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Differences in neurogenic potential in floor plate cells along an anteroposterior location: midbrain dopaminergic neurons originate from mesencephalic floor plate cells

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Directed differentiation and purification of mesencephalic dopaminergic (mesDA) neurons from stem cells are crucial issues for realizing safe and efficient cell transplantation therapies for Parkinson's disease. Although recent studies have identified the factors that regulate mesDA neuron development, the mechanisms underlying mesDA neuron specification are not fully understood. Recently, it has been suggested that mesencephalic floor plate (FP) cells acquire neural progenitor characteristics to generate mesDA neurons. Here, we directly examined this in a fate mapping experiment using fluorescence-activated cell sorting (FACS) with an FP cell-specific surface marker, and demonstrate that mesencephalic FP cells have neurogenic activity and generate mesDA neurons in vitro. By contrast, sorted caudal FP cells have no neurogenic potential, as previously thought. Analysis of dreher mutant mice carrying a mutation in the Lmx1a locus and transgenic mice ectopically expressing Otx2 in caudal FP cells demonstrated that Otx2 determines anterior identity that confers neurogenic activity to FP cells and specifies a mesDA fate, at least in part through the induction of Lmx1a. We further show that FACS can isolate mesDA progenitors, a suitable transplantation material, from embryonic stem cell-derived neural cells. Our data provide insights into the mechanisms of specification and generation of mesDA neurons, and illustrate a useful cell replacement approach for Parkinson's disease.

KEY WORDS: Parkinson's disease, Cell replacement therapy, Dopaminergic neuron, Floor plate cells, Neurogenic activity, Lmx1a, Otx2, Embryonic stem cells, Mouse, Rat

INTRODUCTION

Mesencephalic dopaminergic (mesDA) neurons play important roles in the control of movement and behavior, and degeneration or dysfunction of these neurons can lead to severe neurological disorders such as Parkinson's disease (Olanow and Tatton, 1999). Cell replacement therapy is one of the most promising approaches for the treatment of Parkinson's disease (PD) (Olanow et al., 1996). Understanding the mechanism underlying mesDA neuron development is important for engineering functional mesDA neurons as a material for transplantation from stem cells, and indeed, many researchers have succeeded with in vitro engineering of DA neurons from embryonic stem (ES) cells by controlling extrinsic and intrinsic signals (Andersson et al., 2006b; Barberi et al., 2003; Kawasaki et al., 2000; Lee et al., 2000; Perrier et al., 2004).

Sonic hedgehog (Shh), fibroblast growth factor (FGF) 8 and Wnt1 are essential and sufficient for induction of mesDA neurons (Hynes et al., 1995a; Hynes et al., 1995b; Prakash et al., 2006; Ye et al., 1998). Several transcription factors that are selectively expressed in mesDA neurons and involved in the regulation of mesDA neuron differentiation, such as Nurr1 (also known as Nr4a2 – Mouse Genome Informatics), Lmx1b and Pitx3, have been identified as possible downstream targets of these extrinsic signals (reviewed by Simeone, 2005). Recently, two additional homeodomain factors, Lmx1a and Msx1, which are selectively expressed by mesDA progenitors, have been identified (Andersson et al., 2006b). Gain- and loss-of-function experiments suggest that Lmx1a specifies mesDA neurons. However, the ability of Lmx1a to induce mesDA neurons was restricted to ventral mesencephalic progenitors, indicating that the fate-determining activity of Lmx1a is cellular context-dependent. Thus, factor(s) that cooperate with Lmx1a to specify mesDA progenitor identity might exist; moreover, the mechanism underlying mesDA neuron specification has not been fully elucidated and the upstream signals that determine the regional specificity of Lmx1a expression have not been identified.

In addition to the transcription factors regulating dorsoventral (DV) patterning, anteroposterior (AP) patterning factors are also important for mesDA induction. Otx2 has been shown to be required for mesDA neuron generation independently of controlling isthmic organizer positioning, suggesting that Otx2 may determine the AP identity of neural progenitors that confer mesDA identity (Puelles et al., 2004; Vernay et al., 2005). However, a functional link between DV- and AP-determining factors in mesDA neuron induction has not been revealed.

FP cells are morphologically specialized organizer cells located along the ventral midline of the developing neural tube caudal to the diencephalons (reviewed by Placzek and Briscoe, 2005; Strahle et al., 2004). FP cells play important roles in organizing neural tube patterning and guidance of the commissural axons by secreting diffusible molecules such as Shh and netrin 1 (Ntn1). In mammals, FP cells are thought to be derived from cells of neuroepithelial origin induced by Shh signals secreted from the underlying notochord, and differentiated FP cells lose the potency to give rise to neurons (Jessell, 2000). In the caudal neural tube, FP cells form a cluster at the ventral midline that is not mixed with neural progenitor cells. By

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contrast, histological studies and explant culture experiments have suggested that in the developing mesencephalon, FP cells intermingle with neural cells (Hynes et al., 1995b; Placzek and Briscoe, 2005). Consistent with this, neural progenitors expressing proneural factors, such as Mash1 and Ngn2 (also known as Ascl1 and Neurog2, respectively – Mouse Genome Informatics), which are likely to give rise to mesDA neurons, were observed at the ventral midline of the developing mesencephalon (Andersson et al., 2006a; Kele et al., 2006; Vernay et al., 2005), and the mesDA progenitor-selective factors Lmx1a and Msx1 are expressed by ventral midline cells (Andersson et al., 2006b). Thus, it seems probable that the mechanism underlying induction of mesDA neurons by FP cells is different from that of other ventral neuronal populations. Recent observations that forced expression of Msx1 in the ventral midline cells of the mesencephalon under control of *Shh* enhancer resulted in downregulation of Shh expression and premature mesDA neuron generation suggest that Msx1 can convert FP cells into mesDA progenitors (Andersson et al., 2006b). However, due to the lack of lineage-tracing or fate-mapping experiments, the spatial and functional relationships between FP cells and mesDA progenitors have not been directly revealed.

In the present study, we first demonstrated that *Lmx1a* functions in mammalian mesDA neuron development and identified mesDA progenitors by analyzing *dreher* mutant mice, which carry a mutation in the *Lmx1a* locus. Gene expression studies and fluorescence-activated cell sorting (FACS) experiments revealed that mesencephalic FP (mesFP) cells themselves have neurogenic potential. By contrast, caudal FP (cFP) cells had no neuron-generating activity. Furthermore, a FACS approach directly revealed a lineage relationship between mesFP cells and mesDA neurons. Finally, using a transgenic approach, we demonstrated that the anterior identity determined by Otx2 confers neurogenic potential to FP cells and that the DA phenotype in mesDA neurons is determined by FP identity. Furthermore, an FP or DA progenitor-specific cell surface antigen identified in this study could be used for the isolation of mesDA progenitors, a method that would provide suitable material for transplantation therapy for PD, from ES cell-derived sources.

MATERIALS AND METHODS

Subtractive PCR

The ventralmost (V) region, including mesDA neurons and the basal plate (BP) region, were dissected from an embryonic day 12.5 (E12.5) mouse mesencephalon. Subtractive PCR was performed as described previously (Osada et al., 2005) using the V region as a tester and the BP region as a driver.

Mouse mutant strain and transgenic mice

dreher^{J} mice (Millonig et al., 2000) were obtained from the Jackson Laboratory and maintained in a C3H/B6 mixed background. Embryos were genotyped by amplifying and sequencing the genomic fragments around the mutation.

pFP was constructed by ligating the SV40 poly(A) signal and the genomic fragments for the floor plate-specific enhancer (SFPE1) and promoter of *Shh* gene (Epstein et al., 1999) into the pSP73 vector (Promega). *Myc-Otx2*, *Lmx1a*, *Mash1* and *IRES-Lmx1a* cDNAs were cloned into the pFP vector. Linearized pFP constructs were injected into fertilized eggs and transgenic embryos were collected at E11.5 or 12.5. Embryos were genotyped by PCR. The sequences of primers used for construction and genotyping are available upon request.

Immunohistochemistry and in situ hybridization

Immunohistochemistry was performed as described previously (Nakatani et al., 2004). Hamster anti-Lmx1a and anti-Corin mAbs were raised against GST-Lmx1a [amino acids (aa) 271-308] and the extracellular domain of mouse Corin (aa 161-502), respectively. Rat anti-Pitx3, anti-Nkx6.1 (Nkx6-1) and anti-Nurr1 mAbs were raised against Pitx3 synthetic peptide (aa 1-15), GST-Nkx6.1 (aa 60-122) and GST-Nurr1 (aa 86-248), respectively. A polyclonal rabbit anti-Lmx1b antibody was raised against GST-Lmx1b (aa 271-306) and affinity purified. Other primary antibodies used in this study included: anti-βIII-tubulin (Covance), anti-En1/2, anti-Lim1/2, anti-FP4, anti-Shh (Developmental Studies Hybridoma Bank), anti-BrdU (Roche), anti-MPM2 mAb (Upstate), anti-Th (Chemicon), anti-Nurr1 (Santacruz), anti-MAP2 (Sigma), anti-Mash1 (BD Pharmingen), anti-NG2 (also known as Cspq – Mouse Genome Informatics) (Chemicon), anti-nestin (Chemicon), anti-HuC/D (Molecular Probes), anti-Ngn2 (Santacruz) and anti-Myc (Roche). BrdU labeling experiments were performed as described (Nakatani et al., 2004).

In situ hybridization was performed as described previously (Nakatani et al., 2004). The sequences of primers used for amplifying probes are available upon request.

Cell sorting and culture

Ventral mesencephalons were dissected from E13.5 rat embryos or E9.75 mouse embryos and dissociated using Accumax (Chemicon). Cell suspensions were labeled with anti-Corin monoclonal antibody and PE-labeled anti-hamster secondary antibody (BD Bioscience). Cell sorting was performed on a FACS Aria (BD Bioscience). Sorted cells were plated on a glass chamber coated with poly-L-ornithine, laminin and fibronectin and cultured in DMEM/F12 supplemented with N2 (Invitrogen), 20 ng/ml brain-derived growth factor (BDNF; R&D systems), 200 nM ascorbic acid and either 5-10% KSR (Invitrogen) (for rat cells) or B27 supplement (Invitrogen) (for mouse cells). Cells were fixed with 2% paraformaldehyde and immunostained as described (Nakatani et al., 2004).

Differentiation of ES cells was performed as described previously (Kawasaki et al., 2000), and cell sorting and culture were performed as in primary mesencephalic cells.

RT-PCR

RT-PCR was performed essentially as described previously (Nakatani et al., 2004). Total RNA was isolated from 3×10^4 of the mesencephalic Corin$^+$ cells or 2×10^5 of the ES cell-derived cells using an RNeasy Mini Kit (Qiagen). The numbers of cycles were 40 for *Shh*, *Hnf3β* and *Ntn1*, and 37 for glyceraldehyde-3-phosphate dehydrogenase (*G3PDH*; *Gapdh* – Mouse Genome Informatics) in mesencephalic Corin$^+$ cells, and 38 for *Nanog*, 30 for *Lmx1a*, and 27 for *G3PDH* in ES cell-derived cells. The sequences of primers used for RT-PCR are available upon request.

RESULTS

Isolation of genes selectively expressed in mesDA neurons and their progenitors

To identify the genes that regulate mesDA neuron development, we searched for genes selectively expressed in the ventral midline region of the developing mesencephalon and obtained several genes, including homeodomain transcription factor *Lmx1a*, *Msx1*, *Msx2* and transmembrane domain-containing cell-surface protease *Corin*. Recently, Andersson et al. (Andersson et al., 2006b) reported that Lmx1a is selectively expressed in the mesDA lineage. We further confirmed the selective expression of Lmx1a in the mesDA lineage.

At E12.5, Lmx1a was selectively expressed in the roof plate and the ventral midline region where mesDA neurons emerged, as has been reported (Fig. 1A) (Andersson et al., 2006b). Double immunostaining revealed that virtually all neurons positive for tyrosine hydroxylase (Th) and other known mesDA neuron-selective transcription factors expressed Lmx1a (Fig. 1B-E and see Fig. S1 in the supplementary material). Importantly, virtually all the Lmx1a$^+$ cells in the mantle layer (ML) co-expressed Lmx1b and Nurr1 (Fig. 1C,D and see Fig. S1 in the supplementary material), suggesting that at this stage, Lmx1a$^+$ precursors arising from the ventral midline regions only differentiate into mesDA neurons.

Importantly, Lmx1a was expressed at a high level in the ventricular zone (VZ) cells lying adjacent to mesDA neurons as well as in postmitotic mesDA neurons (Fig. 1B-E), and some of these Lmx1a$^+$ VZ cells co-expressed the M-phase marker MPM2 and efficiently incorporated BrdU injected 2 hours before fixation (Fig. 1G-I). Furthermore, the proneural transcription factors Mash1 and Ngn2, which are required for correct mesDA neuron development (Andersson et al., 2006a; Kele et al., 2006) were co-expressed with Lmx1a in the VZ (Fig. 1J and data not shown), indicating that Lmx1a$^+$ VZ cells are proliferating neural progenitor cells. Together with the observation that Lmx1a and Nkx6.1 (which marks a neighboring domain) form a sharp boundary between the regions in both the VZ and ML (Fig. 1F), these results confirm that the Lmx1a$^+$ progenitor domain represents a proliferative mesDA progenitor domain (Andersson et al., 2006b).

Lmx1a is involved in the generation of mesDA neurons

The function of *Lmx1a* in mesDA neuron development, in chick and mouse ES cell-derived cultures, has previously been reported (Andersson et al., 2006b). To examine the in vivo role of *Lmx1a* in mammalian mesDA neuron development, we analyzed *dreher* mutant mice carrying a loss-of-function mutation in the first LIM domain of Lmx1a (Millonig et al., 2000). We first analyzed mesDA neuron differentiation in *dreher* embryos at E13.5. The numbers of Th$^+$ and Lmx1b$^+$ cells were apparently reduced in homozygous *dreher* embryos (*Lmx1a*$^{dr/dr}$) compared with wild-type controls (Fig. 2A). We observed a more than 30% reduction in the number of Lmx1b$^+$ neurons in *Lmx1a*$^{dr/dr}$ embryos at E13.5 (Fig. 2C). A milder phenotype was observed in heterozygous mutants, indicating the dose-dependent requirement of *Lmx1a* activity for mesDA neuron development. The number of Brn3a$^+$ (also known as Pou4f1 – Mouse Genome Informatics) red nucleus (RN) neurons that emerged from the adjacent dorsal domain was unaffected by the *dreher* mutation (data not shown); suggesting a cell-autonomous defect in mesDA neuron development in the *dreher* mutants.

This reduction in the number of mesDA neurons is possibly caused by the mis-specification of neurons arising from the Lmx1a$^+$ progenitor, or by reduced neurogenesis or cell death. A TUNEL assay showed no detectable increase in cell death in the ventral mesencephalon of *Lmx1a*$^{dr/dr}$ mutants at E12.5 and 13.5 (data not shown), suggesting that increased cell death is not a cause of this phenotype. In both *Lmx1a*$^{dr/dr}$ embryos and wild-type littermates, almost all Lmx1a$^+$ neurons, as well as virtually all nascent precursors near the VZ and migrating differentiated neurons around the ventral midline region, were positive for Lmx1b (Fig. 2A). These observations indicate that neurons generated from Lmx1a$^+$ progenitors are specified to a mesDA fate in *Lmx1a*$^{dr/dr}$ mutants. Finally, we examined whether the rate of mesDA neuron generation is reduced in *Lmx1a*$^{dr/dr}$ mutants. At E11.5, many Th$^+$ neurons began to accumulate at the ventral midline of the ML in wild-type embryos (Fig. 2B). By contrast, only a small number of Th$^+$ cells were detected in the *Lmx1a*$^{dr/dr}$ mesencephalon. TuJ1$^+$Lmx1a$^+$ neurons, which probably emerge from Lmx1a$^+$ progenitors and seem to be fated to become mesDA neurons, were less numerous in *Lmx1a*$^{dr/dr}$ embryos than in wild-type controls (Fig. 2B), supporting the argument against the possibility that a delay of differentiation causes the reduction in Th$^+$ cell numbers. Taken together, we concluded that the generation of mesDA neurons is reduced in *Lmx1a*$^{dr/dr}$ embryos. Thus, *Lmx1a* activity is required for efficient neurogenesis in mesDA progenitor cells. This is consistent with a previous report of an avian study (Andersson et al., 2006b). However, the *dreher* mutant phenotype was milder than that of chick embryos transfected with siRNA for *Lmx1a* (see Discussion).

Lmx1a regulates proneural gene expression and cell growth in mesDA progenitors

To determine the cause of the *dreher* phenotype in mesDA neuron production, we first examined changes in gene expression in Lmx1a$^+$ progenitor cells. The expression levels of Nkx6.1 and *Sim1*, both of which mark progenitor domains adjacent to the Lmx1a$^+$ domain, were not changed, suggesting that the mesDA progenitor domain is normally patterned in *Lmx1a*$^{dr/dr}$ embryos (data not shown). In addition, we could not detect any difference in Shh expression in the ventral midline of the mesencephalon at E10.5 (data not shown). Thus, we reasoned that only neuron generation from the Lmx1a$^+$ domain is affected without any change in progenitor identity. To test this, we analyzed the expression of the proneural factors Ngn2 and Mash1 at E11.5. The intensities of the Ngn2 and Mash1 signals were significantly lower in *Lmx1a*$^{dr/dr}$ embryos than in wild-type embryos and the number of Ngn2$^+$ and Mash1$^+$ cells in the Lmx1a$^+$ domain was reduced to approximately 70% without significant change in the total number of Lmx1a$^+$ progenitor cells in the *Lmx1a*$^{dr/dr}$ mutant, whereas Ngn2 and Mash1

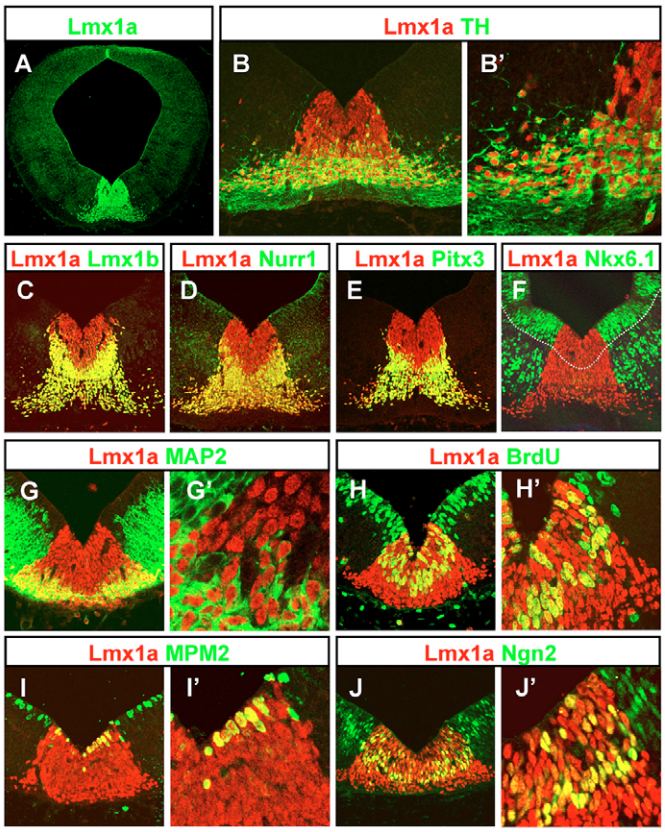

Fig. 1. Lmx1a is selectively expressed in mesDA neurons and their proliferative progenitors. (**A**) Expression of Lmx1a in the mouse mesencephalon at E12.5. (**B-F**) Lmx1a is selectively expressed in the mesDA neurons. Lmx1a is co-expressed with Th (B), Lmx1b (C), Nurr1 (D) and Pitx3 (E) but not with Nkx6.1 (F) in the mouse ventral mesencephalon at E12.5. Only Lmx1a is expressed in the VZ region of the mesDA domain. (**G-J**) Lmx1a is expressed in the proliferative progenitors. Lmx1a was co-expressed with MPM2 (I) and Ngn2 (J), and Lmx1a$^+$ VZ cells incorporate acutely injected BrdU (H) at E11.5.

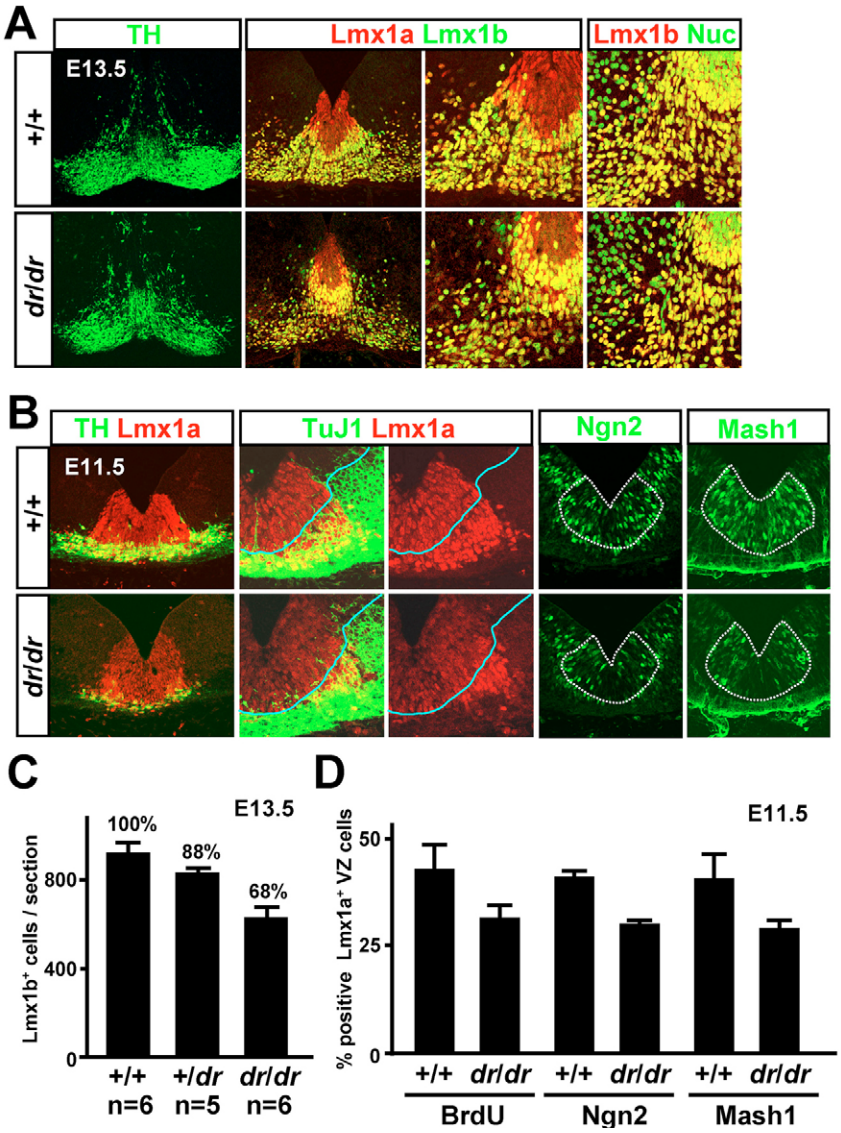

Fig. 2. Lmx1a regulates mesDA neurogenesis by inducing proneural factor expression and cell growth in their progenitors. (**A**) The mesDA number (indicated by Th and Lmx1b expressions) is reduced in dreher mutant embryos compared with wild-type embryos at E13.5, but the fate of Lmx1a$^+$ neurons is not affected. The intensities of anti-Th and anti-Lmx1b staining were lower in dreher mutants. However, almost all Lmx1a$^+$ neurons and neurons generated from ventral midline regions of the mesencephalon, in mutants and wild-type controls, expressed Lmx1b. (**B**) Neurogenesis in Lmx1a$^+$ progenitors is affected by the dreher mutation. The Th$^+$ neuron number is severely reduced in dreher mutants at E11.5. Ngn2 and Mash1 expression levels in the Lmx1a$^+$ VZ region are reduced and, consistently, the generation of Lmx1a$^+$ TuJ1$^+$ neurons is less efficient in mutants. Note that reductions in the expression levels of proneural factors are apparent in the medial part of the Lmx1a$^+$ domain. (**C**) Quantification of Lmx1b$^+$ neurons in the ventral mesencephalon of wild-type, heterozygous and homozygous dreher mutant embryos at E13.5. (**D**) Quantification of BrdU-incorporating, Ngn2$^+$ and Mash1$^+$ cells in Lmx1a$^+$ VZ region.

expression in other progenitor domains in the mesencephalon were not affected (Fig. 2B,D and data not shown). It should be noted that a reduction in the expression of proneural factors was apparent in the medial region of the Lmx1a$^+$ domain (Fig. 2B). This pattern of proneural factor expression in the *Lmx1a*$^{dr/dr}$ mutant at E11.5 was similar to that in wild-type embryos at an earlier stage (compare Fig. 2B with Fig. S2 in the supplementary material), suggesting the possibility that development of ventral midline cells is delayed in the mutant. However, at any later developmental stage, mesDA neuron numbers were lower in *Lmx1a*$^{dr/dr}$ mutants than in wild-type controls, without significant cell death (see Fig. S3 in the supplementary material; data not shown). Thus, the phenotype cannot be simply explained by a delay in mesDA neurogenesis. In addition to the downregulation of proneural factors, the rate of BrdU incorporation was also decreased in *Lmx1a*$^{dr/dr}$ mutants (Fig. 2D). Taken together, these results suggest that Lmx1a is involved in mesDA neuron generation through regulating proneural gene expression in proliferating progenitors and their cell growth. The requirement for Lmx1a activity in proneural gene induction in Lmx1a$^+$ progenitors is consistent with a previous chick study (Andersson et al., 2006b). Moreover, the strong correlation between the selective downregulation of proneural gene expression in the Lmx1a$^+$ progenitor domain and the selective reduction in the number of mesDA neurons generated strongly suggests that mesDA neurons are derived from the Lmx1a$^+$ midline progenitor domain.

Lmx1a is required for the correct specification of postmitotic DA precursors

As noted above, Lmx1b$^+$ Th$^+$ mesDA neurons were generated in *Lmx1a*$^{dr/dr}$ embryos (Fig. 2A). Thus, we could examine the postmitotic role of *Lmx1a* in mesDA development by analyzing *dreher* mutants.

At E13.5, the percentage of Th$^+$/Nurr1$^+$ in mutant embryos was reduced from 54.4±2.6% to 45.0±1.5%. To determine the cause of this phenotype, we analyzed other mesDA neuron markers, but their expression levels in mutants were mostly normal (see Fig. S4 in the supplementary material). Additionally, the projection of Th$^+$ axons to the striatum was also normal in mutants (see Fig. S5 in the supplementary material). However, we found that Lim1/2 (also known as Lhx1/5 – Mouse Genome Informatics), which are expressed in the RN neurons that emerge from the domain adjacent to the mesDA domain (Fedtsova and Turner, 2001), and which were not co-expressed with Lmx1b in wild-type embryos, were co-expressed in some Lmx1b$^+$ neurons emerging near the margin between the mesDA

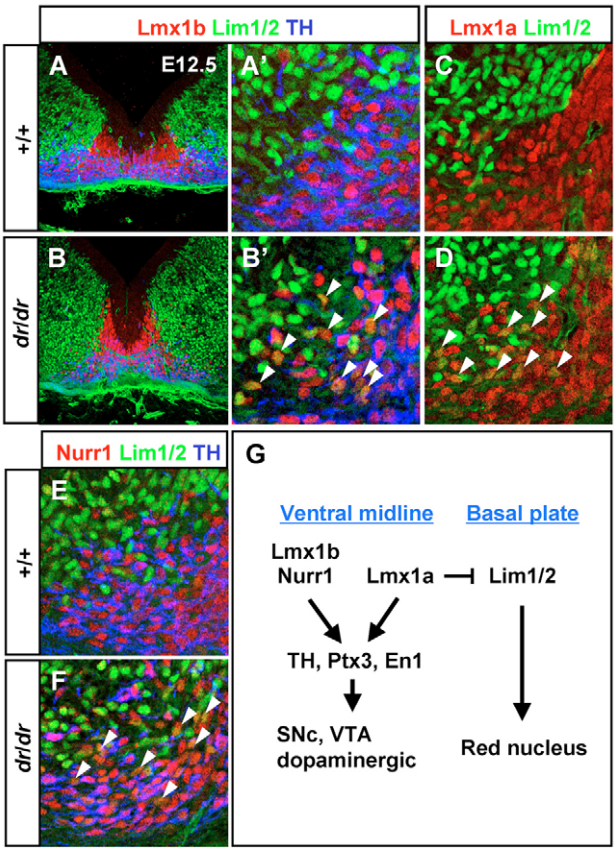

Fig. 3. Mis-specification of mesDA neurons in dreher embryos. Lim1/2 is expressed in RN neurons and is not co-expressed with mesDA markers in wild-type embryos (**A**,**C**,**E**). By contrast, Lim1/2 is ectopically expressed in Lmx1b$^+$ (**B**,**B′**), Lmx1a$^+$ (**D**) and Nurr1$^+$ (**F**) neurons (arrowheads). Note that these Lim1/2$^+$ mesDA precursors do not express Th. (**G**) Model of Lmx1a function in postmitotic mesDA specification.

and RN domains at E12.5 (Fig. 3A,B). Co-expression of Lim1/2 with Lmx1a in mutant embryos suggests that Lim1/2 are ectopically expressed in those precursors fated to mesDA neurons (Fig. 3C,D). These neurons with mixed identity expressed Nurr1 but not Th and Pitx3 (Fig. 3E,F and see Fig. S6 in the supplementary material), indicating that they could not mature into mesDA neurons.

Taken together, these observations suggest that *Lmx1a* is required not only for mesDA neurogenesis, but also for the correct differentiation programming of mesDA neurons by repressing Lim1/2 expression in postmitotic precursors (Fig. 3G).

MesDA progenitors have an FP-like marker expression profile

A recent report suggests the possibility that mesFP cells convert to neural progenitors at the mesDA neurogenesis stage (Andersson et al., 2006b). This is consistent with the above observation that Lmx1a$^+$ mesDA progenitors lay at the ventral midline, where FP cells reportedly exist (Hynes et al., 1995b). However, a lineage relationship between FP cells and mesDA neurons has not been directly indicated by a lineage-tracing or fate-mapping study. To examine a potential lineage relationship between these cell types, we first compared the expression patterns of Lmx1a and FP cell markers. In the caudal neural tube, Shh, Hnf3β (Foxa2 – Mouse Genome Informatics) and FP4 have been used as FP cell markers. However, in the mesencephalon, Shh and Hnf3β were expressed in broad regions, including the Nkx6.1$^+$ neural progenitor domain (see Fig. S7 in the supplementary material). By contrast, FP4 specifically marks ventral midline cells and was not co-expressed with Nkx6.1. Thus, we chose FP4 as an FP cell marker in the mesencephalon.

In E14.5 rat embryos, FP4 expression was detected at the ventral midline region of the mesencephalon near Th$^+$ mesDA neurons, as previously reported (Fig. 4A) (Hynes et al., 1995b). Importantly, the Lmx1a$^+$ domain completely overlapped with the FP4$^+$ domain, and these markers were co-expressed at the single cell level (Fig. 4B,C). Lmx1a was expressed in virtually all neuroepithelial cells in this region (Fig. 4D), indicating that essentially all FP4$^+$ cells in the mesencephalon express Lmx1a. Furthermore, co-expression of FP4 with Ngn2 and Mash1 was observed (Fig. 4E,F). Consequently, expression of the early neural precursor marker *Dll1*, which is indicative of neurogenesis, was detected in a subset of cells in the mesencephalic FP4$^+$ regions (Fig. 4O). By contrast, proneural factors and *Dll1* expressions were not detected in the FP regions of the caudal neural tube (Fig. 4I-Q and data not shown). These observations indicate that at the neurogenesis stage, mesencephalic ventral midline cells with FP-like marker expression have mesDA progenitor characteristics, whereas cFP cells do not produce neurons as previously thought.

We next examined the expression of FP and mesDA lineage markers at an early stage. At E11.5, one day before the onset of proneural gene expression in the Lmx1a$^+$ midline region, FP4 and Lmx1a were co-expressed in the ventral midline as seen later during the neurogenesis stage (Fig. 4G,H). These results suggest that FP cells in the early mesencephalon have mesDA-lineage characteristics and further support a lineage relationship between FP cells and mesDA neurons.

Mesencephalic ventral midline cells with FP-like characters have neurogenic potential

To directly examine whether Lmx1a$^+$ mesencephalic ventral midline cells with FP-like characteristics indeed generate neurons, we performed cell sorting and in vitro cell culture experiments. As the *Corin* gene, one of the genes isolated by a ventral midline-selective gene search in the present study, encodes a cell surface protein (Yan et al., 1999), we reasoned that Corin could be used as an antigen for FACS. In situ hybridization analysis revealed that in the developing neural tube *Corin* was specifically expressed along the ventral midline regions from the mesencephalon to the spinal cord (Fig. 5A and data not shown). Double immunostaining revealed that Corin and FP4 were expressed in essentially identical regions in the mesencephalon and the metencephalon (Fig. 5B), suggesting that Corin specifically marks Lmx1a$^+$ mesencephalic ventral midline cells and cFP cells, at least in these regions.

We first examined whether mesencephalic ventral midline cells can be sorted using Corin as a marker at the mesDA neurogenesis stage. As expected, an antibody against the extracellular domain of Corin could detect the surface expression of this antigen in the E13.5 ventral mesencephalon and metencephalon cells of rat, by flow cytometry, and Corin$^+$ populations could be sorted into approximately 95-98% pure populations (Fig. 5C). Immediate examination of marker expression in sorted Corin$^+$ cells revealed that almost all Corin$^+$ cells derived from mesencephalon expressed Lmx1a and nestin (Nes), and FP4 expression was detected in more than 95% (Fig. 5D); this finding confirms that FP4 and Lmx1a are co-expressed at a single cell level. Furthermore, the expression of other FP marker genes, such as *Shh*, *Hnf3β* and *Ntn1*, in sorted Corin$^+$ cells, was confirmed (Fig.

5E). Similarly, approximately 95% of metencephalic Corin$^+$ cells expressed FP4 (data not shown). Thus, Lmx1a$^+$ ventral midline cells and cFP cells can be sorted from developing mesencephalon and metencephalon.

To examine whether mesencephalic ventral midline cells with FP-like characteristics have the potency to generate neurons, Corin$^+$ cells were sorted from E13.5 rat mesencephalon and cultured in vitro. At 2 days in vitro (DIV), cells positive for a neuronal marker HuC/D (also known as Elavl3/4 – Mouse Genome Informatics) emerged in a mesencephalic Corin$^+$ cell culture (data not shown). At 3-6 DIV, the HuC/D$^+$ ratio reached approximately 65% (65±3% at 6 DIV; Fig. 6A and data not shown). Similarly, expression of other neuronal markers, such as TuJ1 and MAP2 (also known as Tubb3 and Mtap2, respectively – Mouse Genome Informatics), was observed (data not shown), indicating that these cells are differentiated neurons. By contrast, metencephalic Corin$^+$ cells did not efficiently proliferate (compare the cell densities in Fig. 6A) and less than 1% of cells expressed HuC/D or TuJ1 (Fig. 6A and data not shown), indicating that cFP cells cannot generate neurons in vitro as they do in vivo. This finding supports the argument against the possibility that mesencephalic ventral midline cells can acquire the potency to differentiate into neurons by dissociation or by in vitro culture conditions.

However, as the purity of the Corin$^+$ population was 95-98%, it is possible that contaminated Corin$^-$ neural progenitors could efficiently proliferate and differentiate into neurons by co-culture with Corin$^+$ non-neurogenic cells. To test this possibility, Corin$^-$ cells were labeled with DiI and co-cultured with unlabeled Corin$^+$ cells at 5% frequency. If neurons in mesencephalic Corin$^+$ cell culture were mainly derived from contaminated Corin$^-$ populations, more than 50% of the neurons would be expected to be derived from DiI-labeled cells. At 6 DIV of the co-culture experiment, about 3% of total cells were DiI$^+$ and the total HuC/D$^+$ cell number had not increased compared with that in cultures of Corin$^+$ cells alone (data not shown). Furthermore, DiI$^+$ cells contributed to only 3.83± 0.50% of the HuC/D$^+$ cells (Fig. 6E), indicating that contaminated Corin$^-$ cells could only contribute a portion of the neurons generated in a mesencephalic Corin$^+$ cell cultures.

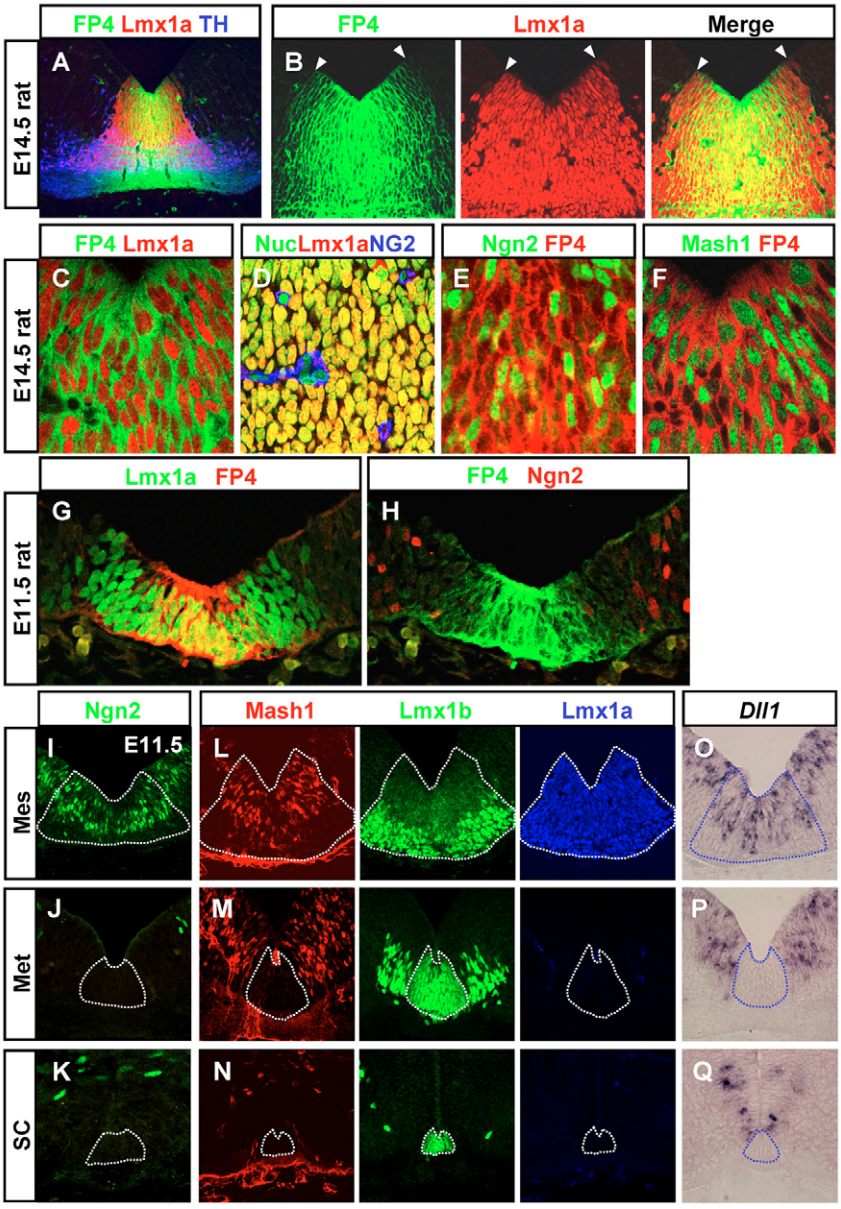

Fig. 4. Mesencephalic ventral midline cells with FP-like marker expression have mesDA progenitor characteristics. (**A-C**) Co-expression of FP4 and Lmx1a in the rat ventral mesencephalon at E14.5. (**D**) Virtually all of the neural progenitor cells at the ventral midline of the mesencephalon express Lmx1a. NG2 marks blood vessels. (**E**,**F**) Ngn2 and Mash1 are expressed in FP4$^+$ cells in the rat mesencephalon. (**G**,**H**) FP cells of the E11.5 rat mesencephalon without proneural gene expression have mesDA characteristics. (**I-Q**) Ngn2, Mash1, Lmx1a and Dll1 are selectively expressed in mesencephalic FP cells, but not in caudal FP cells of E11.5 mouse embryos. Lmx1b is expressed in midline cells at all AP locations. Mes, mesencephalon; Met, metencephalon; Nuc, nuclear stain; SC, spinal cord.

On the basis of these results, we conclude that mesencephalic ventral midline cells with FP-like characteristics have the potency to generate neurons, whereas cFP cells are non-neurogenic. Thus, ventral midline cells that develop in different AP locations have distinct neurogenic potential.

MesDA neurons originate from mesencephalic ventral midline cells with FP-like characteristics

Next, we examined the identity of the neurons generated in mesencephalic Corin$^+$ cell culture. Approximately 85-90% of the HuC/D$^+$ cells were Nurr1$^+$, suggesting that these neurons are mesDA precursors (Fig. 6B). Indeed, 53.6±3.4% and 53.7±3.6% of the neurons derived from mesencephalic Corin$^+$ population expressed Th and Pitx3, respectively, and about 80% of the Th$^+$ neuron co-expressed Pitx3 (Fig. 6C), indicating that these neurons have the correct mesDA identity. Thus, most of the neurons appear to be fated to differentiate into mesDA neurons. By contrast, although Corin$^-$ populations generated similar numbers of neurons, the percentages of Th$^+$ and Pitx3$^+$ cells were 4.0±1.3% and 2.7±0.4%, respectively (Fig. 6C), supporting the argument against the possibility that mesencephalic progenitors are transfated to a mesDA fate under our culture conditions. Additionally, about 80% of the Th$^+$ cells derived from a Corin$^-$ population did not incorporate BrdU during the in vitro culture period (data not shown). These Th$^+$ BrdU$^-$ neurons are likely to be differentiated from the postmitotic precursors that have already exited the cell cycle before cell sorting. As Corin expression is restricted to Nes$^+$ TuJ1$^-$ progenitors, postmitotic precursors derived from Corin$^+$ cells could be included in the Corin$^-$ population, suggesting that Th$^+$ BrdU$^-$ neurons emerging in the Corin$^-$ culture are possibly derived from Corin$^+$ cells.

On the basis of these findings, we concluded that mesencephalic Corin$^+$ cells sorted at the neurogenesis stage give rise to mesDA neurons in vitro. Together with the results obtained from in vivo gene expression studies and analysis of the *dreher* mutants, these fate-mapping experiments suggested that mesDA neurons originate from mesencephalic ventral midline cells with FP-like characteristics, and that Lmx1a$^+$ FP4$^+$ Corin$^+$ cells define mesDA progenitor cells at the neurogenesis stage.

MesDA neurons originate from mesencephalic FP cells

Before the onset of mesDA neurogenesis, FP cells without proneural gene expression exist in the mesencephalon (Andersson et al., 2006b). Comparison of Lmx1a expression and the expression of FP markers

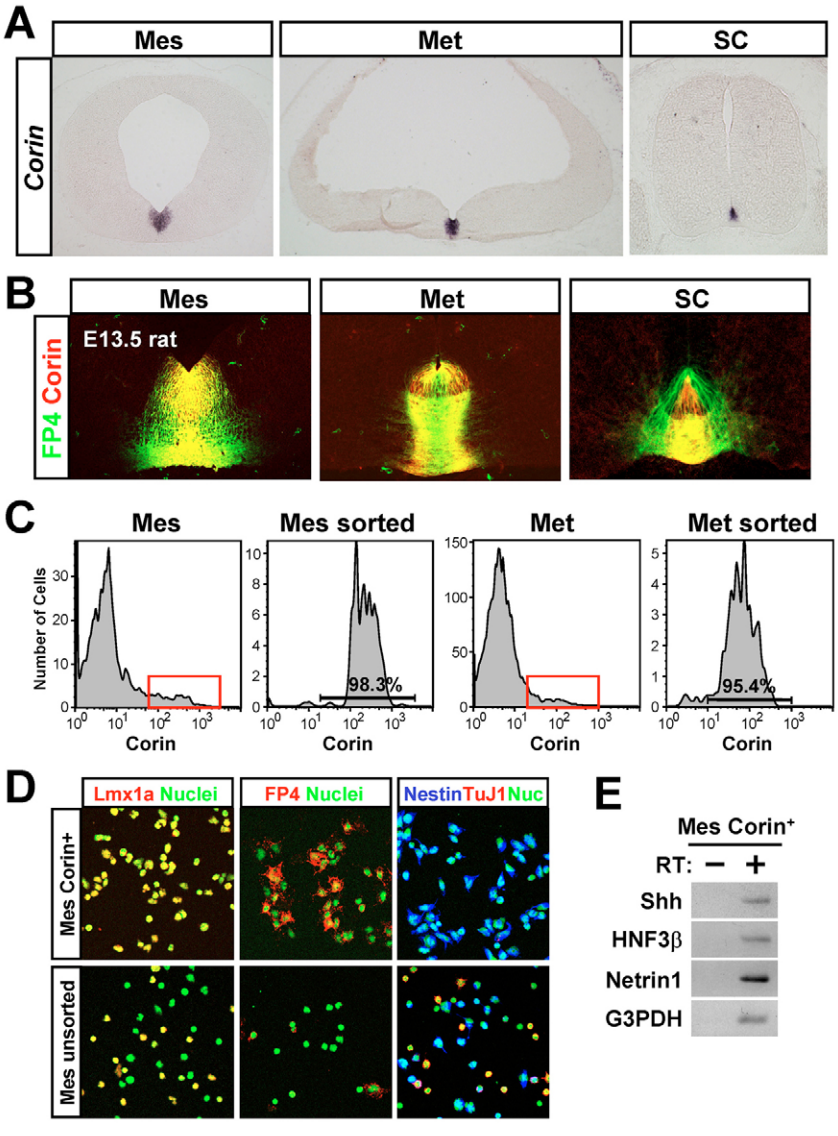

Fig. 5. FACS isolation of mesencephalic ventral midline cells with FP-like characteristics. (**A**) Corin is selectively expressed in ventral midline cells from the mesencephalon to the spinal cord of E12.5 mouse embryos. (**B**) Co-expression of Corin and FP4 in E13.5 rat FP cells. Note that Corin is expressed only in medial FP cells in the spinal cord. (**C**) FACS analysis of E13.5 rat ventral mesencephalon and metencephalon cells. Corin$^+$ cells can be sorted with more than 95% purity. (**D**) Rat mesencephalic Corin$^+$ cells express Lmx1a, FP4, nestin but not TuJ1. Virtually all the Corin$^+$ cells express Lmx1a and nestin. FP4 expression is detected in more than 95% of the Corin$^+$ cells. (**E**) Mesencephalic Corin$^+$ cells express Shh, H*nf*3β and Ntn1 transcripts. G3PDH, glyceraldehyde-3-phosphate dehydrogenase.

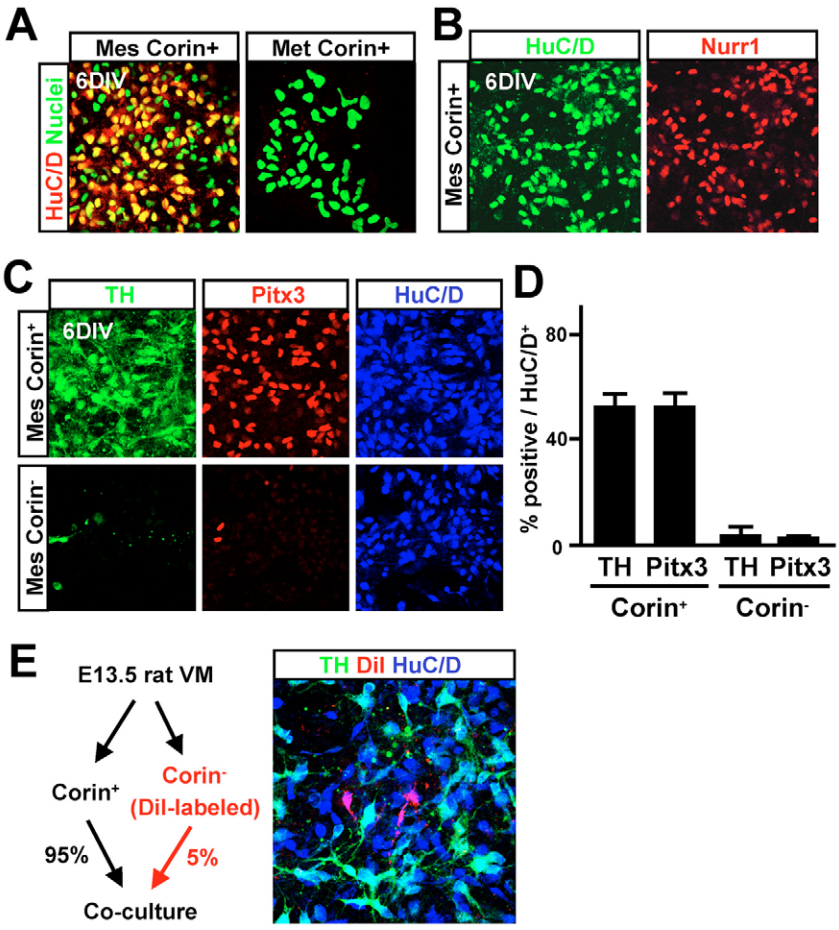

Fig. 6. Sorted mesencephalic ventral midline cells generate mesDA neurons, but caudal FP cells do not differentiate into neurons in vitro. (**A**) HuC/D$^+$ neurons are generated from E13.5 rat mesencephalic Corin$^+$ cells, but not from metencephalic Corin$^+$ cells at 6 DIV. (**B**) Neurons generated in mesencephalic Corin$^+$ cell cultures express Nurr1. (**C**,**D**) Th$^+$ and Pitx3$^+$ mesDA neurons are efficiently generated from mesencephalic Corin$^+$ cells, but not from mesencephalic Corin-negative cells. (**E**) Co-culture of Corin$^+$ cells and DiI-labeled Corin$^-$ cells. At 6 DIV, DiI$^+$ cells do not account for the majority of HuC/D$^+$ and Th$^+$ neurons, suggesting that mesDA neurons generated in Corin$^+$ cell cultures are derived from Corin$^+$ cells, but not from contaminated Corin$^-$ cells.

confirmed that, in the E9.75 mouse mesencephalon, Lmx1a is coincidently expressed in Shh$^+$ FP cells (Andersson et al., 2006b) (Fig. 7A). The above observation from FACS experiments that mesDA neurons originate from midline cells with FP-like characteristics is in line with the previously suggested idea that mesFP cells acquire neural progenitor characteristics to generate mesDA neurons (Andersson et al., 2006b). To directly examine whether mesDA neurons originate from FP cells, we performed cell-sorting experiments using early-stage embryos, before the onset of mesDA neurogenesis. For this purpose we used mouse embryos, as the expression level of Corin in the mesencephalon at this early stage was lower than that at the neurogenesis stage, and our anti-Corin antibody could recognize mouse Corin more sensitively than rat antigen (data not shown). Corin started to be expressed in some midline cells at E9.25; thereafter, Corin expression extended dorsally and became coincident with Lmx1a expression (data not shown). At E9.75, Corin was detected in a midline subpopulation of FP cells that were negative for proneural genes (Fig. 7A). We sorted mesencephalic Corin$^+$ cells at this stage

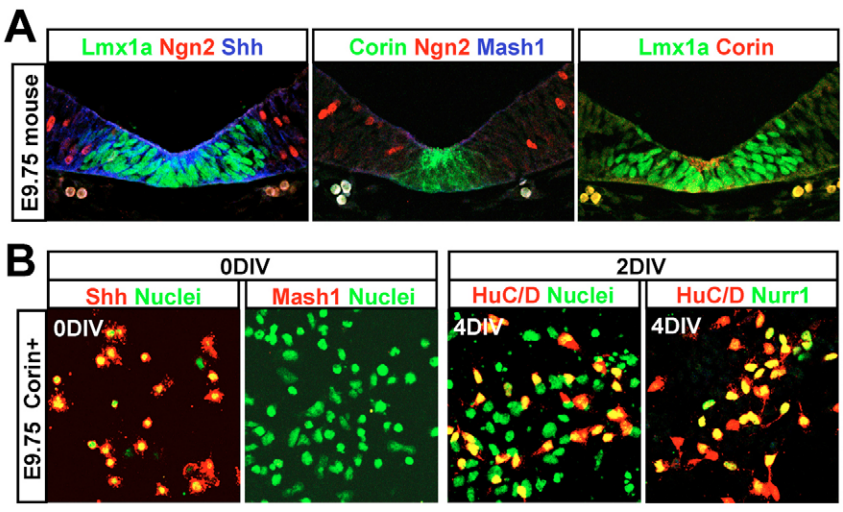

Fig. 7. mesDA neurons originate from FP cells. (**A**) Mouse early mesencephalic FP cells express Corin and Lmx1a but not proneural genes. (**B**) Mesencephalic FP cells sorted from E9.75 mouse embryos generate Nurr1$^+$ mesDA neuron precursors in vitro. Note that Corin$^+$ cells at the sorting period do not express proneural genes (A) and acquire neurogenic activity during culturing, suggesting that mesencephalic FP cells are intrinsically fated to acquire neural progenitor characteristics.

and confirmed that these cells expressed Shh, but not proneural factors, by immediate staining (Fig. 7B and data not shown). When these cells were cultured for 4 days, many HuC/D$^+$ neurons emerged, most of which expressed the mesDA neuron marker Nurr1. By contrast, Corin$^-$ cells gave rise to neurons, but most of these were negative for Nurr1; however, some Nurr1$^+$ neurons were observed, possibly due to the inclusion of Lmx1a$^+$ Corin$^-$ FP cells in Corin$^-$ fraction (data not shown). Altogether, these results strongly suggest that mesDA neurons originate from mesFP cells. The fact that the sorted cells did not express proneural genes during the sorting period suggests that mesFP cells are intrinsically fated to acquire mesDA progenitor characteristics.

Anterior identity determines neurogenic potential in the FP cells

Next, we asked what factor(s) confer neurogenic potential on mesFP cells. One candidate is Otx2, as it is selectively expressed in the neural tube anterior to the midbrain-hindbrain border and is required for mesDA neuron development (Puelles et al., 2004; Vernay et al., 2005). To test this, we generated transgenic mice ectopically expressing *Otx2* under control of the FP enhancer of the *Shh* gene (SFPE1) (Epstein et al., 1999), as Shh is selectively expressed by FP cells in the metencephalon (see Fig. S7 in the supplementary material). As expected, *Otx2* was ectopically expressed in cFP cells of transgenic embryos at E11.5 (Fig. 8A). In the metencephalon and spinal cord of transgenic embryos, Lmx1a was ectopically induced in Corin$^+$ cFP cells (Fig. 8A and data not shown), suggesting that *Otx2* determines the regional specificity of Lmx1a expression in FP cells and that ectopic expression of Otx2 alone can confer an anterior identity on FP cells. Importantly, Ngn2, Mash1 and Msx1 were also expressed in cFP cells in transgenic embryos, and consequently, HuC/D$^+$ postmitotic neurons emerged within the cFP region (Fig. 8A and data not shown). Thus, neurogenic potential are conferred on FP cells by the anterior identity determined by Otx2. Furthermore, neurons generated from cFP cells in transgenic embryos have the mesDA identity, although we could not exclude

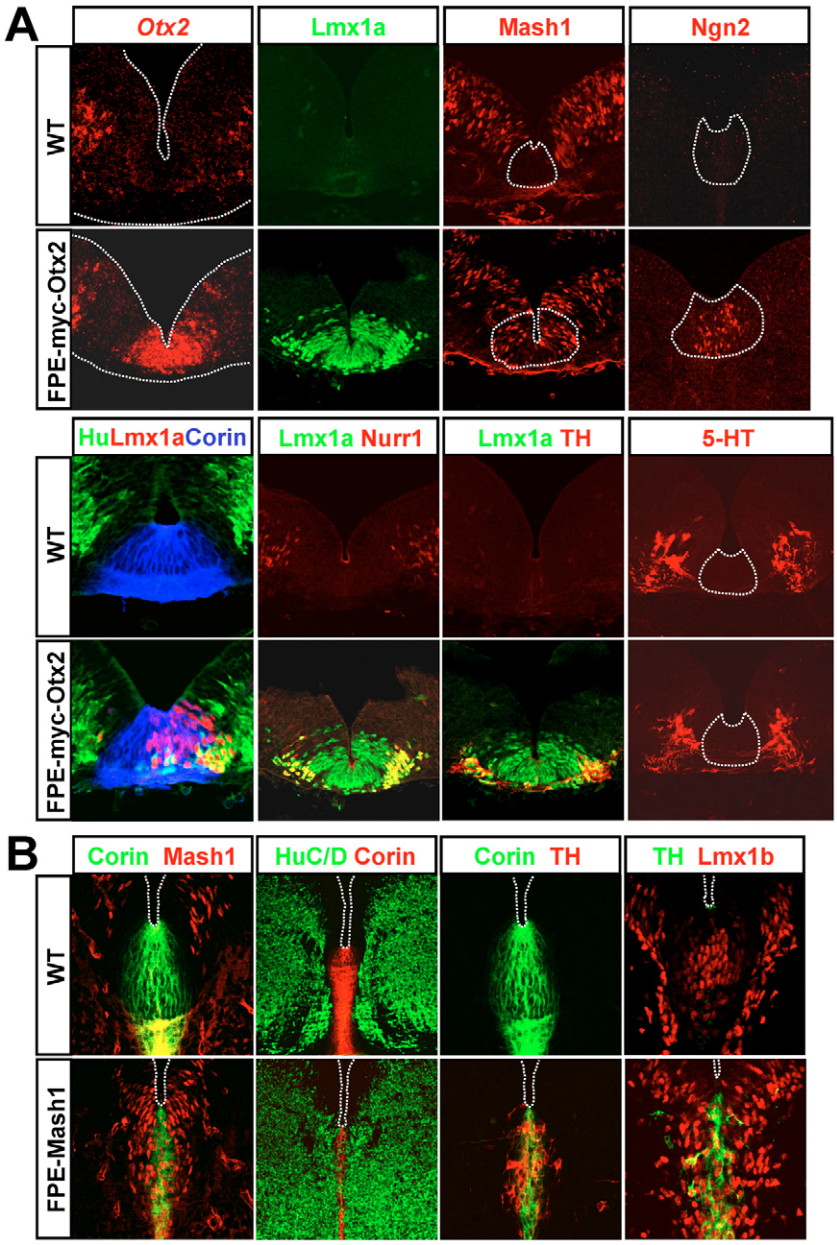

Fig. 8. Anterior identity confers neurogenic potential in the FP cells. Otx2 (**A**) or Mash1 (**B**) are ectopically expressed under the control of the FP-specific enhancer of the Shh gene. Images show the ventral midline region of the metencephalon at E11.5 (A) or at E12.5 (B). The transgene is selectively expressed in the cFP region. In Otx2-transgenic embryos, cFP cells acquire mesFP-like characteristics and mesDA neurogenesis occurs in the cFP region. By contrast, in Mash1-transgenic embryos, cFP cells differentiate into neurons positive for Th and Lmx1b, but negative for other mesDA markers.

the possibility that some mesDA neurons emerge from non-FP regions in the *Otx2*-transgenic embryos (Fig. 8A and data not shown), indicating that Otx2 on its own can specify mesDA progenitor identity in the context of FP cells; further supporting the idea that mesDA neurons originate from FP cells. Importantly, development of 5-HT neurons, which emerge from the region just dorsal to the FP cells, was not affected by the transgene expression.

Lmx1a is not sufficient for the induction of neurogenesis in caudal FP cells

The mesFP-selective expression of Lmx1a and the requirement of Lmx1a activity for mesDA neurogenesis, as revealed by the analysis of *dreher* mutants, raise the question of whether *Lmx1a* is a determinant of neurogenic potential in FP cells as a gene target downstream of Otx2. To test this possibility, we generated transgenic mice ectopically expressing *Lmx1a* in cFP cells (see Fig. S8 in the supplementary material). However, neither ectopic expression of Ngn2, Mash1 or Msx1 nor neurogenesis was observed in transgenic embryos (see Fig. S8 in the supplementary material; data not shown). Thus, although Lmx1a activity is required for neurogenesis in mesFP-derived progenitors, Lmx1a is not sufficient for the induction of neurogenesis in cFP cells.

DA phenotype is determined by the FP identity

mesDA neurons were generated from cFP cells in *Otx2*-transgenic embryos, raising the possibility that mesDA identity is determined by the FP identity, and that only neurogenic potential is regulated by mesencephalic factors including Otx2 and Lmx1a. To test this, the proneural gene *Mash1* was ectopically expressed in cFP cells under control of the *Shh* enhancer (Fig. 8B). Forced expression of *Mash1* induced neurogenesis in the FP cells, as judged by the downregulation of Corin and the ectopic emergence of neurons (Fig. 8B). Thus, cFP cells retain the potential to differentiate into neurons, but cannot initiate the program due to the loss of their potential to initiate the expression of proneural factors.

Importantly, cFP cell-derived neurons in *Mash1*-transgenic embryos expressed Lmx1b and Th (Fig. 8B), suggesting that the DA phenotype is determined by factor(s) selectively expressed in FP cells. However, other mesDA markers, such as Nurr1, Lmx1a and Pitx3, were not expressed in these neurons (data not shown). Thus, mesDA identity is likely to be specified by mesFP-selective factor(s).

A previous report that *Lmx1a* can induce ectopic mesDA neurons in the chick ventral mesencephalon and mouse ES cells (Andersson et al., 2006b) led us to examine whether *Lmx1a* can confer mesDA identity to cFP cell-derived neurons. However, in transgenic mice ectopically expressing both *Mash1* and *Lmx1a* in cFP cells, mesDA markers other than Th and Lmx1b were not induced in the FP cell-derived neurons (data not shown). Thus, Lmx1a and FP factor(s) are not sufficient to specify mesDA identity in cFP cell-derived neurons.

Isolation of mesDA progenitors from an ES cell-derived in vitro differentiated neural cell population

Cell replacement therapy is a promising approach for the treatment of PD. To use ES cell-derived neurons as material for cell transplantation therapy, purification of mesDA neurons and removal of undifferentiated ES cells would be required for an efficient and safe clinical treatment. Therefore, we examined whether FACS sorting with an anti-Corin antibody is suitable for this purpose.

Undifferentiated ES cells did not express Corin, either at the transcript or surface protein levels (Fig. 9A and data not shown). When ES cells were induced to differentiate into mesDA neurons by co-culturing with PA6 stromal cells for 6 days (Kawasaki et al., 2000), cell surface expression of Corin was induced in a subset of the ES cell-derived population (Fig. 9A). Importantly, surface Corin expression was not detected in the population expressing high levels of E-cadherin (cadherin 1), which might contain undifferentiated ES cells (data not shown). Consequently, when Corin$^+$ cells were sorted, undifferentiated ES cells identified by the expression of *Nanog* and *Eras* were completely removed (Fig. 9B and data not shown). Also, the Corin$^+$ population expressed *Lmx1a* at a high level.

Sorted Corin$^+$ cells showed the same characteristic features of neural progenitors as embryonic mesencephalon-derived Corin$^+$ cells. Of these Corin$^+$ cells, 95.7±1.13% were positive for Nes, and indeed, Corin$^+$ cells could proliferate in vitro (data not shown). When these cells were cultured for 6 days, 61.3±0.65% of them were positive for HuC/D, indicating that these Corin$^+$ populations contain neural progenitors (Fig. 9C). Furthermore, 69.7±2.32% of HuC/D$^+$ cells expressed Nurr1 and about 37.4±0.48% of HuC/D$^+$ neurons expressed Th. Co-expression of other mesDA neuron markers, such

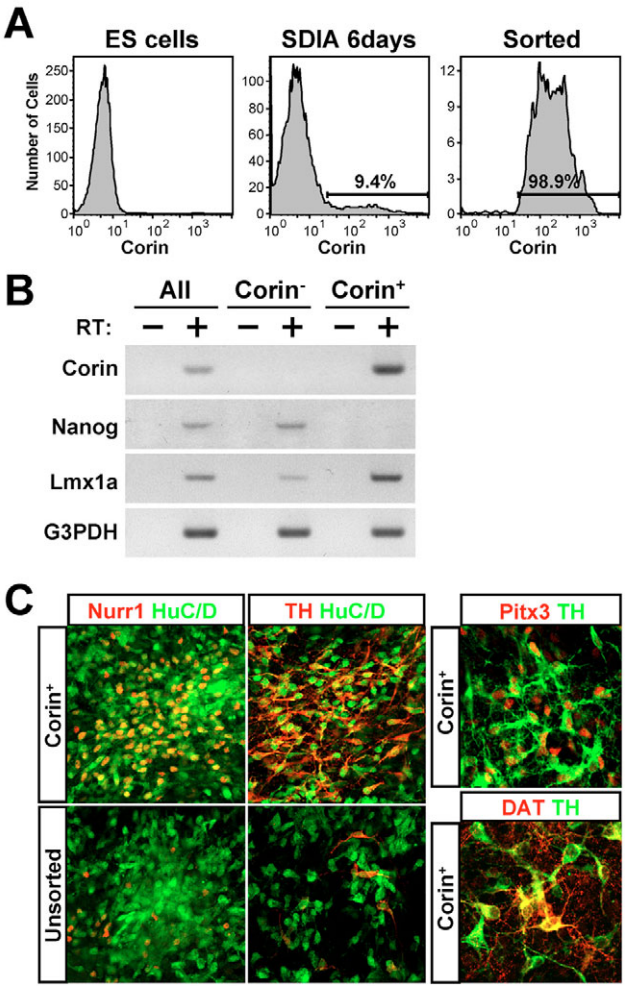

Fig. 9. Isolation of mesDA progenitors from ES cell-derived neural populations. (**A**) Surface expression of Corin in undifferentiated ES cells and SDIA-induced populations. (**B**) RT-PCR analysis of unsorted SDIA-induced cells (all) and sorted Corin$^+$ and Corin$^-$ populations. Expression of undifferentiated ES cell-specific Nanog is not detected in the Corin$^+$ population. (**C**) mesDA progenitors are enriched in the Corin$^+$ population. Neurons generated from Corin$^+$ cells show correct mesDA neuron identity. G3PDH, glyceraldehyde-3-phosphate dehydrogenase.

as Pitx3 and DAT (also known as Slc6a3 – Mouse Genome Informatics), was also detected. Taken together, these results demonstrate the possible applicability of FACS with Corin as a marker to enrich mesDA progenitors as safe and efficient cell materials for transplantation therapy in patients with PD.

DISCUSSION

A previous report suggested that mesFP cells acquire neurogenic activity to generate mesDA neurons, as evaluated by analyzing transgenic mice mis-expressing Msx1 in early mesFP cells (Andersson et al., 2006b). However, the lineage relationship between these cells had not been examined directly. Identification of an FP-specific surface marker in the present study enabled us to directly demonstrate that mesDA neurons originate from mesFP cells. We also show that mesFP-derived DA progenitors, even during the period of neurogenesis, still maintain most of their FP characteristics, including not only marker expression (FP4 and Corin), but also expression of functional factors such as Shh and Ntn1; however, downregulation of Shh expression has been reported (Andersson et al., 2006b). Thus, mesFP cells are a specialized cell population that organizes neural tube patterning and axon guidance, and generates mesDA neurons by themselves. Furthermore, sorting experiments suggested that mesFP cells are intrinsically fated to acquire neurogenic activity. Moreover, our mis-expression experiments consistently showed that Otx2, which patterns the AP axis of the neural plate before FP formation, can confer neurogenic potential on cFP cells, suggesting that mesFP cells are already programmed to become mesDA progenitors at the period of determination into an FP fate. These findings will shed light on the mechanism of mesDA neuron generation and could facilitate the development of novel therapeutic approaches for the treatment of PD. Furthermore, we showed that a cell surface antigen selective for mesDA progenitors is useful for isolating mesDA progenitor-enriched materials for cell transplantation therapy from ES cell-derived sources. Here we discuss the mechanism underlying the specification and generation of mesDA neurons as FP cell derivatives.

Functional differences of FP cells along the AP axis and the origin of mesDA neurons

Although functional differences between FP cells in different AP locations have been suggested by histological and gene expression studies (Andersson et al., 2006a; Andersson et al., 2006b; Kele et al., 2006; Placzek and Briscoe, 2005; Vernay et al., 2005), they have yet to be directly demonstrated. In the present study, in vitro culture experiments using FACS demonstrated that mesFP cells indeed have the potential to generate neurons while cFP cells cannot so differentiate. Importantly, we revealed that mesFP cells and later midline cells with FP-like characteristics express the mesDA lineage marker Lmx1a, and that these cells produce mesDA neurons in vitro. This is consistent with the in vivo observation that all neurons emerging from the FP region appeared to be fated to differentiate into mesDA neurons, as judged by the expression of Lmx1a, Lmx1b and Nurr1. Finally, this potential of mesFP cells to generate mesDA neurons was confirmed by the observation that mesDA neurons were generated from cFP cells, the AP identity of which was changed to a mesencephalic one by the ectopic expression of *Otx2*. Although we have not yet performed a lineage-tracing experiment in vivo, our present results and the data from a previous study by Andersson et al. (Andersson et al., 2006b) strongly suggest that mesDA neurons originate from mesFP cells. Our conclusion is further supported by earlier studies in mice deficient for *Gli2*, a mediator of Shh signaling that is required for FP cell formation, showing that mesDA neurons were dramatically reduced in number whereas most of the other ventral neuronal types developed normally (Matise et al., 1998).

What are the differences in neurogenicity between FP cells in the mesencephalon and those in the caudal neural tube? Gain-of-function studies have revealed that Otx2 can trigger proneural gene expression in FP cells. It has been reported that ectopic *Otx2* expression under the control of the *En1* enhancer induces mesDA neurogenesis in the hindbrain (Brodski et al., 2003). However, in these transgenic mice, isthmus positioning was also affected; thus, Otx2 function in FP cells could not be clarified. By contrast, our transgenic embryos ectopically selectively expressed *Otx2* in FP cells. In addition, ectopic neurogenesis in FP cells was observed not only in the metencephalon, but also in the spinal cord, distant from the isthmus, indicating that this phenotype is likely to be independent of the isthmus positioning activity of Otx2. Otx2 activity for the induction of neurogenesis in FP cells is possibly mediated, at least in part, through the induction of Lmx1a, which is required for proneural gene expression in FP cells. However, Lmx1a alone is insufficient to confer mesencephalic identity to FP cells, indicating that another factor(s), with its expression regulated by Otx2, might be involved in the determination of mesDA identity. Alternatively, Otx2 itself may cooperate with Lmx1a to induce proneural gene expression, as Otx2 activity is required for mesDA neuron production at the neurogenesis stage (Puelles et al., 2004; Vernay et al., 2005), in addition to the requirement for the early mesencephalic specification that might be involved in the induction of *Lmx1a* in FP cells.

Our results indicated that mesDA neurons are specified dorsoventrally as FP cells and anteroposteriorly by Otx2 signals. Furthermore, induction of Lmx1a, an important regulator of mesDA neurogenesis, by Otx2 in the context of FP cells suggests cooperation between the AP determinant Otx2 and FP-specific signals that might be induced by a high concentration of Shh in determining mesDA fate, at least in part, by inducing Lmx1a. Identification of an FP-specific factor that regulates Lmx1a expression will define the molecular cascade of mesDA specification.

The mechanism of mesDA neuron specification

Neural progenitor identity is determined by a combinatorial code of transcription factors such as homeobox and basic helix-loop-helix factors. It can be expected that the LIM-homeobox transcription factor Lmx1a specifies mesDA progenitor identity; indeed, ectopic expression of *Lmx1a* in the chick ventral mesencephalon can induce mesDA neurons (Andersson et al., 2006b). However, the *dreher* mutation did not affect progenitor identity, and consequently most postmitotic precursors emerging from the Lmx1a$^+$ region normally expressed mesDA neuron markers such as Lmx1b and Nurr1. Thus, in mouse, Lmx1a appears to be required mainly for regulating neurogenesis in proliferative progenitors through proneural gene induction, rather than to determine progenitor identity; however, Lmx1a has been reported to have a potency to determine correct mesDA fate in mouse ES cell-derived neurons (Andersson et al., 2006b). Similar neurogenic activity of Lmx1a without changing neuronal subtype identities has been reported previously (Chizhikov and Millen, 2004b). This discrepancy may indicate differences among animal species in their requirements of Lmx1a for mesDA specification, as reported in the case of caudal roof plate formation, for which only Lmx1a is essential in mouse whereas Lmx1b plays an important role in chick (Chizhikov and Millen, 2004a). However, loss-of-function of Lmx1a in avian embryos resulted in the complete inhibition of neurogenesis in the Lmx1a$^+$ progenitor domain, making

it difficult to reveal the requirement of Lmx1a for mesDA progenitor specification. Alternatively, together with the observations described below, differences in the severities of neurogenesis defects in the mesDA domain between mouse and chick embryos may suggest a more likely possibility that *dreher* mutants retain partial *Lmx1a* activity due to a hypomorphic mutation. First, mutant Lmx1a protein was still consistently expressed in *dreher* mutant embryos. Second, a dose-dependent action of Lmx1a was observed. Finally, mutant Lmx1a protein can still weakly interact with the transcriptional co-factor NLI and shows weak transcriptional activity (data not shown). If this is the case, induction of neurogenesis in FP cells might be fully dependent on Lmx1a in both species. However, a recent report demonstrated that the *dreher*^{J} mutant used in this study showed an essentially identical cerebellar phenotype to theoretical null-type *dreher* mutants (*dreher*^{7J}) (Chizhicov et al., 2006). Analysis of mesDA development in Lmx1a-null mice will be needed to resolve this issue.

Lmx1a is expressed not only in proliferative progenitors but also in postmitotic mesDA neurons, and the *dreher* mutation leads to abnormal differentiation in a subset of mesDA precursors, suggesting the involvement of Lmx1a in postmitotic mesDA specification. However, the fact that most mesDA neurons differentiated normally in *dreher* mutants may again suggest residual Lmx1a activity in *dreher* mutants or the existence of compensating factor(s). On the one hand, ectopic expression of Lim1/2 was observed in mesDA precursors expressing Lmx1b at low levels (Fig. 3B and data not shown), suggesting that the postmitotic roles of Lmx1a and Lmx1b may be redundant. On the other hand, a gain-of-function experiment in chick revealed that Lmx1a is sufficient to specify mesDA fate (Andersson et al., 2006b). Again, null mutant mice for Lmx1a will be needed to clarify the requirement of Lmx1a in mesDA specification.

It has been reported that Lmx1a has the potency to intrinsically determine mesDA identity (Andersson et al., 2006b). However, Lmx1a was expressed not only in mesDA lineage cells but also in the glutamatergic domain in the ventral midline region of caudal diencephalons (Andersson et al., 2006b) (T.N., Y.M. and Y.O., unpublished). Moreover, Nurr1 and Lmx1b were also expressed in these Lmx1a^{+} glutamatergic neurons (data not shown), suggesting that Lmx1a alone may be capable of inducing Nurr1 and Lmx1b, but cannot specify a mesDA fate. The candidate cooperative factor might be selectively expressed in FP cells, as they emerge along the ventral midline except in the diencephalons, in which mesDA neurons do not emerge. Supporting this idea, neurons generated from cFP cells by the forced expression of *Mash1* expressed Th. Thus, the DA phenotype appears to be determined by a factor(s) selectively expressed in FP cells along all AP locations. However, other mesDA neuron markers were not expressed in these cFP-derived neurons, even when *Lmx1a* was additionally expressed. These results suggest that another factor(s) that is selectively expressed in the mesencephalon cooperatively confers mesDA identity with Lmx1a and FP factor(s). This idea is consistent with the observation by Andersson et al. (Andersson et al., 2006b) that Lmx1a can induce mesDA neurons only in the ventral mesencephalon. Otx2 is still a candidate for this factor. Further, more precise analyses concerning FP cell development should define the mechanism of mesDA specification.

Isolation of mesDA progenitors for cell replacement therapy

To date, several efficient methods for inducing mesDA neurons from ES cells have been established (Andersson et al., 2006b; Barberi et al., 2003; Kawasaki et al., 2000; Lee et al., 2000; Perrier et al., 2004). However, although the resultant materials were functional in animal model experiments, they have potential risks for teratoma formation or side effects due to contamination from undifferentiated stem cells or neurons from another lineage. Isolation of DA neurons by introducing markers, such as the expression of fluorescent protein under the control of a DA neuron-specific promoter, has been successful (Sawamoto et al., 2001); however, this approach retains a potential risk of tumorigenicity due to the need for gene manipulation, and it is laborious to apply to nuclear-transferred ES cells or stem cells derived from individual patients. Thus, identification of a mesDA-specific cell surface antigen for cell isolation is required for the realization of a cell replacement therapy using stem cell-derived cell materials. The demonstration of FACS of mesDA progenitors from an ES cell-derived mixed population in this study should accelerate the application of stem cell-derived materials for transplantation therapies. However, Corin^{+} cell populations potentially contain cFP cells that cannot differentiate into DA neurons. Thus, optimization of the differentiation procedure or the identification of a co-marker that can distinguish mesFP and cFP cells will be needed to establish more efficient therapeutic methods. A trial involving an application of this approach to human ES cells is presently ongoing.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/134/17/3213/DC1

References

Andersson, E., Jensen, J. B., Parmar, M., Guillemot, F. and Bjorklund, A. (2006a). Development of the mesencephalic dopaminergic neuron system is compromised in the absence of neurogenin 2. *Development* **133**, 507-516.

Andersson, E., Tryggvason, U., Deng, Q., Friling, S., Alekseenko, Z., Robert, B., Perlmann, T. and Ericson, J. (2006b). Identification of intrinsic determinants of midbrain dopamine neurons. *Cell* **124**, 393-405.

Barberi, T., Klivenyi, P., Calingasan, N. Y., Lee, H., Kawamata, H., Loonam, K., Perrier, A. L., Bruses, J., Rubio, M. E., Topf, N. et al. (2003). Neural subtype specification of fertilization and nuclear transfer embryonic stem cells and application in parkinsonian mice. *Nat. Biotechnol.* **21**, 1200-1207.

Brodski, C., Weisenhorn, D. M., Signore, M., Sillaber, I., Oesterheld, M., Broccoli, V., Acampora, D., Simeone, A. and Wurst, W. (2003). Location and size of dopaminergic and serotonergic cell populations are controlled by the position of the midbrain-hindbrain organizer. *J. Neurosci.* **23**, 4199-4207.

Chizhikov, V. V. and Millen, K. J. (2004a). Control of roof plate development and signaling by Lmx1b in the caudal vertebrate CNS. *J. Neurosci.* **24**, 5694-5703.

Chizhikov, V. V. and Millen, K. J. (2004b). Control of roof plate formation by Lmx1a in the developing spinal cord. *Development* **131**, 2693-2705.

Chizhikov, V., Steshina, E., Roberts, R., Ilkin, Y., Washburn, L. and Millen, K. J. (2006). Molecular definition of an allelic series of mutations disrupting the mouse Lmx1a (dreher) gene. *Mamm. Genome* **17**, 1025-1032.

Epstein, D. J., McMahon, A. P. and Joyner, A. L. (1999). Regionalization of Sonic hedgehog transcription along the anteroposterior axis of the mouse central nervous system is regulated by Hnf3-dependent and -independent mechanisms. *Development* **126**, 281-292.

Fedtsova, N. and Turner, E. E. (2001). Signals from the ventral midline and isthmus regulate the development of Brn3.0-expressing neurons in the midbrain. *Mech. Dev.* **105**, 129-144.

Hynes, M., Porter, J. A., Chiang, C., Chang, D., Tessier-Lavigne, M., Beachy, P. A. and Rosenthal, A. (1995a). Induction of midbrain dopaminergic neurons by Sonic hedgehog. *Neuron* **15**, 35-44.

Hynes, M., Poulsen, K., Tessier-Lavigne, M. and Rosenthal, A. (1995b). Control of neuronal diversity by the floor plate: contact-mediated induction of midbrain dopaminergic neurons. *Cell* **80**, 95-101.

Jessell, T. M. (2000). Neuronal specification in the spinal cord: inductive signals and transcriptional codes. *Nat. Rev. Genet.* **1**, 20-29.
Kawasaki, H., Mizuseki, K., Nishikawa, S., Kaneko, S., Kuwana, Y., Nakanishi, S., Nishikawa, S. I. and Sasai, Y. (2000). Induction of midbrain dopaminergic neurons from ES cells by stromal cell-derived inducing activity. *Neuron* **28**, 31-40.
Kele, J., Simplicio, N., Ferri, A. L., Mira, H., Guillemot, F., Arenas, E. and Ang, S. L. (2006). Neurogenin 2 is required for the development of ventral midbrain dopaminergic neurons. *Development* **133**, 495-505.
Lee, S. H., Lumelsky, N., Studer, L., Auerbach, J. M. and McKay, R. D. (2000). Efficient generation of midbrain and hindbrain neurons from mouse embryonic stem cells. *Nat. Biotechnol.* **18**, 675-679.
Matise, M. P., Epstein, D. J., Park, H. L., Platt, K. A. and Joyner, A. L. (1998). Gli2 is required for induction of floor plate and adjacent cells, but not most ventral neurons in the mouse central nervous system. *Development* **125**, 2759-2770.
Millonig, J. H., Millen, K. J. and Hatten, M. E. (2000). The mouse Dreher gene Lmx1a controls formation of the roof plate in the vertebrate CNS. *Nature* **403**, 764-769.
Nakatani, T., Mizuhara, E., Minaki, Y., Sakamoto, Y. and Ono, Y. (2004). Helt, a novel basic-helix-loop-helix transcriptional repressor expressed in the developing central nervous system. *J. Biol. Chem.* **279**, 16356-16367.
Olanow, C. W. and Tatton, W. G. (1999). Etiology and pathogenesis of Parkinson's disease. *Annu. Rev. Neurosci.* **22**, 123-144.
Olanow, C. W., Kordower, J. H. and Freeman, T. B. (1996). Fetal nigral transplantation as a therapy for Parkinson's disease. *Trends Neurosci.* **19**, 102-109.
Osada, A., Kiyozumi, D., Tsutsui, K., Ono, Y., Weber, C. N., Sugimoto, N., Imai, T., Okada, A. and Sekiguchi, K. (2005). Expression of MAEG, a novel basement membrane protein, in mouse hair follicle morphogenesis. *Exp. Cell Res.* **303**, 148-159.
Perrier, A. L., Tabar, V., Barberi, T., Rubio, M. E., Bruses, J., Topf, N., Harrison, N. L. and Studer, L. (2004). Derivation of midbrain dopamine neurons from human embryonic stem cells. *Proc. Natl. Acad. Sci. USA* **101**, 12543-12548.
Placzek, M. and Briscoe, J. (2005). The floor plate: multiple cells, multiple signals. *Nat. Rev. Neurosci.* **6**, 230-240.
Prakash, N., Brodski, C., Naserke, T., Puelles, E., Gogoi, R., Hall, A., Panhuysen, M., Echevarria, D., Sussel, L., Weisenhorn, D. M. et al. (2006). A Wnt1-regulated genetic network controls the identity and fate of midbrain-dopaminergic progenitors in vivo. *Development* **133**, 89-98.
Puelles, E., Annino, A., Tuorto, F., Usiello, A., Acampora, D., Czerny, T., Brodski, C., Ang, S. L., Wurst, W. and Simeone, A. (2004). Otx2 regulates the extent, identity and fate of neuronal progenitor domains in the ventral midbrain. *Development* **131**, 2037-2048.
Sawamoto, K., Nakao, N., Kobayashi, K., Matsushita, N., Takahashi, H., Kakishita, K., Yamamoto, A., Yoshizaki, T., Terashima, T., Murakami, F. et al. (2001). Visualization, direct isolation, and transplantation of midbrain dopaminergic neurons. *Proc. Natl. Acad. Sci. USA* **98**, 6423-6428.
Simeone, A. (2005). Genetic control of dopaminergic neuron differentiation. *Trends Neurosci.* **28**, 62-65; discussion 65-66.
Strahle, U., Lam, C. S., Ertzer, R. and Rastegar, S. (2004). Vertebrate floor-plate specification: variations on common themes. *Trends Genet.* **20**, 155-162.
Vernay, B., Koch, M., Vaccarino, F., Briscoe, J., Simeone, A., Kageyama, R. and Ang, S. L. (2005). Otx2 regulates subtype specification and neurogenesis in the midbrain. *J. Neurosci.* **25**, 4856-4867.
Yan, W., Sheng, N., Seto, M., Morser, J. and Wu, Q. (1999). Corin, a mosaic transmembrane serine protease encoded by a novel cDNA from human heart. *J. Biol. Chem.* **274**, 14926-14935.
Ye, W., Shimamura, K., Rubenstein, J. L., Hynes, M. A. and Rosenthal, A. (1998). FGF and Shh signals control dopaminergic and serotonergic cell fate in the anterior neural plate. *Cell* **93**, 755-766.