeurphologyJ ImageJ plugin, see Methods for quantification details) and we found a higher number of Darpp32+ cells per unit area in the iGOF line than in control cells (from 79.5±26.3 in basal condition to 693±76 in iGOF; number of cells/area; see Methods for quantification details, n=3; Fig. 7c-d, f, n=3).

Finally, we studied if the Gsx2-Ebf1 combination could confer functional electrophysiological properties to the differentiated neurons. While passive properties did not change significantly between doxycycline-treated and non-treated cells (Supplementary Fig. 6a-c), we found interesting results studying sodium currents. In particular, Na+ current density was significantly higher in doxycycline-treated cells (from 30.75±6.58 pA/pF in control cells to 76.06±10.14 pA/pF in Gsx2-Ebf1 over-expressing cells, p<0.001).

To explore whether also the single Gsx2 and Ebf1 iGOF lines facilitate striatal neurons generation, we conducted similar long-term differentiation experiments, by analyzing the cells at day 80 of differentiation. In contrast to the Gsx2-Ebf1 double line, we could not detect an increase of Darpp32+/Ctip2+ cells (Supplementary Fig. 6d-g). While these findings were in agreement with the previous data for the Gsx2 iGOF line (increase cell cycle length and reduced differentiation), we were intrigued by the fact that the Ebf1 iGOF line did not increase striatal neurons generation compared to basal conditions, taking into account the Ebf1 over-expression effects on neuronal differentiation (Fig. 5). To investigate the effects of Ebf1 over-expression during striatal differentiation, we analyzed
Ctip2 expression at day 30 (at the end of the doxycycline treatment) and at day 50 (Supplementary Fig. 7a). Interestingly, Ebf1 over-expression caused Ctip2 up-regulation both at day 30 (Supplementary Fig. 7b-e), when Ebf1 over-expression was at its peak, and at day 50 (Supplementary Fig. 7f,g), 20 days after doxycycline was removed from the medium and Ebf1 over-expression was absent. However, this was not sufficient to trigger a consistent striatal phenotype at day 80 (Supplementary Fig. 6f,g).

In summary, these experiments showed that the Gsx2-Ebf1 iGOF line induced a striatal neuronal identity in differentiating hES cells, while the single lines did not.

**Gsx2-Ebf1 iGOF cells survive and differentiate in vivo after transplantation.**

Next, we wanted to assess long-term survival and differentiation of Gsx2-Ebf1 iGOF cells after transplantation in the striatum of QA-lesioned athymic, adult rats. The transplanted animals were followed up to 2 months, and then sacrificed for Immunohistochemical analysis. To this goal, we decided to induce Gsx2 and Ebf1 expression from day 15 to day 20 of neuronal differentiation, and perform the transplant at day 20 (Fig. 8a). Two months after transplantation, we found many human nuclei+ cells in the transplanted site (Fig. 8b-c, red cells), suggesting optimal survival (average of 53±16% human nuclei+ cells, Fig. 8f). We then analyzed the expression of markers of mature striatal neurons: Ctip2, Gaba, and Darpp32. Interestingly, Ctip2 and Gaba were largely present in the lesioned transplanted site (human nuclei+ cells) (Fig. 8b, arrowheads point to examples of Ctip2+/hNuclei+ cells). In addition, immunostaining for Darpp32 and Ctip2 showed similar results (Fig. 8c), with these two striatal markers expressed in the site of. To further investigate the co-expression of Ctip2 and Darpp32 in human nuclei+ cells, we analyzed the immunostaining for Ctip2/human nuclei (Fig. 8c’) and Darpp32/human nuclei (Fig. 8c’’) on the same section showed in Fig. 8c. Insets in c’ and c’’ show representative human nuclei cells expressing both Ctip2 and Darpp32 markers. We quantified the cells...
that were human nuclei/Ctip2 double positive, and we found 23±6% of cells expressing both markers. These results suggest that Gsx2-Ebf1 iGOF cells were able to differentiate into striatal neurons in vivo as in vitro.
DISCUSSION

This study aimed to achieve two goals: (1) to study Gsx2 and Ebf1 function during human ventral telencephalic development, and (2) to improve MSNs differentiation from hES cells by transcriptional specification. In both efforts, we have succeeded in applying an iGOF system for forcing TFs expression in defined temporal windows, and in combining this approach with a morphogens-driven ventral telencephalic specification.

In making progress toward the first aim, we have demonstrated a dual role for Gsx2 in embryonic human neural progenitors. First, it imparts a regional identity by directly or indirectly down-regulating Pax6 expression. We found this effect during different time windows of Gsx2 induction, suggesting a time independent primary function for this TF. It is also important to note that Gsx2 iGOF cells responded properly to neural induction extrinsic signals as evidenced by the correct expression of early neural plate markers as Otx2 and N-Cadherin. Secondly, Gsx2 has a major role in regulating proliferation, by lengthening the cell cycle in a context-independent manner: we found similar results in cells as different as LT-NES, self-renewing hES cells, and hES-derived neural progenitors. To begin with, we show that in LT-NES cells, a model of human neuroepithelial cells, constitutive Gsx2 over-expression caused a progressive increase in cell cycle length during passages, leading to a proliferation block and to differentiation impairment. Interestingly, Gsx2 time-restricted over-expression showed the same consequences on cell cycle regulation, suggesting that this is a key Gsx2 role in neuronal progenitors. Moreover, since LT-NES cells have a ventral anterior hindbrain identity, and hES-derived neural progenitors express more anterior markers, as Otx2, this Gsx2 activity on cell cycle regulation is context-independent, and likely reflects a primary role. Interestingly, it is well accepted that during mouse development cell cycle lengthening is correlated with enhanced neurogenesis\textsuperscript{22}. Our data about Gsx2-regulated cell cycle lengthening are
somehow in contrast, since we found a reduction in differentiation. Probably Gsx2 retains human neural progenitor cells in a condition that prevents excessive proliferation and differentiation, with implications for the generation of the correct number of differentiated progeny during human development.

A recent paper has evidenced that in adult neural stem cells Gsx2 over-expression promotes the transition from quiescent to activated neural stem cells. Nonetheless, they also pointed out how a high level of Gsx2 blocks the lineage progression toward transit amplifying progenitors, a more differentiated cell population. Our findings obtained in human neural progenitors are in line with the suggestion that fine-tuned Gsx2 levels must be reached to promote neuronal differentiation. The ventral mouse and human telencephalon express at high-level Gsx2 in the VZ, including the LGE proliferative region, and this expression data likely reflects the roles played by this TF. Later during development, the Gsx2 expression is reduced in both the number of Gsx2+ cells and the intensity levels, suggesting that its expression must be down regulated over time to allow neuronal maturation.

Thus, our data point to a role for Gsx2 in restraining cell cycle progression in neural progenitors, while instructing a regional ventral phenotype. Of note, the differentiation defect observed in Gsx2 iGOF cells was rescue by Ebf1: the co-recruitment of Gsx2 and Ebf1 caused a more efficient neuronal differentiation, while preserving the regional patterning activity of Gsx2, as shown by the Pax6 down-regulation. Interestingly, even if these two TFs are not expressed in the same region and time during development, their combination in hES cells-derived neural progenitors allowed a proper cell cycle progression and neuronal differentiation, while maintaining a patterning activity (Pax6 down-regulation). We show here that Ebf1, by using iGOF lines or mmRNAs transfections,
can enhance neuronal differentiation in hES-derived neuronal populations, in term of neuronal numbers and morphological characteristics.

In this work we also identified a temporal window for an efficient iGOF transcriptional activation or mmRNAs transfections leading to improved human neural progenitors patterning and differentiation towards MSNs. In the last few years, the use of specific extrinsic signals in combination with the dual SMAD inhibition strategy resulted in the development of protocols for the derivation of many central and peripheral nervous system lineages from hES and iPS cells. Here we show that TFs with different expression pattern and timing can be combined to efficiently differentiate hES H9 cells towards a striatal phenotype. This study has then provided a working system for combining extrinsic (morphogens) and intrinsic (TFs) players in order to manipulate hES or iPS cell fates. In particular, by combining a ventral-inducer like Gsx2 and a neuronal differentiation-effector as Ebf1 we could shift the differentiation outcome towards MSNs. Of interest for future studies in stem cell therapies for HD, we show that, upon transplantation in HD rat models, Gsx2-Ebf1 iGOF cells can survive, differentiate, and express key striatal markers as Ctip2 and Darpp32.

We also show that hES cells can be harnessed to model human embryonic development and neuronal differentiation by inducible expression of key developmental TFs. This technique allows mimicking and testing the temporal windows of TFs activation during human embryonic development.
Figure Legends

Figure 1. Production of hES H9 inducible lines for Gsx2, Gsx2-Ebf1, and Ebf1. (a-top) Schematic representation of nucleofection of a pCAG-TeON-3G plasmid in hES H9 cells, puromycin selection, isolation of resistant clones, and screening of the best responsive clones after pTRE-Luciferase transient transfections. Chart shows quantification of Luciferase activity in selected clones after doxycycline treatment for 24 hours, compared to basal levels. (a-bottom) Schematic representation of nucleofection of pTRE-Gsx2, pTRE-Gsx2-Ebf1, and pTRE-Ebf1 plasmids in Tet-ON3G hES cells, puromycin selection, isolation of resistant clones, and selection of the best expressing clones. (b-j) Representative immunofluorescent images and their quantifications of stable hES cell lines for Gsx2 (b-d), Gsx2-Ebf1 (e-g), and Ebf1 (g-j) after 72 hours of doxycycline treatment in pluripotency conditions. Quantifications showed transgenes expression of 55±3% (Gsx2 in Gsx2 iOGF), 51±19%; 48±22% (Gsx2 and Ebf1 in Gsx2-Ebf1 iGOF, respectively), and 60±8% (Ebf1 in Ebf1 iOGF). Data are presented as means and distribution of single experiments. Scale bar 75µm.

Figure 2. Gsx2 and Ebf1 roles during patterning of telencephalic progenitors. (a-d) Gsx2-Ebf1 iGOF line down-regulate Pax6 and Nkx2.1 expression during the day20-30 developmental window. Instead, Gsx2 and Ebf1 single lines downregulated only Pax6 (e-l). Representative immunofluorescence images for Pax6 (green) and Nkx2.1 (red) expression. Scale bar: 100µm. (m) Quantification analysis for Pax6 and Nkx2.1 expressing cells, n=3. * p<0.05 ** p<0.01, *** p<0.003 t test analysis. Data are presented as means ± SD.
Figure 3. Gsx2 and Ebf1 modulate cell cycle kinetics.

(a,c,e) Representative images of a BrdU cumulative labeling experiment in Gsx2 (a) Gsx2-Ebf1 (c) and Ebf1 (e) lines in culture condition allowing pluripotency. BrdU has been added to the culture media for 0.5 – 4 – 8 – and 24 hours. (b,d,f) quantification of BrdU+ cells at the different time points. Data are represented as mean±SD.

(g-j) Representative images of neuronal progenitor cells cycle length analysis using BrdU/IdU co-labeling. Day30 hES cells-derived neural progenitors, treated for 10 days with doxycycline, were exposed to IdU at T0 hours (h) and with BrdU at T1.5 h (see experimental design in k). Arrowheads point to cells that left S-phase at T 1.5h (Lcells, green), whereas yellow cells are still in the S-phase at T 2h. Scale bar 75μm. (l) Tc estimation from BrdU/IdU analysis of Gsx2 and Gsx2-Ebf1 iGOF, showing the different effects of adding Ebf1 to Gsx2 over-expression.

Figure 4. Gsx2 constitutive over-expression decreases proliferation and differentiation.

(a-b) Representative images of BrdU-labeled LT-NES cells after a 2-h pulse of BrdU in the control line (a) and in one constitutively over-expressing Gsx2 (b). (c) Graph illustrates the percentage of BrdU positive cells of total population in WT and Gsx2-GOF LT-NES cells after 2, 4, and 24h of BrdU exposure (2h: 36.33±5.06% and 26.76±5.95%; 4h: 41.90±7.71% and 29.21±6.64%; 24h 87.09±6.43% and 58.87±8.73%, respectively). In the time points analyzed: **: p<0.005, ***: p<0.0005 (unpaired, two tails t-test analysis) by comparing WT and Gsx2 GOF. Center lines show the average; whiskers indicate minimum and maximum.

(d-e) Representative images of neuronal monolayers generated from LT-NES WT and Gsx2 GOF lines: seven days after growth factors withdrawal, LT-NES WT cells readily differentiated into βIII-Tubulin+ neurons (d, red signal), whereas LT-NES Gsx2 GOF cells did not (e, red signal). (f) Graph illustrates quantification of βIII-Tubulin+ cells of total
population in the two lines, by cell counts of 10x fields, normalized to cell nuclei. Center lines show the average; whiskers indicate minimum and maximum. ****: p<0.0005

(g-h) Analysis of cell cycle characteristics using IdU/BrdU co-labeling. Monoclonal antibodies specific for both BrdU and IdU (red) and BrdU alone (green) are used to identify cells in the S-fraction (red and green double-labeled cells, \( S_{cells} \)) and cells leaving the S phase (green only cells, \( L_{cells} \), arrowheads in g and h). (i) \( T_c \) estimation from BrdU/IdU analysis of WT and Gsx2 GOF line. LT-NES Gsx2 GOF line increases dramatically the \( T_c \) after a few passages (P0-P5-P10). Center lines show the average; whiskers indicate minimum and maximum. Scale bars 75\( \mu \)m.

**Figure 5.** Ebf1 enhances neuronal differentiation in hES-derived neural progenitors

(a-b) Representative images of day30 neuronal monolayers generated from Ebf1 iGOF hES line in basal conditions (a) and after 10 days of doxycycline treatment (b) showing \( \beta III-Tubulin \) (green) and Ebf1 (red) expression. (c) Graph shows quantification of \( \beta III-Tubulin^+ \)cells in the two conditions. Center lines show the average; whiskers indicate minimum and maximum. (d-l) Ebf1 mmRNA transfections of H9 hES-derived neural progenitor cells. (d-e) Representative images of day30 neuronal monolayers generated from H9 hES cells transfected with 200ng of Ebf1 mmRNA, showing Ebf1 over-expression (red). (f) Quantification of Ebf1\(^+\) cells 48 hours after transfection. (g-h) representative images of \( \beta III-Tubulin \) expression of neuronal monolayers in control cells (g) and after 5 days of Ebf1 mmRNA transfections (h). (i) Quantification of \( \beta III-Tubulin^+ \) neurons. Center lines show the average; whiskers indicate minimum and maximum. (j-l) Neurite attachment points quantification of \( \beta III-Tubulin \) staining performed in (g-h). (l) Graph shows attachment points quantification by NeurphologyJ software. Center lines show the average; whiskers indicate minimum and maximum.
Figure 6. Gsx2 and Ebf1 differentially regulate cell cycle exit
(a-b) Phase-contrast images of neuronal monolayers generated from Ebf1 iGOF (a) and Gsx2-Ebf1 iGOF (b) lines in control and doxycycline-treated cells. (c-h) Representative images of cell cycle exit studies following the experimental design depicted in (i). (j) Quantification of cell cycle exit in Gsx2, Gsx2-Ebf1, and Ebf1 lines after 10 days of doxycycline treatments compared with basal conditions (no doxycycline, dotted line). Center lines show the average; whiskers indicate minimum and maximum. **: p<0.005.

Figure 7. Gsx2-Ebf1 over-expression between day20 and 30 promotes striatal differentiation.
(a-d) Representative images of neuronal monolayers generated from Gsx2-Ebf1 iGOF line: immunofluorescence for Ctip2 (red) and Darpp32 (green) at day80 of striatal differentiation, in day20-day30 doxycycline-treated (b,d) and non-treated cells (a,c); (f-g) 5x magnification, (h-i) 20x magnification. (e) Quantification of Darpp32+/Ctip2+ cells by automated cell counts of 10x fields normalized to the area occupied by nuclear counterstaining. Data are shown as Darpp32+/Ctip2+ cells per Dapi+ (nuclear staining) area. The images in figure represent reproducible results from 4 out of 5 differentiation experiments reaching day80. Scale bar in b,d 250μm; scale bar in a,c, 75μm. (f) Sodium current density of neuronal monolayer cultures at day100 of differentiation in control and doxycycline-treated conditions. Data are represented as mean±SD. Individual round and squared dots represent individual recorded cells. Sodium current density was significantly higher (** p<0.001) in Gsx2-Ebf1 over-expressing cells.
**Figure 8.** Gsx2-Ebf1 over-expressing cells maturate *in vivo* into MSNs.

(a) Experimental design for hES cells-derived neural progenitors transplantation in QA-lesioned athymic rats, after 5 days of doxycycline treatment. (b-e’) representative images of grafted cells 2 months after transplantation, assayed for human nuclei marker and specific MSNs markers. (d’,e’) magnifications of regions depicted in d,e. Arrowheads point to human cells expressing both Ctip2 and Darpp32. Arrows point to grafted human cells expressing either Ctip2 or Darpp32. Scale bar 35 µm.

**Supplementary Figure 1 (Relative to Figure 1).** Quantification of transgenes expression in inducible lines.

(a-d) Representative images of neuronal monolayers generated from Gsx2 and Gsx2-Ebf1 iGOF lines in basal (no doxycycline) conditions, showing that the genetic modifications did not alter neuronal differentiation potential. Immunofluorescence images for (a) Map2 (red) and Calbindin (green) expression, (b) Map2 (red) and Foxp2 (green) expression, (c) Ctip2 (green) expression, and (d) Gaba (green) expression. Scale bar 75µm.

(e) Western blots analyses of Gsx2, Gsx2 and Ebf1, and Ebf1 over-expression after 10 days of doxycycline treatment during the day20-day30 differentiation time window used in the study. (f) Quantification of western blots showed in (a). Error bars show SD. Transcription factors over-expression data are normalized over Tubulin levels.

**Supplementary Figure 2 (relative to Figure 2).** Early neuroectoderm differentiation in Gsx2 and Gsx2-Ebf1 line.

(a-d) Immunostaining for Pax6 (green) and Gsx2 (red) at day15 showing Pax6 down-regulation of Gsx2 in Gsx2 and Gsx2-Ebf1 iGOF lines after 5 days of doxycycline treatment. (e-h) Immunostaining for Otx2 (green) and N-Cadherin (red) of Gsx2 in Gsx2
and Gsx2-Ebf1 iGOF lines after 5 days of doxycycline treatment, showing a correct neural induction. Scale bar: 100μm.

**Supplementary Figure 3 (relative to Figure 3).** Gsx2 negatively regulate differentiation and cell cycle progression.

(a-d) Immunostaining of day30 neuronal monolayers generated from Gsx2 iGOF line for Gsx2 (a-c) and Map2 (b-d), showing reduced Map2 expression in cells over-expressing Gsx2 after 10days of doxycycline (compare b and d).

(e-k) Cell cycle length analysis in two additional clones inducible for Gsx2 (G17 and G18). (e-f, h-i) immunostaining for BrdU and IddU using two antibodies recognizing or BrdU-IddU (green) or only BrdU (red) following the design showed in Figure 3k. (g,k) Estimation of cell cycle length in G18 (g) and G17 (k) clones showing results (Tc increase) similar to Figure 3 for the Gsx2 iGOF clone used throughout the study (G4). Center lines show the average; whiskers indicate minimum and maximum.

**Supplementary Figure 4 (relative to figure 4).** Poor differentiation potential in LT-NES cells constitutively over-expressing Gsx2.

(a-b) Representative images of neuronal monolayers generated from WT and Gsx2 GOF cells at day10 of differentiation after Fgf2 and Egf withdrawal: the mature neuronal marker Map2 was absent in LT-NES-Gsx2 cells. (c-d) Early passage LT-NES-Gsx2 cells express the early neuronal marker βIII-Tubulin (red) but failed to express the lineage specific marker Gaba (green).

**Supplementary Figure 5 (relative to figure 7).** Gsx2 and Ebf1 over-expression induces Isl1 and Ctip2.
(a-b) The two different experimental designs in iGOF line (a) and by using mmRNAs (b).
(c-j) Representative images of neuronal monolayers generated from Gsx2-Ebf1 iGOF line (c-f) and after Gsx2 and Ebf1 mmRNAs transfections (g-j). Graphs illustrates quantification of Isl1$^+$ and Ctip2$^+$ cells of total population in the two experimental approaches, by cell counts of 10x fields, normalized to cell nuclei. Center lines show the average; whiskers indicate minimum and maximum. *: p<0.05, black squares show the individual measurements, unpaired t-test analysis.

**Supplementary Figure 6 (relative to figure 7).** Electrophysiological passive properties of day100 Gsx2-Ebf1 line and MSNs differentiation potential of Gsx2 and Ebf1 single iGOF lines.
(a-c) Membrane resistance, potential, and capacitance in control and doxycycline-treated cells at day100 of differentiation, showing no statistically significative differences. Data are represented as Mean ± SEM. Rounded and squared dots represent individual recorded cells. (d-g) Immunostaining for Darpp32 (green) and Ctip2 (red) in Gsx2 (d-e) and Ebf1 (f-g) iOGF lines, showing at day80 of differentiation no major increase in MSNs markers after doxycycline treatment (day20-day30 treatment).

**Supplementary Figure 7 (relative to figure 7).** Ebf1 overexpression transiently induces Ctip2.
(a) Schema showing the experimental design. (b-e) Immunostaining for Ebf1 (green) and Ctip2 (red) in neuronal monolayers generated from Ebf1 iGOF line showing that after 10 days of doxycycline treatment Ctip2 was over-expressed together with Ebf1. (f-g) Ctip2 overexpression was maintained also at day50.
METHODS

ES and LT-NES cell culture. hES H9 cell line (Wicell, Wisconsin, USA) was cultured on Matrigel™ (BD, Becton Dickinson) or Matrix (Cell Guidance System, Cambridge, UK). Pluripro (Cell Guidance System, Cambridge) medium was changed daily. Cells were passaged enzymatically with Accutase (Invitrogen) every 3 days. LT-NES cells were derived as described in20 and maintained in DMEM/F12 (Life Technologies, USA) supplemented with N2 1:100 (Life Technologies, USA); B27 1:1000 (Life Technologies, USA); Fgf2 and Egf (Peprotech) 10ng/ml. LT-NES neuronal differentiation was triggered by removing Fgf2 and Egf from the medium.

Neuronal differentiation. hES cells were plated for neuronal induction as described in24. Briefly, cells were plated at a density of 0.7 × 10⁵ cells cm⁻² on Matrigel™ coated dishes in Pluripro medium supplemented with 10 µM ROCK inhibitor (Y-27632²⁵, Sigma). Cell cultures were expanded for three days until they were nearly confluent. The starting differentiation medium included DMEM/F12 (LifeTechnologies, USA) with N2 and B27 (Life Technologies, USA), supplemented with 5 µM Dorsomorphin (Sigma, USA) or 500 nM LDN 193189 (Sigma, USA) and 10 µM SB431542 (Tocris, UK), which were used until day 12. Every two days, the medium was replaced with new medium. Starting on day 5, 200ng ml⁻¹ SHHC-25II (R&D, USA) and 100ng ml⁻¹ DKK-1 (Peprotech, USA) were added to the culture and maintained for three weeks. After the appearance of rosettes (around day 15), the entire cell population was detached using Accutase (Millipore, Germany) and re-plated at a cell density of 2.5 × 10⁴ cells cm⁻² on dishes coated with Matrigel™ (BD, Becton Dickinson). The cells were maintained in terminal differentiation medium, which was composed of N2 medium supplemented with B27 and 30ng ml⁻¹ BDNF, until the end of differentiation.
**Generation of hES H9 inducible lines.** To generate an inducible hES cell line, we first modified a pCMV-TetON-3G (Clontech, USA) by removing the TetOn-3G cassette by digestion with EcoRI and HindIII (Biolabs, USA). Then, we removed the CRE cassette of a pCAG-CRE vector (Addgene, USA) and inserted the gel-purified TetON-3G cassette, to generate a pCAG-TetON-3G vector. Next, we inserted in the pTRE3G-IRES responsive vector (Clontech, USA) Gsx2 alone (in the first MCS), Gsx2 (in the first MCS) together with Ebf1 (in the second MCS), and Ebf1 alone (in the first MCS). Gsx2 cDNA was a gift from Kenneth Campbell (Cincinnati), Ebf1 cDNA was a gift from Giacomo Consalez (Milano).

hES H9 cell line was cultured as described. 8x10^6 cells were used for introducing the constructs by Nucleofection (Lonza) using a mouse ES cell nucleofection kit and electroporation protocol B16. 7μg of pCAG-TetON-3G in the first round and 7μg of pTRE-Gsx2 or pTRE-Gsx2-Ebf1 in the second round were used, together with 700ng of linear resistant marker (Clontech, Puromycin during the first round and Hygromycin during the second round). Cells were then plated in two Matrigel-coated 6cm dishes with Pluripro medium supplemented with Rock inhibitor (Y-27632\textsuperscript{25}). After 72 hours antibiotics (Puromycin during the first round and Hygromycin during the second round) were added to the medium for positive selection. Following approximately 2 weeks in selection medium, hES cell colonies were carefully selected and expanded in Matrigel-coated 48-well plates. Clones were then expanded and tested for transgene expression after 48 hours of doxycycline treatment. During the first round, the clones were screened by transient transfections with a pTRE-Luciferase vector (Clontech, USA). During the second round, the clones were screened by 48 hours of doxycycline treatment and immunofluorescence analysis for Gsx2 and Gsx2-Ebf1.

**Immunofluorescence.** Cells were fixed in 4% paraformaldehyde (PFA) for 15 min at room temperature (RT) and washed 3x with PBS. Cells were then permeabilized with 0.5% Triton (Sigma, USA) and blocked with 10% normal goat serum (Vector, USA) for 1 h at RT.
Next, cells were incubated overnight at 4°C with the following primary antibodies and dilutions: anti-OCT4, 1:100 (Santa Cruz, USA); anti-OTX2, 1:500 (Chemicon, USA); anti-PAX6, 1:2000 (Hybridoma Bank, USA); anti-NESTIN 1:200 (R&D, USA); anti-βIII-tubulin, 1:1000 (Promega, USA); anti-MAP2, 1:500 (BD Bioscience, USA); anti-CALBINDIN 1:200 (Swant, Switzerland); anti-GABA 1:500 (Sigma, USA); anti-CTIP2 1:500 (Abcam, UK); anti-DARPP32 1:200 (Epitomics, USA); anti-GSX2 (Millipore, Germany). After 3 washes in PBS 0.1% Triton, appropriate secondary antibodies conjugated to Alexa fluorophores 488 or 568 (Molecular Probes, Invitrogen) were diluted 1:500 in blocking solution and mixed with Hoechst 33258 (5 µg ml⁻¹; Molecular Probes, Invitrogen) to counterstain the nuclei. Images were acquired with a Leica DMI 6000B microscope (5x, 10x and 20x objectives) and analyzed with LAS-AF imaging software, and then processed using Adobe Photoshop, only to adjust contrast for optimal RGB rendering with the same procedure in doxycycline-treated and –untreated cells.

**Cell cycle analysis with IdU and BrdU.** IdU (Sigma, USA) is first added in the culture medium for 1.5 hours followed by BrdU (Sigma, USA) for 30 minutes. Cells are then fixed at the end of the BrdU treatment. For IdU/BrdU double labeling, primary antibodies used were mouse anti-BrdU/IdU (which recognizes both BrdU and IdU, clone B44, 1:100; BD, USA) and rat anti-BrdU (clone BU1/75, 1:100; Abcam, UK). After 4% PFA fixation, cells are first treated with 0.2N HCl for 5’ at RT and then with 2N HCl for 20’ at 37° for BrdU/IdU immunofluorescence. Cell cycle lengths (estimation) were calculated as previously described⁷: cells labeled initially with IdU and leaving S-phase during the interval between IdU and BrdU will be labeled with IdU but not BrdU (leaving fraction).

**Cumulative BrdU labeling.** BrdU is added to the cell culture medium for different time windows in different wells. BrdU immunofluorescence is performed as described above for the cell cycle analysis.
Cell cycle exit study. iGOF cell lines were treated with doxycycline from day20 to day30 of neuronal differentiation. At day25 cells were exposed to BrdU for 2hrs to label cells in the S-phase of the cell cycle. Neuronal differentiation was carried on until day30, when cells were fixed and processed as described above for BrdU immunofluorescence. Cells were also stained for Ki67 to label all proliferating cells at day30. Cell-cycle exit index was calculated by dividing the total number of BrdU$^+$ Ki67$^-$ cells by the total number of BrdU$^+$ cells.

mmRNA transfections. The transfection mix was prepared according to the manual of the StemMACS mRNA Reprogramming Kit (Miltenyi Biotec) using the StemMACS mRNA Transfection Reagent and StemMACS mRNA Transfection Buffer. 200 ng mmRNA of GSX2 and EBF1 (gently provided by Miltenyi Biotec) were used daily for 5 consecutive days. As a transfection control, 100ng of nuclear GFP (Miltenyi Biotec) was used the first day of transfection to monitor the transfection efficiency.

Transplantations. Athymic adult rats (Charles River) were lesioned one week before transplantation with quinolinic acid (QA). The lesion was generated by injection of 210 nmol of freshly made QA using the following stereotaxic coordinates: AP= +0.5, L= +/-2.8, V= 5.0. Gsx2-Ebf1 iGOF cells were differentiated as described above. Cells were treated with doxycycline from day20 to day30 of differentiation to induce Gsx2 and Ebf1 expression. At day30 cells were detached with Accutase supplemented with N2 1:100 for 20-30 minutes at 37°C. Cells were then re-suspended to obtain a single cell suspension at a concentration of 50x10$^3$ cells /μl and then transplanted in complete medium by bilateral stereotaxic transplantation in lesioned adult athymic rats using the following coordinates: AP= +0.9, L= +3.1/-3.1, DV= 5.0. A total of 2x10$^5$ cells (4 μl) per injection site was delivered by a single injection. Two months after transplantation the animals were sacrificed, transcardial perfused, and the brains cryosectioned for Immunohistochemical analyses.
References


