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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.

Funding

This work was supported by grants from the Bio and Medical Technology Development Program (2010-0020232) and the Medical Research Center (2008-0062190). S.-H.Y. was supported by the Basic Science Research Program (2012R1A6A3A01019699), funded by the National Research Foundation of Korea (NRF) of the Ministry of Education, Science and Technology (MEST), Republic of Korea.

Supplementary material

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

References

Abrajano, J. J., Qureshi, I. A., Gokhan, S., Zheng, D., Bergman, A. and Mehler, M. F. (2009). REST and CoREST modulate neuronal subtype specification, maturation and maintenance. *PLoS ONE* **4**, e7936.

Abrajano, J. J., Qureshi, I. A., Gokhan, S., Molero, A. E., Zheng, D., Bergman, A. and Mehler, M. F. (2010). Corepressor for element-1-silencing transcription factor preferentially mediates gene networks underlying neural stem cell fate decisions. *Proc. Natl. Acad. Sci. USA* **107**, 16685-16690.

Adams, C. C. and Workman, J. L. (1995). Binding of disparate transcriptional activators to nucleosomal DNA is inherently cooperative. *Mol. Cell. Biol.* **15**, 1405-1421.

Alon, U. (2007). Network motifs: theory and experimental approaches. *Nat. Rev. Genet.* **8**, 450-461.

Andersson, E. R., Prakash, N., Cajanek, L., Minina, E., Bryja, V., Bryjova, L., Yamaguchi, T. P., Hall, A. C., Wurst, W. and Arenas, E. (2008). Wnt5a regulates ventral midbrain morphogenesis and the development of A9-A10 dopaminergic cells in vivo. *PLoS ONE* **3**, e3517.

Ang, S. L. (2009). Foxa1 and Foxa2 transcription factors regulate differentiation of midbrain dopaminergic neurons. *Adv. Exp. Med. Biol.* **651**, 58-65.

Ang, S. L., Wierda, A., Wong, D., Stevens, K. A., Cascio, S., Rossant, J. and Zaret, K. S. (1993). The formation and maintenance of the definitive endoderm lineage in the mouse: involvement of HNF3/forkhead proteins. *Development* **119**, 1301-1315.

Aranda, A. and Pascual, A. (2001). Nuclear hormone receptors and gene expression. *Physiol. Rev.* **81**, 1269-1304.
Bonilla, S., Hall, A. C., Pinto, L., Attardo, A., Götz, M., Huttner, W. B. and Arenas, E. (2008). Identification of midbrain floor plate radial glia-like cells as dopaminergic progenitors. *Glia* **56**, 809-820.
Chung, S., Leung, A., Han, B. S., Chang, M. Y., Moon, J. I., Kim, C. H., Hong, S., Pruszak, J., Isacson, O. and Kim, K. S. (2009). Wnt1-lmx1a forms a novel autoregulatory loop and controls midbrain dopaminergic differentiation synergistically with the SHH-FoxA2 pathway. *Cell Stem Cell* **5**, 646-658.
Cirillo, L. A., McPherson, C. E., Bossard, P., Stevens, K., Cherian, S., Shim, E. Y., Clark, K. L., Burley, S. K. and Zaret, K. S. (1998). Binding of the winged-helix transcription factor HNF3 to a linker histone site on the nucleosome. *EMBO J.* **17**, 244-254.
Cirillo, L. A., Lin, F. R., Cuesta, I., Friedman, D., Jarnik, M. and Zaret, K. S. (2002). Opening of compacted chromatin by early developmental transcription factors HNF3 (FoxA) and GATA-4. *Mol. Cell* **9**, 279-289.
Davidson, E. H. and Levine, M. S. (2008). Properties of developmental gene regulatory networks. *Proc. Natl. Acad. Sci. USA* **105**, 20063-20066.
Deierborg, T., Soulet, D., Roybon, L., Hall, V. and Brundin, P. (2008). Emerging restorative treatments for Parkinson's disease. *Prog. Neurobiol.* **85**, 407-432.
Eeckhoute, J., Carroll, J. S., Geistlinger, T. R., Torres-Arzayus, M. I. and Brown, M. (2006). A cell-type-specific transcriptional network required for estrogen regulation of cyclin D1 and cell cycle progression in breast cancer. *Genes Dev.* **20**, 2513-2526.
Ferri, A. L., Lin, W., Mavromatakis, Y. E., Wang, J. C., Sasaki, H., Whitsett, J. A. and Ang, S. L. (2007). Foxa1 and Foxa2 regulate multiple phases of midbrain dopaminergic neuron development in a dosage-dependent manner. *Development* **134**, 2761-2769.
Fuentes, P., Cánovas, J., Berndt, F. A., Noctor, S. C. and Kukuljan, M. (2012). CoREST/LSD1 control the development of pyramidal cortical neurons. *Cereb. Cortex* **22**, 1431-1441.
Glass, C. K. and Ogawa, S. (2006). Combinatorial roles of nuclear receptors in inflammation and immunity. *Nat. Rev. Immunol.* **6**, 44-55.
He, X. B., Yi, S. H., Rhee, Y. H., Kim, H., Han, Y. M., Lee, S. H., Lee, H., Park, C. H., Lee, Y. S., Richardson, E. et al. (2011). Prolonged membrane depolarization enhances midbrain dopamine neuron differentiation via epigenetic histone modifications. *Stem Cells* **29**, 1861-1873.
Hurtado, A., Holmes, K. A., Ross-Innes, C. S., Schmidt, D. and Carroll, J. S. (2011). FOXA1 is a key determinant of estrogen receptor function and endocrine response. *Nat. Genet.* **43**, 27-33.
Jacobs, F. M., van der Linden, A. J., Wang, Y., von Oerthel, L., Sul, H. S., Burbach, J. P. and Smidt, M. P. (2009a). Identification of Dlk1, Ptpru and Klhl1 as novel Nurr1 target genes in meso-diencephalic dopamine neurons. *Development* **136**, 2363-2373.
Jacobs, F. M., van Erp, S., van der Linden, A. J., von Oerthel, L., Burbach, J. P. and Smidt, M. P. (2009b). Pitx3 potentiates Nurr1 in dopamine neuron terminal differentiation through release of SMRT-mediated repression. *Development* **136**, 531-540.
Jin, H., Romano, G., Marshall, C., Donaldson, A. E., Suon, S. and Iacovitti, L. (2006). Tyrosine hydroxylase gene regulation in human neuronal progenitor cells does not depend on Nurr1 as in the murine and rat systems. *J. Cell. Physiol.* **207**, 49-57.
Jo, A. Y., Park, C. H., Aizawa, S. and Lee, S. H. (2007). Contrasting and brain region-specific roles of neurogenin2 and mash1 in GABAergic neuron differentiation in vitro. *Exp. Cell Res.* **313**, 4066-4081.
John, S., Sabo, P. J., Thurman, R. E., Sung, M. H., Biddie, S. C., Johnson, T. A., Hager, G. L. and Stamatoyannopoulos, J. A. (2011). Chromatin accessibility pre-determines glucocorticoid receptor binding patterns. *Nat. Genet.* **43**, 264-268.
Jönsson, M. E., Ono, Y., Björklund, A. and Thompson, L. H. (2009). Identification of transplantable dopamine neuron precursors at different stages of midbrain neurogenesis. *Exp. Neurol.* **219**, 341-354.
Kim, J. Y., Koh, H. C., Lee, J. Y., Chang, M. Y., Kim, Y. C., Chung, H. Y., Son, H., Lee, Y. S., Studer, L., McKay, R. et al. (2003a). Dopaminergic neuronal differentiation from rat embryonic neural precursors by Nurr1 overexpression. *J. Neurochem.* **85**, 1443-1454.
Kim, K. S., Kim, C. H., Hwang, D. Y., Seo, H., Chung, S., Hong, S. J., Lim, J. K., Anderson, T. and Isacson, O. (2003b). Orphan nuclear receptor Nurr1 directly transactivates the promoter activity of the tyrosine hydroxylase gene in a cell-specific manner. *J. Neurochem.* **85**, 622-634.
Kim, S. M., Yang, J. W., Park, M. J., Lee, J. K., Kim, S. U., Lee, Y. S. and Lee, M. A. (2006). Regulation of human tyrosine hydroxylase gene by neuron-restrictive silencer factor. *Biochem. Biophys. Res. Commun.* **346**, 426-435.
Kittappa, R., Chang, W. W., Awatramani, R. B. and McKay, R. D. (2007). The foxa2 gene controls the birth and spontaneous degeneration of dopamine neurons in old age. *PLoS Biol.* **5**, e325.
Le, W., Conneely, O. M., Zou, L., He, Y., Saucedo-Cardenas, O., Jankovic, J., Mosier, D. R. and Appel, S. H. (1999). Selective agenesis of mesencephalic dopaminergic neurons in Nurr1-deficient mice. *Exp. Neurol.* **159**, 451-458.
Lee, H. S., Bae, E. J., Yi, S. H., Shim, J. W., Jo, A. Y., Kang, J. S., Yoon, E. H., Rhee, Y. H., Park, C. H., Koh, H. C. et al. (2010). Foxa2 and Nurr1 synergistically yield A9 nigral dopamine neurons exhibiting improved differentiation, function, and cell survival. *Stem Cells* **28**, 501-512.
Li, Q., Lau, A., Morris, T. J., Guo, L., Fordyce, C. B. and Stanley, E. F. (2004). A syntaxin 1, Galpha(o), and N-type calcium channel complex at a presynaptic nerve terminal: analysis by quantitative immunocolocalization. *Neuroscience* **24**, 4070-4081.
Li, Y., Cong, B., Ma, C., Qi, Q., Fu, L., Zhang, G. and Min, Z. (2011). Expression of Nurr1 during rat brain and spinal cord development. *Neurosci. Lett.* **488**, 49-54.
Lin, W., Metzakopian, E., Mavromatakis, Y. E., Gao, N., Balaskas, N., Sasaki, H., Briscoe, J., Whitsett, J. A., Goulding, M., Kaestner, K. H. et al. (2009). Foxa1 and Foxa2 function both upstream of and cooperatively with Lmx1a and Lmx1b in a feedforward loop promoting mesodiencephalic dopaminergic neuron development. *Dev. Biol.* **333**, 386-396.
Livak, K. J. and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **25**, 402-408.
Lupien, M., Eeckhoute, J., Meyer, C. A., Wang, Q., Zhang, Y., Li, W., Carroll, J. S., Liu, X. S. and Brown, M. (2008). FoxA1 translates epigenetic signatures into enhancer-driven lineage-specific transcription. *Cell* **132**, 958-970.
Martinat, C., Bacci, J. J., Leete, T., Kim, J., Vanti, W. B., Newman, A. H., Cha, J. H., Gether, U., Wang, H. and Abeliovich, A. (2006). Cooperative transcription activation by Nurr1 and Pitx3 induces embryonic stem cell maturation to the midbrain dopamine neuron phenotype. *Proc. Natl. Acad. Sci. USA* **103**, 2874-2879.
Metzakopian, E., Lin, W., Salmon-Divon, M., Dvinge, H., Andersson, E., Ericson, J., Perlmann, T., Whitsett, J. A., Bertone, P. and Ang, S. L. (2012). Genome-wide characterization of Foxa2 targets reveals upregulation of floor plate genes and repression of ventrolateral genes in midbrain dopaminergic progenitors. *Development* **139**, 2625-2634.
Monaghan, A. P., Kaestner, K. H., Grau, E. and Schütz, G. (1993). Postimplantation expression patterns indicate a role for the mouse forkhead/HNF-3 alpha, beta and gamma genes in determination of the definitive endoderm, chordamesoderm and neuroectoderm. *Development* **119**, 567-578.
Morizane, A., Li, J. Y. and Brundin, P. (2008). From bench to bed: the potential of stem cells for the treatment of Parkinson's disease. *Cell Tissue Res.* **331**, 323-336.
Nakatani, T., Kumai, M., Mizuhara, E., Minaki, Y. and Ono, Y. (2010). Lmx1a and Lmx1b cooperate with Foxa2 to coordinate the specification of dopaminergic neurons and control of floor plate cell differentiation in the developing mesencephalon. *Dev. Biol.* **339**, 101-113.
Nebbioso, A., Dell'Aversana, C., Bugge, A., Sarno, R., Valente, S., Rotili, D., Manzo, F., Teti, D., Mandrup, S., Ciana, P. et al. (2010). HDACs class II-selective inhibition alters nuclear receptor-dependent differentiation. *J. Mol. Endocrinol.* **45**, 219-228.
Nitsch, D., Boshart, M. and Schütz, G. (1993). Activation of the tyrosine aminotransferase gene is dependent on synergy between liver-specific and hormone-responsive elements. *Proc. Natl. Acad. Sci. USA* **90**, 5479-5483.
Ono, Y., Nakatani, T., Sakamoto, Y., Mizuhara, E., Minaki, Y., Kumai, M., Hamaguchi, A., Nishimura, M., Inoue, Y., Hayashi, H. et al. (2007). Differences in neurogenic potential in floor plate cells along an anteroposterior location: midbrain dopaminergic neurons originate from mesencephalic floor plate cells. *Development* **134**, 3213-3225.
Park, C. H., Kang, J. S., Kim, J. S., Chung, S., Koh, J. Y., Yoon, E. H., Jo, A. Y., Chang, M. Y., Koh, H. C., Hwang, S. et al. (2006). Differential actions of the proneural genes encoding Mash1 and neurogenins in Nurr1-induced dopamine neuron differentiation. *J. Cell Sci.* **119**, 2310-2320.
Park, C. H., Kang, J. S., Yoon, E. H., Shim, J. W., Suh-Kim, H. and Lee, S. H. (2008). Proneural bHLH neurogenin 2 differentially regulates Nurr1-induced dopamine neuron differentiation in rat and mouse neural precursor cells in vitro. *FEBS Lett.* **582**, 537-542.
Park, C. H., Lim, M. S., Rhee, Y. H., Yi, S. H., Kim, B. K., Shim, J. W., Kim, Y. H., Jung, S. J. and Lee, S. H. (2012). In vitro generation of mature dopamine neurons by decreasing and delaying the expression of exogenous Nurr1. *Development* **139**, 2447-2451.
Placzek, M. (1995). The role of the notochord and floor plate in inductive interactions. *Curr. Opin. Genet. Dev.* **5**, 499-506.
Purcell, D. J., Jeong, K. W., Bittencourt, D., Gerke, D. S. and Stallcup, M. R. (2011). A distinct mechanism for coactivator versus corepressor function by histone methyltransferase G9a in transcriptional regulation. *J. Biol. Chem.* **286**, 41963-41971.
Rhee, Y. H., Ko, J. Y., Chang, M. Y., Yi, S. H., Kim, D., Kim, C. H., Shim, J. W., Jo, A. Y., Kim, B. W., Lee, H. et al. (2011). Protein-based human iPS cells efficiently generate functional dopamine neurons and can treat a rat model of Parkinson disease. *J. Clin. Invest.* **121**, 2326-2335.
Saijo, K., Winner, B., Carson, C. T., Collier, J. G., Boyer, L., Rosenfeld, M. G., Gage, F. H. and Glass, C. K. (2009). A Nurr1/CoREST pathway in microglia and astrocytes protects dopaminergic neurons from inflammation-induced death. *Cell* **137**, 47-59.
Sakurada, K., Ohshima-Sakurada, M., Palmer, T. D. and Gage, F. H. (1999). Nurr1, an orphan nuclear receptor, is a transcriptional activator of endogenous tyrosine hydroxylase in neural progenitor cells derived from the adult brain. *Development* **126**, 4017-4026.
Sasaki, H. and Hogan, B. L. (1994). HNF-3 beta as a regulator of floor plate development. *Cell* **76**, 103-115.
Sasaki, H., Hui, C., Nakafuku, M. and Kondoh, H. (1997). A binding site for Gli proteins is essential for HNF-3beta floor plate enhancer activity in transgenics and can respond to Shh in vitro. *Development* **124**, 1313-1322.
Saucedo-Cardenas, O., Quintana-Hau, J. D., Le, W. D., Smidt, M. P., Cox, J. J., De Mayo, F., Burbach, J. P. and Conneely, O. M. (1998). Nurr1 is essential for the induction of the dopaminergic phenotype and the survival of ventral mesencephalic late dopaminergic precursor neurons. *Proc. Natl. Acad. Sci. USA* **95**, 4013-4018.

Shim, J. W., Park, C. H., Bae, Y. C., Bae, J. Y., Chung, S., Chang, M. Y., Koh, H. C., Lee, H. S., Hwang, S. J., Lee, K. H. et al. (2007). Generation of functional dopamine neurons from neural precursor cells isolated from the subventricular zone and white matter of the adult rat brain using Nurr1 overexpression. *Stem Cells* **25**, 1252-1262.

Stott, S. R., Metzakopian, E., Lin, W., Kaestner, K. H., Hen, R. and Ang, S. L. (2013). Foxa1 and foxa2 are required for the maintenance of dopaminergic properties in ventral midbrain neurons at late embryonic stages. *Neuroscience* **33**, 8022-8034.

Tontsch, S., Zach, O. and Bauer, H. C. (2001). Identification and localization of M-CoREST (1A13), a mouse homologue of the human transcriptional co-repressor CoREST, in the developing mouse CNS. *Mech. Dev.* **108**, 165-169.

van Heesbeen, H. J., Mesman, S., Veenvliet, J. V. and Smidt, M. P. (2013). Epigenetic mechanisms in the development and maintenance of dopaminergic neurons. *Development* **140**, 1159-1169.

Wagner, J., Akerud, P., Castro, D. S., Holm, P. C., Canals, J. M., Snyder, E. Y., Perlmann, T. and Arenas, E. (1999). Induction of a midbrain dopaminergic phenotype in Nurr1-overexpressing neural stem cells by type 1 astrocytes. *Nat. Biotechnol.* **17**, 227-228.

Wang, Q., Li, W., Zhang, Y., Yuan, X., Xu, K., Yu, J., Chen, Z., Beroukhim, R., Wang, H., Lupien, M. et al. (2009). Androgen receptor regulates a distinct transcription program in androgen-independent prostate cancer. *Cell* **138**, 245-256.

Wasserman, W. W. and Sandelin, A. (2004). Applied bioinformatics for the identification of regulatory elements. *Nat. Rev. Genet.* **5**, 276-287.

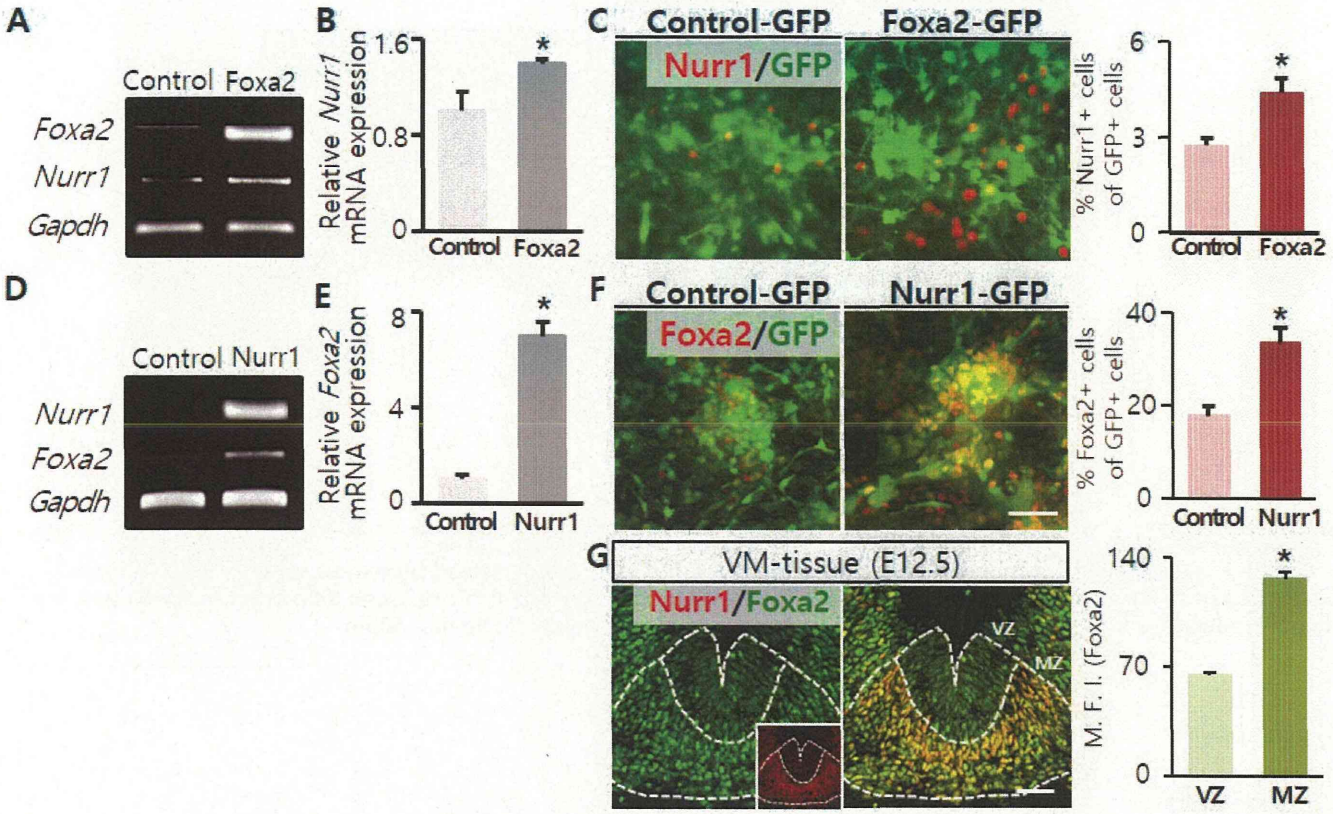
Watakabe, A., Ichinohe, N., Ohsawa, S., Hashikawa, T., Komatsu, Y., Rockland, K. S. and Yamamori, T. (2007). Comparative analysis of layer-specific genes in Mammalian neocortex. *Cereb. Cortex* **17**, 1918-1933.

Xu, L., Glass, C. K. and Rosenfeld, M. G. (1999). Coactivator and corepressor complexes in nuclear receptor function. *Curr. Opin. Genet. Dev.* **9**, 140-147.

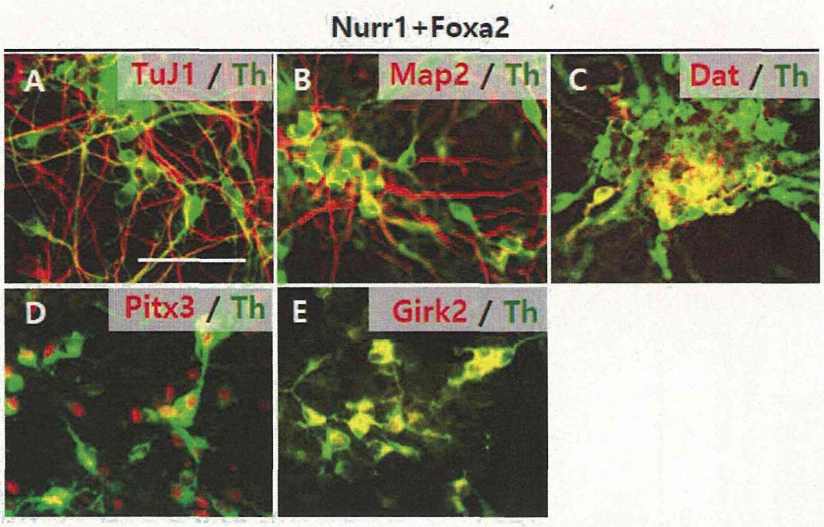
Yang, J. W., Choi, E. Y., Park, M. J. and Lee, M. A. (2011). Expression of tyrosine hydroxylase is epigenetically regulated in neural stem cells. *Biochem. Biophys. Res. Commun.* **414**, 712-718.

Zetterström, R. H., Williams, R., Perlmann, T. and Olson, L. (1996). Cellular expression of the immediate early transcription factors Nurr1 and NGFI-B suggests a gene regulatory role in several brain regions including the nigrostriatal dopamine system. *Brain Res. Mol. Brain Res.* **41**, 111-120.

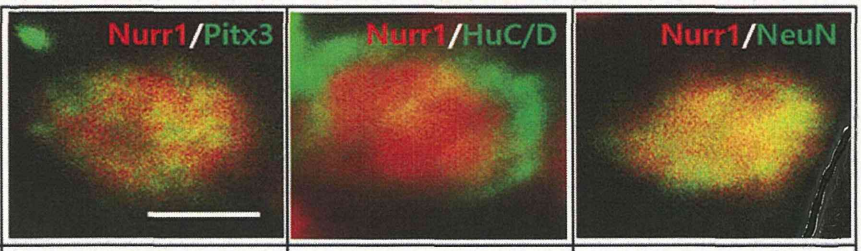
Zetterström, R. H., Solomin, L., Jansson, L., Hoffer, B. J., Olson, L. and Perlmann, T. (1997). Dopamine neuron agenesis in Nurr1-deficient mice. *Science* **276**, 248-250.



Suppl. Fig. S1. Gain-of-function analyses *in vitro* to confirm positive cross-regulatory loop between Nurr1 and Foxa2 expression. NPCs derived from VM at E11 were subcultured to examine gain-of-function effects. (A-F) The passaged VM-NPC cultures yielded fewer Nurr1$^+$ and Foxa2$^+$ cells upon differentiation than unpassaged cultures. The passaged cultures were transduced with virus expressing Foxa2-IRES-GFP (A-C) or Nurr1-IRES-GFP (D-F) and differentiated for 6 days. Control cultures were transduced with virus expressing IRES-GFP. Semi-quantitative PCR (A,D), real-time PCR (B,E), and immunocytochemistry (C,F) analyses were carried out for Nurr1 and Foxa2 expression. (C,F) are representative images for Nurr1$^+$/GFP$^+$ and Foxa2$^+$/GFP$^+$ cells, respectively. Graphs on the right depict percentages of the co-expressing cells out of total GFP$^+$ cells in 20-40 clusters randomly selected from 3 independent culture sets. *Significantly different from control at P<0.05(B), P<0.005(C), and P<0.001(E,F). (G) Comparison of Foxa2 expression levels in the Nurr1-negative ventricular zone (VZ) and Nurr1-positive mantle zone (MZ) of the embryonic mouse VM. VM tissue sections of mouse embryos at E12 were stained with anti-Foxa2 antibody (Inset, identical section Nurr1-stained). Foxa2-stained cells were randomly selected from the VZ and MZ (40 cells each) and Foxa2 expression levels were quantified as mean fluorescence intensities (MFI) of individual anti-Foxa2-stained cells. *P<0.001, Student's t-test. Scale bar, 50 μm.

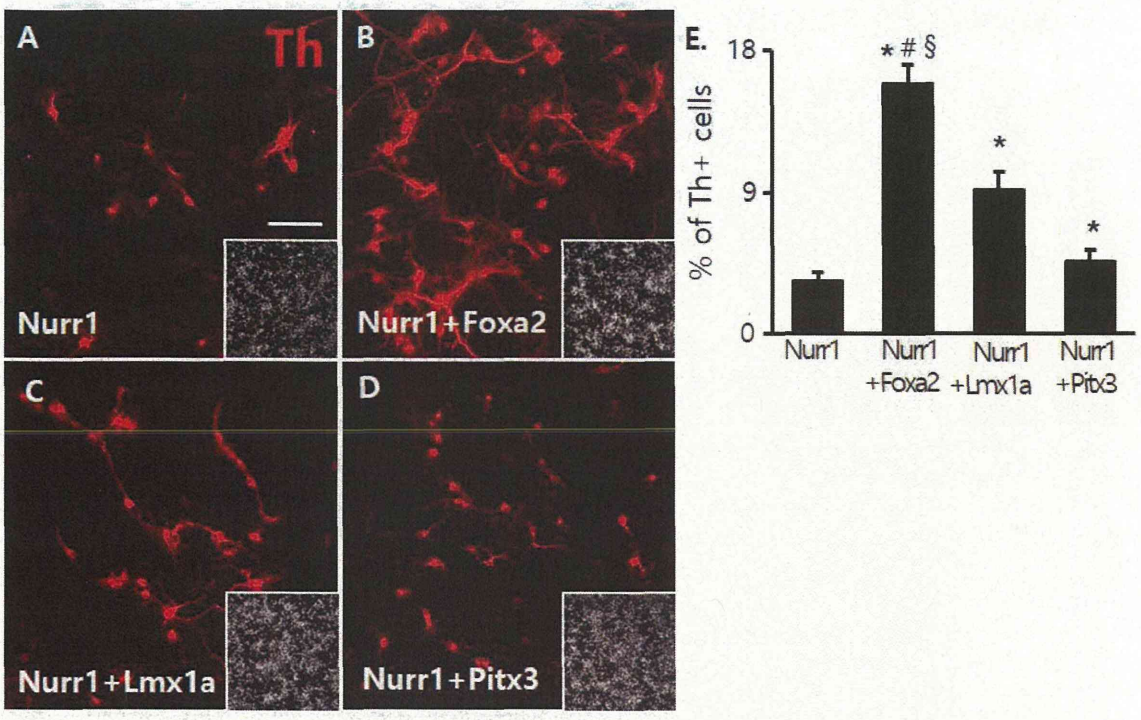


Suppl. Fig. S2. Midbrain-type DA neuronal phenotypes of TH⁺ cells induced by exogenous Nurr1 and Foxa2 co-expression. Non-dopaminergic cortical NPCs were transduced with Nurr1⁺ Foxa2, and differentiated for 6 days. Immunofluorescence staining was conducted using the indicated antibodies. Scale bar, 50μm.



Pearson's Correlation	0.705±0.045	0.122±0.037	0.325±0.132
Overlap Coefficient	0.799±0.073	0.46±0.038	0.565±0.038

Suppl. Fig. S3. Analysis of Nurr1 colocalization with Pitx3, NeuN, and HuC/D by Pearson's correlation and overlap coefficient values. Shown are representative images of single nucleus co-stained with Nurr1/Pitx3, Nurr1/NeuN, and Nurr1/HuC/D from the VM tissue sections at E12. Scale bar, 5μm.



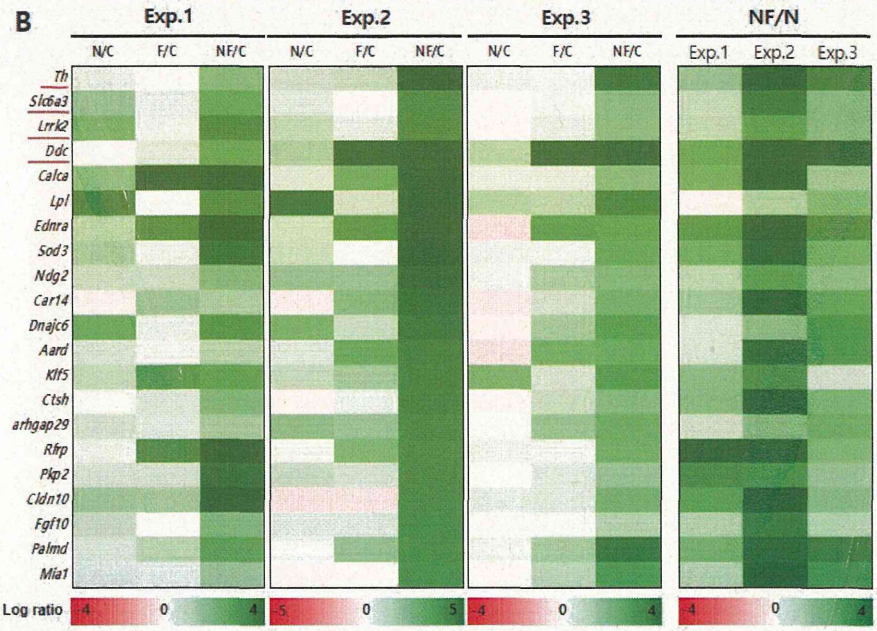
Suppl. Fig. S4. Comparison of the activities of Nurr1 coactivators reported. NPCs derived from mouse embryonic cortices at E12 were co-transduced with the retroviruses containing the control empty vector (A), Foxa2 (B), Lmx1a (C), or Pitx3 (D) along with Nurr1. The coactivator virus titers were carefully adjusted to $1x10^{11}$ virus particles/ml. Graph E represents the percentage of $DAPI^+$ cells that were TH^+. Significance from the control*, Nurr1+Lmx1a# and Nurr1+Pixt3§ at $P<0.001$, Scale bar, 50μm.

Suppl. Table. S1. mRNA expressions of 21 genes selected from microarray data

A

SYMBOL	DEFINITION	Accession No.	Fold Increase			
			N/C	F/C	NF/C	NF/N
Th	Tyrosine hydroxylase	NM_009377	1.71±0.02	1.52±0.43	38.47±1.18	22.513±0.41
Slc6a3	Dopamine transporter, Dat	NM_010020	1.23±0.14	-0.03±1.12	12.2±2.81	9.77±1.2
Lrrk2	Leucine-rich repeat kinase 2	NM_025730	1.96±0.6	1.22±0.02	12.01±3.63	6.13±0.01
Ddc	Dopa Decarboxylase, Aromatic L-amino acid decarboxylase, Aadc	NM_016672	1.66±0.59	26.96±4.83	39.15±6.34	14.3±1.41
Sod3	Superoxide dismutase3	NM_011435	1.96±0.22	0.04±1.12	23.36±7.23	11.67±2.37
Calca	Calcitonin/calcitonin-related polypeptide, alpha	NM_007587	1.86±0.29	4.71±3.3	28.61±14.5	17.04±10.49
Lpl	Lipoprotein lipase	NM_008509	27.16±1.77	6.41±4.7	53.7±6.31	2.0±0.36
Ednra	Endothelin receptor type A	NM_010332	1.59±0.45	9.2±3.7	24.5±9.72	14.88±1.85
Ndg2	Nur77 downstream gene 2	NM_175329	3.11±0.98	2.58±0.96	16.42±8.79	4.88±1.28
Car14	Carbonic anhydrase 14	NM_011797	-1.9±0.53	3.09±1.69	17.18±6.5	24.35±8.42
Dnajc6	DnaJ (Hsp40) homolog, subfamily C, member 6	NM_198412	4.23±2.53	2.12±0.35	18.85±2.55	6.31±3.18
Aard	Alanine and arginine rich domain containing protein	NM_175503	1.3±0.06	5.86±2.21	14.85±3.25	11.34±1.91
Klf5	Kruppel-like factor 5	NM_009769	6.66±4.48	2.2±0.83	17.67±0.17	4.8±3.26
Ctsh	Cathepsin H	NM_007801	0.16±1.34	1.3±0.24	12.67±4.31	12.76±7.19
Arhgap29	Rho GTPase activating protein 29	NM_172525	3.26±1.39	2.96±0.52	13.73±1.93	4.85±1.49
Rfrp	Neuropeptide VF precursor	NM_021892	1.39±0.36	3.15±2.05	14.23±0.09	11.0±2.95
Pkp2	Plakophilin 2	NM_026163	1.8±0.17	1.35±0.1	10.72±4.06	5.8±1.71
Cldn10	Claudin10	NM_021386	-1.65±0.09	-1.34±0.35	12.22±2.49	16.92±3.86
Palmd	Palmdelphin	NM_023245	1.31±0.24	3.26±0.43	26.49±12.01	19.19±5.65
Fgf10	Fibroblast growth factor 10	NM_008002	1.33±0.04	1.12± 0.06	10.13± 3.74	7.7±3.04
Mia1	Melanoma inhibitory activity 1	NM_019394	0.06±1.26	0.35±1.56	15.14±2.85	14.26±0.44

B



High through-put gene expression analyses were done on NPCs transduced with control (C), Nurr1 (N), Foxa2 (F), and Nurr1+Foxa2 (NF). To know co-activator role of Foxa2 in Nurr1-induced gene expression, the microarray data were analyzed for the gene expressions up-regulated (>2 folds) in NPCs expressing Nurr1+Foxa2, compared to those expressing Nurr1 alone. 21 genes fit this criterion and are listed with their expression ratios (A) and heatmaps of log2 transformed expression ratios (B). n= 3 independent microarray analyses. Each microarray analysis was done in the control-, Nurr1-, Foxa2-, Nurr1+Foxa2-transduced cultures, and interested gene expressions in the Nurr1-, Foxa2, and Nurr1+Foxa2-expressing cultures were compared with those of the control culture. Genes associated with DA neuron phenotypes are underlined.

Suppl. Table. S2. PCR primers information used in this study

Gene symbol	Sequence	Product size	Cycles & Annealing temp.
PCR primers for gene expression			
Tyrosine Hydroxylase (Th)	F : gccgtctcagagcaggatac R : agcatttccatccctctcct	196bp	30-32 cycles 60 ℃
Dopamine transporter (Dat)	F : tggcttcgttgtcttctcct R : cagctggaactcatcgacaa	221bp	26-28 cycles 58 ℃
Vesicle monoamine transporter2 (Vmat2)	F : ctttggagttggttttgc R : gcagttgtggtccatgag	300bp	26-28 cycles 58 ℃
Engrailed1 (En1)	F : tcaagactgactacagcaacccc R : ctttgtcctgaaccgtggtggtag	200bp	26-28 cycles 58 ℃
Gbx2	F : atgagcgcagcgttcccgccg R : cggcggtggcggcagcacca	200bp	26-28 cycles 58 ℃
CoREST	F : cacttggtatggacgacacg R : cagcccttaggcagaatgag	210bp	30-32 cycles 60 ℃
Forkhead box protein2 (Foxa2)	F : gacataccgacgcagctaca R : ggcaccttgagaaagcagtc	215bp	26-28 cycles 58 ℃
Nurr1	F : cggtttcagaagtgcctagc R : ttgcctggaacctggaatag	194bp	26-28 cycles 58 ℃
Gapdh	F : ctcatgaccacagtccatgc R : ttcagctctgggatgacctt	154bp	25-28 cycles 60 ℃
PCR primers for ChIP assay			
1. Primers for *Foxa2* promoter			
Foxa2 (Region1)	F : ctgcaggcagagaacacaga R : ctttctggctacccacctca	248bp	40-45 cycles 58 ℃
Foxa2 (Region2)	F : caagaccctccactccaaaa R : cagaggcaggaggatctcag	193bp	40-45 cycles 58 ℃
2. Primers for *Nurr1* promoter			
Nurr1 (Region1)	F : gcggtgggtcattgtttc R : gcgctccggttcattgtc	199bp	40-45 cycles 58 ℃
Nurr1 (Region2)	F : gggcacagtggcttaaaagt R : ctcctctgcaagttccaacc	181bp	40-45 cycles 58 ℃
Nurr1 (Region3)	F : tgaataagacacgcgtcagg R : agccccactgtccttttcttt	212bp	40-45 cycles 58 ℃
Nurr1 (Region4)	F : cagtgtcttaggggccagag R : gaagatcagctactctgctgga	221bp	40-45 cycles 58 ℃

Suppl. Table. S3. Prediction of Nurr1 and Foxa2 binding sites on promoters of DA neuronal marker genes.

TF	Gene promoter (Kb from TSS)	PWM setting		Predicted binding sites			
				Mouse		Rat	
				Sequence	Location	Sequence	Location
Nurr1	*Foxa2* (M:-1085) (R: -1069)	Conservation cutoffs	94%	AAGCTCAC	420~427	AAGCTCAA	428~435
				GTAACCTT	1041~1048	GTAACCTT	1025~1032
		Window size	50				
		Score threshold	80%				
	Th (M:-2505) (R: -2461)	Conservation Cutoffs	70%	AAGGTTAA	356-363	AAGGTTAA	336~343
				GAGGACAC	1399~1406	GAGGACAC	1346~1353
		Window size	50	AAGGTCCC	1511~1518	AAGGTCCC	1454~1461
				GAGGTCAG	1788~1795	GAGGTCAG	1747~1754
		Score threshold	80%	CTGGCCTT	2437~2444	CTGGCCTT	2392~2399
	Dat (M:-2775) (R: -2556)	Conservation Cutoffs	70%	CTGACCTA	560~567	TTGACCTA	246~253
				GTGACCAT	2045~2052	GTGACCAT	1820~1827
		Window size	50	GAGACCTG	2184~2191	GTGACCTG	1955~1962
				GTGGCCTC	2643~2650	GTGGCCTC	2423~2430
		Score thresold	75%				
Foxa2	*Nurr1* (M:-1091) (R: -1010)	Conservation Cutoffs	46%	AATGCAAATGA	212~223	ATACCAAAGAGC	129~140
				GACTGATAATTG	238~249	GAATGTGCAGGG	151~162
		Window size	50	AAATATTTACCT	370~381	CCCCGTTTCCCT	200~211
				AAGCCCCTTTAG	389~400	AAGCATCCTGTG	219~230
		Score threshold	60%				
	Th (M:-2505) (R: -2461)	Conservation Cutoffs	80%	ACACAGACAAAG	230~241	ACACAGACAAAG	211~222
				AAAGCAATATTT	320~331	AAAGCAATATTT	300~311
				CAATATTTGTGT	324~335	CAATATTTGTGT	304~315
		Window size	50	AAATCCACATTC	362~373	AAATCCACACTC	342~353
				GAGCAGGCAGTG	826~837	GAGCAGGCAGTG	783~794
				GAGTAAATAGTC	840~851	GAGTAAATAGTC	797~808
		Score threshold	85%	GAGTAGATAGTA	2000~2011	GAATAGATAGTA	1964~1975
				CTAGATTTATTT	2094~2105	CTAGATTTGTCT	2063~2074
				AATCCAGCATGG	2151~2162	AATCCAGCATGG	2109~2120
	Dat (M:-2775) (R: -2556)	Conservation Cutoffs	80%	GAATAAATGTTT	1357~1368	GAATAAATGTTT	1017~1028
				AAATGTTTGTTG	1361~1372	AAATGTTTGCTG	1021~1032
		Window size	50				
		Score threshold	85%				