Efficient production of mesencephalic dopamine neurons by Lmx1a expression in embryonic stem cells

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Communicated by Thomas M. Jessell, Columbia University College of Physicians and Surgeons, New York, NY, March 13, 2009 (received for review December 10, 2008)

Signaling factors involved in CNS development have been used to control the differentiation of embryonic stem cells (ESCs) into mesencephalic dopamine (mesDA) neurons, but tend to generate a limited yield of desired cell type. Here we show that forced expression of Lmx1a, a transcription factor functioning as a determinant of mesDA neurons during embryogenesis, effectively can promote the generation of mesDA neurons from mouse and human ESCs. Under permissive culture conditions, 75%–95% of mouse ESC-derived neurons express molecular and physiological properties characteristic of bona fide mesDA neurons. Similar to primary mesDA neurons, these cells integrate and innervate the striatum of 6-hydroxy dopamine lesioned neonatal rats. Thus, the enriched generation of functional mesDA neurons by forced expression of Lmx1a may be of future importance in cell replacement therapy of Parkinson disease.

intrinsic determinant | magnetic bead sorting | neuroscience | Parkinson disease | transplantation

Parkinson disease (PD) is characterized by a decline in dopaminergic neurotransmission caused by a progressive degeneration of mesencephalic dopamine (mesDA) neurons of the substantia nigra pars compacta (SN) (1). Previous studies using fetal grafts in PD patients have indicated that cell replacement therapy can result in significant symptomatic relief (2). However, future development of cell therapy must rely on the use of stem cells because the availability of fetal grafts cannot satisfy practical and ethical requirements. Thus, much work has focused on development of methods for engineering mesDA neurons from stem cells (3–6). Such methods usually involve culturing cells with extrinsic signaling factors (e.g., Shh, FGF8, Wnts), which are known to influence the local environment in which mesDA neurons are generated during embryogenesis (7–10). However, these molecules underlie the regional specification of several cell types generated in midbrain (MB) and hindbrain during CNS development. As a consequence, in addition to mesDA neurons, cultures also include other regionally related neuronal subtypes such as serotonergic (i.e., 5-HT) and γ-aminobutyric acid (i.e., GABAergic) neurons. Importantly, when used in cell therapy, some of these contaminating neuronal types may cause unwanted side effects after grafting (11). Thus, heterogeneity complicates development of a therapeutic application of stem cells and emphasizes the importance of developing methods that can generate highly enriched cultures of mesDA neurons.

During development, mesDA neurons are generated immediately rostral of the MB/hindbrain border in the most ventral region of the MB and diencephalon (12). The positional specification of mesDA neurons is initiated by local inductive signals that impose regional character on neural progenitors at early developmental stages. The induction of mesDA neurons requires the activity of Shh, FGF8, and Wnt signaling that initiate the specification of MB territories of the neural tube (7–10). A key role of signaling factors in neuronal fate determination is to regulate the expression of transcription factors in neural progenitors (13–16). Lmx1a is a homeodomain transcription factor that recently was shown to be induced in response to early signaling in the ventral MB and is selectively expressed in proliferating mesDA progenitors (17, 18). The induction of Lmx1a initiates a regulatory cascade involving additional downstream transcription factors, which promote the subsequent differentiation and maturation of mesDA neurons.

Our initial characterization suggested that Lmx1a is an important determinant of mesDA neurons during embryonic development, and further indicated that Lmx1a, under permissive conditions, can promote the differentiation of mesDA neurons in mouse (m)ESC cultures ($mesDA^{mES}$) (17). However, Lmx1a was transiently expressed and only a limited proportion of cultured cells expressed transgenic Lmx1a in these experiments. Thus, cultures contained a mix of different neuronal subtypes, and the efficiency whereby Lmx1a can induce $mesDA^{mES}$ neurons could not be determined. Also, several important functional and physiological properties of these cells were not characterized.

In this study we tested how effectively forced expression of Lmx1a can induce a mesDA neuron phenotype, and to examine if such cells show functional properties similar to endogenous mesDA neurons. We found that forced expression of Lmx1a in stably transformed mESCs provides a remarkably robust strategy for efficient production and enrichment of mesDA neurons. Analysis of the phenotypic and physiological properties of these cells, as well as the ability of grafted cells to integrate into the striatum of mesDA neuron lesioned neonatal rat brains, showed that Lmx1a-induced $mesDA^{mES}$ neurons are essentially indistinguishable from authentic mesDA neurons. Moreover, although our study focuses primarily on mESCs, we provide evidence that Lmx1a can also promote the generation of $mesDA^{hES}$ neurons when expressed in differentiating human (h)ESCs.

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Results

Forced Lmx1a Expression in mESCs Results in Effective $mesDA^{mES}$ Neuron Generation. In experiments designed to test the ability of Lmx1a to promote mesDA neuron generation from ESCs, we used a protocol in which mESCs are induced to differentiate by culturing cells as monolayers in a defined culture medium (17, 19). Under these conditions, the neural progenitor marker Nestin is induced

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Author contributions: S.F., E.A., L.H.T., A.E.M., A.B., T.P., and J.E. designed research; S.F., E.A., L.H.T., M.E.J., J.B.H., E.N., Z.A., U.M., S.K., and N.V. performed research; O.H. contributed new reagents/analytic tools; S.F., E.A., L.H.T., M.E.J., J.B.H., E.N., S.K., A.E.M., A.B., T.P., and J.E. analyzed data; and S.F., E.A., L.H.T., A.E.M., A.B., T.P., and J.E. wrote the paper.

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The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0902396106/DCSupplemental.

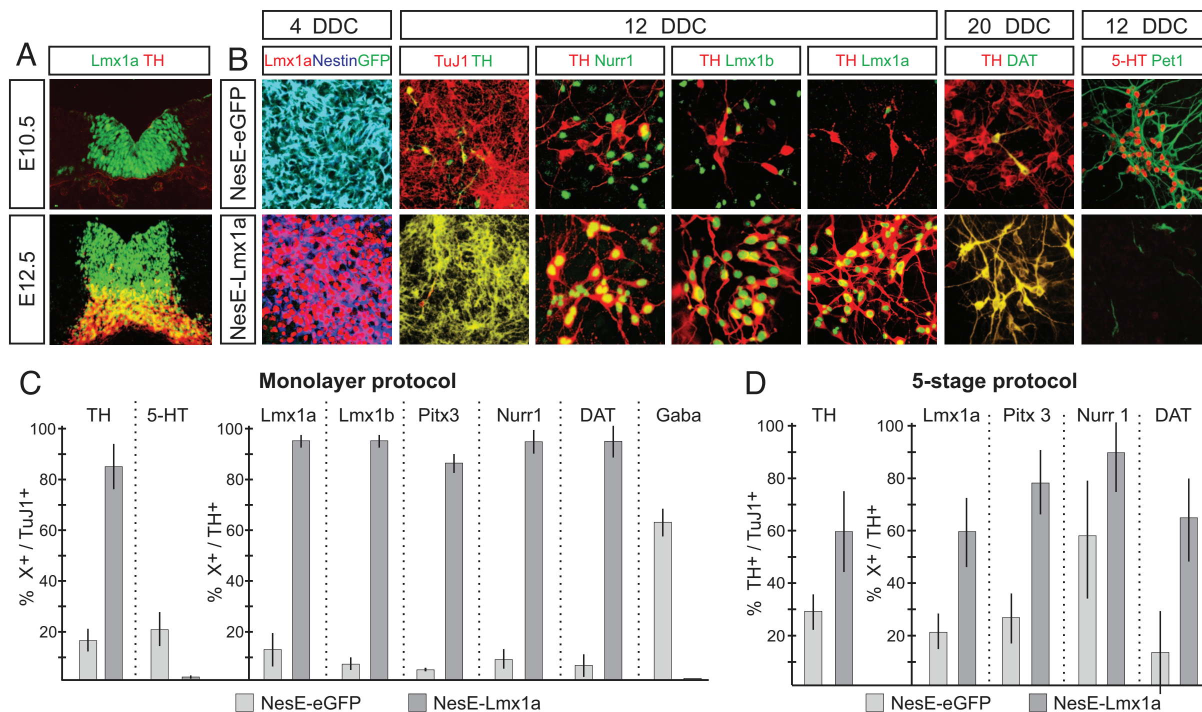

Fig. 1. Lmx1a promotes generation of mesDA$^{\mathrm{mES}}$ neurons. (*A*) Lmx1a is expressed in DA progenitors and postmitotic TH$^+$ neurons at embryonic d 10.5 and 12.5 in mouse ventral MB. (*B*) NesE-Lmx1a and NesE-eGFP mESCs were differentiated and analyzed. At 4 DDC, >85% Nestin$^+$ cells derived from NesE-Lmx1a and NesE-eGFP cells expressed Lmx1a or eGFP, respectively. At 12 DDC, few TuJ1$^+$ cells derived from NesE-eGFP cells expressed TH whereas >75% of TuJ1$^+$ cells derived from NesE-Lmx1a expressed TH and other mesDA markers. Generation of 5-HT neurons was repressed in NesE-Lmx1a cultures. (*C*) Quantification at 12 DDC. (*D*) Quantification of NesE-Lmx1a and NesE-eGFP ESCs differentiated according to the 5-stage protocol. More than 60% of Lmx1a-induced TH$^+$ neurons co-expressed Lmx1a, Nurr1, and Pitx3 at stage 5 d 10 and DAT at d 20. (See Fig. S1 for expression patterns.) (*C*–*D*) Error bars indicate SD, $n = 5$.

within 2 to 5 d in differentiation conditions (DDC). This is followed by differentiation into post-mitotic neurons expressing the pan-neuronal marker TuJ1. To mimic the extrinsic embryonic ventral MB environment, Shh and FGF8 were added during the initial phase of culture under which ESCs differentiate into neural progenitor cells. In vivo, Lmx1a expression is detected in presumptive mesDA cell progenitors from E9.0 (Fig. 1*A*) (17). Transient transfection experiments in mESCs indicated that Lmx1a promotes mesDA$^{\mathrm{mES}}$ neuron differentiation, but the efficiency was not possible to assess as forced expression was achieved in only a subfraction of cells. We generated stably transformed mESCs using a *Nestin* enhancer (*NesE*)-driven expression vector to direct Lmx1a expression to the neural progenitor stage of ESC differentiation (17, 20). As controls, stable cell lines expressing eGFP under the control of the *NesE* were generated.

NesE-Lmx1a and NesE-eGFP cell lines (with expression of Lmx1a or GFP in >85% of Nestin$^+$ cells at 4 DDC, respectively; Fig. 1*B*) were examined at 12 DDC, when most neural cells have differentiated into TuJ1$^+$ postmitotic neurons. Strikingly, after differentiation of NesE-Lmx1a cells, a remarkably high proportion (75%–95%) of all TuJ1$^+$ neurons co-expressed TH (Fig. 1*B*). Ninety to 98% of these TH$^+$ neurons also co-expressed additional mesDA neuron markers, including Lmx1b, Pitx3, Nurr1, Lmx1a, En1/2, and Foxa2 (Fig. 1 *B* and *C*; data not shown) (17, 21–27). At 20 DDC, neurons appeared more mature with elaborate neurite extension, and most TH$^+$ neurons at this stage expressed the dopamine transporter (DAT) and the vesicular monoamine transporter 2 (VMAT2; Fig. 1*B*; data not shown). Extensive but non-overlapping expression of Girk2 and Calbindin at 20 DDC suggested the presence of neurons corresponding to both the SN (A9 neurons) and ventral tegmental area (A10 neurons) subtypes of mesDA neurons (data not shown) (28, 29). In contrast, in control NesE-eGFP cells, 10%–15% of TuJ1$^+$ neurons expressed TH and only a subfraction of those (10%–20%) co-expressed other mesDA neuron markers (Fig. 1 *B* and *C*). mesDA$^{\mathrm{mES}}$ neurons were also efficiently generated when NesE-Lmx1a cells were used in an alternative differentiation protocol [Fig. 1*D* and supporting information (SI) Fig. S1] (6). Lmx1a promotes the generation of mesDA$^{\mathrm{mES}}$ neurons at the expense of other ventral neuronal subtypes (17). Consistent with this, 5-HT neurons were almost completely suppressed (0.1%–1%) in NesE-Lmx1a cultures compared with controls (18%–25%; Fig. 1 *B* and *C*). Thus, the enhanced production of mesDA$^{\mathrm{mES}}$ neurons by Lmx1a occurs at the expense of other neuronal subtypes.

Electrophysiological recordings revealed that a majority of Lmx1a-induced neurons had acquired an electrophysiological profile that is strikingly similar to the properties of native mesDA neurons (30). They fired spontaneous action potentials with a half-width of 2.93 ms ± 0.14, which was significantly larger than that of control cells (1.9 ms ± 0.1; $P < 0.001$; $n = 30$; Fig. 2*A*). Application of dopamine (DA) in NesE-Lmx1a cells induced hyper-polarization of the membrane potential and blocked the spontaneous firing of action potentials, whereas the firing in control cells was unaffected (Fig. 2*A*). Finally, NesE-Lmx1a cells, but not control cells, responded to hyper-polarizing current with a slow depolarizing sag, followed by a rebound firing of action potentials (Fig. 2*A*; $n = 30$). These results indicate that NesE-Lmx1a cells express biophysical properties characteristic of native mesDA neurons (30). In addition, differentiated NesE-Lmx1a cells, but not control cells, contained high amounts of DA and the DA metabolite DOPAC (Fig. 2*B*). Taken together, Lmx1a has a remarkable capacity to effectively promote the differentiation of neurons with the phenotype and behavior of bona fide mesDA neurons.

Lmx1a-induced mesDA$^{\mathrm{mES}}$ neurons were next examined in vivo after transplantation of cells into the striatum of neonatal rats with unilateral 6-hydroxy DA (6-OHDA) lesions at 4 DDC, a stage when

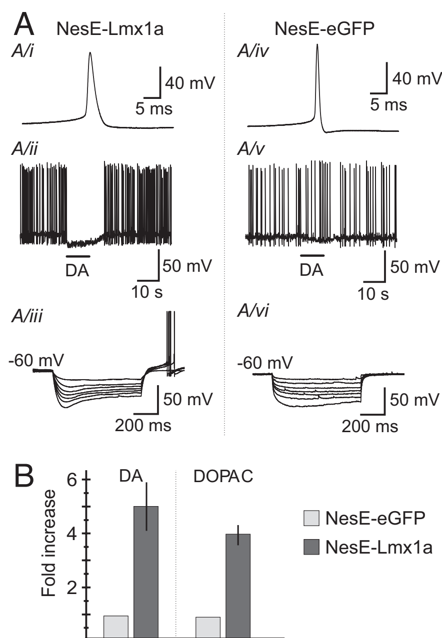

Fig. 2. Lmx1a-induced mesDA^{mES} neurons show the same physiological properties as mesDA neurons in vivo. (*A*) Electrophysiology of randomly chosen neurons showed that 60% of Lmx1a-induced neurons, but only 10% of control neurons, showed properties of mesDA neurons (See *SI Text* for additional information). (*B*) HPLC analysis showed a 4-fold increase in DA and DOPAC content in NesE-Lmx1a-induced neurons compared with NesE-eGFP-derived neurons.

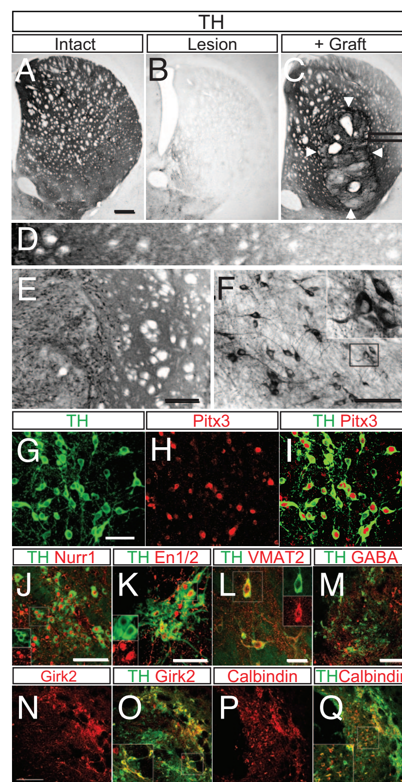

Fig. 3. Lmx1a-induced progenitors give rise to grafts containing many mesDA^{mES} neurons following transplantation into rat striatum. (*A*) Immunohistochemistry for TH illustrates the typical dense staining of DA fibers in the intact striatum; (*B*) almost complete loss of TH fibers is seen in 6-OHDA lesioned striatum; (*C*) there is substantial graft-derived re-innervation of the lesioned striatum 4 weeks after transplantation (arrowheads, graft core). (*D*) The boxed area in *C* is shown at larger magnification. The innervation of the host striatum is most dense in close proximity to the graft and extends over large distances. (*E*) Typical appearance of part of an intra-striatal graft and associated fiber outgrowth revealed by TH staining. The graft core (*Left*) contains many intensely stained TH$^+$ neurons, which innervate the adjacent host striatum and form a dense terminal network (*Right*). (*F*) Most TH$^+$ neurons have a large size and elongated shape typical for mesDA neurons. (*G–I*) Virtually all grafted mesDA^{mES} neurons co-expressed TH and Pitx3. The vast majority also expressed Nurr1 (*J*) and En1/2 (*K*) (boxed areas in *J* and *K* depicted as single colors illustrating cytoplasmic and nuclear localization of TH or transcription factors, respectively). (*L*) Many of the TH$^+$ neurons co-expressed VMAT2 (boxed area depicted as single colors to show the overlap and cytoplasmic distribution). (*M*) Despite large numbers of GABA$^+$ neurons, there were only very rare examples of overlap between TH and GABA. The grafts contained different mesDA neuronal subtypes as indicated by co-expression of Girk2 (*N* and *O*) or Calbindin (*P* and *Q*) in TH$^+$ cells (*O* and *Q*). Boxed areas shown at greater magnification illustrating overlap between TH and Girk2/Calbindin. (Scale bars, 500 μm in *A–C*; 200 μm in *E* and *M–Q*; 100 μm in *F*; 50 μm in *G–I* and *J–L*.)

most cells display a Nestin$^+$ neural progenitor cell identity (17) (data not shown). The survival of mesDA^{mES} neurons was assessed in sections stained for TH, and overall graft survival was assessed in sections stained with the mouse cell-specific M2/M6 antibody. The M2/M6 staining showed that the grafts had failed to survive in approximately 50% of the cases. At 4 weeks after transplantation, all surviving grafts contained numerous TH$^+$ neurons (Fig. 3 *A–F*). On examination of the TH$^+$ cells, NesE-Lmx1a grafts contained large numbers of TH$^+$ neurons with a size and shape resembling the A9 and A10 mesDA neuron subtypes (Fig. 3*F*) (28). Consistent with the presence of authentic nigral mesDA neurons, the NesE-Lmx1a grafts gave rise to extensive TH$^+$ innervation of the host striatum with TH$^+$ fiber outgrowth displaying a clear striatal target preference (Fig. 3 *C–E*).

Double immunostaining with mesDA neuron markers confirmed the authenticity of transplanted mesDA^{mES} neurons and showed that virtually all grafted TH$^+$ neurons co-expressed Pitx3 (Fig. 3 *G–I*), and the vast majority also co-expressed Nurr1 (Fig. 3*J*), En1/2 (Fig. 3*K*), and Lmx1a (data not shown). Many of the TH$^+$ neurons also expressed VMAT2 (Fig. 3*L*). The TH$^+$ neurons, with very rare exceptions, were all GABA$^-$ (Fig. 3*M*). Double staining for TH and the 2 mesDA neuron subtype markers Girk2 (A9) and Calbindin (A10) showed that both subtypes were present in the grafts (Fig. 3 *N–Q*), in a proportion (50:50) similar to that normally present in transplants of fetal ventral mesencephalic tissue. Similar to primary tissue grafts, the TH$^+$Girk2$^+$ neurons tended to cluster at the periphery and the TH$^+$Calbindin$^+$ cells in the core of the graft cell clusters. Thus, our analysis of mESCs in vitro and after grafting in vivo indicates that NesE-Lmx1a-derived TH$^+$ neurons are essentially indistinguishable from bona fide primary mesDA neurons.

Grafts contained cells that continued to proliferate and a variable, and sometimes quite extensive, overgrowth was seen in the animals with surviving grafts (Fig. 3*C*). A significant number of Ki67$^+$ cells could be detected within these grafts, suggesting the presence of proliferating cells. None of these cells co-expressed TH but many were Sox1/2$^+$ and Nestin$^+$, suggesting that they, at the time of analysis, express traits of neural progenitors (Fig. S2). Transplantation of isolated mESC-derived neural progenitors does not give rise to tumors (31, 32). It is therefore likely that overgrowths originate from a small subset of ESCs that escaped differentiation in vitro and retained a pluripotent state at the time of grafting (3).

To try to avoid overgrowth within grafts, we applied magnetic bead sorting and antibodies against the polysialic acid (PSA)-neural cell adhesion molecule (NCAM) (33) to enrich early post-mitotic neurons before grafting. Analysis of cells that were cultured in vitro after enrichment showed that the vast majority of sorted cells

Fig. 4. Grafting of PSA-NCAM-sorted NesE-Lmx1a-derived neurons eliminates overgrowth. (*A*) Eleven DDC NesE-Lmx1a cells were sorted and transplanted into the striatum of 6-OHDA lesioned neonatal rats. (*B*) Most of the TH^+ neurons had a large size and elongated shape typical of mesDA neurons. (*C*) M2/M6 staining showing the size of the graft. (*D*) The majority of the transplanted neurons (HuC/D^+ cells) expressed TH. (*E–H*) Most of the grafted neurons co-expressed TH and other mesDA markers: Pitx3 (*E*), Nurr1 (*F*), En1/2 (*G*), and VMAT2 (*H*). There were few 5-HT (*I*) and GABA (*J*) neurons in the graft.

co-expressed markers for mesDA neurons and TuJ1 (data not shown). Grafting of these cells into lesioned neonatal rats did not generate overgrowth (Fig. 4*A*). Although only relatively few cells survived in these grafts (see *Materials and Methods*), the majority of cells corresponded to TH^+ neurons (65%–70% of HuC/D^+ cells; Fig. 4*D*) that co-expressed Pitx3, Nurr1, En1/2, and VMAT2 (Fig. 4 *E–H*). Very few cells, if any, expressed markers for other neuronal cell types such as 5-HT or GABA (Fig. 4 *I* and *J*). Thus, these results indicate that magnetic sorting of PSA-$NCAM^+$ post-mitotic neurons can be used in grafting experiments to separate post-mitotic $mesDA^{mES}$ neurons from contaminating rapidly dividing cells in ESC cultures.

Expression of Lmx1a in Human ESCs. To directly examine if Lmx1a also promotes differentiation of mesDA neurons in hESCs, lentiviral vectors with the PGK promoter and Lmx1a or eGFP control were generated (L-Lmx1a and L-eGFP, respectively). hESC cultures, differentiated into neural progenitors and neurons (Fig. 5; data not shown) (4), were exposed to Shh and FGF8 at 7 to 20 DDC and then infected as illustrated in Fig. 5*B*. Only a small fraction (<20%) of cells expressed Lmx1a under these conditions (data not shown). Approximately 60% of the neural progenitor cells expressed either Lmx1a or eGFP at 2–4 d post-transduction (pt) with L-Lmx1a or L-eGFP, respectively (data not shown). At 30 d pt, 50%–65% of $TuJ1^+$ neurons in cultures infected with L-Lmx1a co-expressed TH (Fig. 5 *A* and *C*). Moreover, the majority (75%–85%) of TH^+ cells also expressed other mesDA neuron markers at 40 d pt (Fig. 5 *A* and *C*; data not shown). In contrast, although a significant proportion of $TuJ1^+$ neurons in L-eGFP cultures expressed TH (≈25%), only a minority (10%–20%) of these cells co-expressed Lmx1b, Pitx3, Nurr1, En1/2, Foxa2, or DAT. It is possible that some of the TH^+ cells in L-eGFP cultures correspond to oculomotor neurons generated laterally to mesDA neurons in the MB, as these cells appear to transiently express TH during human embryogenesis (34). Also, most $Lmx1b^+$ cells corresponded to 5-HT neurons in L-eGFP transduced cells but to $mesDA^{hES}$ neurons in L-Lmx1a cultures (Fig. 5 *A* and *C*; data not shown) (35, 36). Thus, similar to experiments in mESCs, forced expression of Lmx1a promotes the generation of $mesDA^{hES}$ neurons and suppresses 5-HT neurons in hESC cultures.

Discussion

Development of stem cell-based cell therapy of PD may be realistic as transplantation of primary embryonic MB tissue to patients with PD can restore striatal dopaminergic neurotransmission and provide long-lasting symptomatic relief (2). However, any future therapeutic success will depend on standardized protocols using stem cells that generate a high proportion of mesDA neurons that are well defined with respect to identity and function. Moreover, it will also be important to avoid the co-transplantation of non-mesDA cells that can cause unwanted side effects. Several protocols for generating TH^+ neurons from ESCs have been described and usually depend on extrinsic signaling factors such as Shh and FGF8 in the differentiation medium during the process (3–6). However, without additional modification of cells (37, 38), only a small proportion of neurons express characteristics of mesDA neurons and their ability to project axons and effectively innervate the host striatum after

Fig. 5. Lmx1a promotes $mesDA^{hES}$ neurons in differentiating hESCs. hESC-derived neuroepithelial progenitors were infected with lentiviral (*L*) vectors carrying Lmx1a or eGFP and analyzed at day 30 to 40 pt. (*A*) In L-Lmx1a-infected cultures, >50% of $TuJ1^+$ neurons co-expressed TH at d 30 pt, compared with 25% in L-eGFP-infected cultures. Most TH^+ neurons co-expressed mesDA markers, e.g., Lmx1b, Pitx3, Nurr1, and DAT, whereas markers for 5-HT neurons were suppressed. Few TH^+ neurons derived from L-eGFP-infected cells co-expressed mesDA markers. (*B*) Differentiation scheme. (*C*) Quantification of marker expression. Error bars indicate SD, $n = 4$.

transplantation remains unclear (39). In this study, we show that forced expression of Lmx1a can effectively promote mesDA^{ES} neuron differentiation of both mESCs and hESCs. The *NesE*-driven expression of Lmx1a in differentiating mESCs results in a remarkable enrichment of neurons (>75%) showing mesDA neuron identity. These cells also produce DA and show the electrophysiological profile typical of primary mesDA neurons (Fig. 2). Moreover, after transplantation of NesE-Lmx1a mESC-derived progenitors into neonatal rats, surviving TH^{+} neurons extensively innervate the striatum and appear identical to primary mouse mesDA neurons. Thus, Lmx1a effectively promotes the generation of functional and transplantable mesDA^{mES} neurons from differentiating ESCs.

We found that a significant proportion of NesE-eGFP mESCs exposed to Shh and FGF8 expressed endogenous Lmx1a after 5 to 7 DDC in our differentiation protocol (Fig. S3). Nevertheless, only a minor fraction of TH^{+} neurons were generated, and most of these cells did not co-express other mesDA neuron markers (Fig. 1 *B–D*). These observations raise the question why *NesE*-driven expression of Lmx1a can so potently induce differentiation into mesDA^{ES} neurons. Importantly, we noted that expression of Lmx1a was initiated already at 2 DDC in NesE-Lmx1a mESCs, a time when Nestin^{+} neural progenitors first could be detected in our differentiation protocol (Fig. S3). These data indicate that ESC-derived neural progenitors progressively lose their potential to generate mesDA neurons over time, and that the early expression achieved by the *NesE* serve to shift the expression of Lmx1a into a permissive time window in which Lmx1a still effectively can promote the generation of mesDA^{ES} neurons and suppress alternative fates of neuronal differentiation.

After transplantation of NesE-Lmx1a mESC-derived progenitors into neonatal rats, surviving TH^{+} neurons retain a mesDA identity and show a characteristic morphology and gene expression pattern that appear identical to primary mouse mesDA neurons. Notably, also the core-to-periphery distribution of Calbindin^{+} ventral tegmental area and Girk2^{+} SN neurons was similar to how such cells are distributed in grafts using primary ventral MB cells (Fig. 3 *N–Q*) (28). Importantly, outgrowth of TH^{+} axons was extensive and TH^{+} axons also showed a clear target preference for striatal areas of the brain. A similar preference to innervate striatal target cells has previously been described for primary mesDA neurons (40). Together these in vitro and in vivo analyses strongly suggest that Lmx1a effectively promotes the generation of functional and transplantable mesDA^{mES} neurons from differentiating ESCs, which appear indistinguishable from isolated primary mesDA neurons.

After grafting, extensive overgrowths were formed from cells that continued to proliferate, and many of these cells expressed a neural progenitor cell identity at the time of analysis (Fig. S2). Previous studies suggest that such tumors can arise from a small fraction of undifferentiated ESCs that fail to adopt a neural fate in vitro, and which continue to grow within the brain after transplantation (41). Alternatively, overgrowths may have originated from a small fraction of contaminating forebrain progenitors with an intrinsic capacity to grow for extensive time periods (42). We also observed similar overgrowths after grafting of control ESCs into the rat striatum, or after transplantation of cells differentiated according to the 5-stage protocol (data not shown). Thus, overgrowth appear to reflect a general limitation in controlling ESC differentiation (32, 43) and are not related to the transgenic expression of Lmx1a.

We used magnetic sorting of early post-mitotic neurons using antibodies against PSA-NCAM to eliminate contaminating cells, a strategy that previously has been used to isolate neurons from differentiating ESCs (33). Although the number of surviving neurons was low, the vast majority of surviving cells showed the phenotype and morphology of mesDA neurons and there were no evidence of tumor formation or continued proliferation of cells at the time of analysis (data not shown; Fig. 4). TH^{+} neurons enriched by sorting cells expressing eGFP under the control of Neurogenin2 or Pitx3 regulatory sequences have previously been used for grafting and showed relatively good survival (44, 45). Thus, techniques aimed at improving the survival of Lmx1a-engineered cells, or other sources of mesDA neurons, should facilitate future grafting experiments of enriched mesDA^{ES} neurons.

Stem cell-based protocols have so far mostly focused on the addition of growth factors to control the differentiation of cells into TH-expressing neurons, in part because it appears attractive to avoid methods that involve genetic manipulation of cells (3–6). However, introduction of foreign DNA into stem cells should not be a significant disadvantage as long as such a strategy provides a safe, effective, and functional outcome. Indeed, the ability of transcription factors to intrinsically restrict the developmental potential of ESCs can provide several advantages. First, our data show that Lmx1a expression effectively eliminates the generation of potentially harmful 5-HT neurons (Fig. 1 *B* and *C*) (11). Second, cultured hESCs tend to drift into rapidly growing forebrain progenitors (42), an effect that possibly could be prevented by forced expression of transcriptional determinants (e.g., Lmx1a). Forced expression of transcription factors, either alone or in combination, may therefore provide a powerful tool in stem cell engineering, as illustrated by the recent success in generating induced pluripotent stem cell lines from somatic cells by the forced expression of a combination of ESC-expressed transcription factors (46–49).

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Materials and Methods

DNA Constructs. Mouse Lmx1a or eGFP cDNAs were subcloned into NesE vectors containing a neomycin selection cassette (17) or lentiviral vectors with the PGK promoter (50).

Maintenance and Differentiation of mESCs. mESCs were propagated as described (17). To generate stable lines, mESCs were nucleofected with NesE vectors according to protocol (mouse ESC nucleofector; Amaxa). After selection, individual clones were picked and expanded. Clones were tested for transgene expression at 4 DDC, and 2 independent clones expressing transgenes in >85% of Nestin^{+} cells were selected for further experiments. The reason why Lmx1a was not detected in all Nestin^{+} cells is unclear but may reflect later kinetics of transgene expression, gene silencing, or antibody sensitivity. For monolayer cultures, differentiation was performed as described (17, 19). For the 5-stage protocol, cells were differentiated as described (6). For detailed protocol see *SI Text*. In the text, the Curis Shh agonist Hh-Ag1.3 is referred to Shh. For statistical analysis, the number of positive cells was counted in random squares on the culture slides. Data points represent the average of 3 to 5 independent experiments ± SD.

Maintenance, Differentiation, and Infection of hESCs. hESC lines (401, 293, 351) were maintained and differentiated as described (4). For detailed protocol see *SI Text*. In brief, hESCs were dissociated and induced to differentiate as embryoid bodies in NIM medium. At d 5, embryoid bodies were plated in nucleophosmin (NPM) medium with 10 ng/mL FGF2. After 2 d, 70 nM Hh-Ag1.3 and 100 ng/mL FGF8 were added. Early neuroepithelial colonies were isolated and infected with Lmx1a or eGFP lentiviral vectors (multiplicity of infection, 3–4). Forty-eight hours pt, cells were isolated and re-plated in NPM. Cells were analyzed for neural markers at 4 to 40 d pt. The 3 hESC lines gave similar results. Representative results are from hES 401. Data points represent the average of 4 independent experiments ± SD.

Electrophysiology and HPLC. NesE-Lmx1a and NesE-eGFP cells were differentiated according to the 5-stage protocol and analyzed at stage 5 day 17 to 24. For electrophysiology, cells were recorded using a patch-clamp amplifier (AxoPatch 200A; Axon Instruments) and signals were acquired in the whole-cell configuration (for detailed protocol see *SI Text*). Patch electrodes were filled with intracellular solution. Neurons were continuously perfused with extracellular solution at room temperature. DA (Sigma-Aldrich) was applied at a final concentration of 50 μM. The analysis was performed using Clampfit 8. The results are statistically analyzed using unpaired *t* test and presented as mean ± SEM. For HPLC analysis, cells were trypsinized and cell pellets collected

by centrifugation. Pellets were analyzed by HPLC for DA and DOPAC content as described (51).

Magnetic Sorting. NesE-Lmx1a cells were differentiated as monolayer cultures for 11 DDC. Magnetic sorting was performed according to protocol (Miltenyi Biotec). Primary PSA-NCAM antibody was from Chemicon and secondary antibody from Miltenyi Biotec. Approximately 60% of all cells were $TuJ1^+$ at 11 DDC. The yield after MACS was 8% to 11%. The low yield may be a result of inadequate binding to columns, difficulties with eluting cells, and/or unsatisfactory survival after purification. The estimated purity after sorting was 90%–95% $TuJ1^+$ cells. $Ki67^+$ cells were only rarely detected, and 2 weeks after sorting there was no increase in cell numbers in sorted cells maintained in culture.

6-OHDA Lesioning and Intra-Striatal Transplantation of mESCs. Neonatal rats were used as recipients (Sprague–Dawley; B & K Universal). All animal-related procedures were conducted in accordance with local ethical guidelines and approved animal care protocols. The neonatal stereotaxic procedures were performed as described (52). For detailed protocol see *SI Text*. In brief, on postnatal d 1, rats received unilateral injections of 6-OHDA into the right lateral ventricle. NesE-Lmx1a ESCs were differentiated as monolayers for 4 DDC (no sorting) or 11 DDC (PSA-NCAM sorting) before transplantation. One to 2 μL cell suspension (100,000 cells/μL) was implanted unilaterally into the dorsal, lesioned striatum at postnatal d 3. Four weeks later, rats were perfused with formaldehyde and the brains postfixed, transferred to sucrose, and sectioned. Immunohistochemistry was performed on free-floating sections as described (28). Denervation was confirmed in all grafted animals by ensuring that TH expression was unilaterally lost in the SN.

Immunohistochemistry. Immunohistochemical localization of proteins was performed as described (13). For antibody list see *SI Text*.

ACKNOWLEDGMENTS. We thank M. Smidt for reagents; Q. Deng, M. Carta, A. Josefsson, and U. Jarl for technical assistance; and members of the Ericson, Perlmann, and Björklund laboratories for discussions. This work was supported by funding from the European Community's seventh framework programme under the grant agreement 222943 (to T.P., J.E., A.B.); Knut och Alice Wallenberg Foundation (J.E., T.P., A.B.); the Michael J. Fox Foundation for Parkinson's research (J.E., T.P., A.B.); Vetenskapsrådet via support to the Linné Center for Developmental Biology and Regenerative Medicine, Vetenskapsrådet (J.E., T.P.); Göran Gustafssons Stiftelse; Swedish Foundation for Strategic Research (J.E.); and Edmond J. Safra Foundation (A.B.).

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