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NKX2.1 specifies cortical interneuron fate by activating *Lhx6*

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In the ventral telencephalon, the medial ganglionic eminence (MGE) is a major source of cortical interneurons. Expression of the transcription factor NKX2.1 in the MGE is required for the specification of two major subgroups of cortical interneurons – those that express parvalbumin (PV) or somatostatin (SST) – but direct targets of NKX2.1 remain to be established. We find that electroporation of *Nkx2.1* cDNA into the ventral telencephalon of slice cultures from *Nkx2.1*$^{-/-}$ mouse embryos, followed by transplantation into neonatal cortex to permit postnatal analysis of their fate, rescues the loss of PV- and SST-expressing cells. The LIM-homeobox gene *Lhx6* is induced by this rescue experiment, and gain- and loss-of-function studies suggest that *Lhx6* is necessary and sufficient to rescue these and other interneuron phenotypes in cells transplanted from *Nkx2.1*$^{-/-}$ slices. Finally, NKX2.1 protein binds a highly conserved sequence in the *Lhx6* promoter, and this sequence appears to mediate the direct activation of *Lhx6* by NKX2.1. The slice transfection and transplantation methods employed here are beginning to uncover embryonic mechanisms for specifying neuronal fates that only become definable postnatally.

KEY WORDS: Cell fate determination, GABA, Medial ganglionic eminence, *Nkx2.1*, Parvalbumin, Somatostatin, Mouse

INTRODUCTION

Over the past two decades there has been rapid progress in understanding the molecular mechanisms for neuronal subtype fate determination. Many of the initial discoveries were made in *Drosophila*, with more-recent studies including other model organisms such as *C. elegans*, zebrafish and chick. In the last decade, these studies have been extended to mammals, for which, particularly in the spinal cord and retina (Dasen et al., 2005; Livesey and Cepko, 2001; Shirasaki and Pfaff, 2002), further advances have occurred. Despite these advances, and the tremendous relevance to human neuropsychiatric illnesses, there has been relatively little progress in cracking the 'molecular codes' for specifying neuronal subtypes of the mammalian forebrain (Schuurmans et al., 2004; Zhao et al., 2003).

Beyond the issues of functional pleiotropy and redundancy, a key reason for this lag is the lengthy delay between the final cell cycle, when much of the crucial specification-related signaling is likely to occur (McConnell and Kaznowski, 1991; Xu et al., 2005), and the appearance of the mature neuronal phenotype. This delay dictates that the readout of any manipulation of cell fate in progenitor cells may only be assessable weeks later, in the context of many potential confounding influences of that manipulation on neuronal development. In this paper we present a transfection/transplantation method for studying the genetic control of neuronal fate determination in the developing mammalian forebrain.

Recent evidence indicates that, at least in rodents and ferrets, the large majority of cortical interneurons originate in the subcortical telencephalon (for a review, see Wonders and Anderson, 2006). Despite advances in determining the origins, migratory pathways and the regulation of interneuron migration, little is known about the specification of distinct subgroups of cortical interneurons. Roughly 70% of cortical interneurons can be divided into two practically non-overlapping groups based on their expression of the calcium-binding proteins parvalbumin (PV) or neuropeptide somatostatin (SST) (Gonchar and Burkhalter, 1997; Tamamaki et al., 2003). Both of these subgroups appear to originate mainly within the medial ganglionic eminence (MGE) (Butt et al., 2005; Valcanis and Tan, 2003; Wichterle et al., 2001; Xu et al., 2004), where their fate determination depends on the transcription factors NKX2.1 and LHX6 (Liodis et al., 2007; Xu et al., 2004). Expression of SST and PV matures postnatally, with PV expression only beginning in rodent cortex during the second postnatal week (Alcantara et al., 1996).

Nkx2.1 is expressed in the MGE and preoptic region of the pallidal telencephalon (Sussel et al., 1999) (see Fig. S1 in the supplementary material). Over 90% of S-phase cells express *Nkx2.1* in both the ventricular and subventricular zones of the MGE (Xu et al., 2005). These progenitors produce oligodendrocytes and interneurons of the striatum and cerebral cortex (Kessaris et al., 2006; Marin and Rubenstein, 2001) and projection neurons of the globus pallidus (Xu et al., 2008). As cells migrate out of the MGE, *Nkx2.1* expression is downregulated in cortical interneurons but is maintained in subgroups of striatal interneurons postnatally (Marin et al., 2000). As they exit the proliferative zone, most MGE-derived cells begin to express LHX6 (Grigoriou et al., 1998), a homeodomain-containing transcription factor that is not detectable in the telencephalon of *Nkx2.1*-null embryos (Sussel et al., 1999). *Lhx6* continues to be expressed in many interneurons, including most of those that go on to express PV or SST, as they migrate to and then differentiate within the cerebral cortex (see Fig. S1 in the supplementary material) (Cobos et al., 2005; Fogarty et al., 2007; Gong et al., 2003; Lavdas et al., 1999; Liodis et al., 2007).

Here we use genetic gain- and loss-of-function manipulations in mouse embryonic slice cultures, followed by transplantation into cortical environments in vitro and in vivo, to further examine the role of *Nkx2.1* in the specification of MGE-derived (PV- or SST-expressing) cortical interneuron subgroups. We demonstrate that the LIM-homeodomain transcription factor gene *Lhx6* is activated by and appears to be a direct target of NKX2.1. Like *Nkx2.1* itself, *Lhx6* is sufficient to rescue both neurochemical and morphological aspects of *Nkx2.1*$^{-/-}$ MGE-derived interneurons. In addition, at least for the specification of the SST-expressing phenotype, *Lhx6* is required around the time of cell cycle exit and not postnatally in mature

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interneurons. These results and the system presented lay important groundwork for further studies on the transcriptional regulation of interneuron fate in the mammalian forebrain.

MATERIALS AND METHODS

Animals

Nkx2.1-null mice (Kimura et al., 1996) on a CD1 background (Xu et al., 2004) and non-transgenic CD1 strain mice were used. All animal procedures were undertaken according to the guidelines of the Institutional Animal Care and Use Committee at the Weill Cornell Medical College.

Gene constructs, slice electroporation and transplantation

Slice electroporation (EP) was conducted as described (Stuhmer et al., 2002; Xu et al., 2005) using vectors concentrated with Endotoxin-free DNA Maxiprep Kits (Qiagen). Full-length cDNAs for *Nkx2.1* [from John Rubenstein (UCSF, San Francisco, CA) and Oscar Marin (Universidad Miguel Hernández, Alicante, Spain)] and *Lhx6* (from Vassilis Pachnis, MRC, London, UK) were cloned into pCAG-IRES-GFP (from Connie Cepko, Harvard Medical College, Boston, MA) and their expression confirmed by immunofluorescence (see Fig. 1 for NKX2.1). VP16-Nkx2.1 is a gift from Parvis Minoo (Li et al., 2002). The *Nkx2.1* homeodomain point mutant (Val45Phe) (Krude et al., 2002) was generated using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) and subcloned into the pCAGGS vector to produce pNkx2.1ΔHD.

To generate the *Lhx6* RNAi vector, a sequence encoding a small hairpin (sh) targeting the distal end of exon 2 (sense strand, 5′-GTCAGACG-CAGAGGCCTTGGCCATGGCCAAGGCCTCTGCGTCTGACTTTTTT; antisense strand, 5′-agcttAAAAAAGTCAGACGCAGAGGCCTTGG-CCATGGCCAAGGCCTCTGCGTCTGACggcc; lowercase letters indicate nucleotides used in cloning; underlined nucleotides indicate loop region) was inserted into the *Apa*I and *Hin*dIII sites in pSilencer 1.0 (a gift from Yang Shi) (Sui et al., 2002). This shRNAi species has previously been demonstrated to greatly reduce LHX6 abundance in MGE-derived cells and to reduce interneuron migration to the cortex (Alifragis et al., 2004). To enable visualization of the RNAi-transfected cells with a single plasmid, the pU6-shLhx6 construct was then inserted into the *Spe*I site in pCAG-IRES-GFP (shLhx6-GFP). The Scramble RNAi sequence (Ambion) was also subcloned into the same site in the pCAG-IRES-GFP vector. To ensure that nearly all *Nkx2.1*-transfected cells also received the RNAi vector, 2 mg/ml of this vector and 1 mg/ml of pNkx2.1 were microinjected into the slice for EP. This procedure results in over 90% co-labeling of NKX2.1 and GFP in transplanted cells (data not shown) (see also Stuhmer et al., 2002).

For transplantation studies, 12-16 hours after EP GFP epifluorescence was imaged, then the regions of the MGE with the highest densities of GFP+ cells were dissected out, mechanically dissociated, and counted on a hemocytometer under epifluorescence to calculate the percentage of all cells that were GFP+ (usually 10-15%). In the case of slices from *Nkx2.1*-null embryos, in which a morphologically identifiable MGE does not exist (Sussel et al., 1999), tissue was targeted for EP and dissection from the same approximate dorsal-ventral level as the MGE of control slices, as described (Xu et al., 2004).

For in vitro transplants, the feeder cells were prepared from neonatal cortex as described (Xu et al., 2004; Xu et al., 2005) and the transfected cells were plated at a density of 1000 GFP+ cells per well of a 16-well glass chamber slide (36 mm^2; Lab-Tek). For in vivo transplants, cells were injected into S1 cortex 1 mm below the pial surface of cold-anaesthetized neonatal pups (day of birth or P1), using a microinjector (Nanoinject II, Drummond). Per injection site, 10,000-20,000 cells were placed per hemisphere to obtain at least 1000 GFP+ cells per transplant. At P30 the brains were removed, fixed with 4% paraformaldehyde, and sectioned on a vibratome at 50 μm.

RT-PCR

Slices from *Nkx2.1* nulls were electroporated with pNkx2.1-IRES-GFP or pGFP control (n=3). After 12 hours the MGE-like regions were dissociated and sorted by FACS (Vantage, Becton-Dickinson) producing a yield of 3000-5000 cells. Total RNA was purified (RNeasy Kit, Qiagen) and subjected to reverse transcription (Omniscript reverse transcriptase, Qiagen) and PCR (HotStar Kit, Qiagen). Primers included *Lhx6* (5′-TGATG-GCCCAGCCAG and 5′-GTCCATCTTGCAGTAGATC; 422 bp product), *Nkx2.1* (5′-AACAGCGGCCATGCAGCAGCAC and 5′-CCATGTTCT-TGCTCACGTCC; 315 bp) and β-actin (5′-GAGCTGCCTGACGGCC-AGGT and 5′-TACTCCTGCTTGCTGATCCA; 364 bp).

Immunodetection

Immunofluorescence labeling of cells in dissociated cultures was conducted as described (Xu et al., 2004), and labeling of antigens in postnatal brain sections was conducted floating. Primary antibodies used included GFP (rabbit or chick; Molecular Probes), GABA (rabbit; Sigma), LHX6 [rabbit, a gift from Vassilis Pachnis (Lavdas et al., 1999)], neuropeptide Y (rabbit; Immunostar), NKX2.1 (mouse; Lab Vision), somatostatin (rat; Chemicon), parvalbumin (mouse; Chemicon), calretinin (rabbit; Chemicon), Kv3.1 (rabbit, a gift from Bernardo Rudy, New York University, NY) and PCNA (mouse IgG; Novocastra). Alexa line secondary antibodies (Molecular Probes) were used.

Lhx6 promoter analyses

Phylogenetic sequence comparisons of the *Lhx6* locus were performed using the ECR browser (http://www.dcode.org) sequence alignment and visualization tool (Ovcharenko et al., 2004b).

rVISTA (http://rvista.dcode.org/) (Loots and Ovcharenko, 2004; Ovcharenko et al., 2004a) was used to identify potential transcription factor binding sites (http://zpicture.dcode.org/).

Chromatin immunoprecipitation was conducted on E12.5 MGE samples as per the manufacturer's instructions (Upstate, 17-295), using a mouse anti-NKX2.1 monoclonal antibody (Lab Vision). A 119 bp PCR fragment of the *Lhx6* promoter that includes a consensus NKX2.1 binding sequence at position –240 bp relative to the putative translational start site was identified using primers 5′-AGTCCTAACTTTGTAGTG and 5′-TTTCCCCCTCAG-AGGCTTG.

To generate *Lhx6* reporter constructs, a 2.1 kb fragment of 5′ *Lhx6* genomic region (Fig. 5) was cloned from BAC RP23-2D16 by PCR [5′-ACTAGT(*Spe*I)CAGCCTTTAGAAGCTGGTGC and 5′-TCTAGA(*Xba*I)-CCCTGGCTGGGCCATC]. This fragment was inserted in place of the CAG promoter in pCAG-IRES-GFP to produce p5′-Lhx6-IRES-GFP. Site directed mutagenesis (using the oligo sequence 5′-CCCTCTCCC-TGCACTTAACCCGTGATCGCTTAGTTCCTTTTGCAATCCAAGCC; QuikChange Site-directed Mutagenesis Kit, Stratagene) was then used to remove the putative NKX2.1 binding domain (GCTCTTGAAGTA) from –239 to –250 nt.

RESULTS

Expression of *Nkx2.1* cDNA in *Nkx2.1*$^{-/-}$ slices rescues interneuron fate

Our previous work has shown that progenitors from the MGE-like region of *Nkx2.1*$^{-/-}$ slices (MGE*, Fig. 1B,G; see Fig. S2 in the supplementary material; Materials and methods), cultured on dissociated cells from neonatal cortex, fail to differentiate into the SST- or PV-expressing interneuron phenotype (Xu et al., 2004). To determine whether rescued expression of *Nkx2.1* in the ventral telencephalon of slices from *Nkx2.1*$^{-/-}$ mutants can rescue the PV or SST fate of these cells, telencephalic slices were prepared from *Nkx2.1*$^{-/-}$ mouse embryos at embryonic day (E) 12.5, and an expression vector, pNkx2.1-GFP, was introduced into the MGE* by electroporation (Fig. 1 and see Fig. S2 in the supplementary material). After 1 day in vitro (DIV), subregions of the MGE* with the highest proportion of transfected cells were dissected, dissociated, plated over a feeder culture made from neonatal cortex and maintained for 14-28DIV (see Fig. S2 in the supplementary material). In other experiments, cells were transplanted directly into the cortical plate of the somatosensory cortex of neonatal pups and then examined in tissue sections after 30 days (Fig. 1). After fixation, the fates of cells with neuronal morphology (the vast

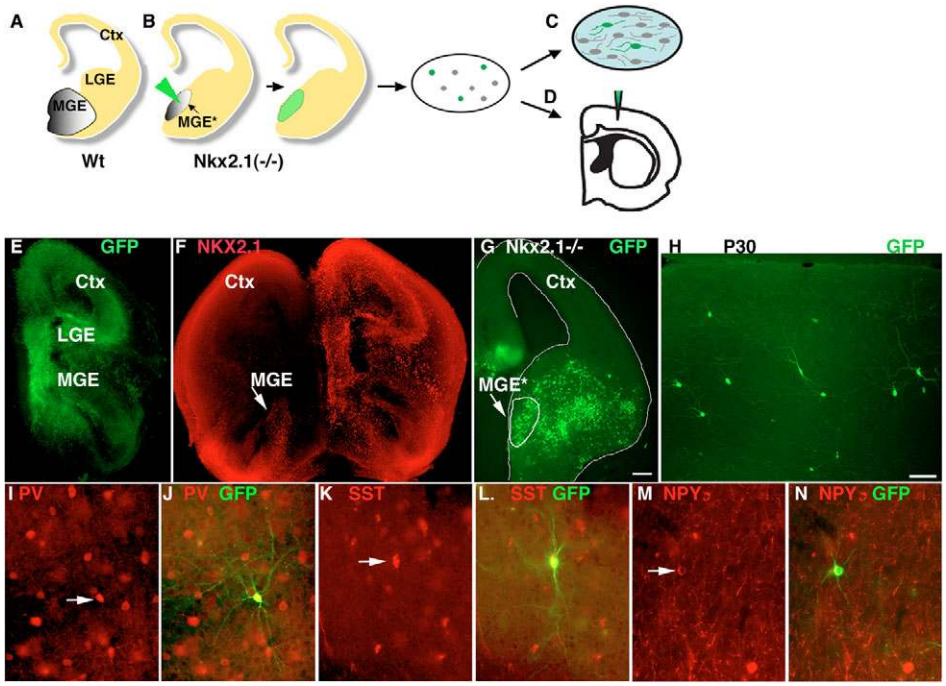

Fig. 1. Transfection of *Nkx2.1*$^{-/-}$ slices with *Nkx2.1* cDNA results in rescue of interneuron phenotypes. (**A-D**) Schematic showing slice electroporation and transplantation paradigm. (A) The *Nkx2.1* domain is shown in the MGE of an E12.5 wild-type (wt) mouse embryo slice. The MGE*, a region that expresses a truncated *Nkx2.1* transcript (Sussel et al., 1999), is shown in the slice from an *Nkx2.1* mutant embryo. (B) This MGE* region is targeted for electroporation, and after 1 day in vitro (DIV) the region is dissected out, dissociated and transplanted (C,D) directly into the neocortex of neonatal pups (as in H-N), or plated onto a high-density culture of neonatal cortical cells [as in Xu et al. (Xu et al., 2004); see Fig. S2 in the supplementary material]. (**E**,**F**) Coronal sections of a slice from an E12.5 wt embryo that was electroporated with pNkx2.1-GFP, maintained 1DIV, then fixed and examined for GFP fluorescence (E) and NKX2.1 immunolabeling (F). The right-hand, electroporated side of the slice has extensive ectopic NKX2.1 expression, whereas only native NKX2.1 expression is seen on the left-hand side of the slice (arrow in F). (**G**) A slice from an *Nkx2.1*$^{-/-}$ embryo was electroporated with pNkx2.1-GFP. After 1DIV, cells from the MGE* (outlined in white) were transplanted into the cortical plate of a neonatal pup and then examined at postnatal day 30 (P30) in 40 μm coronal sections. (**H**) Transplanted GFP-expressing cells scattered through the medial cortex. (**I-N**) Examples of co-labeling for GFP and parvalbumin (PV; I,J), somatostatin (SST; K,L), and neuropeptide Y (NPY; M,N). In control experiments with pGFP vector, almost no cells expressing any of these markers are detected after transplantation of *Nkx2.1*$^{-/-}$ MGE* progenitors (Table 1). MGE, medial ganglionic eminence; LGE, lateral ganglionic eminence; Ctx, cerebral cortex. Scale bars: 100 μm in G,H.

majority of surviving cells from donors at this age of E12.5+1DIV) were determined by immunofluorescence for GFP and the given interneuron subgroup marker. Similar transplantations of cells directly from the MGE of transgenic mice into neonatal cortex have been shown to give rise to neurons with neurochemical and physiological characteristics of inhibitory interneurons (Alvarez-Dolado et al., 2006; Cobos et al., 2005).

Consistent with previous results using in vitro transplants from *Nkx2.1* nulls (Xu et al., 2004), *Nkx2.1*$^{-/-}$ cells transfected with control vector almost never gave rise to PV+, SST+ or NPY+ interneurons (Table 1). In marked contrast, transfection with *Nkx2.1* cDNA resulted in substantial rescue of PV or SST expression (Table 1, Fig. 1I-L). In addition, neurons expressing NPY, a neuropeptide that shares about 50% co-labeling with SST in mouse cortex, were also rescued (Fig. 1M,N). Similar results were obtained whether cells were plated onto a cortical feeder layer or transplanted into the neonatal cortical plate in vivo (Table 1, Fig. 1 and see Fig. S2 in the supplementary material).

Lhx6 is induced when *Nkx2.1* is expressed in the *Nkx2.1*$^{-/-}$ MGE*

The ability of *Nkx2.1* expression to rescue neurochemical aspects of the *Nkx2.1*$^{-/-}$ interneuron phenotype provides an opportunity to identify downstream effectors of cortical interneuron fate determination. A lead candidate for such an effector is LHX6, a LIM-homeodomain-containing transcription factor that is expressed in most MGE-derived interneurons from around the time of their final cell cycle through their maturity in the postnatal cerebral cortex (Cobos et al., 2005; Fogarty et al., 2007; Gong et al., 2003; Lavdas et al., 1999; Liodis et al., 2007) (see Fig. S1 in the supplementary material); LHX6 is not detectable in the MGE* of *Nkx2.1*-null embryos (Sussel et al., 1999). Indeed, RT-PCR detected the induction of *Lhx6* expression in the MGE* of *Nkx2.1*-null embryos transfected with *Nkx2.1* (Fig. 2A).

To further confirm that *Lhx6* can be induced in *Nkx2.1*$^{-/-}$ MGE* progenitors after transfection with pNkx2.1-GFP, rescue- and control-transfected neurons were assessed after in vivo

Table 1. Transfection and transplantation of the MGE-like region in *Nkx2.1*$^{-/-}$ slices with *Nkx2.1* or *Lhx6* cDNAs rescues the expression of MGE-derived interneuron markers

	PV		SST		NPY	
cDNA, transplantation	Control	Rescue	Control	Rescue	Control	Rescue
Nkx2.1, in vitro (*n*=4)	0.4±0.2% (272)	17.0±3.1% (177)	0.6±05% (895)	16.9±4.9% (1802)	0.0±0.0% (1620)	15.1±1.7% (1803)
Nkx2.1, in vivo (*n*=3)	0.0±0.0% (134)	12.7±4.0% (157)	0.0±0.0% (161)	27.6±11.4% (257)	0.0±0.0% (164)	7.1±0.1% (154)
Lhx6, in vivo (*n*=3)	0.0±0.0% (167)	17.5±3.5% (464)	0.0±0.0% (159)	21.9±5.1% (521)	0.0±0.0% (106)	8.5±0.8% (284)

The numbers refer to the percentage of transplanted cells with neuronal morphologies that co-label with the given marker (with total counted neurons shown in parentheses).

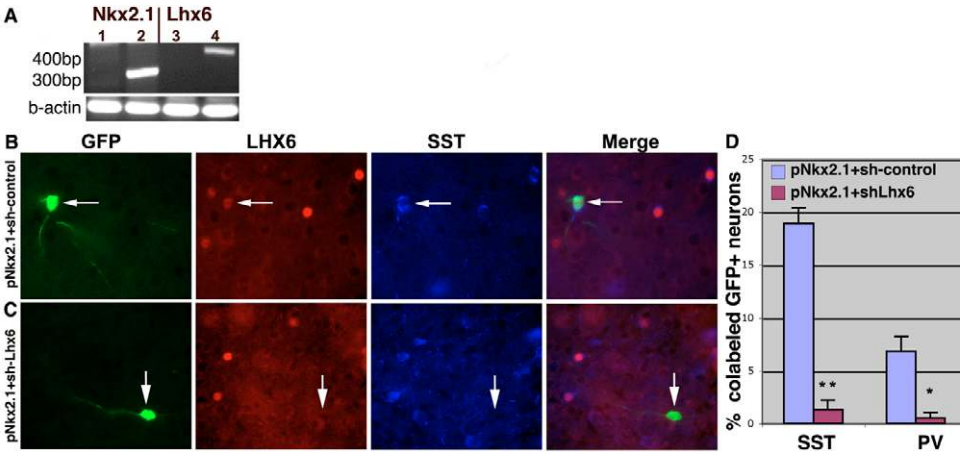

Fig. 2. RNAi knockdown of *Lhx6* blocks the rescue of PV- or SST-expressing cells by *Nkx2.1*. (**A**) The results of RT-PCR for *Nkx2.1* (lanes 1 and 2) and *Lhx6* (lanes 3 and 4) from the MGE-like region (MGE*) of an E12.5 *Nkx2.1*$^{-/-}$ slice that was electroporated with pGFP control vector (lanes 1 and 3) or pNkx2.1-GFP (lanes 2 and 4). *Lhx6* (422 bp band in lane 4) is induced in the *Nkx2.1*-transfected slice. (**B**,**C**) Transfected cells from the MGE* of *Nkx2.1*$^{-/-}$ slices that were cultured from E12.5+1DIV, then transplanted into the parietal cortex of a neonatal mouse in vivo and evaluated in 40 μm sections at P30. The neuron in B was from a transplant that received pNkx2.1+ scramble RNAi control, and co-immunolabels for LHX6 (red) and somatostatin (SST, blue pseudocolored from Cy5 signal). Transfection of pNkx2.1-GFP + shLhx6 RNAi blocks the induction of *Lhx6* and blocks rescue of the SST+ phenotype (C). Note that endogenous SST+ GFP-negative cells in the mouse cortex co-label for LHX6. (**D**) Quantification of the effect of shLhx6 RNAi on the *Nkx2.1* rescue of PV+ and SST+ interneuron fate (n=3 donor samples for each condition transplanted into separate pups, Student's t-test, $^{**}P$<0.01, $^{*}P$<0.03).

transplantation by immunofluorescence labeling with an LHX6-specific polyclonal antibody (Lavdas et al., 1999; Liodis et al., 2007). LHX6 protein was detectable in more than half (54/93=58%, n=3) of the *Nkx2.1*$^{-/-}$ cells transfected with the *Nkx2.1* cDNA. Nearly all of those rescued for expression of PV or SST co-labeled with LHX6 (56/60=93%), whereas LHX6 was detectable in very few of those that received the control vector (3/90).

Lhx6 induction is required for the rescue of the PV or SST phenotype by *Nkx2.1*

The expression pattern of *Lhx6* and its induction in the *Nkx2.1* rescue paradigm raise the possibility that this gene functions in interneuron fate determination. A previous loss-of-function study of *Lhx6* by small hairpin RNA interference (shRNAi) in slice cultures found that *Lhx6* expression is required for normal interneuron migration to the cortex, but not for the expression of GABA (Alifragis et al., 2004). This result has recently been confirmed and extended to the fate determination of PV- and SST-expressing interneuron subgroups in cortex by loss-of-function analysis (Liodis et al., 2007). To determine whether the induction of *Lhx6* is required downstream of *Nkx2.1* in the specification of PV- and SST-expressing interneurons, we conducted loss-of-function studies using the *Nkx2.1* rescue/transplantation paradigm. The same pU6-shRNAi sequence used by Alifragis and colleagues was cloned into a vector that also expresses GFP (p-shLhx6-GFP). The effectiveness of this *Lhx6* RNAi construct at knocking down LHX6 expression was confirmed in primary cultures (see Fig. S3 in the supplementary material).

To examine whether rescue of the *Nkx2.1*$^{-/-}$ interneuron phenotype by *Nkx2.1* expression requires the induction of *Lhx6*, we co-electroporated shLhx6-GFP with pNkx2.1 into the MGE* of *Nkx2.1*$^{-/-}$ slices. As expected, *Lhx6* shRNAi greatly reduced the induction of *Lhx6* in *Nkx2.1*$^{-/-}$ MGE-like cells that were also transfected with pNkx2.1 (Fig. 2B). Remarkably, shLhx6 RNAi nearly eliminated the rescue of SST- and PV-expressing cells by pNkx2.1 (Fig. 2C; n=3, P<0.01 for SST and P<0.03 for PV).

Lhx6 expression also rescues the interneuron fate defect of the *Nkx2.1*$^{-/-}$ MGE*

The ability of *Lhx6* shRNAi to block the *Nkx2.1* rescue experiment raises the possibility that *Lhx6* functions downstream of *Nkx2.1* in the specification of cortical interneurons. To determine whether *Lhx6* itself can rescue the *Nkx2.1*$^{-/-}$ interneuron defect, full-length *Lhx6* cDNA was cloned into the pCAG-IRES-GFP vector. When expressed within the MGE* of *Nkx2.1*$^{-/-}$ slices, pLhx6-GFP also lead to a substantial rescue of PV-, SST- and NPY-expressing cells (Fig. 3, Table 1). In those cells expressing PV, roughly 95% also express the potassium channel Kv3.1 (also known as KCNC1 – Mouse Genome Informatics) (Fig. 3A-D) that is closely associated with PV expression in cortical interneurons (Weiser et al., 1995). Like PV, Kv3.1 was very rarely expressed by control-transfected *Nkx2.1*$^{-/-}$ cells from the MGE* (1/57=1.8%, from three transplantations).

To determine whether, in addition to these neurochemical makers, morphological aspects of interneuron differentiation were rescued in this paradigm, *Nkx2.1*$^{-/-}$ progenitors from the MGE* that were transfected with *Lhx6* or control vector were examined for the presence of dendritic spines. Since cortical interneurons are generally smooth or sparsely spiny, the frequency of heavily spiny versus aspiny or sparsely spiny neurons can provide a measure of whether morphological as well as neurochemical characteristics of *Nkx2.1*$^{-/-}$ MGE*-derived interneurons are rescued by *Lhx6*. Indeed, *Nkx2.1*$^{-/-}$ progenitors from the MGE* that were transfected with *Lhx6* had a significantly lower likelihood than control-transfected cells of being heavily spiny (42% of GFP+ cells in control transfections, 25% in *Lhx6* transfections, P<0.03; Fig. 3I-K). Of *Lhx6*-transfected *Nkx2.1*$^{-/-}$ cells that expressed PV or SST, nearly all (70/72 examined) had a non- or sparsely spiny morphology.

The large majority of Lhx6-transfected Nkx2.1$^{-/-}$ cells also expressed detectable levels of GABA (data not shown), but the interpretation of this result is complicated by the fact that GABA is also expressed by most subcortical projection neurons and is not reduced in the pallidum of *Nkx2.1* mutants (Sussel et al., 1999), in pallidal cells transfected by RNAi for *Lhx6* (Alifragis et al., 2004), or in the cortex of *Lhx6*-null mutants (Liodis et al., 2007). In

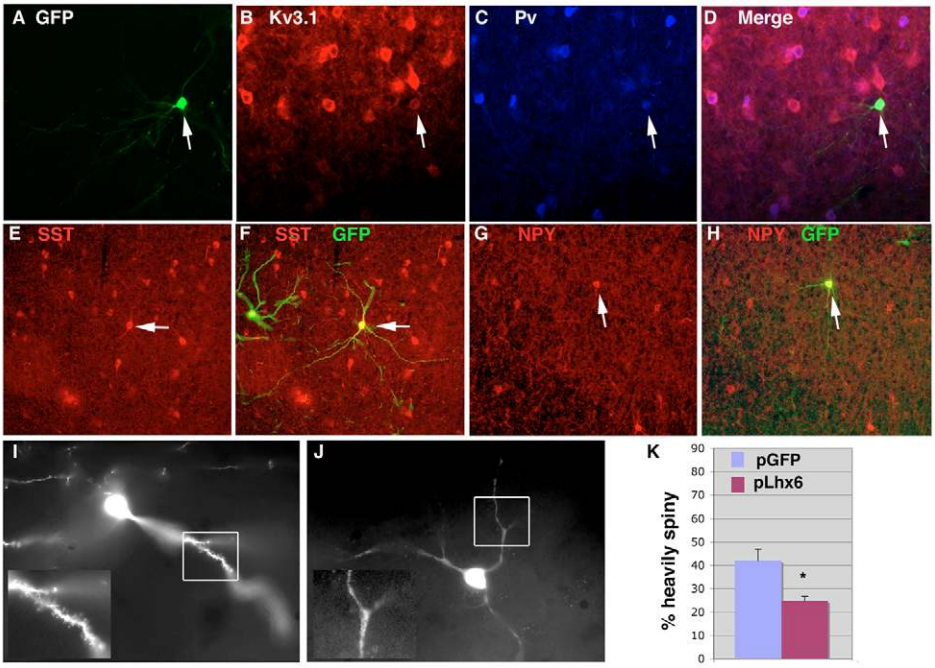

Fig. 3. *Lhx6* expression can rescue interneuron phenotypes in transplanted cells from *Nkx2.1*$^{-/-}$ MGE*. pLhx6-GFP was electroporated into the MGE-like region of E12.5 *Nkx2.1*$^{-/-}$ slices, then after 1DIV the transfected regions were dissociated and transplanted into the cortex of neonatal pups. Shown are coronal sections through a P30 mouse that had received the transplantation into the cortical plate at P1. (**A-H**) Examples of co-labeling for GFP together with Kv3.1 and parvalbumin (PV; A-D), somatostatin (SST; E,F), and neuropeptide Y (NPY; G,H). In control experiments with pGFP vector, few cells expressing these markers are detected after transplantation of *Nkx2.1*$^{-/-}$ MGE-like progenitors (see text and Table 1). (**I**,**J**) Transfected neurons (I, pGFP control; j, pLhx6-GFP) photographed at higher magnification to reveal dendritic spines. Insets show the boxed regions at higher magnification. (**K**) The frequency of heavily spiny neurons is significantly lower in the *Nkx2.1*$^{-/-}$ MGE* cells transfected with *Lhx6* than in controls (41.9% versus 24.7%, n=3, *P<0.03). In addition, those *Nkx2.1*$^{-/-}$ cells 'rescued' for expression of PV or SST by *Lhx6* are nearly all non- or sparsely spiny. These results suggest that *Lhx6* can act downstream of *Nkx2.1* to direct some aspects of both the neurochemical and morphological fates of MGE-derived cortical interneurons.

summary, these results suggest that *Lhx6* directs both neurochemical and morphological aspects of MGE-derived interneuron fate, independently of the expression of GABA.

NKX2.1 appears to directly activate *Lhx6* expression in the MGE

The requirement for *Nkx2.1* for expression of *Lhx6* (Sussel et al., 1999), and the induction of *Lhx6* in the *Nkx2.1* rescue experiment (Fig. 2), raise the question of whether NKX2.1 directly activates *Lhx6* expression. Whereas NKX2.2 regulates neuronal fate in the ventral spinal cord by transcriptional repression (Muhr et al., 2001), NKX2.1 is known to directly activate the transcription of target genes in the lung and thyroid (Li et al., 2000; Mizuno et al., 1991; Tell et al., 1998). To examine this issue we first compared the mouse, human, chicken, fugu and frog sequences over approximately 10 kb of genomic DNA 5′ to the predicted *Lhx6* start site. There are multiple regions of high homology, particularly within 500 bp of the putative translational start site (Fig. 4A). One of these regions includes a consensus NK2 family binding sequence [T(T/C)AAGT(A/G)(G/C)TT] (Watada et al., 2000) located at –240 bp (Fig. 4B). To determine whether NKX2.1 binds this region, chromatin immunoprecipitation was conducted on lysates of MGE from E12.5 embryos. PCR on the DNA pulled down using an anti-NKX2.1 monoclonal antibody (see Materials and methods) indicated that a 119 bp fragment including the above sequence appears to bind NKX2.1 in vivo (Fig. 4C).

To determine whether this sequence promotes the transcription of *Lhx6* within the *Nkx2.1* expression domain, an IRES-GFP construct was cloned into the 3′ end of a 2.1 kb fragment of the *Lhx6* promoter (p5′-Lhx6-GFP). Electroporation (EP) of this construct into the MGE of E13.5 slices resulted in robust expression of GFP (Fig. 5A-C; n=5). By contrast, little expression was apparent upon EP into either the dorsal midline of wild-type embryos (Fig. 5A-C; n=5), or into the lateral ganglionic eminence (LGE) or cortex (Fig. 6A-C). Consistent with the requirement for NKX2.1 to drive the expression of *Lhx6*, no GFP expression was seen after EP into the MGE-like region of *Nkx2.1*$^{-/-}$ slices (Fig. 5D-F; n=5). However, Co-EP of p5′-Lhx6-GFP together with an *Nkx2.1* expression vector restored GFP expression in the MGE-like region of *Nkx2.1*$^{-/-}$ slices (Fig. 5G-I; n=5). Similarly, Co-EP of p5′-Lhx6-GFP together with an *Nkx2.1* expression vector was able to drive p5′-Lhx6-GFP expression in the LGE and cortex of wild-type slices (Fig. 6D-F; n=5).

The above results suggest that expression of this *Lhx6* promoter fragment in the telencephalon requires the presence of *Nkx2.1*. To determine whether the NKX2.1 binding sequence in the promoter fragment of p5′-Lhx6-GFP is necessary for *Lhx6* expression within the MGE, this sequence was removed from the reporter construct, generating pΔ5′-Lhx6-GFP. EP of pΔ5′-Lhx6-GFP into the MGE of wild-type embryos resulted in very limited expression of GFP (Fig. 5J-L; n=5). Since ectopic expression of *Nkx2.1* was able to drive expression of pLhx6-GFP in the LGE and cortex (Fig. 6D-F), and this effect was nearly eliminated when the NKX2.1 consensus binding sequence is removed from the reporter construct (Fig. 6J-L), we next tested whether the DNA-binding region of NKX2.1 is required for this effect. A point mutation that is associated with a hereditary movement disorder in humans (Krude et al., 2002), resulting in a Val45Phe alteration in the homeodomain, was introduced into the *Nkx2.1* expression vector (pNkx2.1ΔHD). This mutation greatly reduced the ability of NKX2.1 to bind to its consensus target sequence. Co-EP of pNkx2.1ΔHD together with pLhx6-GFP into the LGE resulted in minimal activation of the reporter (Fig. 6, compare G-I with D-F). Finally, a vector containing the VP16 transcriptional activation sequence fused to *Nkx2.1* was tested (VP16Nkx2.1) (Li et al., 2002). VP16Nkx2.1 strongly

activated the pLhx6-GFP reporter, suggesting that NKX2.1 does not indirectly activate *Lhx6* transcription by repressing the expression of an intermediate gene.

DISCUSSION

Despite its likely relevance to neuropsychiatric disease, progress in revealing the molecular control of cell fate determination in the mammalian telencephalon has been slow. In this paper we demonstrate that the homeodomain transcription factor NKX2.1 acts to specify neurochemical and morphological aspects of cortical/striatal interneuron fate by directly activating the LIM-homeodomain gene *Lhx6*. The combined use of slice EP and transplantation of transfected cells should be applicable to the study of other characteristics of interneuron subgroups, and possibly to other neurons whose defining characteristics are achieved long after crucial fate-determining events have occurred during embryogenesis.

Nkx2.1-null mice fail to generate normal MGE tissue (Sussel et al., 1999) and are unable to generate cortical interneurons expressing PV or SST (Xu et al., 2004), distinct subgroups that are known to originate primarily from the MGE (Wonders and Anderson, 2006). By transfecting *Nkx2.1* back into the MGE-like region of *Nkx2.1*$^{-/-}$ slices, culturing the slice for 24 hours and then transplanting the transfected cells into the cortex of neonatal pups, both the PV and the SST phenotypes can be rescued in vivo (Fig. 1, Table 1). Parallel experiments in which the *Nkx2.1*-transfected cells are cultured on a feeder layer of dissociated neonatal cortex also show substantial rescue of these neurochemical phenotypes, whereas the expression of these phenotypes in *Nkx2.1*-null cells transfected with the control plasmid is almost non-existent (Table 1; see Fig. S2 in the supplementary material).

In addition to the neurochemical phenotype, two additional lines of evidence suggest that the rescued cells are interneuron-like. First, they display morphological features of interneurons, including curved terminals characteristic of large PV+ and smaller SST+ basket cells, and aspiny or sparsely spiny dendrites. Second, more than 90% of the *Nkx2.1*$^{-/-}$ neurons rescued for the PV or SST phenotype are immunopositive for LHX6, a transcription factor expressed in most MGE-derived interneurons of the striatum and cortex from around the time that they exit the cell cycle through maturity (Cobos et al., 2005; Fogarty et al., 2007; Gong et al., 2003; Lavdas et al., 1999; Liodis et al., 2007) (see Fig. S1 in the supplementary material).

Lhx6 specifies interneuron fates downstream of *Nkx2.1*

The expression patterns of *Nkx2.1* and *Lhx6* (see Fig. S1 in the supplementary material), the loss of *Lhx6* expression in *Nkx2.1* nulls (Sussel et al., 1999), and the induction of *Lhx6* by *Nkx2.1* (Figs 2, 5, 6), raise the possibility that *Lhx6* functions directly downstream of *Nkx2.1* in the specification of PV and SST interneuron fate. Co-transfection of the *Nkx2.1*$^{-/-}$ MGE-like region in slices with expression vectors for both *Nkx2.1* and pLhx6-shRNAi produces a dramatic reduction in the frequency of PV+ and SST+ phenotypes (Fig. 2). This result suggests that *Lhx6* expression is required for the acquisition of these phenotypes, a notion that is strongly supported by the cortical interneuron phenotype observed in *Lhx6* nulls in which GABA expression in cortex is grossly normal but the number of PV- or SST-expressing interneurons is very dramatically reduced (Liodis et al., 2007).

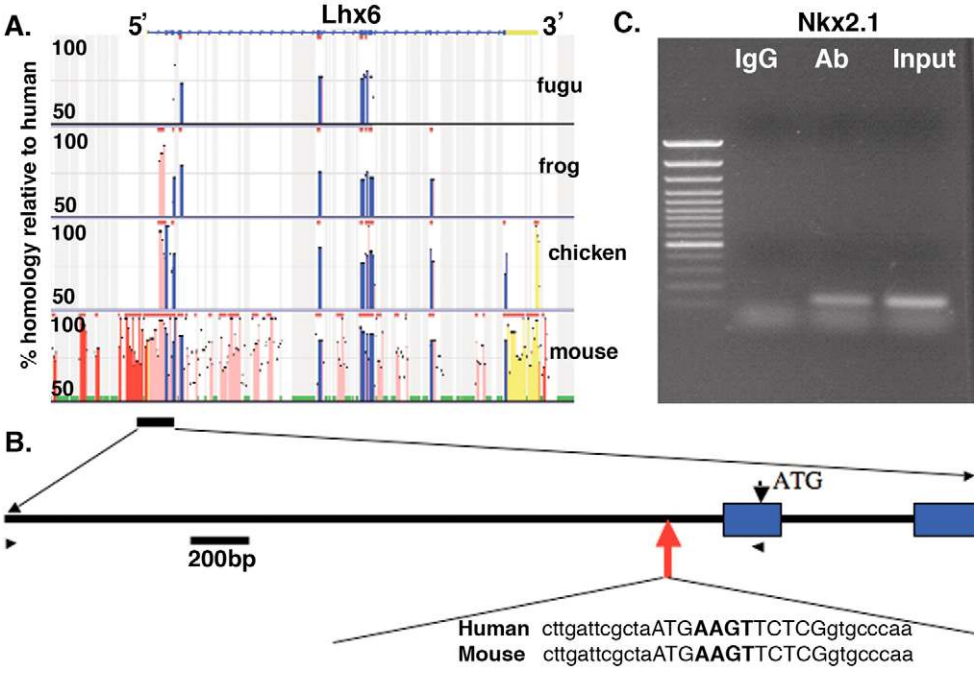

Fig. 4. Binding of NKX2.1 to a conserved consensus binding sequence in the *Lhx6* promoter. (**A**) Evolutionarily conserved region (ECR) visualization of the *LHX6* gene locus in the human genome. The conservation profile of the human sequence in comparison with the mouse, chicken, frog and fugu genomes is shown. In the model, the *Lhx6* loci of the given species are displayed on the horizontal axis and the vertical axis represents the percentage of base-pair identity (from 50 to 100%) between the given species and human. The horizontal bar above each species provides an overview of the distribution of ECRs. A conserved alignment is blue if it overlaps with a coding exon. Yellow, untranslated region; pink, intron (although most of the pink region 5′ to the *Lhx6* translation start site in fact appears to be an intergenic region, see NCBI sequence data); red, intergenic region. The green bars at the bottom indicate repetitive elements in the sequence. (**B**) rVISTA analysis revealed a conserved NKX2.1/TITF1 binding site 240 bp from the translation start site. The arrowheads indicate the locations of the PCR primers used to clone a 2.1 kb promoter fragment of genomic DNA (see Materials and methods and Figs 5, 6). (**C**) The NKX2.1 consensus binding sequence is located within a 119 bp PCR product that was used to probe chromatin immunoprecipitation results on E12.5 MGE. IgG, control mouse IgG; Nkx2.1 Ab, mouse anti-NKX2.1; Input, control PCR on a crosslinked, sonicated MGE sample; left, molecular weight marker. These results suggest that NKX2.1 binds the *Lhx6* promoter at a highly conserved NKX2.1 consensus binding sequence in vivo.

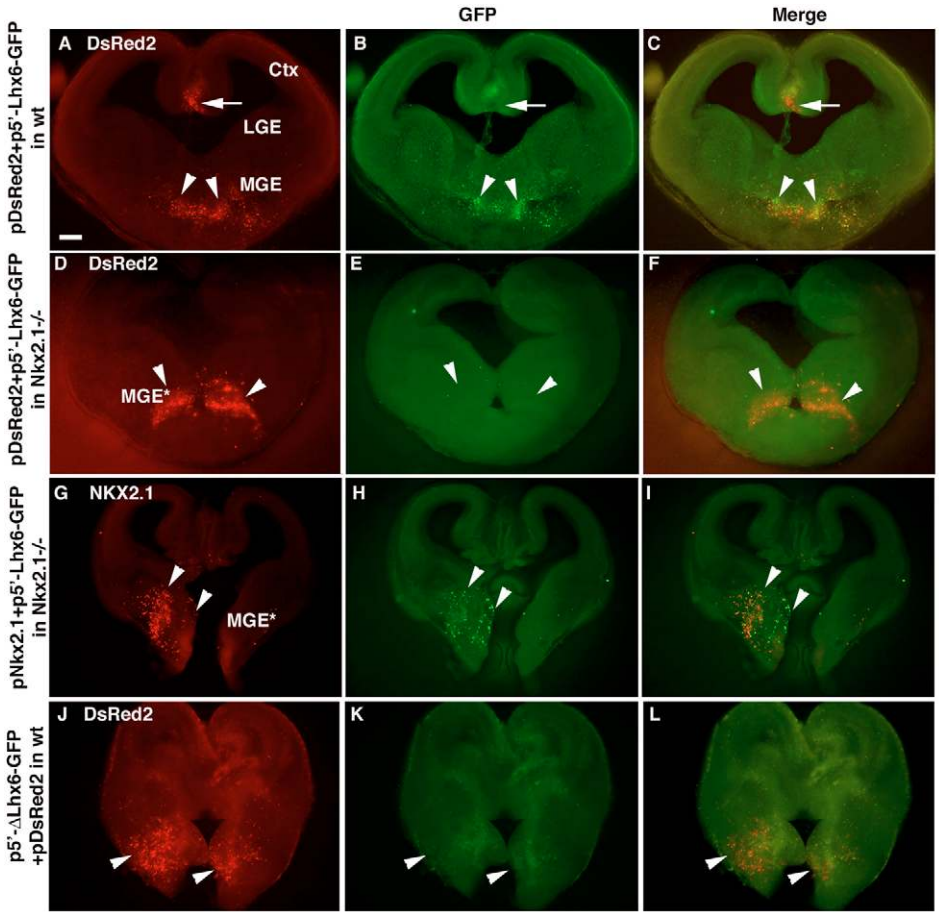

Fig. 5. *Nkx2.1* activates the expression of an *Lhx6* promoter reporter. Shown are examples of coronal, telencephalic slices at E13.5+1DIV that were electroporated with the constructs indicated. (**A-C**) Constitutively expressing pCAG-DsRed2 (A, pDsRed2) was introduced into a wt mouse embryo slice together with a reporter construct that contains 2.1 kb of the *Lhx6* promoter region placed 5′ to IRES-GFP (p5′-Lhx6-GFP, B). The merged image in C shows that the reporter construct is detectable in the ventral, *Nkx2.1*-expressing region (arrowheads) and not in the electroporated region of the medial cortex (arrow). (**D-F**) In marked contrast to B and C, electroporation of p5′-Lhx6-GFP into the MGE-like region (MGE*) of this slice from an *Nkx2.1* null results in no reporter expression (E,F). (**G-I**) However, the expression of p5′-Lhx6-GFP is rescued in an *Nkx2.1*-null slice by the addition of exogenous *Nkx2.1* (red signal in G and I is NKX2.1 immunofluorescence). (**J-L**) A wild-type slice electroporated with a mutated reporter construct in which only the NKX2.1 consensus binding sequence has been deleted (p5′-Δ-Lhx6-GFP; see Materials and methods). Minimal expression of GFP is detected in the MGE with this construct (K-L). *n*=at least five experiments for each result. MGE, medial ganglionic eminence; LGE, lateral ganglionic eminence; Ctx, cerebral cortex. Scale bar: 200 μm in A for A-L.

The loss-of-function evidence raises the question of whether *Lhx6* is not only required for acquisition of the PV+ and SST+ phenotypes, but is also sufficient to restore this phenotype in the *Nkx2.1*-null context. Indeed, expression of *Lhx6* cDNA within the MGE-like region of *Nkx2.1* nulls also results in a substantial restoration of these phenotypes (Fig. 2, Table 1). The apparent rescue extends beyond PV and SST because most of the 'rescued' PV-expressing cells also express the Kv3.1 potassium channel. In addition, in contrast to controls, *Lhx6*-rescued PV- and SST-expressing cells are nearly all aspiny or sparsely spiny, suggesting that *Lhx6* promotes both morphological and neurochemical aspects of interneuron fate (Fig. 3). The control of multiple aspects of MGE-derived interneuron characteristics suggests that *Lhx6* might function to drive multiple transcriptional cascades to direct the specification of several subgroups of this telencephalic neuronal subclass.

NKX2.1 appears to directly activate *Lhx6* expression

The results presented above suggest that NKX2.1 functions to specify interneuron subgroups in the MGE largely or entirely by activating *Lhx6* transcription. Unlike the prominent repressor functions of other Nkx family members in directing cell fate within the ventral spinal cord (Vallstedt et al., 2001), NKX2.1 directly activates target genes in the thyroid and lung (Liu et al., 2002; Mizuno et al., 1991; Moya et al., 2006), although these targets are not known to include LIM-homeodomain transcription factors. Comparative genomic sequence and transcription factor binding site analyses reveal a highly conserved consensus NKX2.1 binding sequence about 240 bp from the *Lhx6* translation initiation site (Fig. 4), and this site is present in a 119 bp fragment identified by chromatin immunoprecipitation. In addition, a 2.1 kb fragment upstream of the translation initiation site drives expression of GFP reporter specifically in *Nkx2.1*-expressing regions of wild-type slices (Figs 5, 6). This expression is lost in *Nkx2.1*-null slices, can be rescued by exogenous addition of *Nkx2.1* to the *Nkx2.1*-null slices, and is abolished when the NKX2.1 binding sequence is removed from the reporter (Fig. 5). Moreover, the ability of ectopic *Nkx2.1* to drive the *Lhx6* reporter construct in the LGE or cortex (Fig. 6) is abolished by a point mutation in the homeodomain that has previously been shown to greatly diminish the ability of NKX2.1 to bind its target DNA sequence (Krude et al., 2002). Although establishment of the definitive role played by the identified NKX2.1 binding sequence requires in vivo confirmation, taken together these results suggest that NKX2.1 drives specification of the SST+ and PV+ phenotypes via the direct activation of *Lhx6*.

Role of *Lhx6* in cortical interneuron specification

Interestingly, ectopic expression of either *Lhx6* or *Nkx2.1* in the ventral half of the E12.5 LGE, which normally gives rise primarily to medium spiny neurons of the striatum (Stenman et al., 2003), does not produce PV+ or SST+ neurons (T.D. and S.A.A., unpublished). This result suggests that, consistent with the residual expression of a truncated *Nkx2.1* transcript within the MGE-like region of *Nkx2.1* nulls (Sussel et al., 1999), the MGE* is molecularly distinct from the LGE proper despite the presence of ventricular zone, subventricular

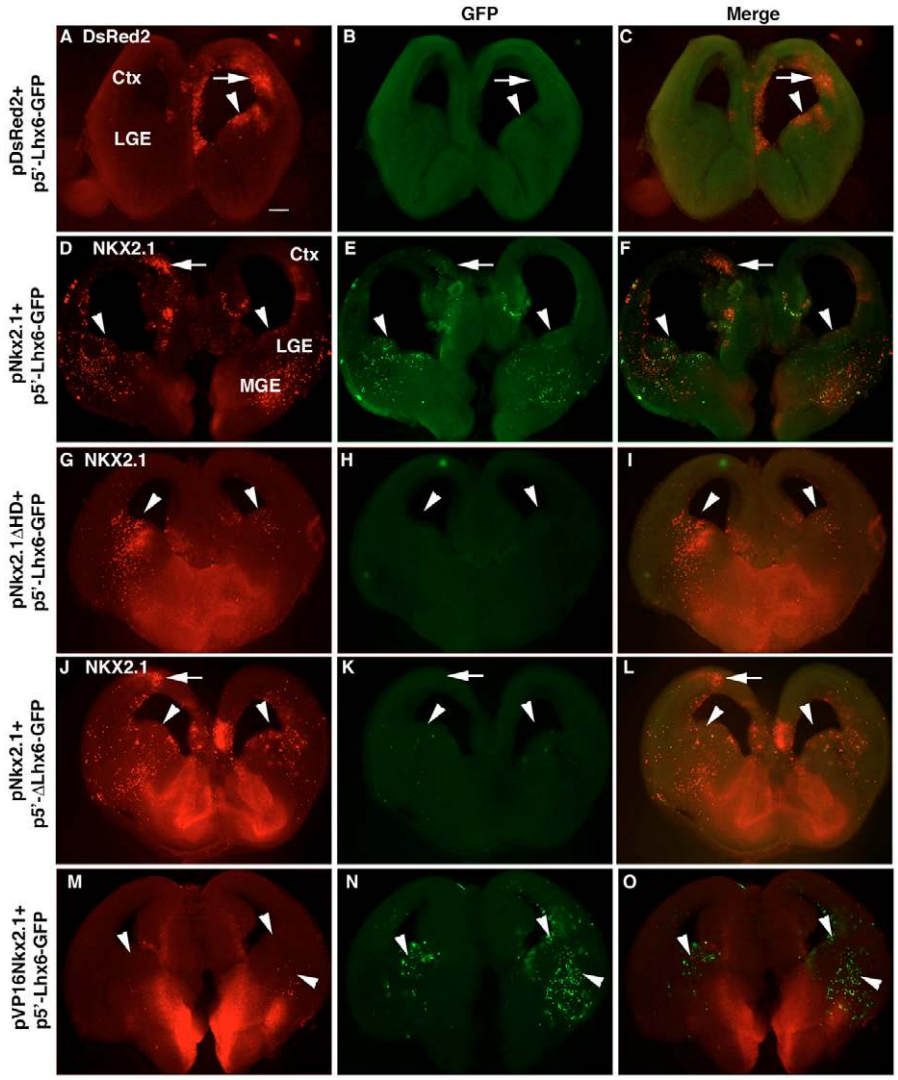

Fig. 6. Ectopic activation of Lhx6-GFP reporter expression by *Nkx2.1*. (**A-C**) In mouse embryo slice cultures maintained from E13.5+1DIV, no expression of GFP is detected when p5′-Lhx6-GFP is introduced into the wt LGE (arrowhead) or lateral cortex (arrow). (**D-F**) Ectopically expressed NKX2.1 drives Lhx6-reporter expression in these regions. (**G-I**) This activation is not present in response to ectopic expression of an altered NKX2.1 construct containing a missense point mutation in the homeodomain that abrogates its ability to bind DNA (Krude et al., 2002). Note that the red signal in G and I is NKX2.1 immunofluorescence that is not affected by the point mutation. (**J-L**) Co-electroporation of pNkx2.1 and the *Lhx6* promoter reporter construct that lacks the NKX2.1 consensus binding sequence (pΔ5′-Lhx6-GFP) results in little expression of GFP. (**M-O**) As with *Nkx2.1* cDNA, fusion of the VP16 activator domain to *Nkx2.1* strongly induces the reporter GFP expression in wt LGE or cerebral cortex. In this case, there is reduced detection of the altered NKX2.1 protein by immunofluorescence (M,O). MGE, medial ganglionic eminence; LGE, lateral ganglionic eminence; Ctx, cerebral cortex. Scale bar: 200 μm in A for A-O.

zone and mantle zone gene expression that is normally restricted to the LGE (Sussel et al., 1999). The absence or presence of such a factor would supply competence to attain a PV+ or SST+ phenotype in response to *Lhx6* expression despite the absence of *Nkx2.1*.

Although these results indicate that *Nkx2.1* function, as it pertains to some crucial aspects of cortical interneuron specification, acts via the activation of *Lhx6*, they do not address the extent to which other aspects of *Nkx2.1* function in the MGE depend on *Lhx6*. For example, *Nkx2.1* is also required for the expression of *Lhx7* (also known as *Lhx8* – Mouse Genome Informatics) (Sussel et al., 1999), and both genes are required for the specification of most cholinergic neurons of the basal forebrain (Fragkouli et al., 2005; Marin et al., 2000; Sussel et al., 1999; Zhao et al., 2003). However, although the MGE or the underlying preoptic/anterior endopeduncular region gives rise to cholinergic interneurons of the striatum (Marin et al., 2000), cholinergic phenotypes are not seen in transplants of these regions into cortex (T.D. and S.A.A., unpublished results), such that a role for *Lhx6* in the specification of these cells was not tested in this study. Interestingly, although essentially all cholinergic interneurons of the striatum derive from *Nkx2.1*-expressing progenitors, a bipolar-morphology *Nkx2.1*-lineage-negative cholinergic interneuron has recently been described in mouse neocortex (Xu et al., 2008).

Our previous work showed that sonic hedgehog signaling during the age range of neurogenesis is required to maintain *Nkx2.1* expression within, and interneuron generation by, cycling progenitors of the MGE (Xu et al., 2005). This paper extends that work in suggesting that *Nkx2.1* specifies PV+ or SST+ interneuron subgroups and other neurochemical, as well as morphological, aspects of MGE-derived interneuron fates by directly activating *Lhx6*. Several lines of evidence suggest that progenitors giving rise to these subgroups might be partially segregated on the dorsal-ventral axis of the MGE (Flames et al., 2007; Fogarty et al., 2007; Ghanem et al., 2007; Wonders et al., 2008). As *Lhx6* itself does not appear to be differentially expressed along the dorsal-ventral axis of the MGE, a key remaining question is how *Lhx6* function is modified to differentially specify the MGE-derived interneuron subgroups of the cerebral cortex.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/8/1559/DC1

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