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Lmx1a and Lmx1b cooperate with Foxa2 to coordinate the specification of dopaminergic neurons and control of floor plate cell differentiation in the developing mesencephalon

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ABSTRACT

Mesencephalic dopaminergic (mesDA) neurons control movement and behavior, and their loss causes severe neurological disorders, such as Parkinson's disease. Recent studies have revealed that mesDA neurons originate from mesencephalic floor plate (FP) cells, which had been thought of as non-neurogenic organizer cells regulating regional patterning and axonal projections. Otx2 and its FP-specific downstream factor Lmx1a have been shown to be sufficient to confer neurogenic activity on FP cells and determine a mesDA fate. However, the mechanism underlying how these factors control mesDA development and how FP cells and mesDA neurons are coordinately specified are still largely unknown. In the present study, we obtained evidence that Lmx1a and Lmx1b cooperate with Foxa2 to specify mesDA neuron identity by gain-of-function approaches using transgenic mice. Lmx1a/b appeared to select a mesDA fate by suppressing red nucleus fate in the context of Foxa2-positive progenitors, at least in part, through repressing the Sim1-Lhx1 and Ngn1 pathways that inhibit proper mesDA differentiation. We also found that, in the mesencephalon, FP cell fate is primarily determined by Foxa2 with a supportive action of Lmx1a/b through repressing Nkx6.1, which inhibits FP cell differentiation. Thus, FP and mesDA identities are determined by distinct specification pathways, both of which are controlled by the same combination of transcription factors, Lmx1a/b and Foxa2, and, as a consequence, mesDA neurons are generated from mesencephalic FP cells.

Introduction

The mesencephalic dopaminergic (mesDA) neuron system controls body movement and reward-based behaviors, and loss of mesDA neuron activity causes severe motor defects characteristic of Parkinson's disease, or psychiatric disorders (Olanow and Tatton, 1999; Tzschentke and Schmidt, 2000). Stem cell-based transplantation therapy is a promising approach for the treatment of Parkinson's disease (Mendez et al., 2008; Olanow et al., 1996). How to engineer mesDA neurons with the correct identity is a fundamental issue in regenerative medicine research (Smidt and Burbach, 2007).

MesDA neurons arise from the ventral midline of the developing mesencephalon. The mesDA identity is induced by the combinatorial signals of sonic hedgehog (Shh), fibroblast growth factor (FGF) 8 and Wnt1 (Prakash et al., 2006, Ye et al., 1998), and as a downstream event, mesDA neurons acquire expression of selective transcription factors, such as Nurr1 (Nr4a2), Lmx1b, Pitx3, En1/2 and Foxa1/2, all of which are required for the correct differentiation or survival of postmitotic mesDA neurons (Alberi et al., 2004; Ferri et al., 2007; Smidt et al., 2000, 2004; Zetterstrom et al., 1997). Transcription factors, which are induced by patterning cues in mitotic mesDA progenitors and comprise the 'transcription factor code', should trigger differentiation programs in postmitotic precursors (Jessell, 2000). Recent studies have identified several transcription factors selectively expressed in proliferative mesDA progenitors, including Otx2, Lmx1a/b, Msx1/2, Ngn2 (Neurog2) and Foxa1/2 (Andersson et al., 2006a, 2006b; Ferri et al., 2007; Kele et al., 2006; Kittappa et al., 2007; Ono et al., 2007; Puelles et al., 2003, 2004; Smidt et al., 2000; Vernay et al., 2005). Loss of any one of these genes, except for Lmx1b, which was recently suggested to be non-autonomously involved in mesDA specification (Guo et al., 2008), results in neurogenesis defects, specifically in a mesDA lineage. Blockade of neurogenesis by loss of these gene functions makes it difficult to determine whether these factors are involved in the specification of mesDA fate.

One of the characteristic properties of mesDA development is its origin from floor plate (FP) cells (Andersson et al., 2006b; Bonilla et al., 2008; Joksimovic et al., 2009; Kittappa et al., 2007; Ono et al., 2007). FP cells are morphologically specialized organizer cells that develop at the ventral midline of the neural tube; they control regional patterning and axonal projection (Placzek and Briscoe, 2005). Classically, FP cells are characterized as non-neurogenic cells, but recently, it has been revealed that mesencephalic FP cells are

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fated to acquire neurogenic potential for generating mesDA neurons retaining FP characteristics (Andersson et al., 2006b; Ono et al., 2007). Thus, control of neurogenic activity in FP cells is one important regulatory step in mesDA development (Andersson et al., 2006b; Omodei et al., 2008; Ono et al., 2007). Otx2, which is known to control anteroposterior patterning of the neural plate, induces Lmx1a expression in FP cells, which, in turn, upregulate the proneural factor Ngn2 to generate mesDA neurons. In addition to this neurogenesis control activity, the Otx2-Lmx1a pathway is involved in the specification of mesDA identity, as revealed by gain-of-function analysis (Andersson et al., 2006b; Brodski et al., 2003; Ono et al., 2007). The observation that Otx2 is sufficient for induction of mesDA neurons only in the context of FP cells might suggest that specifying FP cell fate in a mesencephalic progenitor would be sufficient for acquisition of mesDA identity (Ono et al., 2007). Lmx1a has been identified as a mesDA specifier acting downstream of Otx2. However, the mesDA-inducing activity of Lmx1a is again context dependent, as ectopic mesDA neurons were induced only in the ventral mesencephalon by forced expression of Lmx1a using chick electroporation (Andersson et al., 2006b), suggesting that the factor(s) that cooperate with Lmx1a in mesDA specification remain to be identified. Furthermore, the mechanism of action of Lmx1a in the cell fate determination of mesDA progenitors also remains obscure.

In the present study, we took advantage of gain-of-function approaches to analyze the activities of transcription factors in mesDA specification. Our results suggest that Foxa2 plays important roles in mesDA specification by cooperating with Lmx1a. Mechanistically, Foxa2 partly induces the differentiation programs for both mesDA and red nucleus (RN) neurons, and in this context, Lmx1a selects mesDA fate by suppressing RN fate, at least in part by repressing the Sim1-Lhx1 and Ngn1 pathways, which induce RN fate. By contrast, FP identity is primarily determined by Foxa2, but Lmx1a also contributes by repressing Nkx6.1, which inhibits FP differentiation. Thus, Lmx1a and Foxa2 coordinate the specifications of FP cells and mesDA neurons by regulating independent differentiation pathways.

Materials and methods

Mice

Dreher^{J} mice (Millonig et al., 2000) were obtained from the Jackson Laboratory and maintained as previously described (Ono et al., 2007).

Transgenic constructs were obtained by ligating each cDNA amplified by PCR into pNE vector in which transgene expression is driven by the nestin enhancer (NE) (Nakatani et al., 2007). The primer sequences used for amplification of the cDNA fragments are available upon request. Linearized pNE constructs were injected into fertilized eggs and founder embryos were collected at E12.5. The embryos were genotyped by PCR and tested for transgene expression by immunostaining. We chose transgenic embryos expressing transgenes at similar levels for further analyses and observed essentially the same phenotypes in all chosen embryos. The numbers of transgenic embryos analyzed were as follows: NE-Lmx1a, $n=9$; NE-Lmx1b, $n=4$; NE-Foxa2, $n=4$; NE-Foxa2-IRES-Lmx1a, $n=7$; NE-Nkx6.1, $n=5$; NE-myc-Sim1, $n=3$; NE-Helt, $n=4$.

Immunohistochemistry and in situ hybridization

Immunohistochemistry was performed as described previously (Nakatani et al., 2004). A hamster anti-Lmx1b mAb was raised against GST-Lmx1b (aa 271–306). The other primary antibodies used were: anti-Corin, anti-Lmx1a, anti-Lmx1b, anti-Pitx3, anti-Nkx6.1 and anti-Nurr1 (Ono et al., 2007); anti-En1, anti-Shh and anti-Pax3 (Developmental Studies Hybridoma Bank); anti-HuC/D and anti-GFP (Molecular Probes); anti-Myc, anti-Lhx1, anti-Ngn1, anti-Ngn2 and anti-Foxa2 (Santa Cruz Biotechnology); and anti-tyrosine hydroxylase and anti-Brn3a (Chemicon).

In situ hybridization was performed as described previously (Nakatani et al., 2004).

The m1–m7 domain borders were determined based on the patterns of marker expression in the transgene-negative progenitor cells that intermingled with the transgene-expressing cells in each domain; these patterns were revealed by immunostaining of the same or adjacent sections.

Cell sorting and culture

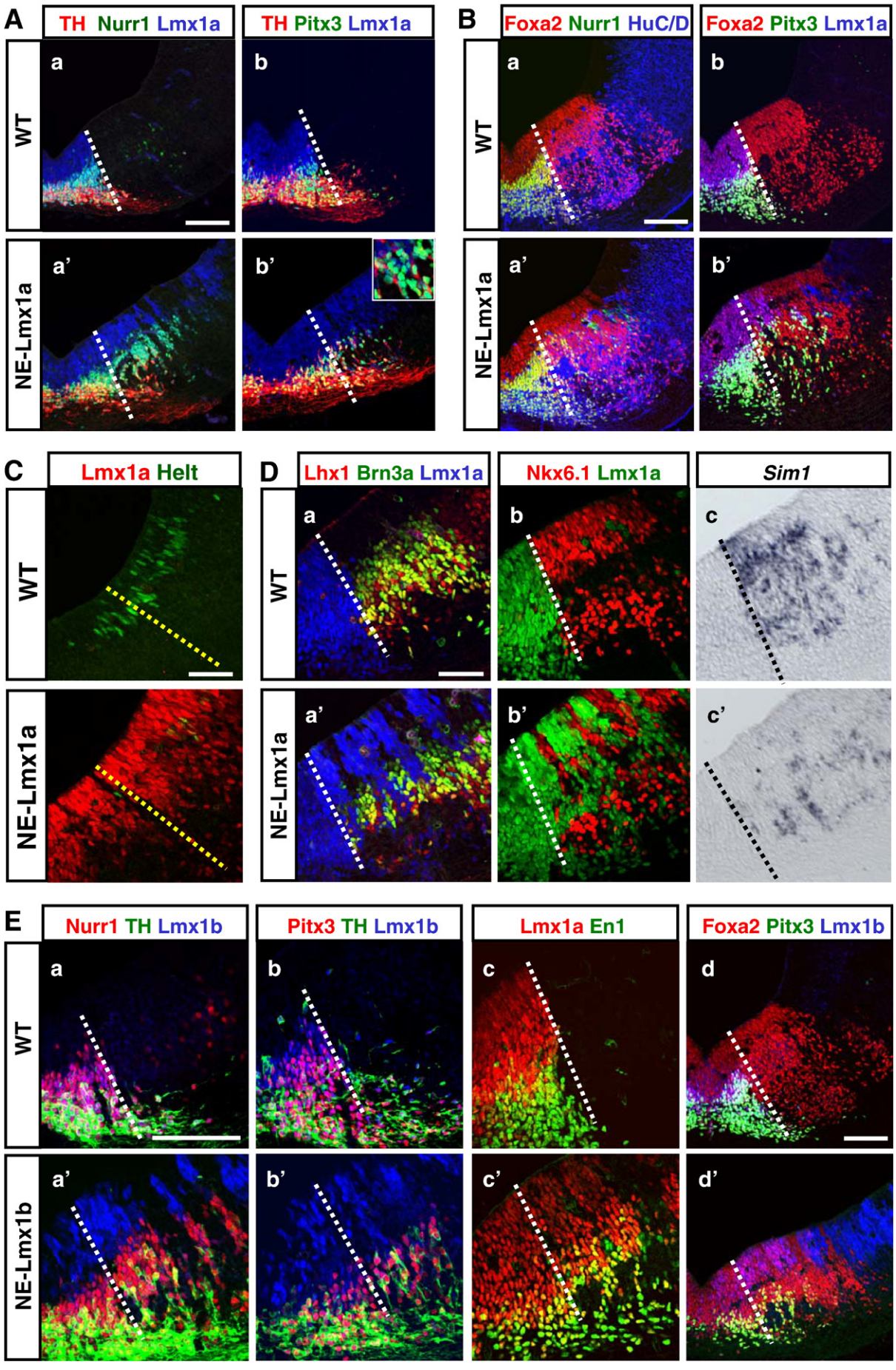
Corin^{+} cells were sorted from E12.5 mouse ventral mesencephalons and cultured as described previously (Ono et al., 2007). Retroviruses expressing *GFP*, *myc-Lhx1*, *Ngn1-IRES-GFP* or *Ngn2-IRES-GFP* were prepared from 293E cells using the RetroMax Retroviral System (Imgenex). Sorted Corin^{+} cells were plated on glass chambers coated with poly-L-ornithine, laminin and fibronectin and cultured for 30 min; they were then infected with retrovirus using ViroMag R/L 100 (OZ Bioscience) and cultured for 4 days.

Results

Lmx1a can specify mesDA fate only within Foxa2^{+} mesencephalic progenitors

To examine how Lmx1a specifies mesDA neuron fate, we generated transgenic mice expressing *Lmx1a* under the control of the *nestin* enhancer (NE-Lmx1a). In the mesencephalons of transgenic embryos at E12.5, ectopic mesDA neurons positive for tyrosine hydroxylase (Th), Nurr1, Pitx3, Lmx1a, Lmx1b and En1 were observed in the ventral region (Fig. 1A and data not shown). Thus, Lmx1a can confer a mesDA fate in the context of the mesencephalon in mammals, as reported in chick embryos (Andersson et al., 2006b). In addition, although a postmitotic role for Lmx1a in mesDA neuron specification has been suggested (Ono et al., 2007), the fact that the *nestin* enhancer used here is active only in proliferative progenitors (Nakatani et al., 2007) suggests that Lmx1a is sufficient to specify progenitor cells into a mesDA fate that consequently induces the postmitotic program determining mesDA neuron identity. Consistently, the mesDA progenitor factor Msx1/2 was ectopically induced by exogenous Lmx1a (Fig. S1). Importantly, however, postmitotic mesDA factors were induced only in the region near the m7 domain that originally generates mesDA neurons, even when exogenous Lmx1a was expressed in all dorsoventral locations in the mesencephalons of the transgenic embryos (Fig. 1A). These results suggest that Lmx1a requires the context conferred by factor(s) selectively expressed in ventral regions to determine mesDA fate. The aim of

Fig. 1. Lmx1a specifies mesDA fate by repressing m6 fate in Foxa2^{+} mesencephalic progenitors. *Lmx1a* (A–D) or *Lmx1b* (E) is ectopically expressed under the control of the *nestin* enhancer (NE-Lmx1a and NE-Lmx1b, respectively). Images show the ventral mesencephalon region at E12.5. (A) Ectopic mesDA neurons positive for Th, Nurr1, Pitx3 and endogenous Lmx1a are induced by Lmx1a. Inset shows magnified images of the m6 domain. Note that these ectopic mesDA neurons are observed only within the Foxa2^{+} regions (B). (C) Helt expression is repressed by exogenous Lmx1a both in the Foxa2^{+} basal plate and the Foxa2^{-} alar plate regions. (D) Lmx1a suppresses the RN fate in the m6 domain. Note that Lmx1a represses expression of *Sim1* and Nkx6.1 in both the VZ and postmitotic precursors. Note that the transgenic embryos shown in the figures expressed exogenous Lmx1a in a proportion of progenitors resulting in a striped pattern. (E) Lmx1b can specify mesDA fate like Lmx1a. Essentially the same activity of Lmx1b in inducing mesDA neurons compared with Lmx1a is observed, suggesting a redundant role for these factors in mesDA specification. Note that Lmx1a is induced by exogenous Lmx1b in the VZ. White and yellow dashed lines indicate the m6/m7 and m4/m5 boundaries, respectively. Bars: A, B and E, 100 μm; C and D, 50 μm.


A
TH Nurr1 Lmx1a
TH Pitx3 Lmx1a
WT
NE-Lmx1a
a
b
a'
b'
B
Foxa2 Nurr1 HuC/D
Foxa2 Pitx3 Lmx1a
C
Lmx1a Helt
D
Lhx1 Brn3a Lmx1a
Nkx6.1 Lmx1a
Sim1
c
c'
E
Nurr1 TH Lmx1b
Pitx3 TH Lmx1b
Lmx1a En1
Foxa2 Pitx3 Lmx1b
d
d'
NE-Lmx1b

the present study was to identify these factor(s) and unmask the mechanism of mesDA specification. To this end, we first determined the precise domain restriction of ectopic mesDA production by exogenous Lmx1a by comparing the expression of mesDA markers with that of regional markers in the NE-Lmx1a mesencephalon, according to a previously reported domain map of the developing mesencephalon (m7, mesDA domain; m6, RN domain; m3-m5, GABAergic domains; m1-m2, GABAergic and glutamatergic domains; for a detailed description of the m1–m7 nomenclature, see Nakatani et al., 2007). We found that one of these key markers, Foxa2, was normally expressed in transgenic embryos, and that ectopic mesDA neurons were restricted within the Foxa2$^+$ m5/m6 regions (Fig. 1B). Ectopic mesDA neurons were observed within Nkx6.1$^+$ regions (Figs. 1A-C), demonstrating that exogenous Lmx1a can induce mesDA neurons, at least in the m6 domain. We could not determine whether the m5 progenitors generate mesDA neurons in the transgenic embryos because Helt, a marker that distinguishes the m5 domain from the m6 domain (Nakatani et al., 2007), was completely repressed in the Foxa2$^+$ progenitors of transgenic embryos (Fig. 1C). However, ectopic mesDA generation was observed only in the ventral part of the Foxa2$^+$ domain, suggesting that Lmx1a can induce ectopic mesDA neurogenesis only in the m6 domain, although Nurr1$^+$ Th$^-$ Pitx3$^-$ neurons were generated at more dorsal regions (Fig. 1Ba'). In addition, Helt expression in the m2–m4 domains was also repressed (Fig. 1C), suggesting that Lmx1a appeared to be active at least in these alar plate and basal plate progenitors, although ectopic mesDA neurons were induced only in restricted regions. Taken together, these results suggest that the m6 context is required for exogenous Lmx1a to induce mesDA fate.

The above observations suggest a possible model in which Lmx1a specifies mesDA fate by repressing alternative fates only in the progenitor cells with potential to differentiate into mesDA neurons. To test this possibility, we analyzed the expression patterns of markers for RN neurons generated from the m6 domain. In NE-Lmx1a embryos, the RN progenitor markers *Sim1* and Nkx6.1 (Nakatani et al., 2007) were completely repressed by Lmx1a in the ventricular zone (VZ) (Figs. 1Db', Dc'). Consistently, generation of postmitotic neurons positive for the RN markers *Sim1*, Nkx6.1, Lhx1 and Brn3a (Pou4f1; Fedtsova and Turner, 2001; Nakatani et al., 2007) was suppressed in the m6 domain, although a certain portion of the postmitotic neurons in the m6 domain still retained RN identity even in the case of transgenic embryos, in which most m6 progenitors expressed exogenous Lmx1a (Figs. 1Da'–c' and data not shown). These results support a model in which Lmx1a specifies mesDA fate by suppressing m6 regional identity.

Lmx1a and Lmx1b act redundantly to specify mesDA fate

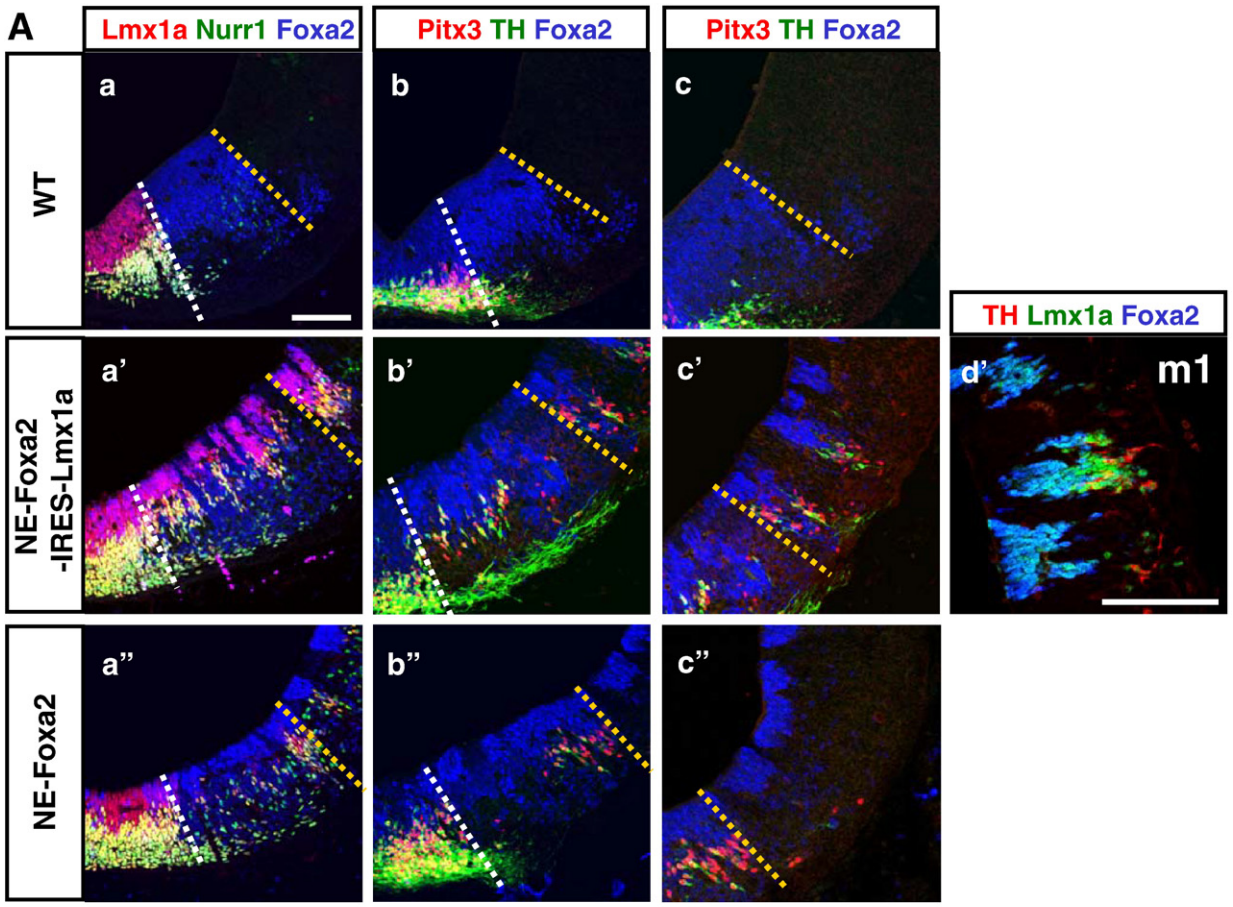
In spite of the potent mesDA-specifying activity of Lmx1a, a previous report analyzing *dreher* mice with a loss-of-function mutation in the *Lmx1a* locus suggested that Lmx1a is largely dispensable for mesDA specification and suppression of m6 fate, although it is important for mesDA neurogenesis (Ono et al., 2007). The observation that the closely related family member Lmx1b was upregulated in the m7 domain of homozygous *dreher* embryos suggested a redundant role for Lmx1a and Lmx1b in mesDA specification (see Fig. S2). To test this possibility, we generated NE-Lmx1b transgenic mice and found that Lmx1b showed essentially identical activity to Lmx1a in terms of mesDA induction (Fig. 1E) and suppression of m6 fate (Fig. S3). Thus, at least in progenitor fate determination, Lmx1a and Lmx1b appear to play highly redundant roles. However, it is also possible that endogenous Lmx1a, which was ectopically induced in the VZ by exogenous Lmx1b (Fig. 1Ec'), conferred mesDA identity on the m6 progenitors rather than direct specification by Lmx1b. By contrast, induction of Lmx1b expression was not detected in the VZ of NE-Lmx1a embryos, even though ectopically generated postmitotic DA neurons expressed Lmx1b (Fig. S4). However, we could not rule out the possibility that exogenous Lmx1a induces endogenous Lmx1b expression in the VZ during the early developmental stages and that this confers mesDA identity. In any case, future studies using loss-of-function approaches for both the Lmx1a/b genes are required to clarify the proposed mechanism of action of these genes in mesDA specification suggested by the gain-of-function approaches in the present study.

Lmx1a and Foxa2 cooperatively determine mesDA fate

The above observations suggest the possibility that Foxa2, which has been reported to be required for mesDA neurogenesis and RN development (Ferri et al., 2007; Kittappa et al., 2007), is also required for Lmx1a function in mesDA induction. To test this possibility, we generated transgenic mice expressing both *Lmx1a* and *Foxa2* under the control of the *nestin* enhancer (NE-Foxa2-IRES-Lmx1a). In the transgenic embryos, ectopic mesDA neurons positive for Lmx1a, Nurr1, Th and Pitx3 were induced not only in the m6 domain, but also in more dorsal domains including the m1 domain in some cases (Figs. 2Aa'–Ad'). Consistently, the mesDA progenitor factor Msx1/2 was ectopically induced in NE-Foxa2-IRES-Lmx1a embryos (Fig. S1). In the transgenic embryos expressing only *Foxa2* (NE-Foxa2), mesDA neurons were not ectopically generated from the m6 domain or alar plate, although in the case of transgenic embryos expressing high levels of Foxa2, mesDA generation was induced in a probable m5 domain (Figs. 2Aa-Ac). In these cases, Lmx1a expression in the VZ was induced in the m5 domain where Th$^+$ Pitx3$^+$ mesDA neurons were ectopically generated (Fig. 2Aa). These results suggest that Foxa2 potentiates the responses of mesencephalic progenitor cells to Lmx1a to specify mesDA progenitor fate, and that this cooperative action of Lmx1a and Foxa2 is sufficient to determine mesDA fate in the context of mesencephalic progenitors. This idea is further supported by the previous observations that deletion of the Foxa1/2 genes resulted in loss of mesDA identity of the neurons generated from the m7 domain despite the fact that Lmx1a expression was retained in m7 progenitors (Ferri et al., 2007).

To unmask the mechanism of action of Foxa2 in mesDA specification, we analyzed the expression patterns of other mesDA and RN markers. In NE-Foxa2 embryos, Nurr1 and Lmx1a were upregulated in some postmitotic neurons generated from the m6 domain and more dorsal domains, even though definitive mesDA identity, as judged by expression of Th and Pitx3, was not acquired by most of these neurons (Figs. 2Aa, Ab). This might be due, at least in part, to the absence of Lmx1a/b induction in the VZ (Figs. 2Aa and S5).

Fig. 2. Lmx1a and Foxa2 cooperatively determine mesDA fate in the mesencephalon. Both *Lmx1a* and *Foxa2* or *Foxa2* alone are ectopically expressed under the control of *nestin* enhancer (NE-Foxa2-IRES-Lmx1a and NE-Foxa2, respectively). Images show the ventral mesencephalon region at E12.5. (A) Exogenous expression of a combination of Foxa2 and Lmx1a can induce mesDA neurons not only in the m6 domain, in which Lmx1a alone can induce mesDA fate, but also in more dorsal regions. By contrast, Foxa2 alone cannot induce ectopic mesDA neurons in most mesencephalon regions, suggesting that Foxa2 potentiates the responses of mesencephalic progenitors to Lmx1a for mesDA induction. Note that in the probable m5 domains of NE-Foxa2 embryos, ectopic Lmx1a expression is induced in the VZ and mesDA neurons emerge from the progenitor domain. By contrast, Lmx1a$^+$ Nurr1$^+$ mesDA precursor-like cells are induced in some regions other than the m5 domain, but these cells cannot acquire a DA phenotype. (B) Foxa2 partially induces the differentiation programs for both mesDA and RN, and in this context, Lmx1a selects a mesDA fate by repressing RN fate. In NE-Foxa2 embryos, RN progenitor markers and Lhx1$^+$ Brn3a$^+$ RN-like neurons are also induced in the regions where Lmx1a$^+$ Nurr1$^+$ mesDA precursor-like cells are induced. By contrast, in NE-Foxa2-IRES-Lmx1a embryos, RN fate is suppressed and mesDA fate is selected. Insets show magnified images of the m2 domains. White and yellow dashed lines indicate the m6/m7 and m4/m5 boundaries, respectively. Note that the NE-Foxa2-IRES-Lmx1a transgenic embryos shown in the figures expressed transgenes in a proportion of progenitors resulting in a striped pattern. Bars: 100 μm.


A
Lmx1a Nurr1 Foxa2
Pitx3 TH Foxa2
Pitx3 TH Foxa2
TH Lmx1a Foxa2
WT
NE-Foxa2 -IRES-Lmx1a
NE-Foxa2
a
b
c
a'
b'
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d'
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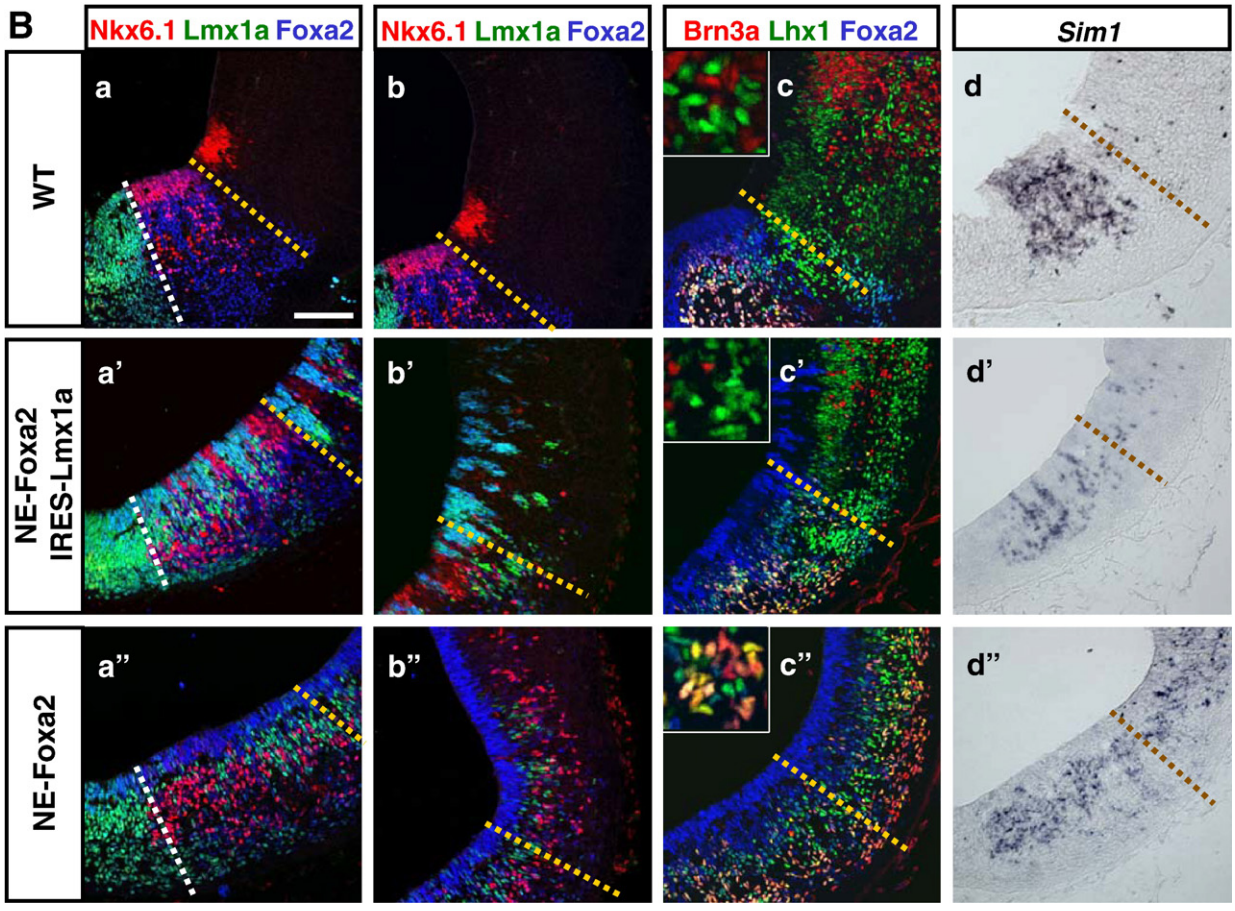
b''
c''


B
Nkx6.1 Lmx1a Foxa2
Nkx6.1 Lmx1a Foxa2
Brn3a Lhx1 Foxa2
Sim1
WT
NE-Foxa2 IRES-Lmx1a
NE-Foxa2
a
b
c
d
a'
b'
c'
d'
a''
b''
c''
d''

These observations suggest that Foxa2 can partially control the mesDA differentiation program, but that Lmx1a in the VZ is required for proper determination of the mesDA fate in this context. Consistently, RN markers, such as *Sim1*, Nkx6.1 and coexpression of Lhx1 and Brn3a were also induced by exogenous Foxa2 without induction of the m6 progenitor factors in the VZ (Figs. 2Ba–Bd). By contrast, in NE-Foxa2-IRES-Lmx1a embryos, ectopic neurons with RN marker expression were not efficiently induced (Figs. 2Ba'–Bd'). Taken together, these observations suggest that Foxa2 restricts mesencephalic progenitors to an m6/m7 fate by inducing a partial differentiation program, and that, only in this context, Lmx1a/b selects a mesDA fate by repressing m6 identity. This idea is consistent with the previous observations by loss-of-function approaches that the differentiations of both mesDA and RN neurons require Foxa1/2 activity (Ferri et al., 2007).

Sim1 but not Nkx6.1 suppresses mesDA differentiation

Our model predicts that Lmx1a/b represses a factor(s) that inhibits mesDA specification or differentiation to determine mesDA fate. The observations that overexpression of Foxa2-induced Nurr1^{+} Lmx1a^{+} neurons in the m5/m6 regions, but only m5-derived neurons adopted a proper mesDA fate, suggest that a factor(s) that inhibits mesDA differentiation is selectively expressed in the m6 domain. At

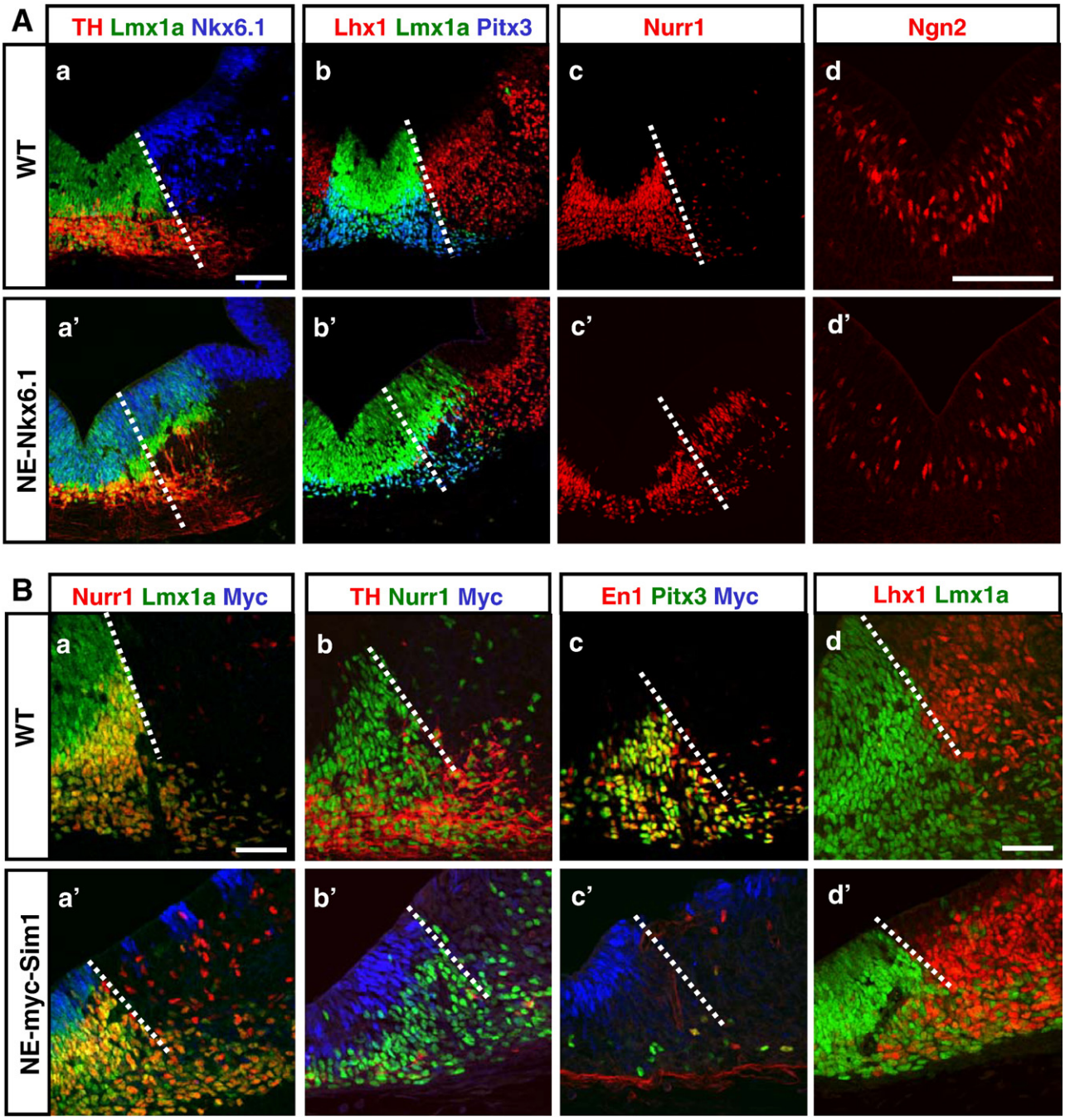


Fig. 3. Sim1 but not Nkx6.1 inhibits mesDA differentiation. *Nkx6.1* (A) or *myc-Sim1* (B) is ectopically expressed under the control of *nestin* enhancer (NE-Nkx6.1 and NE-myc-Sim1, respectively). Images show the ventral mesencephalon region at E12.5. Nkx6.1 does not inhibit correct mesDA differentiation although it partially suppresses mesDA neurogenesis by repressing Ngn2 expression (A). Note that the identity of the m6 domain appears to be transfated to that of the m7 domain by overexpression of Nkx6.1. By contrast, myc-Sim1 expression in the VZ inhibits mesDA differentiation by conferring a partial m6 identity (B). Dashed lines indicate the m6/m7 boundaries. Bars: A, 100 μm; B, 50 μm.

present, the factors known to be selectively expressed in the m6 VZ are Nkx6.1 and *Sim1*, expression of which can be suppressed by Lmx1a/b (Figs. 1Db', Dc' and S3); thus, these factors are candidate inhibitors of mesDA differentiation. We first examined whether Nkx6.1, which has been reported to be involved in neuronal fate determination in other CNS regions (Pattyn et al., 2003; Sander et al., 2000), has a potency to suppress mesDA differentiation. In transgenic embryos expressing Nkx6.1 under the control of the *nestin* enhancer (NE-Nkx6.1), mesDA neurons positive for Th, Pitx3, Lmx1a and Nurr1 were normally generated (Figs. 3Aa'–Ac'), although the numbers of mesDA neurons generated from the m7 domain appeared slightly decreased and the proneural factor Ngn2 was consistently slightly downregulated (Fig. 3Ad'). Specification of mesDA progenitors in the m7 domain also appeared normal because Lmx1a expression was not affected. In addition, Nkx6.1 could not induce m6 fate as the m6-specific VZ marker *Sim1* was not induced, and consequently, the m6 neuronal markers Lhx1 and Brn3a were not ectopically expressed in the m7-derived neurons (Figs. 3Ab' and 6, and data not shown). These results indicate that Nkx6.1 does not have a role in suppressing mesDA fate in the m6 domain, and repression of Nkx6.1 by Lmx1a/b is not required for mesDA specification. Surprisingly, however, despite the fact that Nkx6.1 is expressed in the m6 domain in the wild-type condition, the m6 progenitors acquired Lmx1a expression and consistently generated mesDA neurons with the correct marker profile in NE-Nkx6.1 embryos, although some neurons with the RN marker profile (Lhx1 and *Sim1*) were still generated from the same domain (Figs. 3Ab' and 6), similar to the case for NE-Lmx1a embryos. One possible explanation for this phenotype is that the m6 factor(s) that restricts Lmx1a expression to the m7 domain was downregulated by the overexpression of Nkx6.1, and consequently, Lmx1a was derepressed in m6 progenitors, thereby conferring m7 identity. One candidate m6 factor is Sim1, and consistent with this notion, *Sim1* expression was lost in the m6 VZ of NE-Nkx6.1 embryos (Fig. S6).

To examine the role of Sim1 activity in mesDA/RN fate determination, we ectopically expressed myc-tagged Sim1 under the control of *nestin* enhancer. In NE-myc-Sim1 transgenic embryos, Lmx1a expression in the m7 VZ was not affected and Lmx1a$^+$ Nurr1$^+$ postmitotic mesDA precursors were normally produced from the m7 domain as in wild-type embryos (Fig. 3Ba'), suggesting that Sim1 does not have a potency to suppress m7 identity and that loss of Sim1 expression in the m6 VZ alone is not a cause of the expansion of the mesDA domain in NE-Nkx6.1 (see Discussion). However, we found that most of these precursors with mesDA identity emerging from the anterior m7 domain were negative for Th, En1 and Pitx3 (Figs. 3Bb', Bc'), although the phenotype was less clear in the posterior m7 domain (data not shown). Thus, expression of *Sim1* in progenitors inhibits proper maturation into mesDA neurons. Instead, the m6 markers Lhx1 and Brn3a were ectopically expressed in these Lmx1a$^+$ Nurr1$^+$ Th$^-$ cells generated from the anterior m7 domain (Fig. 3Bd' and data not shown), suggesting that Sim1 expression in the m7 VZ suppressed mesDA differentiation by conferring m6 identity over mesDA identity. Thus, repression of Sim1 appears to be a prerequisite for proper mesDA differentiation. Taken together, these results suggest that one of the roles for Lmx1a/b in mesDA progenitor specification is the repression of *Sim1* to suppress m6 identity.

Lhx1 inhibits mesDA differentiation

Next, we asked whether repression of Lhx1 expression in postmitotic neurons is required for proper mesDA differentiation, because Lhx1 expression was induced by the mis-specified neurons that could not mature into mesDA neurons in *dreher* (Ono et al., 2007) and NE-myc-Sim1 transgenic embryos (Fig. 3Bd'). For this purpose, we performed in vitro culture experiments using Corin$^+$ mesDA progenitors sorted from E12.5 mouse mesencephalons (Ono et al., 2007). When Corin$^+$ progenitors were transduced with retrovirus expressing *GFP* under the control of the *PGK* promoter and cultured for 4 days, 62.3 ± 5.6% of GFP+ cells were positive for HuC/D, and 65.6 ± 4.1% and 30.0 ± 3.9% of GFP$^+$ neurons expressed Pitx3 and Th, respectively (Figs. 4A, B and data not shown). By contrast, 42.0 ± 4.2% of exogenous Lhx1-expressing cells were HuC/D+ neurons, and only 16.0 ± 2.7% and 5.5 ± 3.4% of the Lhx1-expressing neurons derived from Corin$^+$ mesDA progenitors expressed Pitx3 and Th, respectively, demonstrating that Lhx1 inhibits correct maturation into mesDA neurons. Thus, repression of Lhx1 by Lmx1a/b, indirectly, through repressing Sim1 in the VZ, and/or directly in postmitotic neurons (Ono et al., 2007), is a prerequisite for proper mesDA differentiation. Taken together, these data support the above model that Foxa2 confers differentiation potential to m6/m7 neurons, and, in this context, Lmx1a/b selects mesDA fate, at least in part, by repressing the Sim1-Lhx1 pathway that inhibits mesDA differentiation.

Lmx1a and Foxa2 regulate expression of Ngn genes

The above observation that Lmx1a can repress Helt raised the question of whether this pathway is physiologically relevant in

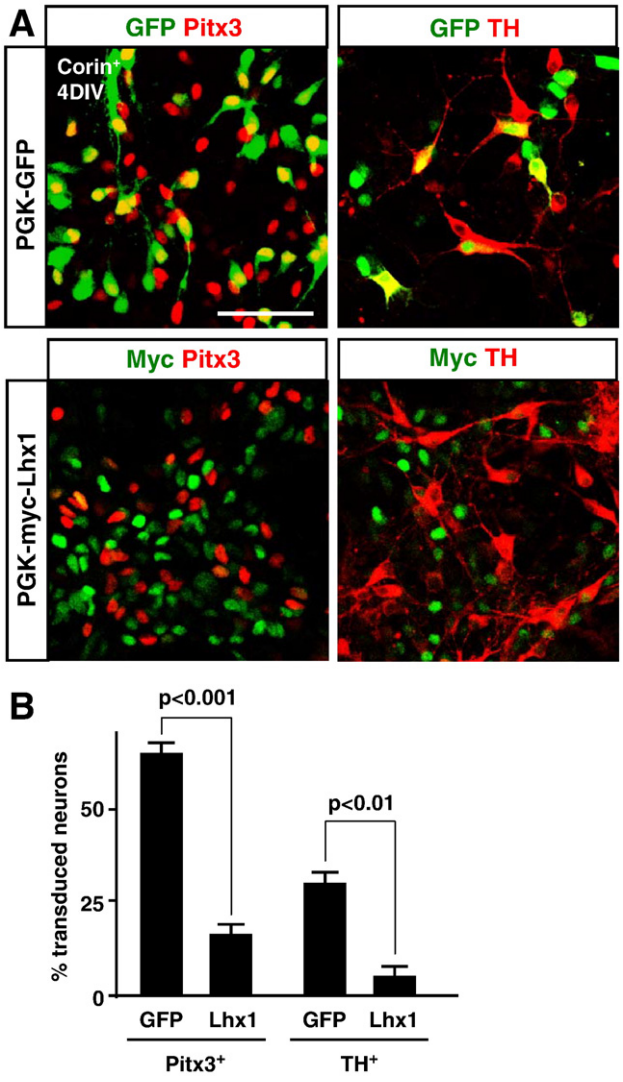


Fig. 4. Forced expression of Lhx1 inhibits mesDA differentiation in vitro. Corin$^+$ mesDA progenitors sorted from E12.5 mouse mesencephalons are transduced with *GFP*- or *Lhx1*-expressing retroviruses and cultured for 4 days in vitro. Neither transgene inhibits neuronal differentiation, but Lhx1 inhibits correct maturation into mesDA neurons. Bar: 50 μm.

mesDA development. A previous report showed that Helt is both necessary and sufficient for repressing Ngn genes (Nakatani et al., 2007). Because Ngn2 was shown to be essential for mesDA generation, it is possible that Helt is repressed in the m7 domain to induce mesDA neurogenesis. To test this possibility, we analyzed the effect of forced expression of Helt in the m7 domain using NE-Helt embryos (Nakatani et al., 2007). As expected, Ngn2 expression was significantly downregulated with no change in Lmx1a expression; consequently, generation of Nurr1$^+$ postmitotic DA neurons was consistently reduced (Fig. S7). These results, together with our previous observations (Nakatani et al., 2007), indicate that Helt is sufficient to prevent proper mesDA neurogenesis in the m7 domain and suggest that suppression of Helt expression in m7 progenitors is a prerequisite for mesDA generation.

If expression of Ngn genes is solely controlled by Helt, as previously suggested, both Ngn1 and Ngn2 could be expressed in the m7 VZ. However, mesDA progenitors expressed only Ngn2 (Kele et al., 2006; Nakatani et al., 2007). Ngn1 is coincidently expressed by glutamatergic progenitors in the mesencephalon and has been shown to have a potency to determine a glutamatergic phenotype (Nakatani et al., 2007). We examined the effect of forced expression of Ngn1 on mesDA differentiation. Because Ngn1 has a proneural function, it is difficult to examine Ngn1's activity in ventral mesencephalic progenitors using a transgenic approach (Nakatani et al., 2007); thus, we used an in vitro culture system. When Ngn1 or Ngn2 were transduced into Corin$^+$ mesDA progenitors, both genes similarly induced neuronal differentiation at 3DIV (HuC/D$^+$/GFP$^+$%: GFP control, 58.5 ± 5.7%; Ngn1-GFP, 94.0 ± 2.3%; Ngn2-GFP, 93.0 ± 2.3%). Importantly, however, these factors differentially affected the differentiation fate; Pitx3 was expressed in 53.6 ± 4.5% of Ngn2-induced neurons, which was comparable to GFP-transduced control (58.1 ± 3.9%) whereas only 24.9 ± 3.0% of Ngn1-transduced neurons expressed Pitx3 (Figs. 5A, B). These results demonstrate that Ngn1 has a potency to suppress mesDA differentiation. Thus, repression of Ngn1 in m7 progenitors appears to be required for proper mesDA specification.

We next addressed the question of what factor(s) controls Ngn1/2 expression in the mesDA lineage. In NE-Lmx1a embryos, Ngn2 expression was induced in broad domains, which is consistent with the proposed activity of Lmx1a in Ngn2 induction and with the observation that Helt was repressed by Lmx1a (Fig. 6a'). Ngn1 expression was also induced in dorsal domains by exogenous Lmx1a (Fig. 6b'). Importantly, however, Ngn1 expression in the m6 domain was significantly reduced, consistent with the ectopic induction of mesDA neurogenesis (Fig. 6b'). The induction of Msx1 by exogenous Lmx1a (Fig. S1) suggests the possible involvement of Msx1 in these activities of Lmx1a. Expression of Otx2, another regulator of Ngn2 expression in the m7 domain, was not affected by exogenous Lmx1a, as expected from its broad VZ expression in the mesencephalon (data not shown). Lmx1b showed essentially identical activity to Lmx1a (data not shown). These results suggest that Lmx1a/b induces Ngn1 and Ngn2 by repressing Helt as we previously observed that both Ngn genes appear to be de-repressed in the context of mesencephalic progenitors by loss of *Helt* (Nakatani et al., 2007), but that only in the m6/m7 context can Lmx1a/b repress Ngn1 expression. When Lmx1a and Foxa2 were coexpressed, Ngn1 was repressed but Ngn2 was induced in more dorsal domains (Figs. 6a, b), indicating that this repression of Ngn1 by Lmx1a is dependent on Foxa2 activity. Taken together, these results suggest that Lmx1a and Foxa2 cooperatively induce Ngn2 and suppress Ngn1 to determine a mesDA fate. We also examined whether Mash1 expression is affected by exogenous Lmx1a and Foxa2. As expected from the fact that Mash1 is expressed

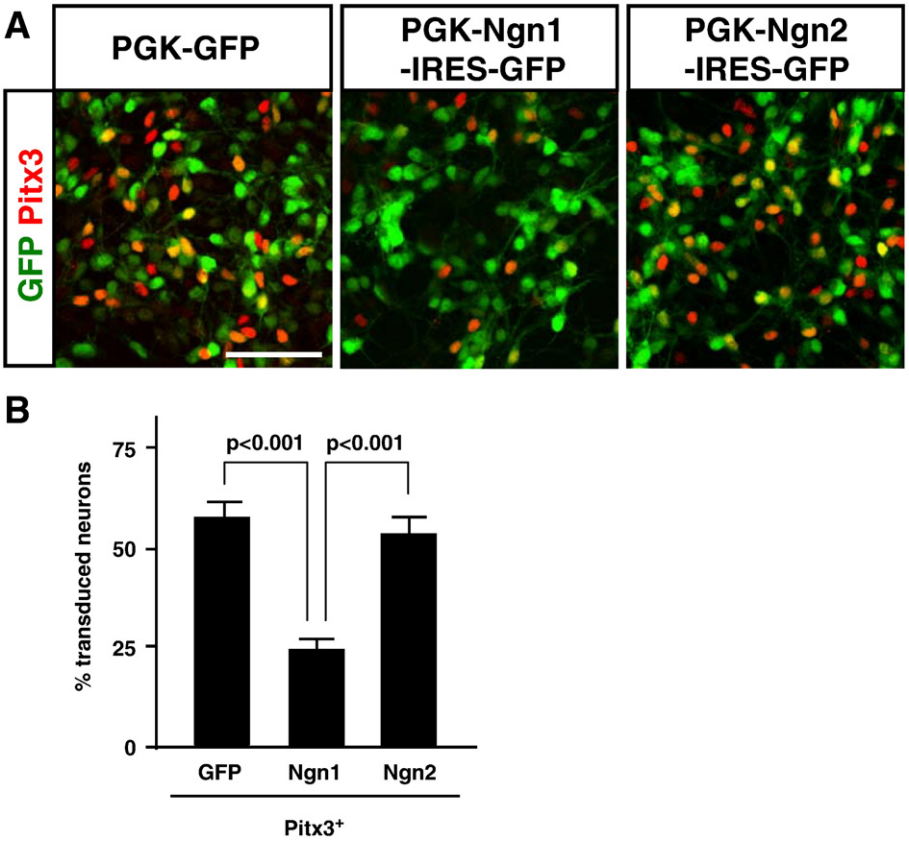


Fig. 5. Forced expression of Ngn1 inhibits mesDA differentiation in vitro. Corin$^+$ mesDA progenitors sorted from E11.5 mouse mesencephalons are transduced with *GFP*-, *Ngn1-IRES-GFP*- or, *Ngn2-IRES-GFP*-expressing retroviruses and cultured for 3 days in vitro. Ngn2 induces correct mesDA differentiation. By contrast, Ngn1 induces general neuronal differentiation but suppresses mesDA fate. Bar: 50 μm.

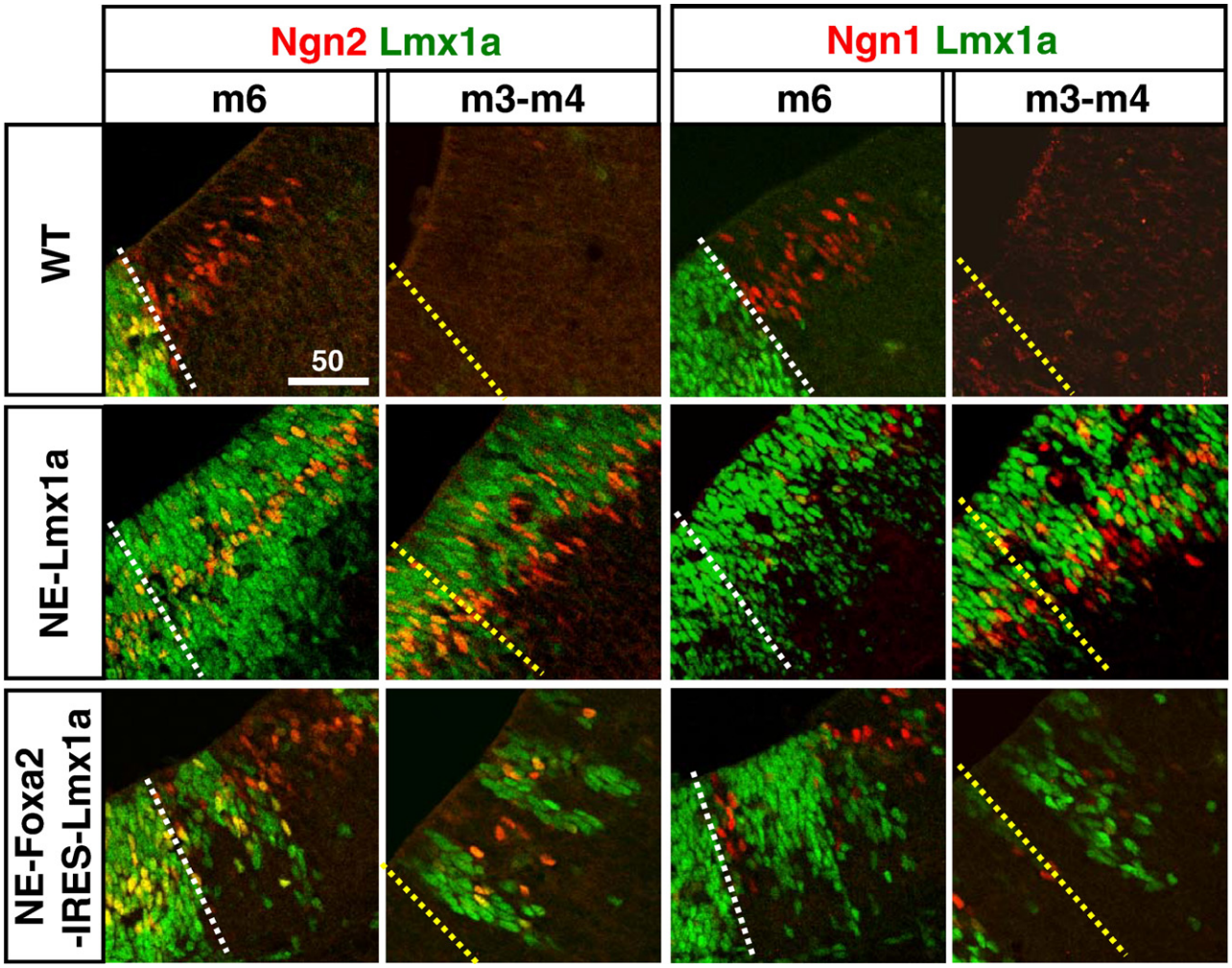


Fig. 6. Lmx1a induces Ngn2 but represses Ngn1 expression by cooperating with Foxa2. Both *Lmx1a* alone and a combination of *Lmx1a* and *Foxa2* are ectopically expressed under the control of *nestin* enhancer (NE-Lmx1a and NE-Foxa2-IRES-Lmx1a, respectively). Images show the ventral (m6) and medial (m3/m4) mesencephalon regions at E12.5. Exogenous Lmx1a can induce Ngn2 expression in both domains. Ngn1 expression is also induced in the m3–m4 domains but repressed in the m6 domain by exogenous Lmx1a. Exogenous expression of a combination of Foxa2 and Lmx1a can repress Ngn1 expression in all domains. White and yellow dashed lines indicate the m6/m7 and m4/m5 boundaries, respectively. Bar: 100 μm.

throughout all mesencephalon regions (Nakatani et al., 2007), Mash1 expression was unchanged in both types of transgenic embryos studied (data not shown).

Lmx1a/b can induce FP differentiation in the ventral mesencephalon

Previously, we and others reported that mesDA neurons originate from FP cells (Andersson et al., 2006b; Ono et al., 2007). The above observations that Lmx1a/b and Foxa2 specify mesDA fate suggest two alternative possibilities: these factors cooperatively specify FP cells that consequently generate mesDA neurons, or mesDA fate and FP identity are independently specified in the same cell. To distinguish between these possibilities, we analyzed FP cell differentiation in the transgenic embryos described above. For this purpose, we used Corin as a definitive FP marker, whose expression is sustained until mesDA neurogenesis (Ono et al., 2007).

In NE-Lmx1a embryos, Corin expression was ectopically induced in the m6 domain (Fig. 7Aa'), where ectopic mesDA neurogenesis was also induced. Although we only examined Corin expression as an FP marker because Corin is the only definitive FP marker for the mesencephalon identified to date, these results suggest that Lmx1a can induce FP differentiation, at least in the context of the ventral mesencephalon. In addition, Shh expression in the m6 domain was decreased by ectopic Lmx1a, similar to the case for the wild-type m7 domain (data not shown).

To examine whether Lmx1a is required for FP specification in the mesencephalon, we analyzed FP marker expression in *dreher* mice. Corin expression was not affected by the mutation (Fig. 7B) as progenitor patterning was mostly normal in *dreher* embryos (Ono et al., 2007). This might again be due to a compensatory role of Lmx1b as Corin expression was induced in the m6 domains of NE-Lmx1b embryos (Fig. 7C).

Foxa2 alone can induce FP differentiation in the mesencephalon

FP marker induction by exogenous Lmx1a/b was restricted to the Foxa2$^+$ region (Fig. 7Ab' and data not shown), suggesting that Foxa2 is again required for this Lmx1a/b activity. Consistent with this idea, Corin was induced in all dorsoventral locations where ectopic mesDA neurons were generated in NE-Foxa2-IRES-Lmx1a embryos (Fig. 8c'), and Shh was induced at low levels as in the m7 domain of wild-type embryos (Fig. 8b'), suggesting that Lmx1a can determine FP identity in the presence of Foxa2. Unexpectedly, however, overexpression of Foxa2 alone induced Corin as well as high level of Shh in the VZ without inducing Lmx1a expression in the VZ or ectopic mesDA production in all dorsoventral locations of the mesencephalon, with the exception of the m5 domain (Figs. 8b and c). Importantly, ectopic

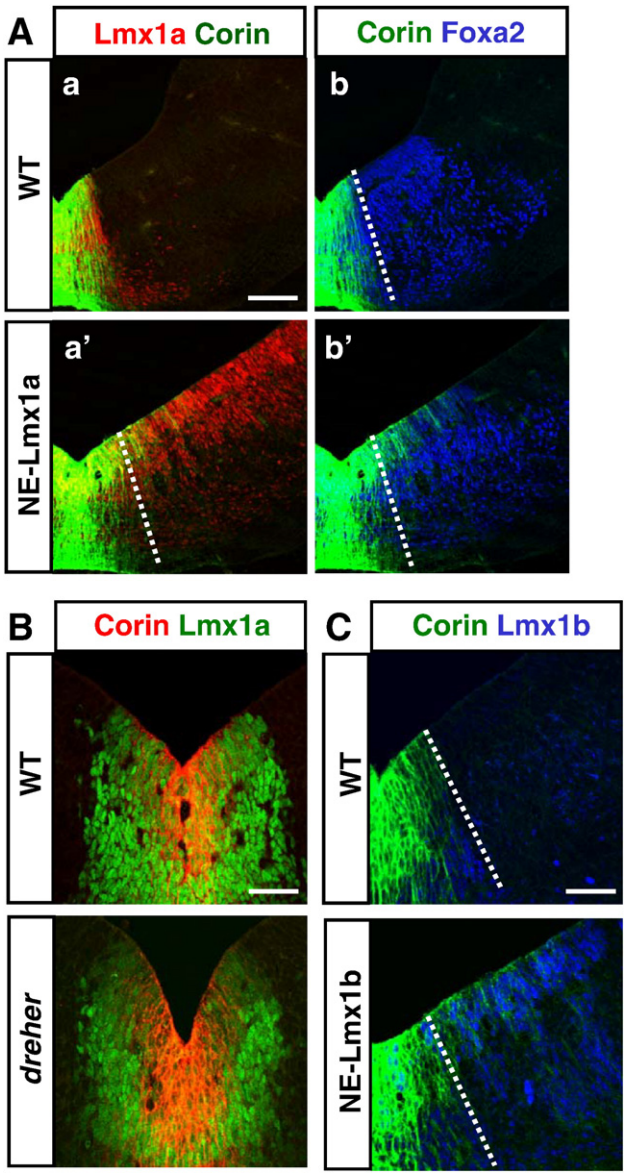


Fig. 7. Lmx1a induces FP differentiation within mesencephalic Foxa2$^+$ domains. Images show the ventral mesencephalon region of NE-Lmx1a embryos (A), homozygous *dreher* mutants (B) and NE-Lmx1b embryos (C) at E12.5. Expression of the FP marker Corin is induced by Lmx1a (A) and Lmx1b (C) within Foxa2+ regions but is not affected by the *dreher* mutation (B), suggesting redundant roles for Lmx1a and Lmx1b in FP induction. Note that FP markers and mesDA neurons are similarly induced by Lmx1a/b (see Fig. 1). Bars: A, 100 μm; B and C, 50 μm.

FP marker induction was only observed in cells expressing exogenous Foxa2 (Fig. 8d), suggesting that this effect is caused by a cell-autonomous action of Foxa2. Thus, Foxa2 appears to primarily control FP differentiation and Lmx1a might support this Foxa2 activity when present at endogenous levels. In addition, these observations suggest that acquisition of FP identity is not sufficient for mesDA production, even in the context of the mesencephalon, with the exception of the m5 domain.

Nkx6.1 but not Sim1 suppresses FP differentiation

The fact that FP differentiation does not occur in the Foxa2$^+$ m6 region in wild-type embryos and the observation that exogenous Lmx1a can induce FP marker expression in the m6 domain suggest that a factor(s) that is selectively expressed in the m6 domain and repressed by Lmx1a might inhibit Foxa2's FP-inducing activity in the m6 domain of wild-type embryos. To test this possibility, we analyzed FP differentiation in NE-Nkx6.1 and NE-myc-Sim1 transgenic embryos. Corin expression was not significantly affected in NE-myc-Sim1 embryos (Fig. 9A), indicating that Sim1 does not have an FP differentiation-inhibiting activity despite its activity in suppressing mesDA differentiation. By contrast, in the NE-Nkx6.1 mesencephalon, Corin expression was mostly abolished, even though mesDA neurons were still generated (Fig. 9B). Thus, repression of Nkx6.1 appears to be a prerequisite for proper FP differentiation. This idea is further supported by the observations that Nkx6.1 expression in the VZ was repressed in the NE-Foxa2 mesencephalon where FP marker expression was induced (Figs. 2Ba and b). Furthermore, the observation that NE-Nkx6.1 embryos still generated mesDA neurons with the correct identity from progenitors without proper FP marker expression suggests that FP differentiation is not a prerequisite for mesDA specification. Taken together, these results support the model that mesDA and FP identities are determined by distinct pathways that are controlled by the same regulators, Lmx1a/b and Foxa2, and that, as a consequence, mesDA neurons are generated from progenitors with FP identity.

Discussion

The mechanism of mesDA neuron specification

Our analyses of a series of transgenic embryos revealed cooperative actions of Lmx1a/b and Foxa2 in the specification of mesDA neurons. The question arises as to how these factors cooperate. As previously reported and we confirmed, Foxa2 induced Shh, which is an essential inductive signal for mesDA neurons (Ye et al., 1998), suggesting the possible involvement of a non-cell autonomous action of Foxa2 in cooperation with Lmx1a/b. However, this is unlikely because our analyses used *nestin* enhancer, which starts to be active only around the time of neural tube closure, when progenitors lose their competency to respond to extrinsic patterning signals. Indeed, in the dorsal regions of NE-Foxa2 and NE-Foxa2-IRES-Lmx1a embryos, progenitors negative for transgene expression neighboring Foxa2-positive cells maintained dorsal identity, as judged by Pax3 expression (see Fig. S8). In addition, previously reported transgenic mice expressing Foxa2 under the control of the *En2* enhancer showed ectopic generation of DA neurons in the dorsal-most regions of the mesencephalon near the induced FP-like cells (Hynes et al., 1995). This is in contrast to the observation in our NE-Foxa2 embryos that ectopic DA generation only occurred in the m5 ventral domain, suggesting the possibility that only when Foxa2 is ectopically expressed at early neural plate stage can it non-autonomously induce DA possibly through induction of Lmx1a by Shh. Thus, it is likely that coexpression of Lmx1a and Foxa2 in mesencephalic progenitor cells is necessary and sufficient for mesDA fate determination. However, no downstream target genes for Lmx1a/b and Foxa2, which are involved in mesDA specification, have been identified to date; thus, we could not address whether these factors cooperatively control transcription of downstream target genes. Indeed, Lmx1a can induce Ngn2 expression and repress Helt expression without Foxa2 activity in NE-Lmx1a embryos, suggesting that Foxa2 is not involved in all transcriptional regulation by Lmx1a. By contrast, repression of Ngn1 and induction of definitive DA markers, such as Th, occurred only in the presence of Foxa2. Thus, Lmx1a and Foxa2 are likely to control some targets cooperatively and others independently. Future studies aimed at identifying the downstream target genes and mechanisms of transcriptional control of these factors are needed to clarify this issue.

Foxa2 is selectively expressed in mesDA and RN lineages and is required for neurogenesis and differentiation of mesDA neurons as well as for RN formation, although the mechanism of action is largely unknown (Ferri et al., 2007). Our results revealed that overexpression of Foxa2 alone can induce generation of neurons with mesDA-like

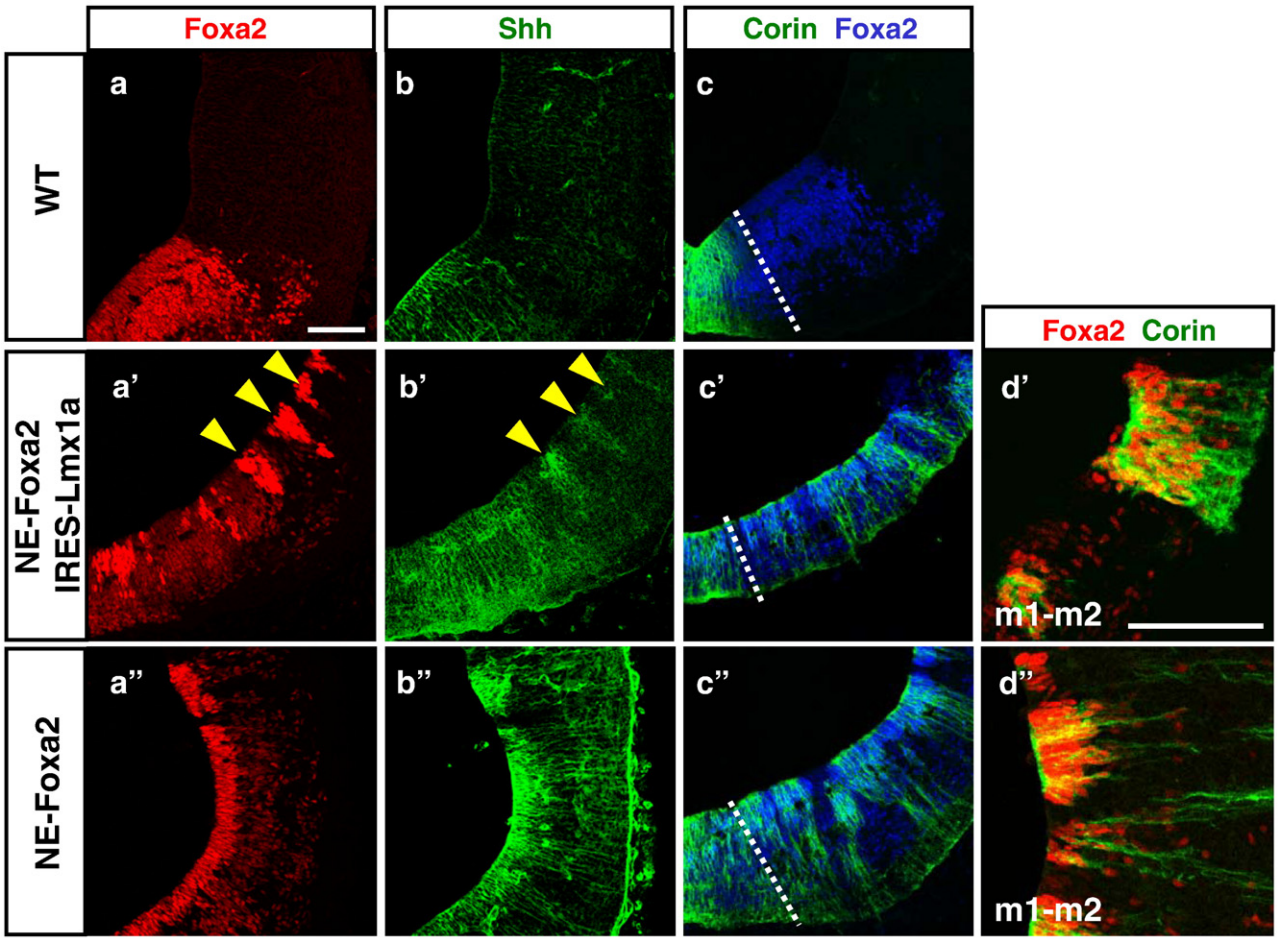


Fig. 8. Foxa2 alone can induce FP differentiation in the mesencephalon. Images show the ventral mesencephalon region of NE-Foxa2 and NE-Foxa2-IRES-Lmx1a embryos at E12.5. Ectopic induction of FP markers (Corin and Shh) is observed not only in the ectopic mesDA-generating progenitors induced by a combination of exogenous Foxa2 and Lmx1a, but also in the progenitors overexpressing Foxa2 alone that cannot produce mesDA neurons. Note that the NE-Foxa2-IRES-Lmx1a transgenic embryos shown in the figures expressed transgenes in a proportion of progenitors resulting in a striped pattern. Bars: 100 μm.

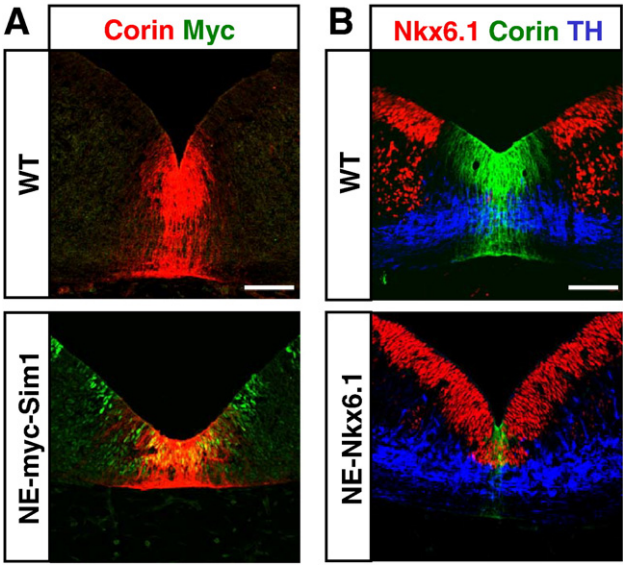


Fig. 9. Nkx6.1 but not Sim1 inhibits FP differentiation in the mesencephalon. Images show the ventral mesencephalon region of NE-Sim1 (A) and NE-Nkx6.1 (B) at E12.5. FP differentiation is not affected by ectopic *Sim1* expression. By contrast, FP marker expression is nearly completely repressed by forced expression of Nkx6.1, even though mesDA neurons are still generated. Bars: 50 μm.

characters, although these neurons are not properly assigned, and, as a consequence, they could not mature into mesDA neurons. Foxa2 also induces neurons with RN identity. These observations suggest that Foxa2 has a potency to control a partial program of differentiation in both of these neuron types. However, in the condition where Foxa2 is expressed at endogenous levels, this activity is strictly controlled by factors that are selectively expressed in regions generating each neuronal subtype. Lmx1a/b appears to select a mesDA fate by suppressing an RN fate in progenitor cells that are partially committed to differentiate into DA and RN neurons by Foxa2. This suppression of RN fate is likely, at least in part, to be mediated by repression of two inhibitory pathways, the Sim1-Lhx1 pathway that inhibits proper mesDA maturation and the Ngn1 pathway that suppresses mesDA fate by conferring a glutamatergic phenotype. However, we used ectopic and overexpression approaches to provide these possible mechanisms of DA specification. Thus, future studies using more physiological conditions, such as loss-of-function approaches, will be needed to confirm these ideas.

The observation that overexpression of Foxa2 in the m5 domain activated Lmx1a expression in the progenitors and consequently induced mesDA generation suggests that Foxa2 acts upstream of Lmx1a, and that some factor(s) selectively expressed in the m5/m6 domains suppresses Foxa2's Lmx1a-inducing activity in the wild-type condition. The question arises as to what factor(s) restrict Lmx1a expression to the m7 domain within the Foxa2^{+} region. In NE-Nkx6.1 embryos, the area of Lmx1a expression expanded into the presumptive m6 domain. Because Nkx6.1 is expressed in the m7 domain in the

early stage of development (Andersson et al., 2006b), it is possible that Nkx6.1 by itself is involved in the induction of Lmx1a expression in the m7 domain. However, this possibility does not explain why Lmx1a is not expressed in m6 progenitors positive for Foxa2 and Nkx6.1 in the wild-type condition. Rather, these observations suggest a more likely possibility that overexpression of Nkx6.1 represses some m6 factor(s) that inhibits Lmx1a induction by Foxa2, and that, as a consequence, Lmx1a is ectopically upregulated in the m6 domain. Sim1 is a candidate for this m6 factor, because Sim1 expression was suppressed in the VZ of NE-Nkx6.1 embryos, although it is also possible that the repression of *Sim1* was caused as a result of the ectopically induced Lmx1a expression. However, Sim1 could not suppress Lmx1a expression in the m7 domain even when ectopically expressed in the early stage of development under the control of the *Shh* enhancer (data not shown). Thus, Sim1 alone cannot control Lmx1a expression in the mesencephalon. However, we could not rule out the possible requirement of *Sim1* in the restriction of Lmx1a expression to the m7 domain, which would need to be examined by loss-of-function studies. Another candidate is Nkx2.2, which is expressed in the m6 domain at high levels at E9.75, when the region of Lmx1a expression is determined (data not shown). However, ectopic expression of Nkx2.2 under the control of the *nestin* enhancer did not affect Lmx1a expression nor mesDA generation in the m7 domain (unpublished observation), suggesting that Nkx2.2 alone cannot suppress Lmx1a expression. Also, it has been reported that Nkx2.2-null mice normally generated mesDA neurons (Prakash et al., 2006). Thus, at present, the mechanisms underlying the regulation of Lmx1a induction in the m7 domain and repression in the m6 domain are unknown.

Our results suggest redundant roles for Lmx1a and Lmx1b in mesDA specification in mice. This may explain the mild phenotype of the *dreher* mutant compared with the strong patterning activity of Lmx1a in gain-of-function experiments (this study; Andersson et al., 2006b; Ono et al., 2007). However, the level of Lmx1b expression in the VZ decreases as development proceeds (Andersson et al., 2006b; Guo et al., 2008; Ono et al., 2007; Smidt et al., 2000), suggesting that in the wild-type condition, Lmx1a appears to mainly determine the mesDA progenitor fate at late stages. In this context, Lmx1a may repress Lmx1b expression in the VZ, since upregulation of Lmx1b was observed in *dreher* embryos. Nevertheless, the onset of Lmx1b expression in the mesencephalic ventral midline precedes Lmx1a expression. The present observation that Lmx1b can induce Lmx1a expression in the m6 VZ of NE-Lmx1b transgenic embryos suggests that Lmx1b acts upstream of Lmx1a and specifies mesDA neurons mainly by inducing Lmx1a. Nevertheless, generation of Th^+ $Nurr1^+$ mesDA neurons in Lmx1b null mutants (Smidt et al., 2000) cannot be simply explained by this idea. In addition, a recent report suggested that Lmx1b is dispensable for mesDA generation in their progenitors (Guo et al., 2008). Precise analysis of mesDA progenitor specification defects in Lmx1b-null and Lmx1a/Lmx1b double mutants will clarify this point.

The mechanism of FP cell specification in the mesencephalon

FP cells develop at the ventral midline of the neural tube and the ventralizing signal Shh has been revealed to be both necessary and sufficient for induction of FP cells (Strahle et al., 2004, Placzek and Briscoe, 2005). However, the intrinsic signals downstream of Shh that determine FP identity are still largely unknown. We observed cell autonomous induction of FP cells by Foxa2, although a loss-of-function experiment will be needed to address whether Foxa2 is indeed required for mesencephalic FP differentiation. Thus, by comparison with mesDA specification, Foxa2 appears to primarily control FP differentiation in the mesencephalon. In spite of our results demonstrating that Foxa2 is sufficient for FP induction in transgenic embryos, $Foxa2^+$ m6 progenitors do not acquire an FP identity in the wild-type condition. This can be explained by the fact that the m6 progenitors expressed Nkx6.1, which can inhibit FP differentiation even in the presence of Foxa2. It has been reported that Nkx6.1 is expressed throughout the ventral mesencephalon, including the ventral midline, at an early stage of development (around E9), and that Lmx1a, which is induced later, activates Msx1 expression, which in turn represses Nkx6.1 expression in the midline (Andersson et al., 2006b). This repression of Nkx6.1 by the Lmx1a-Msx1 pathway appears to be a prerequisite for FP formation as Corin expression in the mesencephalic midline starts after the downregulation of Nkx6.1 (Andersson et al., 2006b; Ono et al., 2007), and ectopic expression of Lmx1a represses Nkx6.1 in the m6 domain and consequently induces FP marker expression (this study). Thus, the $Foxa2^+$ $Nkx6.1^-$ context seems to be a determinant for FP cell fate in the mesencephalon, although analysis of Nkx6.1 null mice will be needed to conclude that only Nkx6.1 needs to be repressed for FP differentiation.

FP cells are formed at the ventral midline in the mesencephalon at around E9.75 (Andersson et al., 2006b). At this stage, FP cells in the mesencephalon are non-neurogenic, as in caudal regions. Importantly, these mesencephalic FP cells appear to have already been intrinsically fated to acquire neurogenic activity at the time of specification (Ono et al., 2007). Furthermore, it has been suggested that FP identity is sufficient to generate mesDA neurons in the context of the mesencephalon because Otx2, which can confer mesencephalic identity on FP cells, is sufficient to induce mesDA neurons from caudal FP cells (Ono et al., 2007). In support of this idea, Lmx1a/b can coincidently induce FP differentiation and mesDA generation in $Foxa2^+$ m6 progenitors. However, FP fate acquisition and mesDA specifications could be separated in the case of transgenic embryos expressing Foxa2, in which FP cells that did not generate correct mesDA neurons were induced, and in the case of transgenic embryos expressing Nkx6.1, in which FP differentiation was nearly completely inhibited but proper mesDA neurons were still generated, suggesting that proper differentiation of FP cells is neither required nor sufficient for mesDA progenitor specification. Nevertheless, in normal conditions, mesDA neurons emerge only from cells with FP identity (Ono et al., 2007). This can be explained by the proposed model that cooperative actions of Lmx1a/b and Foxa2 independently regulate the FP and mesDA determining pathways, and that as a consequence, FP and mesDA identities are coordinately specified in the same cells expressing both factors, although complementary loss-of-function studies will be necessary to prove this idea. Whether a similar mechanism of FP specification is used in the caudal neural tube is currently unknown. Future analyses concerning FP cell fate determination, especially identification of FP cell-specific factors, will be needed to elucidate the whole picture of FP and mesDA neuron development.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2009.12.017.

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