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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

Sang-Hoon Yi[1,2,‡], Xi-Biao He[1,2,‡], Yong-Hee Rhee[1,2,3], Chang-Hwan Park[2,3], Takumi Takizawa[4,*], Kinichi Nakashima[4] and Sang-Hun Lee[1,2,§]

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.

[1]Department of Biochemistry and Molecular Biology, College of Medicine, Hanyang University, Seoul 133-791, Korea. [2]Hanyang Biomedical Research Institute, Hanyang University, Seoul 133-791, Korea. [3]Graduate School of Biomedical Science and Engineering, Hanyang University, Seoul 133-791, Korea. [4]Laboratory of Molecular Neuroscience, Graduate School of Biological Sciences, Nara Institute of Science and Technology, Ikoma, Nara 630-0192, Japan. *Present address: Department of Pediatrics, Graduate School of Medicine, Gunma University, 3-39-22 Showa-machi, Maehashi, Gunma 371-8511, Japan. ‡These authors contributed equally to this work

§Author for correspondence (leesh@hanyang.ac.kr)

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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 isthmic 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

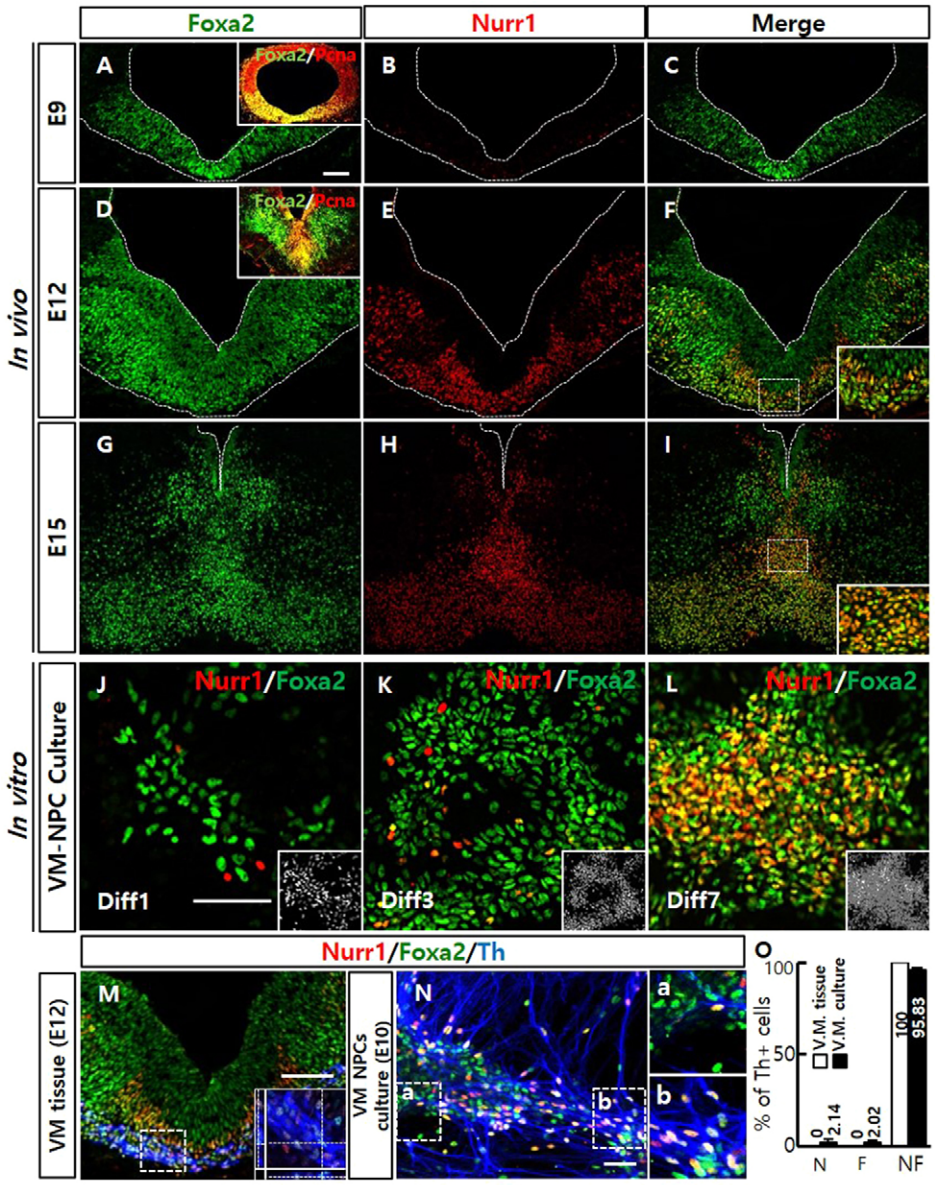

Fig. 1. Colocalization of Foxa2 and Nurr1 in late-stage mDA neuronal development. (A-I) The ventral midbrain (VM) of E9 (A-C), E12 (D-F) and E15 (G-I) mouse embryos was cryosectioned (12 µm) and subjected to immunofluorescence staining for Foxa2 (A,D,G) and Nurr1 (B,E,H). Insets in A and D show colocalization 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 µm.

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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 (shFoxa2) 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 shNurr1 (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, $P<0.001$;

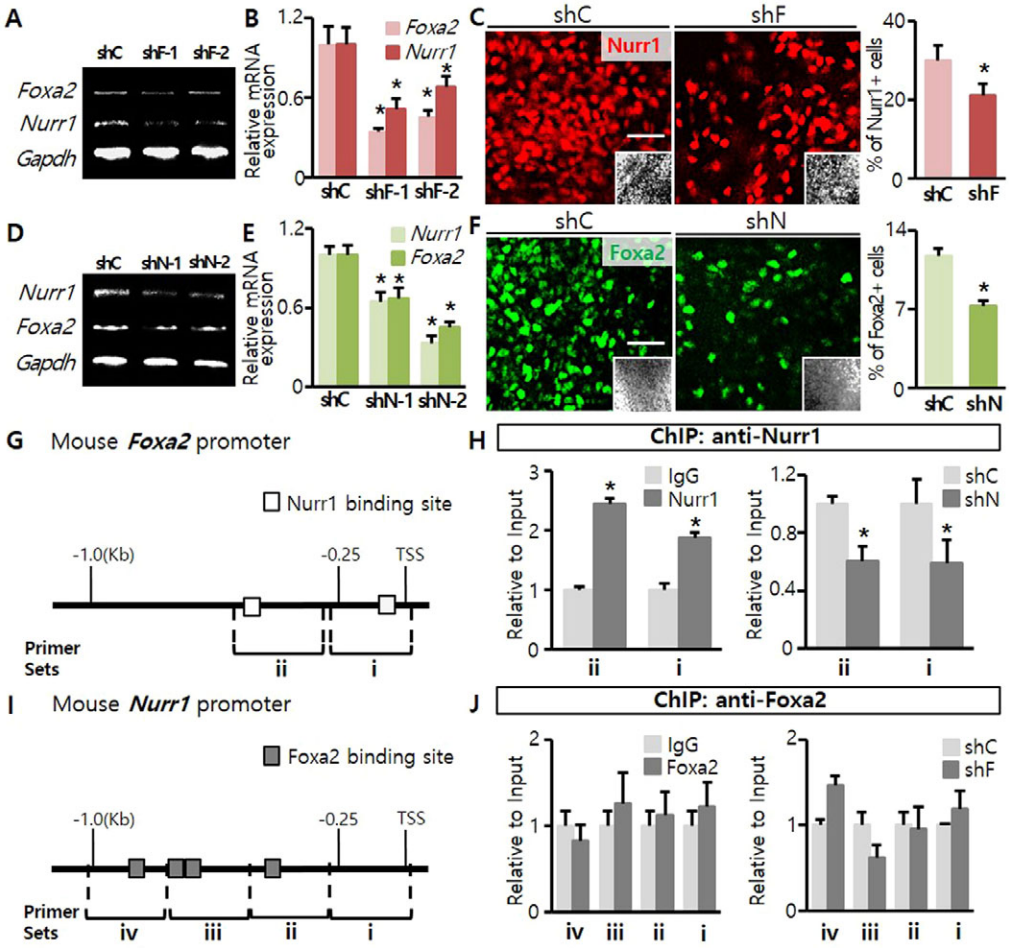

Fig. 2. Positive cross-regulatory loop between Nurr1 and Foxa2. (A-F) NPCs were directly isolated and cultured from VM of mouse embryos at E11, and treated with shFoxa2 (shF; A-C), shNurr1 (shN; D-F) or shControl (shC). Semi-quantitative (A,D) and real-time PCR (B,E) analyses of *Nurr1* and *Foxa2* mRNA expression were carried out 6 days after differentiation. Gapdh, glyceraldehyde 3-phosphate dehydrogenase (housekeeping gene). Reduced Nurr1 expression by shFoxa2 and Foxa2 expression by shNurr1 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 µm. (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.

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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

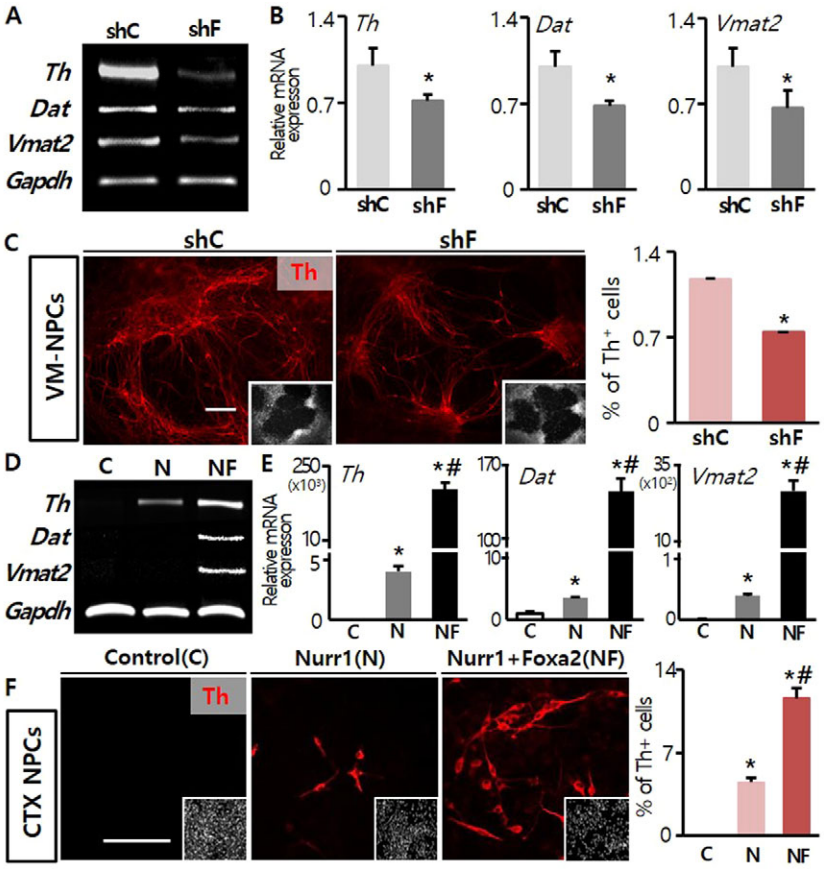

Fig. 3. Foxa2 roles in activating DA phenotype expression. (A-C) Loss-of-function studies of Foxa2. NPCs were directly cultured from VMs of E11 mouse embryos and treated with shFoxa2 RNA (shF). The control cultures were transduced with control shRNA (shC). Semi-quantitative (A) and real-time (B) PCR 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 representative 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 transduced 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 μm.

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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 nucelosome 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

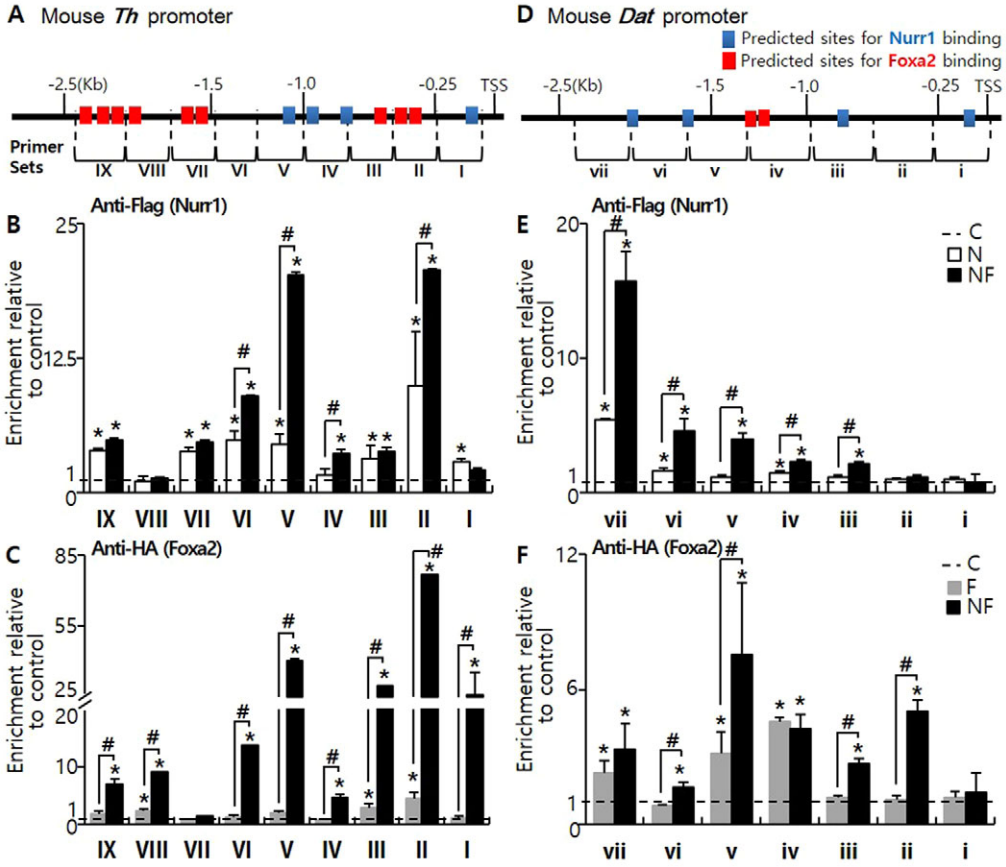

Fig. 4. Enrichment of Nurr1 and Foxa2 proteins at *Th* and *Dat* promoter regions. (A,D) Schematics of mouse *Th* (A) and *Dat* (D) promoters with predicted Nurr1 (blue boxes) and Foxa2 (red boxes) consensus binding sites. The promoter regions encompassing −2.5 kb from the TSS were divided into nine (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.

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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 (Eeckhoute 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

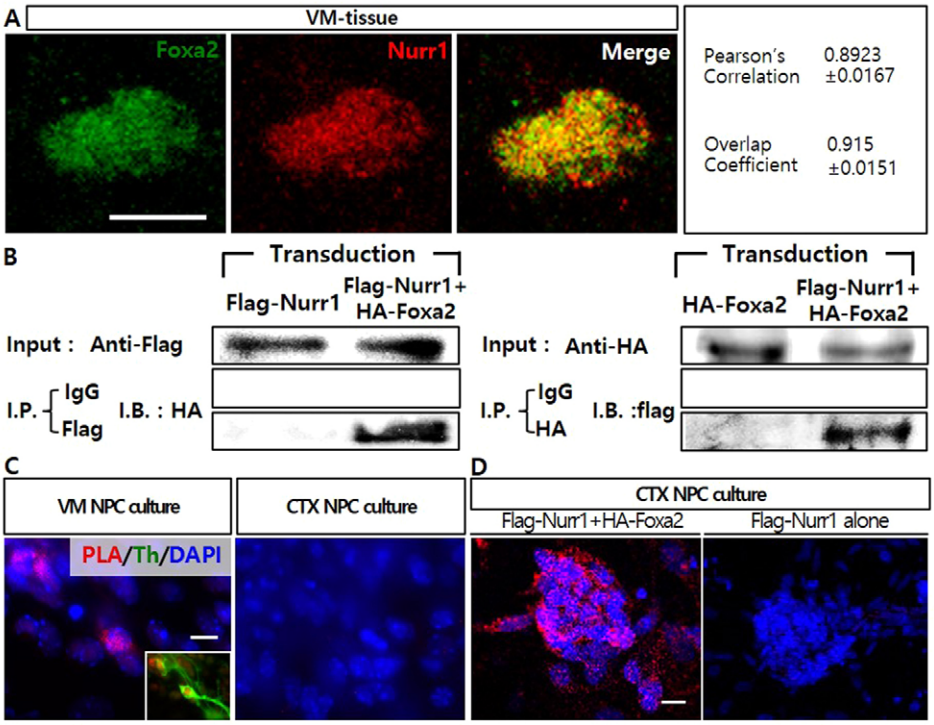

Fig. 5. Protein-protein interactions between Foxa2 and Nurr1 in mDA neuron precursors. (A) Confocal microscopic images of nuclei stained with Nurr1 and Foxa2. Nuclei were selected from Th^+ cells in the VM tissues of mouse embryos at E12 *in vivo* (Fig. 1M). Shown are representative images of a single 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 µm. (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 µm.

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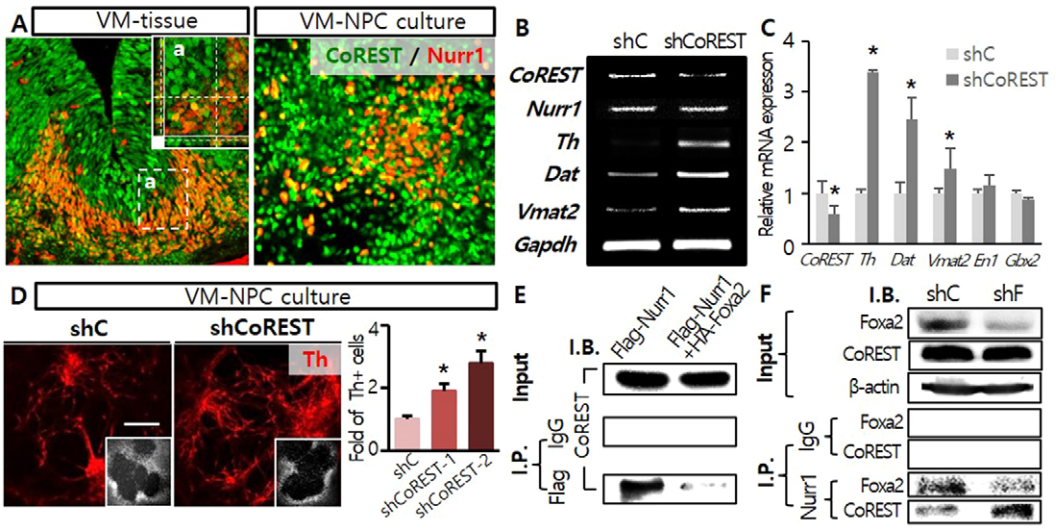

Fig. 6. Inhibitory role of CoREST in mDA neuron development. (A) Colocalization of CoREST with Nurr1 in the developing (E12.5) VM tissue *in vivo* (left) and in VM-derived cultures *in vitro* (right). Inset of left image shows *z*-stacked confocal image of the boxed area along the *y*-axis (left) and *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 µm. (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 (Nebbioso 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

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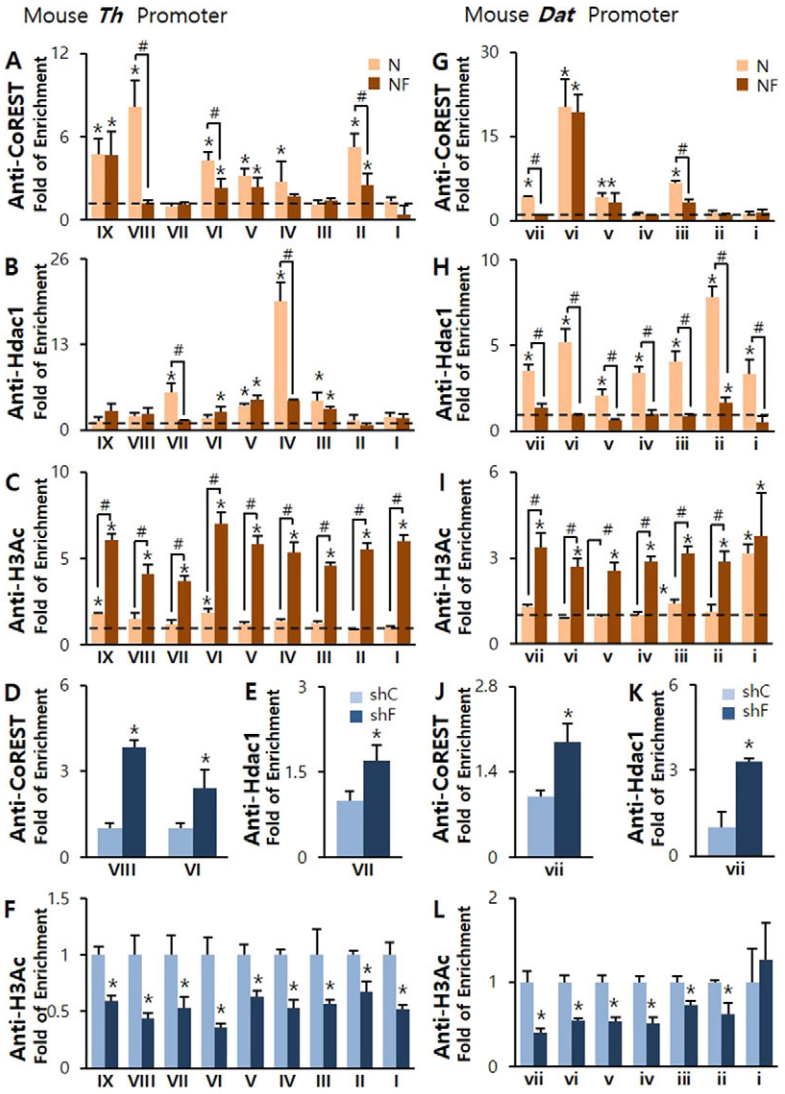

Fig. 7. Foxa2 induces histone 3 acetylation on the *Th* and *Dat* promoters by removing CoREST-Hdac1 repressors from those promoter regions. (A-C,G-I) ChIP analyses with exogenous Nurr1 and Foxa2 overexpression. NPCs derived from mouse cortices at E12 were transduced with viruses expressing Nurr1 (N), 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

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analysis of *Nurr1*$^{-/-}$ mice (Jacobs et al., 2009a). Conditional knockout of Foxa1/2 during development drastically reduces Nurr1 expression (Ferri et al., 2007), probably by a direct expression control, but possibly owing to general developmental defects in the knockout mice. Deletion of Foxa1/2 in adult mouse midbrains resulted in a slight but insignificant decrease of Nurr1^{+} cell numbers (5%) (Stott et al., 2013). Thus, the cross activation of Nurr1 and Foxa2 expression shown in this study and its physiological implications need to be further substantiated in future studies.

Foxa family proteins are involved in tissue-specific gene transcription in multiple tissues. The tissue specificity of Foxa2 activity is likely to be determined by factors specific to each tissue. Specifically, Foxa family proteins interact with nuclear steroid hormone receptors in various tissues. For example, Foxa1 interacts with the glucocorticoid hormone receptor in the liver (Eeckhoute et al., 2006; Nitsch et al., 1993) and pituitary gland (John et al., 2011), with estrogen receptors in breast cancers (Eeckhoute et al., 2006; Hurtado et al., 2011), and with androgen receptors in prostate cancers (Lupien et al., 2008; Wang et al., 2009). We demonstrated in this study another example, namely Foxa2 interacting with the developing VM-specific nuclear receptor Nurr1. A common role of Foxa family proteins is to open compacted chromatin containing tissue-specific genes, thereby facilitating binding of nearby transcription factors (Cirillo et al., 2002). Our ChIP data showed that more Nurr1 protein is recruited to the promoter regions of DA gene in the presence of Foxa2. Notably, a recent paper has shown that DA phenotypes are also lost in adult midbrain by knocking out Foxa1/2 (although the effects by Foxa1/2 deletion are not so drastic as those observed in the developing midbrain), along with reduced Nurr1 binding to the *Th* promoter (Stott et al., 2013), implicating Foxa2 as an epigenetic activator of DA gene expression regardless of developmental stage. Conversely, Nurr1 also enhanced Foxa2 recruitment to DA genes. These findings indicate that Nurr1 also acts as an epigenetic activator to initially recruit Foxa2 to specific DA gene promoters. Based on these findings, we conclude that Nurr1, bound to DA genes, first opens the chromatin neighboring DA neuron-specific genes allowing Foxa2 to be recruited. Foxa2 and/or a Nurr1-Foxa2 complex facilitate further opening of the chromatin-DNA structure, making it more accessible to other transcription factors and RNA polymerase. This gradual unzipping of histone-DNA contact may be a common mechanism of allowing first-bound transcription factors and co-activators to interact (Adams and Workman, 1995).

Nurr1 interaction with an epigenetic repressor, CoREST, has been shown at inflammatory cytokine (Saijo et al., 2009) and DA phenotype promoters (He et al., 2011). We observed Nurr1 binding to CoREST on *Th* and *Dat* promoter regions in the absence of Foxa2. Hdac1 was also recruited to the promoter regions, suggesting that Nurr1-CoREST-Hdac1 form a protein complex, and that Nurr1-mediated DA gene transcription is epigenetically repressed by repressor-mediated histone deacetylation. This mechanism explains why physiological Nurr1 levels without co-activators are inefficient at inducing DA gene expression (Park et al., 2012). Co-expression of Foxa2 generated the Nurr1-Foxa2 protein complex. At the same time, Foxa2 interfered with Nurr1-CoREST-Hdac1 inhibitory complex formation and reduced CoREST and Hdac1 occupancy at DA gene promoters. Consequently, the chromatin of DA genes was opened by repressing Hdac1-mediated histone deacetylation and increasing histone acetylation. Nurr1 is also strongly expressed in neuronal cells in the hippocampus and cortical layer VI (Li et al., 2011; Watakabe et al., 2007). However, without Foxa2 co-expression, the Nurr1-expressing cells in those regions are not dopaminergic, probably owing to the repressive role of CoREST-Hdac1. Jacobs and colleagues (Jacobs et al., 2009b) have reported another epigenetic control mechanism mediated by the Nurr1 activator Pitx3, in which Pitx3 potentiates Nurr1-induced DA gene expression by releasing an Smrt (Ncor1 – Mouse Genome Informatics)-mediated repressor complex (Smrt/Sin3a/Hdac). In addition, Nrsf (Rest – Mouse Genome Informatics) (Kim et al., 2006) and MeCP2 (Yang et al., 2011) have been suggested to be the central negative regulators of *Th* gene transcription during mDA neuron development, possibly by repressing Nurr1 actions (van Heesbeen et al., 2013). It remains to be seen whether all these repressor proteins form a large repressor complex, or if different combinations of the proteins are generated on different regions of the promoters or in different cellular contexts. Similarly, functional interactions among the Nurr1 co-activators Pitx3, Foxa2 and Lmx1a need to be identified.

A common mechanism for determining cell fate during development is the ‘feed-forward induction cascade’, in which developmental transcription factors, expressed in early development, induce additional transcription factors that cooperatively execute further differentiation (Alon, 2007; Davidson and Levine, 2008). We have identified another example of feed-forward involving mDA determination by Foxa2 and Nurr1. In summary, Foxa2 initially induces Nurr1 expression, and then cross-activation maintains colocalization of these factors in mDA neuron precursors. Nurr1 and Foxa2 proteins form an activator complex on DA gene promoters and cooperatively induce the DA neuron fate through epigenetic gene regulation. These findings should facilitate further understanding of mDA neuron development and contribute to stem cell engineering as a treatment for Parkinson’s disease.

MATERIALS AND METHODS

NPC cultures

NPCs were isolated and cultured from embryonic VMs (for loss- and gain-of-function experiments) and cortices (for gain-of-function experiments) from embryonic mice (Imprinting Control Region, ICR) at days 10-12 (E10-12) as previously described (Park et al., 2008). NPCs were expanded *in vitro* by adding basic fibroblast growth factor (bFGF; 20 ng/ml; R&D Systems) and epithelial growth factor (EGF; 20 ng/ml; R&D Systems) in serum-free N2 medium supplemented with ascorbic acid (AA; 200 μM; Sigma) and B27 (Invitrogen Life Technologies). For gain-of-function experiments with viral transductions, NPCs expanded *in vitro* were passaged into freshly prepared dishes, followed by cell proliferation. Viral transductions were carried out as described below. Differentiation of NPCs was induced by withdrawing bFGF and EGF for 5-7 days.

Virus production and transduction

Retroviral vectors expressing Flag-tagged Nurr1 (Flag-Nurr1) or HA-tagged Foxa2 (HA-Foxa2) were constructed by inserting the respective cDNA into pCL or pCL-IRES-GFP (Park et al., 2006). The empty pCL (or pCL-IRES-GFP) vector was used as a negative control. Retrovirus was produced as described previously (Park et al., 2006). For loss-of-function experiments, two lentiviral vector constructs (pGIPZ) with sh-Foxa2 (shFoxa2-1, V3LHS_400600; shFoxa2-2, V3LHS_306420), three with sh-Nurr1 (shNurr1-1, V3LHS_411033; shNurr1-2, V3LHS_377293; shNurr1-3, V2LHS_238950) and four with sh-CoREST (shCoREST-1, V3LMM_472638; shCoREST-2, V3LMM_472634; shCoREST-3, V2LMM_7624; shCoREST-4, V3LMM_472636) were purchased from Open Biosystems (Thermo Scientific). sh-Nurr1-3, sh-CoREST-3 and sh-CoREST-4 were not used, as they only marginally downregulated target gene expression in cultured VM-NPCs (<40%) using real-time PCR analyses. The empty shRNA backbone vector (pGIPZ) was used as a control for the sh-Nurr1, sh-Foxa2 and sh-CoREST effects. The lentiviral vectors were introduced into H293T cells with packaging particles by transfection with Lipofectamine2000 (Invitrogen). Supernatant fractions were harvested

2 and 3 days after transfection, and stored at 70°C until use. Virus titers were determined using QuickTiter Retrovirus Quantitation Kit (Cell Biolabs) and QuickTiter HIV Lentivirus Quantitation Kit (HIV p24 ELISA) (Cell Biolabs). For viral transduction, NPCs cultured *in vitro* were incubated with the viral supernatant [1×10^{11} virus particles (VP)/ml of retrovirus; 10^6 transducing units (TU)/ml of lentivirus] containing polybrene (hexadimethrine bromide: 1 µg/ml, Sigma) for 2 hours (retrovirus) or 6 hours (lentivirus), followed by a medium change.

Immunofluorescence staining

Cultured cells and cryosectioned mouse brain slices were stained as previously described (Rhee et al., 2011). The following primary antibodies were used: Nurr1 (1:500, rabbit, E-20, Santa Cruz Biotechnology; 1:1000, mouse, PP-N1404-00, R&D Systems); Foxa2 (1:500, goat, M-20, sc-6554, Santa Cruz Biotechnology); Th (1:250, rabbit, P40101, Pel-Freez); green fluorescent protein (GFP; 1:2000, rabbit, 819579, Life Technologies); Pcna, (1:50, mouse, 05-347, Millipore); CoREST (1:1000, rabbit, 07-455, Millipore). To visualize the antibodies, secondary antibodies tagged with Cy3 or Cy5 (Jackson ImmunoResearch Laboratories) or Alexa488 (Life Technologies) were used.

Semi-quantitative and real-time PCR analyses

RNA preparation, cDNA synthesis and PCR analysis were performed as previously described (He et al., 2011). The PCR primers and conditions are summarized in supplementary material Table S2.

Immunoprecipitation (IP)

NPCs transduced with Flag-Nurr1 or HA-Foxa2 were harvested with an IP lysis buffer (Thermo Scientific) supplemented with protease inhibitors (Roche Applied Science). Cell lysates were incubated with anti-Flag (3-5 µg, mouse, F3165, Sigma) or anti-HA antibody (3-5 µg, mouse, MMS-101R, Covance) for 18-24 hours at 4°C, and then reacted with anti-mouse magnet beads (Life Technologies) for 1-2 hours at room temperature. After washing the beads, immunoprecipitated proteins were eluted in sample buffer, and subjected to immunoblot (IB) analyses with anti-HA, -Flag or -CoREST antibodies (Millipore).

In situ proximity ligation assay (PLA)

Direct protein interactions between Nurr1 and Foxa2 were further examined using the Duolink *in situ* PLA kit (Olink Bioscience), which is designed to detect protein-protein interactions by emanating red fluorescence (579 nm) when two target proteins are within 40 nm of each other. NPCs cultured from mouse VM at E10 were treated with primary anti-Nurr1 and -Foxa2 antibodies to detect interactions between endogenous protein in the developing VM. The same antibody treatments were used on cortical NPCs as a negative control. Protein interactions were visualized by secondary antibody treatment, ligation, polymerization and detection, according to the manufacturer's protocol. Nurr1-Foxa2 interactions were further examined in cortical NPCs transduced with Flag-Nurr1 and HA-Foxa2 using anti-Flag (Sigma) and -HA (Covance) antibodies. For negative controls, protein interactions were assayed in cortical NPCs that were untransduced or transduced with Flag-Nurr1 alone or HA-Foxa2 alone.

Prediction of consensus Fox2 and Nurr1 binding sites in multiple gene promoters

Foxa2 and Nurr1 binding sites were identified using the Jaspar database (http://jaspar.genereg.net/). Conservation of the binding sites was assessed using the ConSite system (http://consite.genereg.net/), where mouse sequences containing putative Foxa2 or Nurr1 binding sites were compared with corresponding rat sequences. Predicted consensus Foxa2 and Nurr1 binding sites in the promoter DNA of *Nurr1*, *Foxa2*, *Th* and *Dat* were coupled with phylogenetic footprinting to eliminate spurious predictions with specified position weight matrix (PWM) settings, as described previously (Wasserman and Sandelin, 2004).

Chromatin immunoprecipitation (ChIP) assays

ChIP assays were performed as previously described (He et al., 2011). Briefly, chromatins were sheared to an average 400-500 bp long using a sonication Bioruptor (Cosmo Bio Co.) and immunoprecipitated with antibodies against HA (Covance), Flag (Sigma), Nurr1 (E-20, Santa Cruz Biotechnology), Foxa2 (M-20, Santa Cruz Biotechnology; rabbit, ab83517, Abcam), H3Ac (Millipore), Hdac1 (Millipore) or CoREST (Millipore). Immunoprecipitated DNA fragments were collected by magnetic beads (Life Technologies), purified, and subjected to real-time PCR. The comparative cycle threshold method was used to quantify the results. Data were normalized to the input DNA. ChIP data analyzed by real-time PCR were produced in triplicate (n=3) and calculated as fold changes with respect to the control using the $2^{-\Delta\Delta C(T)}$ method (Livak and Schmittgen, 2001). $2^{-\Delta\Delta C(T)}$ values from multiple independent tests (usually two to three) were then compared using one-way ANOVA with Tukey's post hoc analysis (PASW statistics 18; SPSS).

Cell counting and statistical analysis

Immunoreactive or DAPI-stained cells were counted in at least 20 random areas of each culture coverslip using an eyepiece grid at a magnification of 200 or 400×. Data are expressed as the mean ± s.e.m. of three to six independent cultures. Statistical comparisons were made using Student's two-tailed t-test or one-way ANOVA with Tukey post hoc analysis (PASW statistics 18; SPSS).

Microarray

Microarray analysis was requested and carried out by Macrogen Inc. (Seoul, Korea) using Illumina MouseRef-8 v2 expression BeadChip (Illumina Inc.) as described previously (Rhee et al., 2013). Array data were deposited at the Gene Expression Omnibus (National Center for Biotechnology Information) with series accession number GSE54086, and sample accession numbers GSM1307469, GSM1307470, GSM1307471 and GSM1307472.

Competing interests

The authors declare no competing financial interests.

Author contributions

S.-H.Y., X.-B.H. and S.-H.L. developed the concepts and approaches. S.-H.Y., X.-B.H. and Y.-H.R. performed experiments. S.-H.Y., X.-B.H. and S.-H.L. analyzed the data. C.-H.P. constructed Nurr1 and Foxa2 retrovirus. T.T. and K.N. supported the ChIP technique. S.-H.Y., X.-B.H. and S.-H.L. prepared and edited the manuscript.

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Supplementary material

Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.095802/-/DC1

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