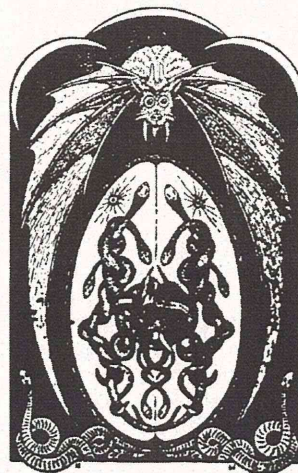
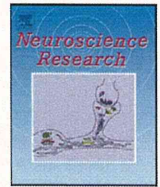


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Induction of superficial cortical layer neurons from mouse embryonic stem cells by valproic acid

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ABSTRACT

Within the developing mammalian cortex, neural progenitors first generate deep-layer neurons and subsequently more superficial-layer neurons, in an inside-out manner. It has been reported recently that mouse embryonic stem cells (mESCs) can, to some extent, recapitulate cortical development *in vitro*, with the sequential appearance of neurogenesis markers resembling that in the developing cortex. However, mESCs can only recapitulate early corticogenesis; superficial-layer neurons, which are normally produced in later developmental periods *in vivo*, are under-represented. This failure of mESCs to reproduce later corticogenesis *in vitro* implies the existence of crucial factor(s) that are absent or uninduced in existing culture systems. Here we show that mESCs can give rise to superficial-layer neurons efficiently when treated with valproic acid (VPA), a histone deacetylase inhibitor. VPA treatment increased the production of Cux1-positive superficial-layer neurons, and decreased that of Ctip2-positive deep-layer neurons. These results shed new light on the mechanisms of later corticogenesis.

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1. Introduction

The mammalian cerebral cortex displays a complex structure with a high diversity of neuronal subtypes (Molyneaux et al., 2007). Within this structure, approximately 80% of the neurons are pyramidal excitatory cells which are derived from radial glial progenitors in the ventricular zone (VZ), and are generated in a well defined spatio-temporal manner (Guillemot et al., 2006; Leone et al., 2008). The first neurons generated are Cajal-Retzius cells, which will reside in the most superficial cortical layer (layer I). Subsequent neurogenesis proceeds in an inside-out fashion, by producing deep-layer neurons (layers V and VI) and then progressively more superficial-layer neurons (layers II–IV).

We have devised an adherent monolayer culture of mouse embryonic stem cells (mESCs) for the efficient generation of neuroectodermal precursors and neural stem cells (Ying et al., 2003; Conti et al., 2005). This system reduces the limitation and complexity of neural induction that are characteristic of multicellular aggregation (Bain et al., 1995; Wiles and Johansson, 1999) and/or

co-culture methods (Kawasaki et al., 2000), and permits direct observation and manipulation of the cells under study. Recently, a better understanding of cortical neurogenesis was attained using our monolayer culture system. Gaspard et al. (2008) showed that, in the presence of sonic hedgehog (Shh) inhibitor, mESCs generate cortical neurons in a sequential fashion similar to that observed in the developing cortex. Despite this breakthrough result, the experiment failed to completely recapitulate later aspects of cortical development. Gaspard et al. (2008) had better success in generating early-born or deep-layer neurons than in generating superficial-layer neurons. Earlier, Shen et al. (2006) also reported that fewer superficial-layer neurons than deep-layer neurons were generated when they cultured cortical neural stem cells isolated at different gestational time points. This inefficiency in reproducing later events of cortical neurogenesis *in vitro* implies the existence of crucial factors which are not present or induced in current experimental systems.

Epigenetic mechanisms such as DNA methylation and histone modification including acetylation are now known to be critical intrinsic programs that dictate fate specification and differentiation of stem cells. Histone acetylation and deacetylation are mediated by histone acetyltransferases (HATs) and histone deacetylases (HDACs), respectively. In general, an increase of histone acetylation

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RESEARCH ARTICLE

STEM CELLS AND REGENERATION

Foxa2 acts as a co-activator potentiating expression of the Nurr1-induced DA phenotype via epigenetic regulation

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ABSTRACT

Understanding how dopamine (DA) phenotypes are acquired in midbrain DA (mDA) neuron development is important for bioassays and cell replacement therapy for mDA neuron-associated disorders. Here, we demonstrate a feed-forward mechanism of mDA neuron development involving Nurr1 and Foxa2. Nurr1 acts as a transcription factor for DA phenotype gene expression. However, Nurr1-mediated DA gene expression was inactivated by forming a protein complex with CoREST, and then recruiting histone deacetylase 1 (Hdac1), an enzyme catalyzing histone deacetylation, to DA gene promoters. Co-expression of Nurr1 and Foxa2 was established in mDA neuron precursor cells by a positive cross-regulatory loop. In the presence of Foxa2, the Nurr1-CoREST interaction was diminished (by competitive formation of the Nurr1-Foxa2 activator complex), and CoREST-Hdac1 proteins were less enriched in DA gene promoters. Consequently, histone 3 acetylation (H3Ac), which is responsible for open chromatin structures, was strikingly increased at DA phenotype gene promoters. These data establish the interplay of Nurr1 and Foxa2 as the crucial determinant for DA phenotype acquisition during mDA neuron development.

KEY WORDS: Foxa2, Nurr1, Midbrain dopamine neuron, Development, Neural precursor cell, Epigenetic control, CoREST, Hdac, Mouse

INTRODUCTION

Midbrain dopamine (mDA) neurons play important roles in voluntary movement, emotion and reward-based behaviors. Dysfunction or degeneration of this neuronal subtype is related to major neuropsychiatric disorders such as Parkinson's disease (PD), schizophrenia and drug addiction. Owing to the pathophysiological implications, mDA neurons are the most extensively studied cells. A molecular understanding of mDA neuron development is of high clinical interest as replacing this cell population in diseased brains is considered to be one of the most promising therapeutic approaches for PD (Deierborg et al., 2008; Morizane et al., 2008). In addition, developmental information can be exploited to establish optimal bioassays for mDA neuron-related disorders.

mDA neurons arise from floor plate cells at the ventral midline of the embryonic midbrain (Bonilla et al., 2008; Ono et al., 2007). Sonic hedgehog (Shh), secreted initially by the notochord and later by floor plate cells, induces expression of forkhead family of winged-helix transcription factor 2 (Foxa2; also known as hepatocyte nuclear factor 3 beta), in the midbrain floor plate cells [mouse embryonic day (E) 8.5] (Ang et al., 1993; Monaghan et al., 1993; Placzek, 1995; Sasaki and Hogan, 1994; Sasaki et al., 1997). Foxa2 acts as a master regulator to induce expression of developmental factors specifying mDA neuron precursors such as Nurr1, Pitx3, Lmx1a, Msx1, neurogenin 2 and Mash1 (Ascl1 – Mouse Genome Informatics) (Ang, 2009; Ferri et al., 2007; Kittappa et al., 2007; Lee et al., 2010; Metzakopian et al., 2012). The early inductive role of Foxa2 is probably achieved by cooperation with the Wnt-Lmx1a/b regulatory loop from the isthmus organizer (Chung et al., 2009; Nakatani et al., 2010). The mDA neuron precursors equipped with a battery of developmental factors finally differentiate into mDA neurons during the late stages of ventral midbrain (VM) development.

Nurr1 (Nr4a2 – Mouse Genome Informatics) is an orphan nuclear receptor expressed in late mDA neuron precursors (Saucedo-Cardenas et al., 1998; Zetterström et al., 1996). Mice lacking Nurr1 lack DA phenotype gene expression in the midbrain (Le et al., 1999; Saucedo-Cardenas et al., 1998; Zetterström et al., 1997). Nurr1 has been reported to act as a transcription factor for expression of tyrosine hydroxylase (Th), the rate-limiting enzyme for DA synthesis (Sakurada et al., 1999). Gain-of-function studies have demonstrated that forced Nurr1 expression induces complete DA phenotype gene expression in naive, non-dopaminergic neural precursor cells (NPCs) (Kim et al., 2003a; Shim et al., 2007; Wagner et al., 1999). Thus, Nurr1 is regarded as the most crucial factor in acquiring the DA phenotype during late mDA neuron development. However, Nurr1 expression alone frequently fails to induce the DA phenotype in NPCs (Jin et al., 2006; Lee et al., 2010; Park et al., 2008). In addition, controlled Nurr1 expression at levels and timing similar to physiological levels and timing in the developing midbrain inefficiently induces DA phenotype gene expression (Park et al., 2012). These findings, combined with common properties of nuclear hormone receptor proteins, which are regulated by co-activators and co-inhibitors (Aranda and Pascual, 2001; Purcell et al., 2011; Xu et al., 1999), indicate that the Nurr1-induced DA phenotype expression may require other proteins in the developing VM. Several co-activators have been reported to cooperate with Nurr1 in mDA neuron development (Jacobs et al., 2009b; Lee et al., 2010; Martinat et al., 2006).

Foxa2 expression continues in late mDA precursors and neurons, suggesting that it plays a role in late developmental stages, but how Foxa2 functions in late mDA neuron development is unknown. In this study, we demonstrate that Foxa2 colocalizes with Nurr1 in mDA neuron precursors and acts as a potent co-activator in Nurr1-

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induced DA phenotype gene expression. We further identified a mechanism of interplay between these proteins, in which Foxa2 physically interacts with Nurr1 to form a protein complex on DA phenotype gene promoters and activates epigenetic regulation of gene transcription.

RESULTS

Foxa2 and Nurr1 colocalize in mDA neuronal precursors and post-mitotic neurons *in vivo* and *in vitro*

Consistent with Foxa2 as an early marker in developing VM, it was detected in mouse embryonic VM at E9 (Fig. 1A,C) (Ferri et al., 2007) and colocalized with the proliferating cell marker, proliferating cell nuclear antigen (Pcna) (Fig. 1A, inset). By contrast, Nurr1 was not expressed in early embryonic VM (Fig. 1B). At a later embryonic stage (E12), Nurr1 was localized to the intermediate and mantle zone (MZ) of the VM, and absent from the proliferating ventricular zone (VZ) (Fig. 1E) (Andersson et al., 2008; Jönsson et al., 2009). At E12, Foxa2 was expressed in broader regions of the VM, including regions in which Nurr1 was expressed, which extended to the VZ (but weaker expression here than in the mantle zone) and to the lateral portions

(Fig. 1D,F). In the Nurr1-expressing domain, Foxa2 was largely colocalized with Nurr1 (Fig. 1F) (Ferri et al., 2007), and cells expressing both Nurr1 and Foxa2 in the MZ acquired the DA phenotype, characterized by Th expression (Fig. 1M). Virtually all Th⁺ cells in the developing VM expressed both Nurr1 and Foxa2, whereas no cells expressing Nurr1 or Foxa2 alone acquired the DA phenotype (Fig. 1O). As development proceeded (E15), the Foxa2 expression domain became narrower and was restricted to the domain of Nurr1⁺ cells (Fig. 1G-I). In the adult midbrain, Nurr1 and Foxa2 were almost exclusively colocalized in Th⁺ DA neurons (data not shown). By comparison, Foxa2 expression was not detected in Nurr1⁺ *cornu ammonis* (CA) neurons of the hippocampus, which are not dopaminergic (data not shown).

NPC cultures derived from rodent VM in early embryonic development maintain DA neurogenic potential and embryonic VM-specific gene expression (Jo et al., 2007; Park et al., 2012). Thus, VM-NPC cultures were used as a bioassay system to understand mDA neuron development. NPCs isolated from mouse VM at E10-11 were allowed to proliferate *in vitro* and differentiate. As in the developing VM, Foxa2 expression was detected from the

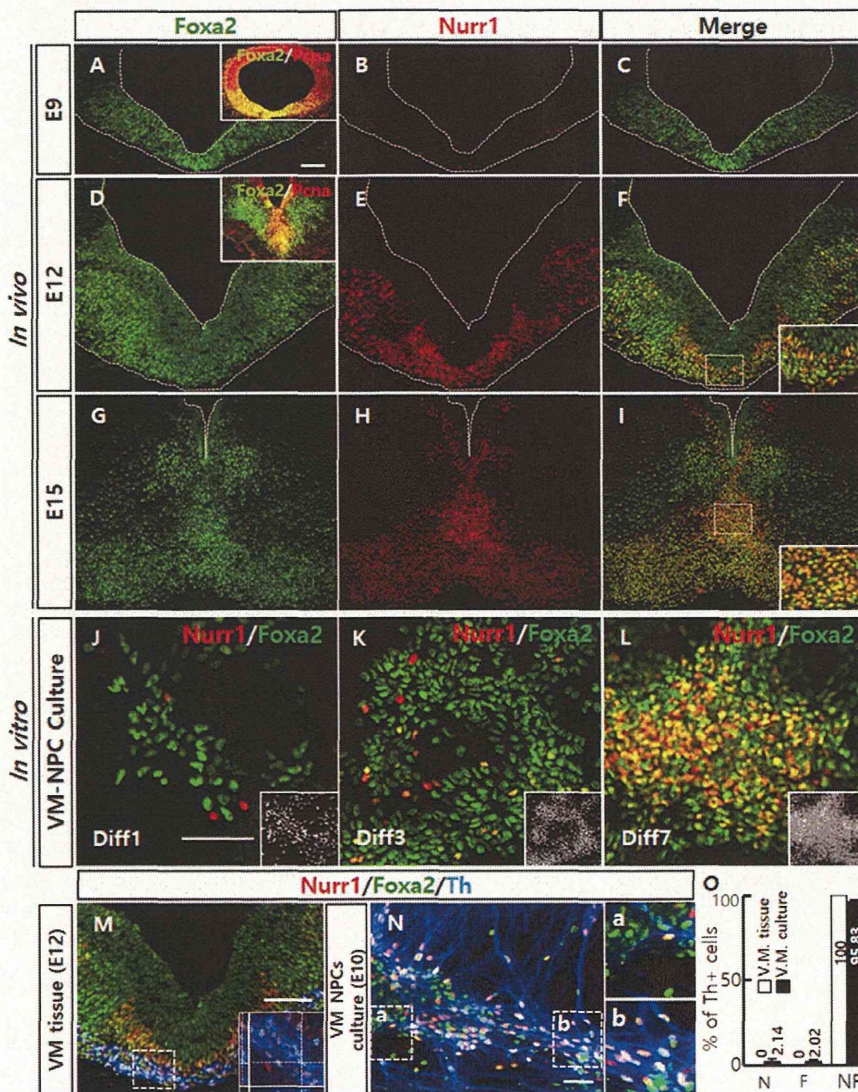


Fig. 1. Colocalization of Foxa2 and Nurr1 in late-stage mDA neuronal development.

(A-I) The ventral midbrain (VM) of E9 (A-C), E12 (D-F) and E15 (G-I) mouse embryos was cryosectioned (12 μ m) and subjected to immunofluorescence staining for Foxa2 (A,D,G) and Nurr1 (B,E,H). Insets in A and D show colocalization of Foxa2 with the proliferating cell marker Pcna in the neighboring sections. Shown in C,F and I are merged images for Nurr1/Foxa2-colabeled cells. Boxed areas in F and I are enlarged in insets. (J-L) NPCs cultured from mouse VM at E11, and stained with Foxa2 and Nurr1 1 (J), 3 (K) and 7 (L) days after NPC differentiation *in vitro*. Insets show DAPI⁺ cells. (M-O) Localization of the DA neuronal marker TH in cells expressing both Nurr1 and Foxa2. VM tissue sections (M) and VM-NPC cultures at differentiation day 7 (N) were triple-stained with Foxa2 (green), Nurr1 (red) and Th (blue) antibodies. Inset of M shows z-stacked confocal image of the boxed area along the y-axis (left) and x-axis (lower). Areas with abundant Th-positive cells (a) and Th-negative cells (b) in N are enlarged at high magnification in the panels to the right. Graph in O depicts percentage of TH-positive cells expressing Nurr1 only (N), Foxa2 only (F) and Nurr1+Foxa2 (NF) *in vivo* (white bars) and *in vitro* (black bars). Scale bars: 50 μ m.

proliferation and early differentiation periods, whereas *Nurr1* was expressed 2-3 days after induction of differentiation (Fig. 1J,K); later, *Nurr1* was abundantly expressed and localized to *Foxa2*⁺ cells in differentiating VM-NPC cultures (Fig. 1L). Th⁺ cells were detected 1-2 days after *Nurr1* expression began (3-4 days after differentiation) (Park et al., 2012) and reached a maximum 7-9 days after induction of differentiation (Fig. 1N). The DA neuronal properties of the Th⁺ cells were confirmed by colocalization of other markers specific for DA homeostasis [dopamine transporter (Dat; Slc6a3 – Mouse Genome Informatics) and vesicle monoamine transporter 2 (*Vmat2*; Slc18a2 – Mouse Genome Informatics)] and neuronal cells [TuJ1 (Tubb3 – Mouse Genome Informatics) and microtubule-associated protein 2 (Map2)] (data not shown) (He et al., 2011). Of all Th⁺ DA neurons on differentiation day 7, most (95.8±1.8%) expressed both *Nurr1* and *Foxa2* (*Nurr1*⁺, *Foxa2*⁺), whereas only 2.1±1.5% expressed *Nurr1* only (*Nurr1*⁺, *Foxa2*⁻) and 2.0±1.3% expressed *Foxa2* only (*Nurr1*⁻, *Foxa2*⁺) (Fig. 1O). These findings, taken together, indicate that *Nurr1* and *Foxa2*, which

colocalize in mDA neuron precursors and neurons, may functionally interact to induce mDA neuronal differentiation.

Foxa2 and *Nurr1* expression establish a positive cross-regulatory loop

When *Foxa2* expression was downregulated in the VM-NPC cultures by short hairpin *Foxa2* (sh*Foxa2*) RNA treatment, *Nurr1* mRNA expression decreased significantly (Fig. 2A,B). This result is consistent with the idea that *Foxa2* is a master regulator inducing expression of a battery of transcriptional factors specific for midbrain development (including *Nurr1*) (Ang, 2009; Ferri et al., 2007; Kittappa et al., 2007; Lee et al., 2010; Metzakopian et al., 2012). The opposite was also true: *Foxa2* mRNA expression decreased in VM-NPC cultures treated with sh*Nurr1* (Fig. 2D,E). Similarly, *Foxa2* protein expression in the *Nurr1*-expressing intermediate/mantle zone was greater than in the *Nurr1*-negative VZ [mean fluorescence intensity (MFI) of individual *Foxa2*-stained cells: 126.78±4.01 versus 64.76±1.86; 40 cells per group, Student's *t*-test, *P*<0.001;

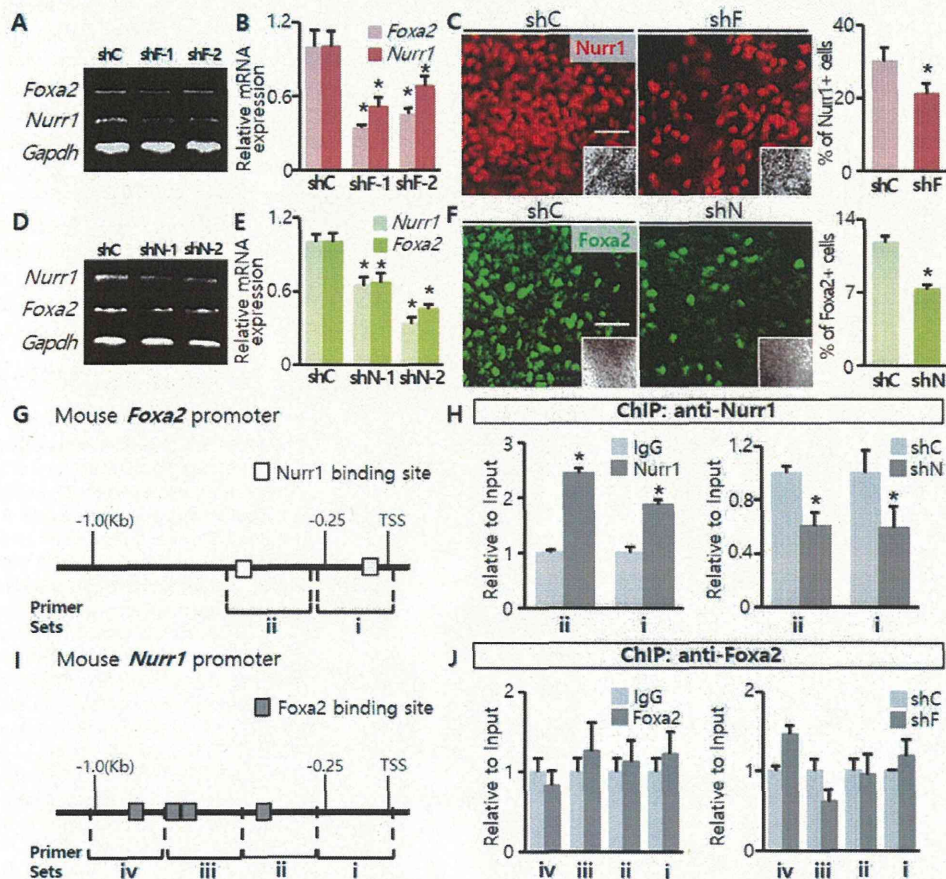


Fig. 2. Positive cross-regulatory loop between *Nurr1* and *Foxa2*. (A-F) NPCs were directly isolated and cultured from VM of mouse embryos at E11, and treated with sh*Foxa2* (shF; A-C), sh*Nurr1* (shN; D-F) or shControl (shC). Semi-quantitative (A,D) and real-time PCR (B,E) analyses of *Nurr1* and *Foxa2* mRNA expression were carried out 6 days after differentiation. *Gapdh*, glyceraldehyde 3-phosphate dehydrogenase (housekeeping gene). Reduced *Nurr1* expression by sh*Foxa2* and *Foxa2* expression by sh*Nurr1* were confirmed by immunocytochemical analyses (C,F). Images are representative clusters of *Nurr1*⁺ (C) and *Foxa2*⁺ (F) cells. Insets, DAPI⁺ cells in the same microscopic fields. Graphs on the right depict the percentage of immunoreactive cells in 20-40 clusters randomly selected from three independent cultures. **P*<0.01(C) or *P*<0.001(F) compared with shControl. Scale bars: 30 μ m. (G-J) ChIP analyses for *Nurr1* protein enrichment in the *Foxa2* promoter region (H) and *Foxa2* protein enrichment in the *Nurr1* promoter region (J). G and I show schematics of the *Foxa2* (G) and *Nurr1* (I) promoter regions within -1 kb from the TSS with the predicted *Nurr1* (*Foxa2*) binding sites and ChIP-PCR primer sets. *Nurr1* (and *Foxa2*) protein occupancies at the consensus binding sites were estimated by comparing ChIP values in the immunoprecipitates generated by anti-*Nurr1* (and anti-*Foxa2*) antibody with IgG (left-hand graphs in H and J). Protein binding in the promoter regions was confirmed in the VM-NPC cultures treated with shRNA (right-hand graphs in H and J). **P*<0.01 compared with respective controls, *n*=3-6. Error bars represent s.e.m.

supplementary material Fig. S1G]. In addition, the number of cells immunoreactive for Nurr1 and Foxa2 was significantly reduced by shFoxa2 and shNurr1 treatments, respectively (Fig. 2C,F). A gain-of-function study further confirmed the cross-activation of gene expression between Nurr1 and Foxa2 (supplementary material Fig. S1). Two regions are predicted to be Nurr1 binding sites within -1 kb of the transcription start site (TSS) of the mouse *Foxa2* promoter (Jaspar database; <http://jaspar.genereg.net>) (supplementary material Table S3; Fig. 2G). Chromatin immunoprecipitation (ChIP) assays in VM-NPC cultures demonstrated enriched Nurr1 protein at the consensus binding sites in precipitates generated with anti-Nurr1 antibody compared with IgG (Fig. 2H). Furthermore, the Nurr1 protein occupancy in those promoter regions was significantly reduced by shNurr1 treatment, indicating that Nurr1 activates *Foxa2* transcription by directly binding *Foxa2* promoter regions. Four consensus sites for Foxa2 binding are predicted within -1 kb of the mouse *Nurr1* promoter (supplementary material Table S3; Fig. 2I). However, we did not detect Foxa2 protein occupancy in the *Nurr1* promoter region by ChIP (Fig. 2J). Thus, unlike Foxa2 expression activated by Nurr1, the role of Foxa2 in activating Nurr1 expression is probably mediated through other regions of the *Foxa2* gene or may be indirect without acting on the promoter. Regardless, our data suggest that Foxa2 and Nurr1 reciprocally activate expression of each other, and that this positive cross-regulatory loop is likely to contribute to maintaining colocalization of these factors in mDA neuron precursors and neurons.

Foxa2 potentiates Nurr1-induced DA phenotype gene expression during NPC differentiation

As described in Fig. 1, DA phenotypes are acquired in late mDA neuronal precursors expressing both Nurr1 and Foxa2. In microarray

analyses of 25,697 genes, forced Nurr1 expression significantly increased (greater than twofold change) the expression of 375 genes, and Foxa2 increased the expression of 271 genes in differentiating NPC cultures. DA phenotype genes, such as *Th* and *Dat*, were increased only slightly by Nurr1 and Foxa2 (1.37- to 1.94-fold) (supplementary material Table S1; data not shown), suggesting that Nurr1 and Foxa2 expression alone is insufficient to induce differentiation into a DA phenotype. As Nurr1 is a major transcription factor for dopaminergic gene expression (Sakurada et al., 1999), we postulated that Foxa2 activates Nurr1-induced effects. To test this hypothesis, we next compared genes induced in NPCs by co-expressing Nurr1 and Foxa2 with expression of Nurr1 alone. mRNA expression of the DA genes *Th*, aromatic L-amino acid decarboxylase (*Aadc*; *Ddc* – Mouse Genome Informatics) and *Dat* was dramatically higher in cultures expressing Nurr1 and Foxa2 (Nurr1+Foxa2) than in those expressing Nurr1 alone (supplementary material Table S1). This result suggests that Foxa2 strongly potentiates the DA phenotype in the presence of Nurr1. The microarray data were confirmed by PCR and immunocytochemical analyses in Foxa2 loss- and gain-of-function assays (Fig. 3). Foxa2 downregulation in VM-NPC cultures significantly decreased mRNA expression of the DA genes *Th*, *Dat* and *Vmat2* (Fig. 3A,B). In addition, shFoxa2 treatment significantly reduced the number of Th^+ DA neurons that differentiated from VM-NPCs (Fig. 3C). Because total cell numbers were not significantly altered by shFoxa2 treatment (data not shown), the Foxa2 effect on cell proliferation (Lee et al., 2010) is not likely to have influenced the DA gene expression data, although this possibility cannot be completely excluded. Forced Nurr1 expression yielded a few TH^+ DA cells in NPC cultures derived from non-dopaminergic embryonic cortical tissues (Fig. 3F). Co-expression of Foxa2 and Nurr1 in NPCs

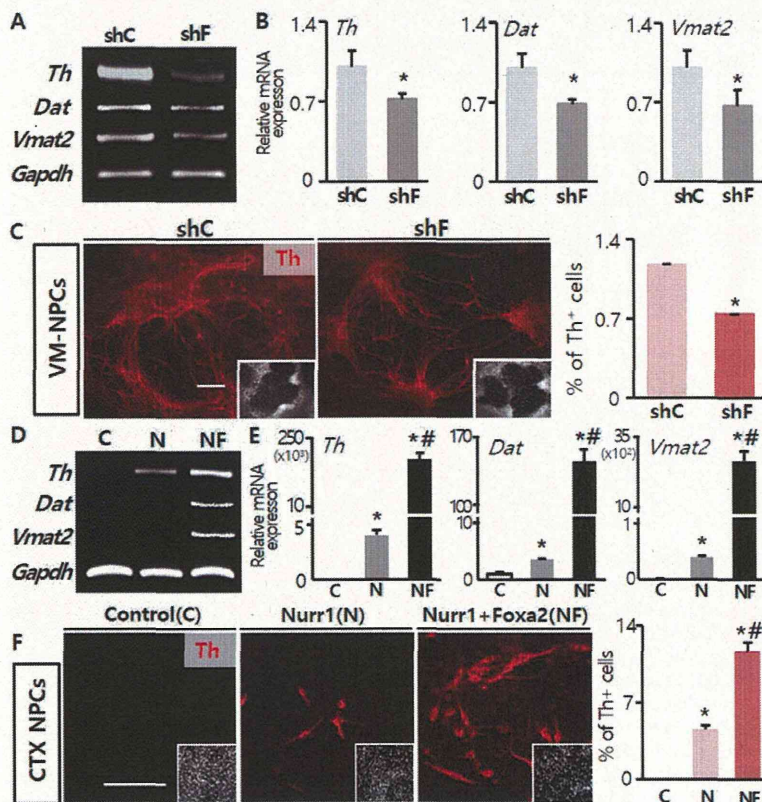


Fig. 3. Foxa2 roles in activating DA phenotype

expression. (A-C) Loss-of-function studies of Foxa2. NPCs were directly cultured from VMs of E11 mouse embryos and treated with shFoxa2 RNA (shF). The control cultures were transfected with control shRNA (shC). Semi-quantitative (A) and real-time (B) PCR analyses were carried out for mRNA expression of DA genes *Th*, *Dat* and *Vmat2* six days after differentiation. * $P < 0.05$ compared with shC-treated control ($n=6$). Foxa2 downregulation effects on DA gene expression were further assessed by immunocytochemical analyses of Th^+ cells (C). Images represent Th^+ DA neurons in shControl and shFoxa2-treated cultures. Insets show DAPI+ cells in the same microscopic fields. The graph on the right depicts the percentage of Th -immunoreactive cells from three independent cultures. * $P < 0.005$ compared with control shRNA. (D-F) Gain-of-function analyses of Foxa2. NPCs derived from E12 mouse cortices were transfected with Nurr1 (N), Nurr1+Foxa2 (NF) or empty vector (C). Effects of Foxa2 co-expression on DA phenotype expression were assessed by semi-quantitative PCR (D), real-time PCR (E) and immunocytochemistry (F). * $P < 0.001$ compared with control; # $P < 0.001$ compared with Nurr1-transduced cultures ($n=3$ for each). Error bars represent s.e.m. Scale bars: 100 μ m.

dramatically increased Th⁺ cell yields (Fig. 3F) along with DA phenotype mRNA expression (Fig. 3D,E). The midbrain-type DA neuronal phenotypes of the Th⁺ cells were confirmed by the co-expression of markers specific for neurons (TuJ1, Map2), mature DA neurons (Dat) and midbrain-type DA neurons [Pitx3, Girk2 (Kcnj6 – Mouse Genome Informatics)] (supplementary material Fig. S2) (Lee et al., 2010).

Foxa2 facilitates Nurr1 recruitment to the *Th* and *Dat* promoters

The synergistic increase in transcription of DA phenotype genes by these factors (Fig. 3) indicates that Nurr1 and Foxa2 interact on the gene promoters. To examine this interaction, NPCs were transduced with HA-tagged Foxa2 (HA-Foxa2), Flag-tagged Nurr1 (Flag-Nurr1) or HA-Foxa2+Flag-Nurr1, and their interactions with exogenous proteins on the promoter DNA were assessed using HA or Flag antibodies. These gain-of-function assays allow determination of pure and direct Nurr1-Foxa2 protein interactions, compared with interactions between endogenous Nurr1 and Foxa2 in VM-NPC cultures where endogenous expression of one protein is affected by overexpression/downregulation of the other (Fig. 2). Because of possible Foxa2-Nurr1 interactions occurring far from the

TSS, we analyzed relatively long promoter segments of the DA genes (up to -2.5 kb). Multiple consensus Foxa2 and Nurr1 binding sites are predicted within -2.5 kb of the TSS of the *Th* promoter (Fig. 4A; supplementary material Table S3). Whereas Foxa2 binding sites are located within 2.5 kb upstream of the TSS, Nurr1 binding sites are predicted in regions close to the TSS (within 1.2 kb) (Fig. 4A; supplementary material Table S3). In ChIP assays, Nurr1 protein was enriched in multiple regions comprising not only consensus binding sites but also unpredicted sites far from the TSS (Fig. 4B). These findings indicate that in addition to direct protein-DNA binding to consensus sequences, Nurr1 proteins can be recruited to *Th* promoter regions indirectly via other proteins. Indirect Nurr1 binding to promoter DNA has previously been reported (Glass and Ogawa, 2006; Saijo et al., 2009). Foxa family proteins decompact DNA from the nucleosome to increase accessibility of transcription factors to the DNA (Cirillo et al., 2002; Cirillo et al., 1998). Consistent with this, Nurr1 recruitment to *Th* promoter regions (II, IV, V, VI) in the presence of Foxa2 expression was much higher than with Nurr1 alone (Fig. 4B). This result suggests that Foxa2 functions as an epigenetic activator to promote *Th* gene transcription by enhancing Nurr1 access to the promoter regions. ChIP assays revealed significantly higher Foxa2 protein

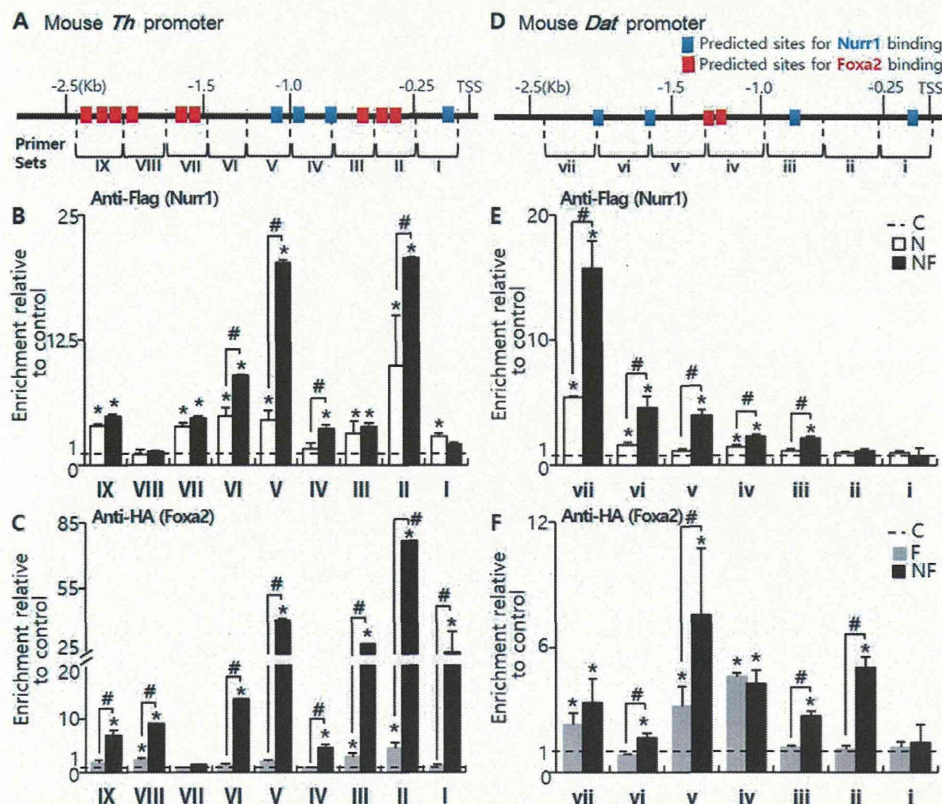


Fig. 4. Enrichment of Nurr1 and Foxa2 proteins at *Th* and *Dat* promoter regions. (A,D) Schematics of mouse *Th* (A) and *Dat* (D) promoters with predicted Nurr1 (blue boxes) and Foxa2 (red boxes) consensus binding sites. The promoter regions encompassing -2.5 kb from the TSS were divided into nine (A; I-IX) or seven (D; i-vii) sub-regions spanning ~200 bp each. (B,E) ChIP analyses to determine Nurr1 protein enrichments in the *Th* (B) and *Dat* (E) promoters. NPCs derived from non-dopaminergic cortical tissues of mouse embryos were transduced with Flag-Nurr1 (N), Flag-Nurr1+HA-Foxa2 (NF) or an empty control vector (C), and immunoprecipitated with Flag antibody at differentiation day 3 *in vitro*. Immunoprecipitated DNA fragments were subjected to real-time PCR analyses using primers designed to detect the promoter regions. In cultures transduced with Nurr1 alone (N), regions with significantly greater values than the controls (transduced with the empty vector, dashed horizontal line) were considered to be real protein-binding regions ($*P < 0.01$). $\#P < 0.05$ [significantly greater in Nurr1+Foxa2-cotransduced cultures (NF) than N], $n=3$, one-way ANOVA. (C,F) Foxa2 protein enrichment in the *Th* and *Dat* promoter regions. NPCs were transduced with HA-Foxa2 (F), Flag-Nurr1+HA-Foxa2 (NF) or empty control vector, and immunoprecipitated with HA antibody. Symbols represent Foxa2 binding values significantly different from the control ($*P < 0.05$) and from F alone ($\#P < 0.05$) in triplicate determinations. Error bars represent s.e.m.

levels at the predicted sites in cultures transduced with HA-Foxa2 than when transduced with a control vector (Fig. 4C). Foxa2 recruitment to the Foxa2 binding regions (II, III, V, VIII, IX) was also remarkably enhanced by Nurr1 (Fig. 4C). Collectively, these findings suggest that Nurr1 and Foxa2 mutually and reciprocally facilitate protein recruitment to promoter DNA. Flag-Nurr1 and HA-Foxa2 also occupied multiple promoter regions of *Dat*, another DA phenotype gene (Fig. 4E,F). As with the *Th* promoter, recruitment of exogenous protein was dramatically enhanced in cultures co-expressing HA-Foxa2+Flag-Nurr1 (Fig. 4E,F).

Formation of an activator complex of Nurr1 and Foxa2 proteins on the *Th* promoter

In the ChIP analyses shown in Fig. 4B,C, regions II, III, V and IX of the *Th* promoter were co-occupied by Foxa2 and Nurr1. Similarly, multiple *Dat* promoter regions were co-occupied by Foxa2 and Nurr1 in cultures expressing Foxa2+Nurr1 (Fig. 4E,F). Collectively, these findings suggest that Nurr1 and Foxa2 proteins interact physically and generate a functional protein complex on these DA phenotype gene promoters. This idea is consistent with previous reports of crosstalk between nuclear hormone receptors and Foxa family proteins (Eeckhoutte et al., 2006; Hurtado et al., 2011; John et al., 2011; Lupien et al., 2008; Nitsch et al., 1993; Wang et al., 2009). Both Foxa2 and Nurr1 showed a granular distribution

within the nucleus (Fig. 5A). Overlaying Nurr1 and Foxa2 confocal images revealed that the two proteins colocalize within the same nuclear speckles. We performed a detailed quantitative colocalization analysis using an intensity correlation algorithm (Li et al., 2004). Foxa2 and Nurr1 showed mutually dependent localization. The overlap of Foxa2 and Nurr1 staining is reflected by a high Pearson's correlation (0.8923 ± 0.0167) and overlap coefficient (0.915 ± 0.0151), indicating strong colocalization (Fig. 5A). In immunoprecipitation (IP) analyses of precursor cells transduced with Flag-Nurr1+HA-Foxa2, Nurr1 was detected in HA antibody (Foxa2) precipitates, and Foxa2 was present in Flag (Nurr1) precipitates (Fig. 5B). Endogenous Nurr1 and Foxa2 binding was also manifested by IP assays in VM-NPC cultures (Fig. 6F). The physical protein-protein interaction between these two proteins was further confirmed by an *in situ* proximity ligation assay (PLA), which allows visualization of protein-protein binding by fluorescence emanating from two proteins in close proximity (Fig. 5C,D).

Inhibitory role of CoREST in DA gene expression by protein interaction with Nurr1

CoREST (also known as Rcor2 – Mouse Genome Informatics) is a common epigenetic repressor that is widely expressed in the developing brain (Fuentes et al., 2012; Tontsch et al., 2001) and is

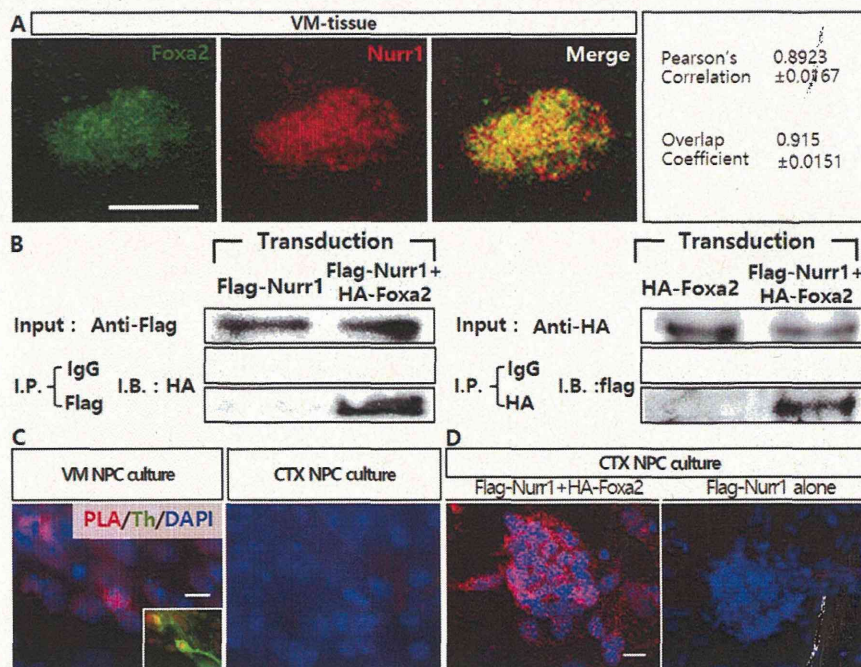


Fig. 5. Protein-protein interactions between Foxa2 and Nurr1 in mDA neuron precursors. (A) Confocal microscopic images of nuclei stained with Nurr1 and Foxa2. Nuclei were selected from Th^+ cells in the VM tissues of mouse embryos at E12 *in vivo* (Fig. 1M). Shown are representative images of a single nucleus co-stained with Foxa2 (green) and Nurr1 (red). Colocalization was assessed by Pearson's correlation and overlap coefficient values ($n=6$, shown on the right). Nurr1 colocalization with other nuclear proteins (Pitx3, NeuN, HuC/D) are also quantitatively analyzed in supplementary material Fig. S3. Scale bar: 5 μ m. (B) IP assays for physical interactions between Foxa2 and Nurr1 proteins. NPCs derived from embryonic cortices were co-transduced with Flag-Nurr1+HA-Foxa2, and subjected to IP assays. As negative controls, NPCs were transduced with Flag-Nurr1 alone (left) or HA-Foxa2 alone (right). Nurr1-Foxa2 protein binding was detected by immunoblot (IB) analysis using an anti-HA antibody in the immunoprecipitate (IP) generated by anti-Flag antibody treatment (left) as well as Flag-IB assay followed by HA-IP (right). (C,D) *In situ* proximity ligation assay (PLA) designed to detect protein-protein interactions by fluorescence (red). (C) Endogenous Nurr1-Foxa2 protein interactions were assessed in differentiating E10 VM-NPCs (left) or cortical NPCs (right, negative control) after 5 days of *in vitro* differentiation using anti-Nurr1 and anti-Foxa2 antibodies. After the PLA reaction, immunofluorescence staining for *Th* (green) was carried out. Inset of left image, Th^+ cells emanating red fluorescence (PLA*) in the same microscopic field. (D) PLA assays demonstrating interaction between exogenous Nurr1 and Foxa2. NPCs were transduced with Flag-Nurr1+HA-Foxa2 (left) or Flag-Nurr1 alone (right, control), and then PLA reactions were carried out using anti-Flag and anti-HA antibodies. Scale bars: 10 μ m.

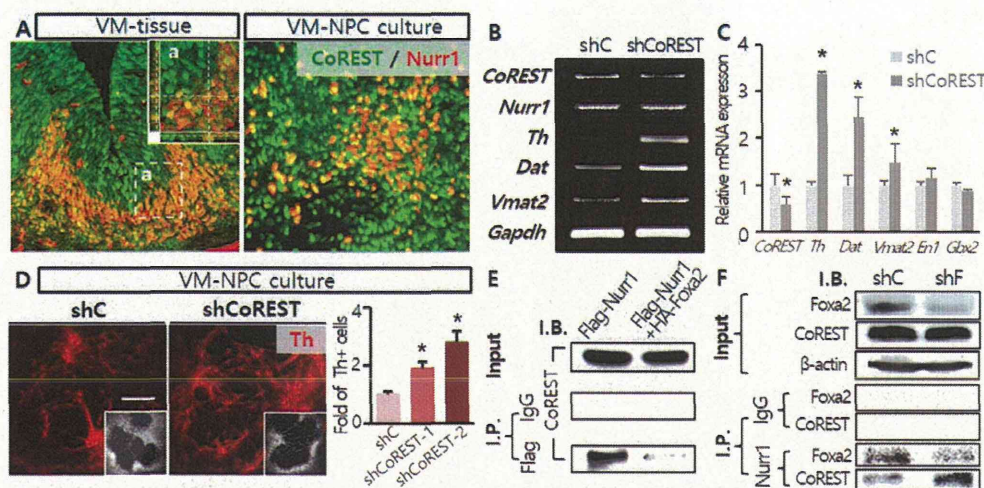


Fig. 6. Inhibitory role of CoREST in mDA neuron development. (A) Colocalization of CoREST with Nurr1 in the developing (E12.5) VM tissue *in vivo* (left) and in VM-derived cultures *in vitro* (right). Inset of left image shows z-stacked confocal image of the boxed area along the *y*-axis (left) and *x*-axis (lower). (B-D) Effect of CoREST knockdown on VM-NPC cultures. NPCs derived from mouse VM at E11, were transduced with shCoREST or control shRNA (shC), then induced to differentiate for 6 days. CoREST knockdown effects on DA neuron differentiation were estimated by mRNA expression of the DA genes *Th*, *Dat* and *Vmat2* using semi-quantitative (B) and real-time (C) PCR analyses, and Th^+ DA neuronal yields after immunostaining (D). Insets in D show DAPI⁺ cells in the same microscopic fields. * $P < 0.005$ compared with control ($n=6$; C) and * $P < 0.05$ compared with control ($n=3$; D). Scale bar: 100 μ m. (E, F) IP assays for CoREST-Nurr1 protein binding, which is abolished in the presence of Foxa2. (E) NPCs derived from mouse embryonic cortices were transduced with Flag-Nurr1 (right lanes) or Flag-Nurr1+HA-Foxa2 (left lanes). Cell lysates were immunoprecipitated (IP) with anti-Flag antibody (or IgG as the negative control) and then subjected to immunoblot (IB) analyses with CoREST antibody. Note that the CoREST band precipitated by Flag antibody (left lane) disappeared in the presence of Foxa2 co-expression (right lane). (F) Alteration of endogenous Nurr1-CoREST protein binding by Foxa2 was further confirmed in NPC cultures derived from mouse VM at E10 exposed to shControl (shC, left lane) or shFoxa2 (shF, right lane). Error bars represent s.e.m.

involved in fate decisions of NPCs and proximate progenitor species (Abrajano et al., 2010), including neuronal subtype specifications (Abrajano et al., 2009). CoREST protein was localized to DA gene promoters in our previous study (He et al., 2011). In addition, CoREST has been reported to interact with Nurr1 (Saijo et al., 2009). Based on these previous findings, we postulated that CoREST inhibits mDA neuron development, probably by inhibiting Nurr1-induced DA gene transcription. Indeed, CoREST protein was ubiquitously expressed and colocalized with Nurr1 in mouse embryonic VM *in vivo* and VM-NPC cultures *in vitro* (Fig. 6A). Downregulation of CoREST in VM-NPC cultures with shCoREST significantly increased mRNA levels specific for the DA phenotype (Fig. 6B,C), and increased DA neuronal yields after differentiation (Fig. 6D). Transcript levels of genes unrelated to DA phenotype determination, such as engrailed 1 (*En1*, involved in midbrain development and mDA neuron survival) and *Gbx2* (caudal brain marker), were not altered by CoREST knockdown (Fig. 6C), indicating a specific CoREST role in DA phenotype gene expression. Nurr1 expression was also not significantly altered by CoREST knockdown (Fig. 6C; data not shown), suggesting that the CoREST-mediated inhibition of DA phenotype gene expression is not mediated by controlling Nurr1 expression. CoREST proteins were co-precipitated with Nurr1 in IP assays (Fig. 6E). Interestingly, the amount of CoREST that precipitated with Nurr1 was greatly reduced by Foxa2 (Fig. 6E). The Foxa2-mediated effect on Nurr1-CoREST protein interaction was further confirmed in VM-NPC cultures with shFoxa2 treatment (Fig. 6F). These findings collectively suggest that CoREST, in the absence of Foxa2, binds to Nurr1 and blocks Nurr1-induced DA gene expression, but the Nurr1-CoREST complex dissociates in the presence of Foxa2 and/or converts into a Nurr1-Foxa2 activator complex (Fig. 5).

The Nurr1-Foxa2 activator complex promotes histone acetylation of chromatin surrounding the *Th* and *Dat* promoters by releasing CoREST-Hdac1 from the promoter

We next examined CoREST protein occupancy at DA gene promoters. ChIP assays revealed that CoREST in cultures transduced with Nurr1 was enriched in *Th* and *Dat* promoters compared with cultures transduced with control empty vector (Fig. 7A,G). The regions occupied by CoREST comprised Nurr1 binding sites (II, IV, V, VI, IX of *Th* promoter; vii of *Dat* promoter) and regions neighboring Nurr1 binding sites (VIII of *Th* promoter). This result suggests that CoREST proteins are recruited with Nurr1 to DA gene promoters in a Nurr1-CoREST complex, and is consistent with the observation that CoREST is recruited to pro-inflammatory cytokine promoters only in the presence of Nurr1 (Saijo et al., 2009). Foxa2 co-expression significantly reduced CoREST enrichment in the *Th* (II, VI, VIII) and *Dat* (ii, iii, vii) promoter regions (Fig. 7A,G). Histone deacetylases (Hdacs) are epigenetic regulators of nuclear receptor-dependent differentiation (Nebbio et al., 2010). In addition, Hdac1 is a common component of the CoREST-mediated epigenetic repressor complex (He et al., 2011; Saijo et al., 2009). Similar to CoREST, Hdac1 was also recruited to the *Th* and *Dat* promoter regions, and Hdac1 recruitment was significantly reduced in the presence of Foxa2 (Fig. 7B,H). Consequently, histone 3 acetylation (H3Ac), a histone modification associated with open chromatin structures, was greatly increased at almost all *Th* and *Dat* promoter regions tested, in cultures transduced with Nurr1+Foxa2 compared with Nurr1 alone (Fig. 7C,I). Foxa2-mediated regulation of repressor protein recruitment and histone acetylation was confirmed by Foxa2-knockdown experiments in dopaminergic VM-NPC cultures. CoREST and Hdac1 proteins were enriched in several promoter regions of *Th* (VIII, VI or VII) and *Dat* (vii) in VM-NPCs as a result

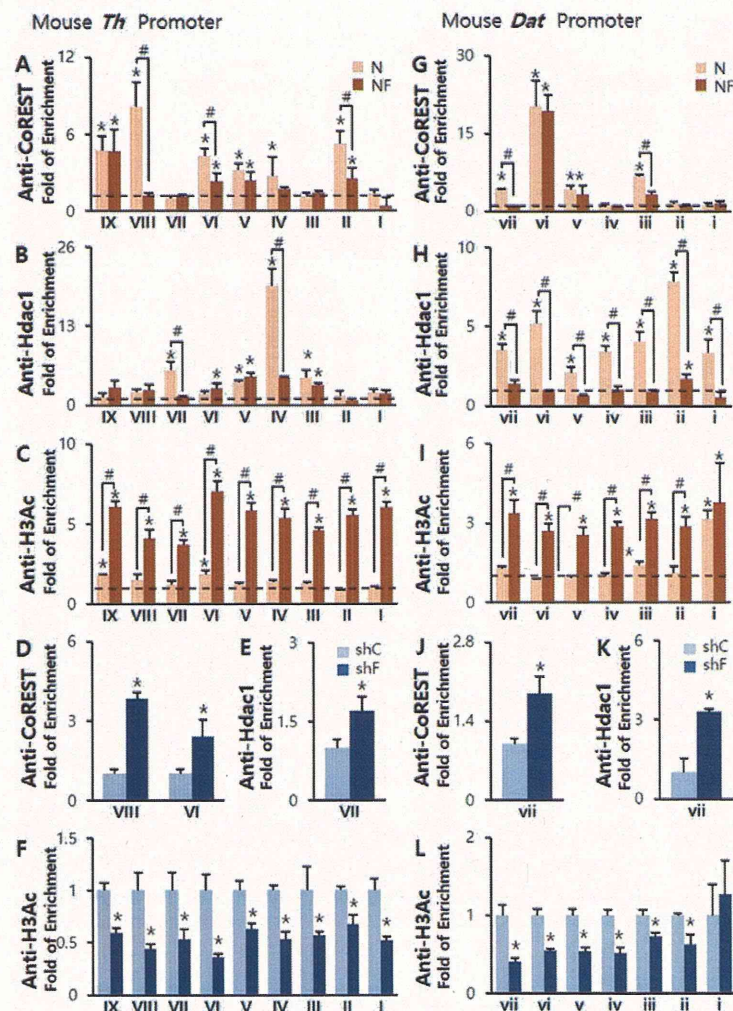


Fig. 7. Foxa2 induces histone 3 acetylation on the *Th* and *Dat* promoters by removing CoREST-Hdac1 repressors from those promoter regions. (A-C,G-I) ChIP analyses with exogenous Nurr1 and Foxa2 overexpression. NPCs derived from mouse cortices at E12 were transduced with viruses expressing Nurr1 (N), Nurr1+Foxa2 (NF) or control empty vector (dashed line), and immunoprecipitated with anti-CoREST (A,G), anti-Hdac1 (B,H) and anti-H3Ac (C,I) antibodies on differentiation day 3. Immunoprecipitated DNA fragments were subjected to real-time PCR analysis using primers designed to detect the promoter regions of *Th* (A-C) and *Dat* (G-I). * $P < 0.05$ compared with control or # $P < 0.05$ compared with N, $n = 3$, one-way ANOVA. (D-F,J-L) ChIP analyses for CoREST, Hdac1 and H3Ac in dopaminergic VM-NPC cultures expressing endogenous Nurr1 and Foxa2. NPCs derived from mouse embryonic VM at E11 were treated with shFoxa2 (shF) or shControl (shC), and subjected to ChIP analysis. * $P < 0.05$ compared with shC-treated control, $n = 3$, one-way ANOVA. Error bars represent s.e.m.

of shFoxa2 treatment (Fig. 7D,E,J,K). Similarly, levels of H3Ac at DA gene promoters were lower in VM-NPCs treated with shFoxa2, than in those treated with shControl (Fig. 7F,L). Collectively, these findings indicate that Nurr1 in the absence of Foxa2 inefficiently induces DA gene expression in the developing VM by forming a repressor complex with CoREST and Hdac1 that induces compact chromatin structures surrounding the DA gene promoters by histone deacetylation. In the presence of Foxa2, the repressor complex of Nurr1-CoREST-Hdac1 switches to an activator complex composed of Nurr1-Foxa2, in which Foxa2 (or both proteins) decompacts chromatin surrounding DA phenotype genes by increasing H3Ac levels. In these open chromatin structures, RNA polymerase and other transcriptional activators are efficiently recruited to the DA phenotype gene.

DISCUSSION

Understanding NPC differentiation into mDA neurons is important for establishing bioassay systems for drug discovery, as well as for regenerative medicine for mDA neuron-related disorders. Given the limited mDA neuronal differentiation of NPCs, these cells have been engineered with genes specific for mDA neuron development. Nurr1, a transcription factor that specifically induces DA gene expression in NPCs (Kim et al., 2003b; Sakurada et al., 1999), has

been the most promising candidate (Kim et al., 2003a; Shim et al., 2007; Wagner et al., 1999). However, like many nuclear receptors that are controlled by co-activators and co-repressors, Nurr1 alone is frequently insufficient to induce DA gene expression (Jin et al., 2006; Lee et al., 2010; Park et al., 2008). Several co-activators, such as Pitx3, Lmx1a and Foxa2, have previously been reported to potentiate Nurr1-induced DA gene transcription (Jacobs et al., 2009b; Lee et al., 2010; Martinat et al., 2006). When we examined and directly compared the co-activator activities of Pitx3, Lmx1a and Foxa2 in Nurr1-expressing NPCs *in vitro*, each of them increased Nurr1-induced Th^+ DA neuron yields, but Foxa2 had the strongest co-activator activity (supplementary material Fig. S4; data not shown). Given the role of Foxa2 in inducing Pitx3 (Lee et al., 2010) and Lmx1a (Lin et al., 2009; Metzakopian et al., 2012) expression, a portion of the Foxa2 co-activator activity observed may be indirectly mediated via Pitx3 and Lmx1a induction and their co-activator roles. More significantly, we obtained strong evidence that Foxa2 directly interacts with Nurr1 to induce DA gene expression during mDA neuron development.

We show that Foxa2 and Nurr1 reciprocally activate expression of each other. The cross activation, however, has not been clearly demonstrated in previous studies analyzing knockout mice. Foxa2 was not included in the gene list significantly changed in microarray