RESEARCH ARTICLE

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). Sonic hedgehog (Shh), secreted initially by the notochord and later by floor plate cells, induces expression of forhead 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; Placek, 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 (Kim et al., 2003a; Shim et al., 2007; Wagner et al., 1999). 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-
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...
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;
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...
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.
leveled 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 Nur1 (Fig. 4C). Collectively, these findings suggest that Nur1 and Foxa2 mutually and reciprocally facilitate protein recruitment to promoter DNA. Flag-Nur1 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-Nur1 (Fig. 4E,F).

**Formation of an activator complex of Nur1 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 Nur1. Similarly, multiple Dat promoter regions were co-occupied by Foxa2 and Nur1 in cultures expressing Foxa2+Nur1 (Fig. 4E,F). Collectively, these findings suggest that Nur1 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., 1993; Wang et al., 2009). Both Foxa2 and Nur1 showed a granular distribution within the nucleus (Fig. 5A). Overlaying Nur1 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 Nur1 showed mutually dependent localization. The overlap of Foxa2 and Nur1 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-Nur1+HA-Foxa2, Nur1 was detected in HA antibody (Foxa2) precipitates, and Foxa2 was present in Flag (Nur1) precipitates (Fig. 5B). Endogenous Nur1 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 Nur1**

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
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 (Hdac3) 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 of CoREST knockdown 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 VMat2 then induced to differentiate for 6 days. CoREST knockdown effects on DA neuron differentiation were estimated by mRNA expression of the DA genes (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), derived from mouse VM at E10 exposed to shControl (shC, left lane) or shFoxa2 (shF, right lane). Error bars represent s.e.m. 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.
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, we have previously been reported to potentiate Nurr1-induced DA gene transcription (Jacobs et al., 2009b; 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; Park et al., 2008). 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
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 (Sajjo 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; Watake 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 epidermal 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_41033; 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 produced 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-Freeze); green fluorescent protein (GFP; 1:2000, rabbit, #1879579, 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
Fox2 and Nurr1 binding sites were identified using the Jaspar database (http://jaspar.genereg.net/). Conservation of the binding sites was assayed 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, chromatin was 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^(-ΔΔCt) method (Livak and Schmittgen, 2001). 2^(-ΔΔCt) values from multiple independent experiments (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

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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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