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Foxa1 and Foxa2 function both upstream of and cooperatively with Lmx1a and Lmx1b in a feedforward loop promoting mesodiencephalic dopaminergic neuron development

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ABSTRACT

Mesodiencephalic dopaminergic neurons control voluntary movement and reward based behaviours. Their dysfunction can lead to neurological disorders, including Parkinson's disease. These neurons are thought to arise from progenitors in the floor plate of the caudal diencephalon and midbrain. Members of the Foxa family of forkhead/winged helix transcription factor, Foxa1 and Foxa2, have previously been shown to regulate neuronal specification and differentiation of mesodiencephalic progenitors. However, Foxa1 and Foxa2 are also expressed earlier during regional specification of the rostral brain. In this paper, we have examined the early function of Foxa1 and Foxa2 using conditional mutant mice. Our studies show that Foxa1 and Foxa2 positively regulate Lmx1a and Lmx1b expression and inhibit Nkx2.2 expression in mesodiencephalic dopaminergic progenitors. Subsequently, Foxa1 and Foxa2 function cooperatively with Lmx1a and Lmx1b to regulate differentiation of mesodiencephalic dopaminergic neurons. Chromatin immunoprecipitation experiments indicate that *Nkx2.2* and *TH* genes are likely direct targets of Foxa1 and Foxa2 in mesodiencephalic dopaminergic cells in vivo. Foxa1 and Foxa2 also inhibit GABAergic neuron differentiation by repressing the *Helt* gene in the ventral midbrain. Our data therefore provide new insights into the specification and differentiation of mesodiencephalic dopaminergic neurons and identifies Foxa1 and Foxa2 as essential regulators in these processes.

Introduction

Mesodiencephalic (mdDA) dopaminergic neurons control voluntary movement and reward based behaviour and their dysfunction can lead to neurological diseases. These neurons have been extensively studied since loss of mdDA neurons is correlated with Parkinson's disease (Hirsch et al., 1988; Lang and Lozano, 1998). One promising approach for the treatment of Parkinson's disease is cell replacement therapy (reviewed in Lindvall and Bjorklund, 2004; Astradsson et al., 2008). Understanding the genetic network regulating mdDA neuron development will facilitate successful engineering of these neurons from embryonic stem cells, which could then serve as a source of cells for transplantation. mdDA neurons are thought to arise from floor plate progenitors in the caudal diencephalon and midbrain (Marin et al., 2005). Some key determinants of mdDA progenitor specification and differentiation have been identified, including the bicoid class homeobox transcription factor (TF) Otx2 (Puelles et al., 2004; Vernay et al., 2005; Omodei et al., 2008), the LIM homeodomain TF Lmx1a (Andersson et al., 2006b; Ono et al., 2007), the forkhead/winged helix TF Foxa1 and Foxa2 (Ferri et al., 2007; Kittappa et al., 2007) and the proneural basic helix–loop–helix (bHLH) TF Neurogenin2 (Ngn2) (Andersson et al., 2006a; Kele et al., 2006). Lmx1a and Otx2 can induce ectopic dopaminergic neurons in gain-of-function experiments, in a context-dependent manner. Over-expression of Lmx1a by electroporation can induce mdDA neurons only from basal and not alar plate progenitors in chick embryos, suggesting that a ventral determinant must act in concert with Lmx1a to induce mdDA neuronal fate (Andersson et al., 2006b). Similarly, Otx2 can induce ectopic dopaminergic neurons only in the floor plate region of the hindbrain (Ono et al., 2007).

Members of the Foxa subfamily of forkhead/winged helix transcription factors, Foxa1 and Foxa2 (Foxa1/2) are expressed in

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floor plate progenitors as early as embryonic days 8.0 (E8.0) (Ang et al., 1993), making them good candidates to contribute to the specification of mdDA progenitor identity. Previous studies have shown that Foxa1/2 regulate neuronal specification in mdDA progenitors by regulating the expression of proneural bHLH transcription factors, Ngn2 and Mash1 (Ferri et al., 2007). In addition, Foxa1/2 are also required for the expression of Nurr1 in immature mdDA neurons and for the differentiation of mature mdDA neurons expressing tyrosine hydroxylase (TH), the rate-limiting enzyme for dopamine synthesis (Ferri et al., 2007). However, whether Foxa1/2 play an earlier role in specifying mdDA progenitor identity has not been addressed.

In this paper, we have determined the role of Foxa1/2 in midbrain progenitors by analyzing the phenotype of conditional *Foxa1/2* double mutant embryos. Our results demonstrate that Foxa1/2 positively regulate Lmx1a and Lmx1b (Lmx1a/b) and inhibit expression of the homeodomain TF Nkx2.2 in neural progenitors, resulting in the specification of mdDA progenitor identity. Foxa1/2 bind to upstream regulatory sequences of the *Nkx2.2* and *TH* genes in chromatin immunoprecipitation (ChIP) assays, suggesting a direct regulation of these genes. We also over-expressed Foxa2 and/or Lmx1a into lateral regions of the midbrain of mouse embryos and found that cooperative interactions between these genes are sufficient to induce ectopic dopaminergic neurons. Moreover, Foxa1/2 inhibit GABAergic neuron differentiation in the ventral midbrain by repressing the *Helt*/*Heslike*/*Megane* gene (Guimera et al., 2006; Miyoshi et al., 2004; Nakatani et al., 2007). These results support a model of feedforward and combinatorial interactions of transcription factors in the development of mdDA neurons.

Materials and methods

Generation and genotyping of mutant embryos and animals

All mouse strains were maintained in a mixed MF1-129/SV background. $En1^{KICre/+}$, $Foxa2^{flox/flox}$ and $Foxa1^{loxp/loxp}$ mouse strains were generated as described (Sapir et al., 2004; Hallonet et al., 2002; Gao et al., 2008 respectively). In this paper, we will refer to the $Foxa1^{loxp}$ allele as $Foxa1^{flox}$. $Foxa2^{flox/flox}$;$Foxa1^{flox/flox}$ mice were generated by crossing $Foxa2^{flox/flox}$ with $Foxa1^{flox/flox}$ animals. To obtain conditional *Foxa1/2* double mutants, we first crossed $En1^{KICre/+}$ mice with $Foxa2^{flox/flox}$; $Foxa1^{flox/flox}$ animals. Subsequently, $En1^{KICre/+}$;$Foxa1^{fl1ox/+}$;$Foxa2^{flox/+}$ F1 male animals were then mated to $Foxa2^{flox/flox}$;$Foxa1^{flox/flox}$ females to generate $En1^{KIcre/+}$;$Foxa2^{flox/flox}$;$Foxa1^{flox/flox}$ double mutants. The $Foxa2^{flox}$ and $Foxa1^{flox}$ alleles were detected by PCR (Hallonet et al., 2002; Gao et al., 2008), whereas the *Cre* transgene was detected by using a pair of primers and PCR conditions as described by Indra et al. (1999). $Shh^{flox/flox}$ (Lewis et al., 2001) animals, also referred to $Shh^{tm2Amc/tm2Amc}$, were purchased from the Jackson Laboratory. $En1^{KIcre/+}$;$Shh^{flox/flox}$ embryos were generated by crossing $En1^{Kicre/+}$;$Shh^{flox/+}$ males with $Shh^{flox/flox}$ females. The Shh^{flox} allele was genotyped by PCR as described on the website of the Jackson Laboratory (http://jaxmice.jax.org). At all times, animals were handled according to the Society of Neuroscience Policy on the Use of Animals in Neuroscience Research, as well as the European Communities Council Directive.

In situ hybridization and immunohistochemistry of brain sections

Embryos were fixed for overnight at 4 °C in 4% paraformaldehyde in 0.1 M PBS and cryoprotected with 30% sucrose in PBS, embedded in OCT compound (VWR International, Poole, UK), and cryosectioned on a cryostat (CM3050S; Leica, Nussloch, Germany). A minimum of three control and three mutant embryos were analyzed, except where indicated. Section in situ hybridization was performed as described previously (Vernay et al., 2005). The following mouse antisense RNA probes have been used: *Gata2* (Nardelli et al., 1999), *Lim1* (Shawlot and Behringer, 1995), *Helt* (Miyoshi et al., 2004), *GAD1* (Behar et al., 1994), Brn3a also known as Pou4f1 — Mouse Genome Informatics (Puelles et al., 2004) and *Islet1* (Puelles et al., 2004).

For immunohistochemistry, sections were incubated overnight at 4 °C with the appropriate primary antibody diluted in 1% BSA in PBS. Sections were then extensively washed in PBS plus 0.1% BSA and incubated 1 h at room temperature with a secondary antibody conjugated with a fluorochrome (Molecular Probes). Sections were then washed and mounted in Vectashield H-1000 (Vector Laboratories, Burlingame, CA). The following primary antibodies were used: rabbit anti-Foxa2 (1:1000) (Filosa et al., 1997), goat antiFoxa2 (1:100) (sc-6554, Santa Cruz), guinea-pig anti-Foxa1 (1:500) (Besnard et al., 2004), rabbit anti-Lmx1a (1:1000) (gift from M. German, unpublished), guinea-pig anti-Lmx1b (1:20,000) (gift from T. Müller and C. Birchmeier, Max-Delbruck-Center for Molecular Medicine, Berlin, Germany), mouse anti-Nkx2.2 (1:5) (74.5A5 Developmental Studies Hybridoma Bank), rabbit anti-TH (1:200) (AB152, Chemicon), rabbit anti-Shh (1:100) (sc9024, Santa Cruz), mouse anti-Brn3a (1:100) (sc-8429, Santa Cruz), mouse anti-Islet1 (1:20) (40.2D6, Developmental Studies Hybridoma Bank), rabbit anti-Nurr1 (1:200) (sc990, Santa Cruz), and rabbit anti-Caspase3 active (1:1000, R&D systems). In some cases, staining of nuclei with Toto-3 iodide (1:1000, Molecular Probes) was performed. All images were collected on a Leica TCS SP2 confocal microscope and processed with Adobe Photoshop 7.0 software (Adobe Systems, San Jose, CA).

BrdU labeling

Pregnant females were injected intraperitoneally with a solution of BrdU (B-5002, at 10 mg/ml in physiological serum; Sigma) at 100 μg for 1 g of body weight and killed 1 h later. Proliferating cells were revealed by immunohistochemistry with a rat anti-BrdU antibody (1:20, Immunologicals Direct) on frozen sections.

ChIP assays

Mouse E12.5 midbrains were dissected and cross-linked in 1% formaldehyde for 10 min while rotating. Cross-linking was quenched by adding glycine to a final concentration of 0.125 M for 5 min while rotating. The tissue was rinsed in cold PBS and homogenized with a plunger in cold whole cell lysis buffer (10 mM Tris–Cl, pH 8.0, 10 mM NaCl, 3 mM MgCl2, 1% NP-40, 1% SDS) and protease inhibitors. Cells were incubated at 4 °C for 10 min. Lysate was sonicated using the Diagenode Bioruptor for 15 min on high, using 30 s intervals. Cell debris were removed by centrifugation at 13,000 rpm for 10 min, and the supernatant was collected and snap frozen in liquid nitrogen. A 10 μl aliquot of the supernatant was reversely cross-linked by the addition of NaCl to a final concentration of 192 mM, incubated overnight at 65 °C, and purified using a PCR purification kit (Qiagen, CA, USA). The chromatin concentration was determined using a NanoDrop 3.1.0 nucleic acid assay (Agilent Technologies, Santa Clara, CA, USA). 10 μg of chromatin per sample was immunoprecipitated with 2 μg of rabbit anti-Foxa2 (Besnard et al., 2004) or normal rabbit anti-IgG antibody (Millipore #12-370). The immunoprecipitated DNA was analyzed by real-time quantitative PCR (qPCR). Foxa2 binding sites in the *Nkx2.2* and *TH* promoters were determined using position weight matrices from the JASPER database (http://jaspar.cgb.ki.se/).

Real-time qPCR

qPCRs were assembled using Platinum SYBR Green Super mix (Invitrogen). Reactions were performed in triplicates using the ABI 7500 PCR System (ABI). The enrichment of target genes was calculated using the MBP locus as a reference for non-specific DNA, and was calculated by comparing input (sheared genomic DNA) to ChIP material. The ChIP primers for quantitative PCR are as follows: for