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Generation of *Nkx2.2:lacZ* mice using Recombination-Mediated Cassette Exchange Technology

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Abstract

Nkx2.2 encodes a homeodomain transcription factor required for the correct specification and/or differentiation of cells in the pancreas, intestine and CNS. To follow the fate of cells deleted for *Nkx2.2* within these tissues, we generated *Nkx2.2:lacZ* knockin mice using a recombination-mediated cassette exchange (RMCE) approach. Expression analysis of *lacZ* and/or β -galactosidase in *Nkx2.2^{lacZ/+}* heterozygote embryos and adults demonstrates that *lacZ* faithfully recapitulates endogenous *Nkx2.2* expression. Furthermore, the *Nkx2.2^{lacZ/lacZ}* homozygous embryos display phenotypes indistinguishable from the previously characterized *Nkx2.2^{-/-}* strain. *LacZ* expression analyses in the *Nkx2.2^{lacZ/lacZ}* homozygous embryos indicate that *Nkx2.2*-expressing progenitor cells within the pancreas are generated in their normal numbers and are not mislocalized within the pancreatic ductal epithelium or developing islets. In the central nervous system of *Nkx2.2^{lacZ/lacZ}* embryos, *LacZ*-expressing cells within the ventral P3 progenitor domain display different migration properties depending on the developmental stage and their respective differentiation potential.

Keywords

Pancreas development; beta cells; ghrelin cells; transcriptional regulation; islet cell lineage

Introduction

Nkx2.2 is a member of the vertebrate homeodomain transcription factor gene family homologous to the *Drosophila NK2/ventral nervous system defective* (*vnd*) gene (Kim and Nirenberg, 1989; Price *et al.*, 1992). *Nkx2.2* is expressed in the central nervous system (CNS), pancreas and intestine, where it plays essential roles in the specification of progenitor cell identity and regulation of cell fate decisions (Briscoe *et al.*, 1999; Desai *et al.*, 2008; Sussel *et al.*, 1998). In the developing pancreas, *Nkx2.2* protein expression is initiated with Pancreatic and duodenal homeobox (Pdx1) and Pancreas transcription factor 1 subunit alpha (Ptf1a) in the dorsal and ventral pancreatic epithelium at e8.75 and e9.5, respectively (Jorgensen *et al.*, 2007). After e11.5, *Nkx2.2* expression is maintained throughout the epithelium, but is produced at varied intensities, with obvious subsets of low and high expressing cells emerging during the secondary transition. The high expressing cells predominantly correlate with the hormone-producing populations. *Nkx2.2* is also found in a large number of endocrine progenitors (Jorgensen *et al.*, 2007), where it has been proposed to modulate the expression of Neurogenin 3 (*Ngn3*) (Anderson *et al.*, 2009b). In

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the adult pancreas, *Nkx2.2* expression is maintained in alpha, beta and pancreatic polypeptide (PP) cells but not in delta cells (Sussel *et al.*, 1998). *Nkx2.2* cannot be detected within the exocrine compartment at any point during development or in the adult.

In the developing CNS, *Nkx2.2* is expressed in specific subsets of neurons along the anterior-posterior axis. *Nkx2.2* has been extensively studied in the ventral spinal cord where it is induced by high levels of Sonic hedgehog (Shh) to specify V3 interneurons and repress motor neuron differentiation (Briscoe *et al.*, 2000; Ericson *et al.*, 1997). After e12.5, a second wave of *Nkx2.2*-expressing progenitor cells gives rise to oligodendrocytes (Qi *et al.*, 2001; Zhou *et al.*, 2001). In the hindbrain, *Nkx2.2* is expressed in the ventral precursor cells that first give rise to the visceral motor neurons (vMN) and then to serotonergic neurons (Briscoe *et al.*, 1999; Pattyn *et al.*, 2003a). In the intestine, *Nkx2.2* can be detected in a subset of *Ngn3*-expressing cells, endocrine progenitor cells located in the crypt compartment (Desai *et al.*, 2008; Wang *et al.*, 2009b) and is expressed in a large number of enteroendocrine-producing cell populations, including the glucagon-like peptide 1 (GLP-1) - expressing L cells, serotonin, somatostatin and cholecystokinin (CCK) cells (Desai *et al.*, 2008).

Interestingly, in each of the three tissues expressing *Nkx2.2*, the loss of *Nkx2.2* induces cell fate conversions. In the pancreas of *Nkx2.2*^{-/-} (null) mice, insulin-producing beta cells are completely absent and glucagon-producing alpha cells are reduced (Prado *et al.*, 2004; Sussel *et al.*, 1998). Similarly, in the intestine of mice lacking *Nkx2.2*, many different hormone-producing populations are reduced, including those expressing CCK, GLP-1, gastrin and serotonin (Desai *et al.*, 2008). In both the pancreas and intestine, the loss of hormone-producing cells is compensated by the upregulation of another endocrine population, the *ghrelin*-expressing cells (Desai *et al.*, 2008; Prado *et al.*, 2004). In the CNS, *Nkx2.2*^{-/-} mice have reduced numbers of V3 interneurons, oligodendrocytes, and serotonergic neuronal populations. Analogous to the pancreas and gut, there is a concomitant upregulation of the dorsally located motor neuron domains in the spinal cord (Briscoe *et al.*, 1999; Pattyn *et al.*, 2003b). Although it is apparent that *Nkx2.2* is essential for the specification of specific cell types in each tissue, it is not clear whether the cell conversions result from the loss of specific *Nkx2.2*-producing progenitor populations, a failure of *Nkx2.2*-expressing cells to migrate or proliferate appropriately, and/or the redirection of *Nkx2.2*-expressing cells to alternative cell fates.

In this study, we describe the generation and characterization of an *Nkx2.2:lacZ* knock-in allele generated by Recombination-Mediated Cassette Exchange (RMCE) (Chen *et al.*, 2011; Long *et al.*, 2004). Analyses of the *Nkx2.2*^{lacZ/+} heterozygous mice demonstrate the faithful recapitulation of *lacZ* and beta-galactosidase in the endogenous *Nkx2.2* expression domains and *Nkx2.2*^{lacZ/lacZ} homozygous mice phenocopy the *Nkx2.2*^{-/-} mice (Prado *et al.*, 2004; Sussel *et al.*, 1998). Furthermore, the ability to track *lacZ*-expressing cells in the absence of *Nkx2.2* suggest that the majority of *Nkx2.2*-derived populations in the pancreas and CNS are maintained in the absence of functional *Nkx2.2* protein and appear to be respecified into alternative cell fates, as previously hypothesized (Briscoe *et al.*, 1999; Prado *et al.*, 2004). However, these studies also revealed that a population of progenitors in the spinal cord fails to migrate from the ventricular zone after e12.5 and thus may not become respecified. The detection of beta-galactosidase expression in the *Nkx2.2*^{lacZ/+} mice has also allowed us to monitor the fate of *Nkx2.2*-expressing cells in the absence of *Pdx1* or *Ngn3*. In each case, *Nkx2.2:lacZ*-expressing cells are present, indicating that *Pdx1* and *Ngn3* are not required to initiate or maintain *Nkx2.2* expression.

Results

We generated the *Nkx2.2:lacZ* allele using a two step RMCE approach (Chen *et al.*, 2011). The first step involved the generation of an *Nkx2.2* acceptor allele using standard homologous recombination methods. The *Nkx2.2* acceptor allele (*Nkx2.2^{LCA}*) was engineered in TL-1 ES cells (Labosky *et al.*, 1994) and has a 5.115 kb region of the *Nkx2.2* genomic region, including both coding exons, replaced with a *pUΔTK-EM7kan* cassette that is flanked with heterologous lox sites (Figure 1A and materials and methods). Electroporated ES cells were screened by southern analysis using external 5' and 3' probes to identify 8 correctly targeted *Nkx2.2^{LCA}* ES cells (Figure 1A,C; data not shown). To generate the *Nkx2.2:lacZ* cassette, the full-length *lacZ* gene containing a N-terminal nuclear localization signal (nls) (Picard and Yamamoto, 1987) was cloned in frame with the *Nkx2.2* ATG, replacing most of the *Nkx2.2* two coding exons and the intervening intron (Figure 1B, materials and methods). RMCE of the *Nkx2.2:lacZ* cassette was performed in ES cell clones 2G6 and 4E9 with an exchange efficiency of 47% and 41%, respectively. RMCE clones were screened by PCR (Figure 1D). One clone from each correctly recombined ES cell line was injected into blastocysts; mice generated from clone 4E9/4D2 were validated by PCR and selected for further analysis (Figure 1E; 53004 and 53005).

Nkx2.2 is expressed in the developing CNS, intestine and pancreas (Briscoe *et al.*, 1999; Desai *et al.*, 2008; Jorgensen *et al.*, 2007; Sussel *et al.*, 1998). To confirm that *lacZ* expression in the *Nkx2.2^{lacZ/+}* mice accurately recapitulated endogenous *Nkx2.2* expression, we characterized *lacZ* expression and/or beta-galactosidase activity in *Nkx2.2^{lacZ/+}* heterozygous embryos throughout embryogenesis (Figure 2). Critical to this analysis, heterozygous *Nkx2.2^{+/-}* or *Nkx2.2^{lacZ/+}* embryos express *Nkx2.2* mRNA transcript at levels comparable to *Nkx2.2* wild type embryos and appear phenotypically wild type (Supplemental figure 1e and 4). Whole mount X-gal staining of e10.5 and e12.5 *Nkx2.2^{lacZ/+}* embryos demonstrated LacZ expression along the entire anterior-posterior axis of the CNS and in the pancreatic bud region, as expected (Figure 2a and 2b) (Jorgensen *et al.*, 2007; Shimamura *et al.*, 1995). At both stages, X-gal staining is apparent in ventral regions of the forebrain, midbrain, hindbrain, and spinal cord, while *lacZ* expression is not detected in control wild type littermates (Supplemental figure 1a and b). Sections at comparative levels from e12.5 embryos demonstrate that LacZ expression corresponds to endogenous *Nkx2.2* mRNA expression in the CNS and dorsal and ventral pancreatic buds (compare Figure 2c to 2d). A ventral view of whole e12.5 brain shows concentrated X-gal staining along the ventral midline, with staining spreading laterally within *Nkx2.2*-expressing cells of the anterior subventricular zone that ultimately migrate rostrally to give rise to oligodendrocyte populations in the olfactory bulb (Figure 2e) (Aguirre and Gallo, 2004; Tonchev *et al.*, 2006). The dorsal view of the embryo shows two pronounced stripes of expression in the spinal cord flanking the midline, which is also characteristic of endogenous *Nkx2.2* expression (Figure 2f). In the e17.5 pancreas, X-gal staining can be detected strongly throughout the epithelial ductal tree and becomes downregulated in the peripheral cells that give rise to the exocrine tissue (Figure 2g). Scattered X-gal staining can be detected throughout the *Nkx2.2^{lacZ/+}* intestine, representing *Nkx2.2*-expressing enteroendocrine cells (Figure 2h) (Desai *et al.*, 2008). Intestine from *Nkx2.2* wild type embryos display only diffuse X-gal staining that could arise from endogenous beta-galactosidase activity (Supplemental figure 1c). Transverse sections of e9.5 and e10.5 whole mount embryos reveal LacZ expression in the CNS and the dorsal and ventral pancreatic buds at 10.5 (Figure 2i, Supplemental figure 1d). Strong X-gal staining can also be detected in the P3 region of the ventral spinal cord, adjacent to the floorplate, as previously described (Briscoe *et al.*, 2000; Briscoe *et al.*, 1999) (Figure 1i', I''', Supplemental figure 1e', e'''). X-gal staining on sections of embryonic and adult pancreata confirms extensive expression of LacZ throughout the pancreatic epithelium at e12.5 and restricted expression of LacZ in the

adult pancreatic islet (Figure 2j and 2k). With the exception of rare X-gal signal observed in isolated centroacinar cells and juxta-ductal cells, X-gal staining is predominantly excluded from the acinar tissue, as expected (Figure 2k, Supplemental figure 1f, Supplemental figure 2) (Sussel *et al.*, 1998).

To confirm appropriate pancreatic *Nkx2.2:lacZ* expression at the cellular level, we performed co-immunostaining with anti-beta-galactosidase antibody in combination with pancreatic cell lineage markers in *Nkx2.2^{lacZ/+}* embryos or adult animals. At e12.5, beta-galactosidase expression can be detected throughout the pancreatic epithelium, extensively overlapping with E-Cadherin, Pdx1 and Ptf1a expressing cells (Figure 3a,3b and Supplemental figure 3). Similar to endogenous *Nkx2.2* protein expression, there appears to be populations of low and high expressing beta-galactosidase-expressing cells in the *Nkx2.2* expression domain, with the high expressing cells predominantly representing a Pdx1-negative, glucagon-positive population (Figure 3b–3f; arrows; Supplemental figure 3). This would suggest that *Nkx2.2* becomes upregulated in the cells that are transitioning from a multipotent progenitor state to a hormone-producing endocrine cell. At e15.5, beta-galactosidase is extensively co-expressed with *Ngn3* in the endocrine progenitor cells and co-expressed with insulin, glucagon and ghrelin in the differentiating endocrine populations. By this stage, beta-galactosidase expression is excluded from Ptf1a-expressing exocrine cells (Figure 3g–3k). At e18.5, beta-galactosidase continues to be co-expressed with insulin, glucagon, ghrelin and PP, but is also unexpectedly found in a small number of somatostatin-expressing delta cells (Figure 3l–3p). Since endogenous *Nkx2.2* is not normally co-expressed with somatostatin and does not appear to function in the delta cell population (Sussel *et al.*, 1998), the observed co-expression may be due to either the perdurance of beta-galactosidase protein or disruption of a delta cell-specific repressor element in the *Nkx2.2:LacZ* allele. Consistent with the former hypothesis, the co-expression LacZ with somatostatin is transient and beta-galactosidase is excluded from the somatostatin-expressing delta cells in the adult islet (Figure 3t). Beta-galactosidase can be detected in alpha, beta, PP and the majority of Pdx1-expressing cells in the adult islet (Figure 3q–3s,3u).

We have previously shown that the *Nkx2.2^{-/-}* mice display altered endocrine cell ratios: insulin-producing beta cells are undetectable at all time points and there is a reduction in the glucagon-producing alpha and pancreatic polypeptide-producing PP cell populations. In the place of these cell types, there is a corresponding increase in the ghrelin-producing epsilon cells (Prado *et al.*, 2004; Sussel *et al.*, 1998). Both *Nkx2.2^{lacZ/lacZ}* and *Nkx2.2^{lacZ/-}* mice display an identical phenotype to the *Nkx2.2^{-/-}* mice confirming that insertion of the *lacZ* gene into the *Nkx2.2* genomic locus creates a null allele (Figure 4 and Supplemental Figure 4). To determine the fate of the *Nkx2.2*-producing cell population in the absence of functional *Nkx2.2* protein, we assessed beta-galactosidase expression in the *Nkx2.2^{lacZ/lacZ}* mice. Although there are fewer alpha and PP cells at all stages examined, beta-galactosidase is co-expressed with glucagon and PP in the cells that do form (Figure 4a,b,g,h), suggesting that although these cells are capable of expressing *Nkx2.2*, their formation is independent of *Nkx2.2* function. In addition, the ghrelin-expressing population, which now represents the majority of cells in islets lacking *Nkx2.2*, also express beta-galactosidase (Figure 4f). This would indicate that the mutant ghrelin-producing population is derived from a respecified *Nkx2.2*-expressing progenitor cell.

Interestingly, in *Nkx2.2^{lacZ/lacZ}* pancreas we continue to observe similar ratios of high and low *Nkx2.2*/beta-galactosidase-expressing cells, suggesting that *Nkx2.2* expression may not be autoregulated as previously suggested (Watada *et al.*, 2003). In addition, at early developmental stages we do not detect alterations in the total numbers of *lacZ*-expressing cells or their spatial or cellular distribution within the pancreatic epithelium (Figure 4a–d). Beta-galactosidase is also still extensively co-expressed with E-cadherin and Pdx1, although

Pdx1 is expressed at lower levels per cell, as previously reported (Figure 3b,d vs. 4d; 4e, 1; (Prado *et al.*, 2004; Sussel *et al.*, 1998). Furthermore, the numbers of beta-galactosidase and *Ngn3*-co-expressing endocrine progenitor cells are equivalent to those present in wild type mice, although the amount of *Ngn3* expressed per cell is reduced as previously described (Figure 4e; (Anderson *et al.*, 2009b). These findings suggest that the altered endocrine cell ratios caused by the absence of *Nkx2.2* is not due to the mislocalization or loss of the *Nkx2.2*-expressing progenitor cell population. Quantification of the total number of each endocrine hormone-producing population at e18.5 confirms that the *Nkx2.2^{lacZ/lacZ}* mice are phenotypically identical to mice homozygous for the *Nkx2.2^{-/-}* allele and also demonstrates that the altered islet cell ratios is not caused by a loss of an *Nkx2.2*-expressing population (Figure 4k and l; Supplemental figure 2c-d).

Comparison of gene expression levels between *Nkx2.2^{LacZ/+}* and *Nkx2.2^{LacZ/LacZ}* were consistent with the cell quantification analysis (Figure 5). There was no difference in the amount of *lacZ* mRNA expression between the heterozygous and homozygous mutant pancreas at all stages of development, confirming that that loss of *Nkx2.2* does not affect the regulation of the *Nkx2.2* locus (Figure 5a). In agreement with our previous studies of the *Nkx2.2^{-/-}* allele, the *Nkx2.2^{lacZ/lacZ}* pancreas has reduced *Ngn3* expression throughout the secondary transition (Figure 5b) (Anderson *et al.*, 2009b). The changes in hormone gene expression are also consistent with the observed altered endocrine cell ratios (Figure 5c).

Pancreatic expression of *Nkx2.2* is initiated just after the onset of *Pdx1* expression in both pancreatic buds, and co-expression of *Nkx2.2* with *Pdx1*, *Ptf1a*, *Nkx6.1* defines the progenitor cell population within the pancreatic epithelium (Jorgensen *et al.*, 2007). In the *Pdx1* null mice, the pancreatic primordia forms, but bud outgrowth and cell differentiation fails to proceed (Offield *et al.*, 1996). To determine whether *Nkx2.2* expression is retained in the *Pdx1*-deficient pancreatic primordia, we assessed *Nkx2.2:lacZ* expression in *Pdx1* null mice (*Nkx2.2^{lacZ/+}; Pdx1^{TA/TA}*; (Holland *et al.*, 2002). At e10.5, a small number of beta-galactosidase cells were detected within the remaining pancreatic primordia, indicating that initiation of *Nkx2.2* pancreatic expression is not dependent on *Pdx1* activity (Supplemental figure 5).

It has been previously demonstrated that *Nkx2.2* and *Ngn3* play intersecting regulatory roles in the developing pancreas (Anderson *et al.*, 2009a; Anderson *et al.*, 2009b; Wang *et al.*, 2008). The two transcription factors directly and indirectly co-regulate a number of pancreatic transcription factors and possibly cross-regulate each other (Anderson *et al.*, 2009b; Wang *et al.*, 2008). In addition, although *Nkx2.2* is expressed prior to the expression of *Ngn3* and is required to maintain wild type levels of *Ngn3* expression, the number of *Ngn3*-expressing endocrine progenitor cells is not affected in *Nkx2.2^{-/-}* mice (Anderson *et al.*, 2009b). In the *Ngn3* null mice (*Ngn3^{TA/TA}*), all endocrine cells fail to differentiate (Wang *et al.*, 2009a; Gradwohl *et al.*, 2000; Heller *et al.*, 2005), but surprisingly *Nkx2.2* mRNA expression is only decreased approximately 50% (Figure 6e). There was no statistical difference ($p=0.1544$; two-tailed student's t test) in the reduction of *Nkx2.2* expression in the *Ngn3* null pancreas on either the *Nkx2.2^{+/+}* or *Nkx2.2^{LacZ/+}* genetic background (Figure 6e), confirming that the observed phenotype is not due haploinsufficiency that could be associated with loss of one *Nkx2.2* allele on the sensitized *Ngn3* null background. To determine which cell types maintain the expression of *Nkx2.2* in the absence of *Ngn3*, we characterized the fate of *Nkx2.2:lacZ* expressing cells in *Ngn3* null mice (*Nkx2.2^{lacZ/+}; Ngn3^{TA/TA}*). In normal pancreata at e18.5, *LacZ* is predominantly expressed in the differentiated endocrine cells as they move away from the ductal epithelium to form islets, although a small number of ductal cells still retain low level expression of *LacZ* (Figure 6a,a',c,c'; arrows). In contrast, in the *Ngn3^{TA/TA}* mice lacking all endocrine cells, beta-galactosidase remains expressed at high levels in almost all cells of the ductal

epithelium, similar to *Nkx2.2:LacZ* expression in the ductal epithelium at earlier stages of pancreas development (Figure 3a-k). Interestingly, expression can also be detected at low levels in the nuclei of exocrine cells in the *Ngn3* null pancreas (Figure 6b, b', d, d'; arrowheads).

In addition to its expression in the pancreas, *Nkx2.2* is expressed in the CNS where it is required for the appropriate differentiation of serotonergic neurons in the hindbrain (Briscoe *et al.*, 1999; Pattyn *et al.*, 2003a), and the ventral V3 interneurons and oligodendrocytes in the spinal cord (Briscoe *et al.*, 2000; Briscoe *et al.*, 1999; Qi *et al.*, 2001). In the absence of *Nkx2.2*, the P3 progenitor domain forms, however the P3 progenitors appear to undergo a ventral-to-dorsal transformation to generate motor neurons in place of the *Sim1* positive V3 interneurons (Briscoe *et al.*, 1999). Consistent with this idea, in e10.5 *Nkx2.2^{lacZ/lacZ}* embryos, beta-galactosidase-producing cells continue to migrate laterally out of the P3 ventricular zone, similar to *Nkx2.2^{lacZ/+}* control embryos (Figure 7d), although in the absence of *Nkx2.2* the laterally located cells are ectopic motor neurons that express *Lim3*, *HB9* and *Isl1/2* (Briscoe *et al.*, 1999). By e12.5, the *Nkx2.2*-expressing P3 progenitor cells give rise to oligodendrocyte precursors, which fail to differentiate in the *Nkx2.2^{-/-}* mice (Qi *et al.*, 2001). Interestingly, at this stage of development the spatial distribution of beta-galactosidase expressing cells in *Nkx2.2^{lacZ/lacZ}* embryos is altered compared to *Nkx2.2^{lacZ/+}* littermates: beta-galactosidase expressing cells predominantly remain within the ventricular zone and do not migrate laterally (Figure 7e, 7f and data not shown). This is consistent with the hypothesis that *Nkx2.2* is necessary for the appropriate differentiation of oligodendrocyte progenitors (Qi *et al.*, 2001).

In *Nkx2.2^{lacZ/+}* mice, expression of beta-galactosidase in the intestine and stomach also reflects the known expression of endogenous *Nkx2.2* (Supplemental figure 6; (Desai *et al.*, 2008). In the stomach, beta-galactosidase is co-expressed with ghrelin as well as several other hormone producing populations (Supplemental figure 6a-b). In the intestine, beta-galactosidase expression can also be detected in the enteroendocrine cell populations, including ghrelin-expressing cells (Supplemental figure 6c). Furthermore, beta-galactosidase expression is present in the expanded ghrelin population that forms in the absence of *Nkx2.2*, indicating that similar to the pancreas, these cells are derived from *Nkx2.2*-expressing populations (Supplemental figure 6d).

Discussion

In this study, we describe the generation of mice carrying an *Nkx2.2:lacZ* knockin allele. To generate this new mouse line, we utilized recombination mediated cassette exchange (RMCE) technology. The RMCE technology consists of two sequential ES cell manipulations that ultimately produces an ES cell line that allows us the generation of multiple allele variants with high efficiency (Chen *et al.*, 2011; Feng *et al.*, 1999; Long *et al.*, 2004). With this technology, we first generated an ES cell line containing the *Nkx2.2* cassette acceptor allele (*Nkx2.2^{LCA}*) using homologous recombination. The *Nkx2.2^{LCA}* allele positions heterologous *loxP* sites within the *Nkx2.2* genomic locus flanking a deletion of the two *Nkx2.2* coding exons. These ES cells allowed us to introduce the *Nkx2.2:lacZ* allele described in this study into the *Nkx2.2* locus with 40–50% efficiency. The *Nkx2.2^{LCA}* allele also facilitates the generation of a series of *Nkx2.2* targeted alleles with high efficiency. This is particularly useful since homologous recombination at the *Nkx2.2* genomic locus has proven to occur with low efficiency due to the high G-C content and numerous repetitive elements associated within essential regions of this locus. To date, we have successfully used the *Nkx2.2^{LCA}* ES cells to generate several new *Nkx2.2* alleles, including two mutant alleles that disrupt distinct protein domains and alleles containing *Cre-EGFP*, *rtTA* and *mCherry*. Overall, the RCME technology has allowed us to overcome

many of the challenging steps associated with gene targeting in mice and has facilitated further analysis of *Nkx2.2* function in the development of the pancreas, intestine and CNS.

The *Nkx2.2:lacZ* allele was generated by replacing the *Nkx2.2* coding exons with the *lacZ* gene. Expression of the beta-galactosidase protein faithfully recapitulates *Nkx2.2* expression and provides a surrogate marker that allows us to monitor the fate of *Nkx2.2*-expressing cells when *Nkx2.2* is deleted. The ability to follow the *Nkx2.2*-derived lineage in the absence of *Nkx2.2* enabled us to directly test our previous hypothesis that cell-respecification is occurring in the *Nkx2.2*^{-/-} mice. In the pancreatic islet and in the small intestine, elimination of *Nkx2.2* resulted in the loss or reduction of several endocrine populations with the concomitant increase of the ghrelin-expressing cell population. In the *Nkx2.2^{lacZ/lacZ}* mice, there was no discernable decrease in the numbers of *lacZ*-expressing cells, supporting the idea that loss of specific cell types was not due to the absence of a population of *Nkx2.2*-derived progenitor cells. Furthermore, the fact that the ghrelin cells in the *Nkx2.2^{lacZ/lacZ}* pancreas also express LacZ suggests that the mutant ghrelin population is derived from *Nkx2.2*-producing progenitors rather than from non-*Nkx2.2* expressing cell populations that expand in the absence of *Nkx2.2* or the other mature hormone-producing cells. These studies also revealed LacZ expression in the populations of PP and glucagon cells that form in the absence of *Nkx2.2*, indicating that these cells are also derived from *Nkx2.2*-expressing progenitor cells, although it appears that their specification is independent of *Nkx2.2* function.

In the CNS, *Nkx2.2* is also required for the differentiation of several neuronal populations (Briscoe *et al.*, 1999; Qi *et al.*, 2001). This has been particularly well studied in the spinal cord, where V3 neurons and oligodendrocytes are sequentially derived from the *Nkx2.2*-expressing P3 progenitor domain. In the spinal cord prior to e12.5, it has been proposed that the loss of *Nkx2.2* activity results in a “ventral-to-dorsal” switch in progenitor fate, such that the P3 progenitors now give rise to motor neurons. The observation that LacZ-expressing cells continue to migrate laterally out of the P3 progenitor domain of the *Nkx2.2^{lacZ/lacZ}* embryos early in development validates the idea that the P3 progenitors are respecified to form the ectopic motor neurons, rather than an alternative mechanism by which the more dorsally located motor neurons expand and migrate ventrally into the space created by the loss of the V3 interneuron population. Interestingly, however, after e12.5 when P3 progenitors give rise to the oligodendrocyte lineage, beta-galactosidase-producing progenitor cells of *Nkx2.2^{lacZ/lacZ}* embryos fail to migrate laterally and remain in the ventricular zone within a region of the rostral spinal cord. These cells likely delineate the oligodendrocyte precursor population that fails to differentiate in the absence of *Nkx2.2*. Future studies utilizing *Nkx2.2:Cre* mediated lineage tracing will be necessary to determine the ultimate fate of the *Nkx2.2* P3 domain cells.

The creation of the *Nkx2.2^{lacZ/+}* mice also facilitated assessment of *Nkx2.2* regulation by two essential pancreatic proteins, Pdx1 and Ngn3. Currently, the commercially available *Nkx2.2* antibodies do not reliably detect *Nkx2.2* protein expression in the developing pancreas, whereas X-gal staining is highly sensitive and there are several commercially available anti-beta-galactosidase antibodies made in different species that facilitate co-immunofluorescence analysis of different antigen combinations. We took advantage of these reagents to determine the fate of *Nkx2.2*-expressing cells in mice lacking either *Pdx1* or *Ngn3*. *Pdx1* and *Nkx2.2* are two of the earliest genes to be expressed in the pancreatic progenitor domain, although *Pdx1* expression arises shortly prior to *Nkx2.2* (Jorgensen *et al.*, 2007). Furthermore, *Pdx1* null mice display almost complete pancreatic agenesis, while *Nkx2.2* appears to function at later endocrine cell specification steps of pancreatic development (Offield *et al.*, 1996; Sussel *et al.*, 1998). Although, the respective expression patterns and mutant phenotypes indicate that Pdx1 functions upstream of *Nkx2.2*, it appears

that Pdx1 is dispensable for initiation of *Nkx2.2* expression in the pancreatic primordium that does form.

The functional relationship between *Ngn3* and *Nkx2.2* is less well-defined. *Nkx2.2* expression precedes *Ngn3* during pancreatic development and *Ngn3* expression is reduced in the absence of *Nkx2.2*. Conversely, deletion of *Ngn3* leads to the complete loss of all islet endocrine cells and therefore appears to function upstream of *Nkx2.2*, which only affects the specification of a subset of endocrine cells. Interestingly, analysis of *Nkx2.2* (beta-galactosidase) expression in the *Ngn3^{TA/TA}* pancreas indicated that *Nkx2.2* expression is maintained at unexpectedly high levels in the epithelial ducts, which reflects wild type *Nkx2.2* expression in earlier staged ductal cells. This may indicate that the ductal epithelial cells within the *Ngn3^{TA/TA}* pancreas are blocked at a more immature stage of development. Another unexpected observation was the identification of low levels of *Nkx2.2* (beta-galactosidase) expression in the acinar cells of the *Ngn3^{TA/TA}* pancreas. Wang et al., (Wang *et al.*, 2010) previously described that inactivation or reduction of *Ngn3* levels, caused the reallocation of a subset of *Ngn3*-expressing endocrine progenitors to the exocrine lineage. *Ngn3* and *Nkx2.2* are expressed both individually and together in different populations of ductal epithelial progenitor cells. The identification of beta-galactosidase expression in the nuclei of amylase-expressing acinar cells of the *Ngn3^{TA/TA}* pancreas suggests that the *Ngn3*-expressing progeny that become redirected to the exocrine lineage in the absence or reduction of *Ngn3* are derived from the *Ngn3* and *Nkx2.2* co-expressing progenitor population.

In summary, the *Nkx2.2:LacZ* mouse provides a useful tool to study the function of *Nkx2.2* in the pancreas, intestine and CNS. Furthermore, the creation of *Nkx2.2* RMCE acceptor ES cells will facilitate the generation of additional *Nkx2.2* alleles for *in vivo* analyses.

Materials and methods

Generation of *Nkx2.2* RMCE ES cells and *Nkx2.2:lacZ* mice

A gene targeting vector for *Nkx2.2* was constructed using the recombineering approach and utilizing the Cre-lox system for future RMCE manipulations as described in Chen et al. (Chen *et al.*, 2011). The first step involved the generation of an *Nkx2.2* acceptor allele (*Nkx2.2^{LCA}*) in TL-1 ES cells (Labosky *et al.*, 1994) using traditional homologous recombination. The targeting construct was derived from an *Nkx2.2*-encoding RP22 BAC clone (#183-A20). The short and long arm *Nkx2.2* fragments (SA & LA) are 4,298 base pairs and 9,668 base pairs respectively. A 5,115 base pair region between these arms was replaced by the floxed *tk-Kan* cassette, a puromycin- Δ thymidine kinase fusion gene driven by the mouse phosphoglycerol kinase promoter (pU Δ TK) and a kanamycin resistant gene driven by the bacterial EM7 promoter (EM7-kan) flanked by minimal (34 bp) tandemly oriented lox71 and lox2272 sites (Cre-recombinase recognition sequences). The construct also contains a diphtheria toxin negative selection cassette (*pgk-DT-A*). All plasmids and their sources have been described in detail (Chen *et al.*, 2011). The resulting *Nkx2.2^{LCA}* gene targeting vector was linearized with *NotI* and used to electroporate TL-1 ES cells. 318 puromycin resistant colonies were screened by Southern blot using 5' and 3' external probes (Figure 1a). For 5' probe analysis, genomic DNA was digested with *NsiI* to produce a wild type fragment of 17,819 base pairs and targeted fragment of 4,991 base pairs. For 3' probe analysis, genomic DNA was digested with *KpnI* to generate a wild type fragment of 17,026 base pairs and targeted fragment of 15,504 base pairs. Eight clones (2.5% efficiency) contained the correctly targeted locus (Figure 1C). Three clones (2D4, 2G6 and 4E9) were used for subsequent RMCE targeting.

To generate the *Nkx2.2:lacZ* targeting vector, an *Nkx2.2* basal exchange vector (pNkx2.2.Ex1.H; Supplemental figure 1) was first created. pNkx2.2.Ex1.H was generated by inserting the 5,115 base pair DNA fragment of the *Nkx2.2* genomic locus that was removed from the *Nkx2.2^{LCA}* targeting allele into pMCS.66/2772 (Chen *et al.*, 2011). A smaller 3491 base pair *HpaI* DNA fragment from within the 5,115 base pair genomic fragment was then subcloned into a pGL3 vector to allow for efficient manipulation of the DNA using overlap PCR. The *nls-lacZ* gene (Picard and Yamamoto, 1987) was fused in frame with the *Nkx2.2* start ATG on the 5' end and 115 base pairs upstream of the stop codon on the 3' end of *Nkx2.2*. The modified *Nkx2.2:nls-lacZ* fusion DNA was sequence verified and cloned back into the pNkx2.2.Ex1.H vector using the *HpaI* sites.

RMCE was performed in two parental clones (2G6 and 4E9) using a positive-negative selection strategy previously described (Chen *et al.*, 2011; Long *et al.*, 2004). In brief, approximately 6×10^6 mESCs containing the *Nkx2.2^{LCA}* allele were co-electroporated with 40 μ g of an exchange plasmid and 40 μ g of pBS185, a *Cre*-expression plasmid (Sauer, 1998). 200 μ g/ml neomycin (Invitrogen) was used for positive selection and 8 μ M gancyclovir (Sigma, St Louis, MO) for negative selection. Surviving clones were screened by DNA PCR using the following primers: Primer 1 (Nkx.A-3') 5'-CTGAGCAGCTCGGAGTGAGGCGCT-3', Primer 2 (hygro 5') 5'-ACGAGACTAGTGAGACGTGCTACT-3'; Primer 3 (Nkx2.2.P2) 5'-CGTCAACCTCAAATCTGGAGCCTA-3'; Primer 4 (Nkx.B-3') 5'-ACTTGGTACGCAGATATTGAGA-3'. Primers 1 and 2 amplify a 606 bp DNA fragment only in the exchanged allele. Primers 3 and 4 detect a 556 bp fragment in the *Nkx2.2:nls-lacZ* (hereafter referred to as *Nkx2.2:lacZ*) allele and a 479 bp fragment in the *Nkx2.2* wild type locus. Correctly exchanged ES cell clones were expanded from the master plate and used to inject C57BL/6J blastocysts to generate *Nkx2.2^{lacZ/+}* mouse lines. Four of the eight parental clones gave germline transmission; one clone was subsequently maintained and used for all studies. The chimeras were initially back-crossed to C57BL/6J mice and subsequently maintained on an outbred Black Swiss (Taconic) background in order to replicate studies with the *Nkx2.2^{-/-}* mice, which are also maintained on a Swiss Black background. No phenotypic variations have been observed throughout the breeding process.

Animals

Nkx2.2^{lacZ/+} (Nkx2-2^{tm3Suss}; MGI:5302422), *Nkx2.2^{+/-}* (Nkx2-2^{tm1Jlr}; MGI:1932100), *Pdx1^{TA/+}* (*Pdx1*^{tm1Macd}; MGI:2388676) and *Ngn3^{TA/+}* (*Neurog3*^{tm2(tTA)Ggu}; MGI:3850054) knockin mice were maintained on a Black Swiss (Taconic) background. Genotyping of mice and embryos was performed by PCR analysis. For *Nkx2.2^{lacZ}* allele, 5'-3 forward': gagtggcgcttcgcctggtt and 5'-3' reverse: agggagtagcagccggtggg primers were used. *Nkx2.2^{+/-}*, *Pdx1^{TA/+}* and *Ngn3^{TA/+}* alleles were previously described (Holland *et al.*, 2002; Sussel *et al.*, 1998; Wang *et al.*, 2009a). All mice were housed and treated according to the Columbia University IACUC approval protocol. All procedures to generate the *Nkx2.2^{lacZ}* mice were approved by the Vanderbilt University Animal Care and Use Committee. The *Nkx2.2^{LacZ/+}* mice are available to the research community upon request.

Immunohistochemistry

Tissues were fixed in 4% PFA for four hours or overnight, washed in cold PBS, incubated in 30% sucrose and cryopreserved. Immunofluorescence was performed on 8 μ M sections. Sections were blocked in the appropriate serum (5% serum in 1x PBS + 0.5% triton-X) for 30 minutes. Primary antibodies were diluted in blocking buffer and incubated on tissue sections overnight at 4 degrees Celsius. The primary antibodies used were: anti-amylase (rabbit, 1:500; Sigma-Aldrich), anti-E-cadherin (rat, 1: 500; Zymed), anti- β -galactosidase (chicken, 1:500; Abcam), anti-ghrelin (goat, 1:500; Santa Cruz Biotechnologies, Inc), anti-

glucagon (guinea pig, 1:1000; Linco), anti-insulin (guinea pig, 1:200; Linco; rabbit, 1:200; Cell Signaling Technology), anti-neurogenin3 (rabbit, 1:500; Beta Cell Biology Consortium), anti-Pdx1 (rabbit, 1:1000; guinea pig, 1:1000; Millipore), anti-PP (rabbit, 1:500; Zymed), anti-Ptf1a (rabbit, 1:500; a gift from Ray Macdonald), anti-somatostatin (rabbit, 1:500; Phoenix Pharmaceuticals, Inc). Sections were incubated with appropriate secondary antibodies (Jackson Immunoresearch). DAPI (1:1000; Invitrogen) was applied for 30 minutes following secondary antibody incubation. Fluorescent images were obtained with a Nikon Eclipse 80i microscope, Q-image camera and ImagePro software (Media Cybernetics). Confocal images were captured using a Zeiss LSM 510 NLO Multiphoton. For quantification purposes, stained cells were counted manually on every seventh section throughout the entire pancreas for wild type (n=3) and mutant (n=3). All values are expressed as means $\pm$ SEM. Statistical analysis was performed using a two-tailed Student's unpaired t test. Results were considered significant when $p < 0.05$.

X-gal staining

Dissected tissues or whole embryo were briefly fixed with 4% PFA, rinsed in wash solution (0.1 M sodium phosphate, 0.1% deoxycholic acid, 0.2% NP40, 2 mM magnesium chloride) and stained in wash solution containing 1 mg/ml X-gal (5-bromo-4-chloro-3-indolyl-b-D-galactoside).

After the whole-mount samples were photographed under the dissecting microscope, transverse frozen sections were prepared for histological analysis.

RNA Analysis

Total RNA was isolated from whole pancreatic tissue from the respective embryonic ages (RNeasy, QIAGEN), and cDNA was prepared using the SuperScript III kit (Invitrogen) and random hexamer primers. Quantitative PCR was performed using 200 ng cDNA, PCR master mix (Eurogentec) and Taqman probes (ABI Assays on Demand) for *ghrelin* (Mm00445450_m1), *glucagon* (Mm00801712_m1), *insulin 2* (Mm00731595_gH), *somatostatin* (Mm00436671_m1), *pancreatic polypeptide* (Mm00435889_m1), *Nkx2.2* (probe 5': ccattgactctgccccatcgctct, forward primer, 5'-3': cctccccgagtgccagat, reverse primer 5'-3': gagtctatcctctccaaagtcaaa) and *neurogenin 3* (probe 5': cctgcgctcgcccaact, forward primer, 5'-3': gacgccaaacttacaaag, reverse primer 5'-3': gtcagtgccagatg t). *LacZ* mRNA expression was tested by Sybr-Green qPCR (forward primer, 5'-3': gagtggcgttcgcctggt, reverse primer, 5'-3': agggagtagcagccggtggg). All genes were normalized to *Cyclophilin B* and were quantified with ABI prism software. An $n = 4$ was obtained for all mouse populations. All values are expressed as means $\pm$ SEM. Statistical analysis was performed using a two-tailed Student's unpaired t test. Results were considered significant when $p < 0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Generation of the *Nkx2.2^{LCA}* ES cells and *Nkx2.2:lacZ* knockin allele
 (a) Schematic of the strategy used to create the *Nkx2.2^{LCA}* allele in ES cells. P1 and P2 indicate the location of the 5' and 3' external southern probes. The blue boxes labeled "1" and "2" represent the two *Nkx2.2* coding exons. "SA" indicates the short arm of homology and "LA" indicates the long arm of homology. (b) Schematic of the *Nkx2.2:lacZ* cassette and *Nkx2.2:lacZ* knockin allele. The *nls-lacZ* gene was cloned in frame at the *Nkx2.2* ATG codon and just upstream of the stop codon, effectively replacing the *Nkx2.2* coding sequence and intervening intron. The *Nkx2.2:lacZ* cassette replaces the *Nkx2.2^{LCA}* allele using RMCE. The Flpe-recombinase is used to remove the FRT-flanked hygromycin cassette. (c) Representative southern blot analysis of targeted ES cells using the 3' (P2) probe. ES cell DNA was digested with *KpnI*. The *Nkx2.2* wild type allele is 17,026 bp and *Nkx2.2^{LCA}* is 15,504 bp. TL1 is the parent ES cell line; the remaining lanes show correctly targeted ES cells. The wild type band from the TL1 parental ES cells migrates at a slightly faster rate due to a difference in DNA abundance and salt concentration in ES cell DNA preparation. (d) Representative PCR amplification results of *Nkx2.2^{LCA}* ES cells containing Cre-mediated recombination of the *Nkx2.2:nls-lacZ* allele. For each RMCE ES cell clone, two sets of primers were used. Lanes labeled "a" indicate a PCR reaction for the targeted *Nkx2.2:lacZ* allele using primers 1 and 2 (606 bp) and lanes labeled "b" indicate the reaction using primers 3 and 4 (556 bp for the *Nkx2.2:nls-lacZ* allele and 479 bp for the *Nkx2.2* wild type allele). (e) Representative PCR amplification results of *Nkx2.2:lacZ* chimeric mice and one of the correctly targeted ES cell clones (4E9/4D2). Lanes are labeled "a" and "b" as above. Chimera #53004 and 53005 were correctly targeted.

Figure 2. The *Nkx2.2:lacZ* allele reproduces endogenous *Nkx2.2* expression pattern

(a,b) X-gal staining in whole mount *Nkx2.2^{lacZ/+}* embryos. The dotted line indicates the level of the section shown in c. (c) A transverse section through an *Nkx2.2^{lacZ/+}* e12.5 embryo stained with X-gal. The level of the section is indicated by the dotted line in b. (d) An *Nkx2.2* mRNA *in situ* performed on a section from an e12.5 wild type embryo. (e,f) Ventral (e) and dorsal (f) views showing X-gal staining of whole mount *Nkx2.2^{lacZ/+}* brain indicates LacZ expression in the dorsal and ventral CNS. (g) X-gal staining of dissected e17.5 pancreas. (h) X-gal staining of dissected e17.5 intestine. (i) Transverse section at e10.5 indicates *Nkx2.2:lacZ* expression in the CNS and the dorsal and ventral pancreas in e10.5 embryos. Boxed regions are magnified in i'-i'''. (j) A section through an e12.5 pancreas; X-gal staining can be detected throughout the pancreatic epithelium. (k) Section through an adult pancreas; X-gal staining is restricted to pancreatic islet in adults. CNS, central nervous system; FB, forebrain; MB, midbrain; HB, hindbrain; SC, spinal cord; DP, dorsal pancreas; VP, ventral pancreas.

Figure 3. *Nkx2.2:lacZ* spatio-temporal expression analysis in *Nkx2.2^{lacZ/+}* embryos
 (a–f) At e12.5 adjacent sections in the pancreatic epithelium show beta-galactosidase staining in the pancreatic domain marked by E-cadherin and Pdx1. (b–f) Beta-galactosidase is extensively co-expressed with Pdx1, except for a population of *Nkx2.2: LacZ^{high}* expressing cells that co-stain with glucagon. The small insets in b and c highlight the fields magnified in d and e respectively. (g–k) At e15.5, beta-galactosidase is co-expressed with insulin-, glucagon- and ghrelin. By this stage the exocrine lineage and LacZ-expressing cells are mutually exclusive. (k) *Nkx2.2: lacZ* is also expressed in Ngn3-expressing endocrine progenitor cells. Filled arrowhead indicates Ngn3⁺/beta-galactosidase⁺ and empty arrowhead indicates Ngn3⁺/beta-galactosidase⁻. (l–p) At e18.5, beta-galactosidase is expressed in most of the endocrine cell populations within the forming islet. (q–u) In adult islets, beta-galactosidase is restricted to α , β and PP cells and in the majority of Pdx1-expressing cells. l–u are confocal images. DAPI staining was used to visualize the nuclei in a, f, g, h, i, k, l, o, p, and t. Magnification 20X otherwise indicated. Insets show higher magnification. All staining was performed in *Nkx2.2^{lacZ/+}* embryos and animals.

Figure 4. Number and location of *Nkx2.2:lacZ* expressing cells in the developing pancreas is indistinguishable between *Nkx2.2^{lacZ/lacZ}* and *Nkx2.2^{lacZ/+}* littermates
 (a) Immunofluorescence analysis beta-galactosidase in the *Nkx2.2^{lacZ/lacZ}* embryos shows that LacZ is expressed in glucagon-producing cells at e10.5. (b-e) At e12.5 in the *Nkx2.2^{lacZ/lacZ}* embryos, beta-galactosidase is co-expressed with Pdx1 within the pancreatic epithelium, with the exception of a population of beta-galactosidase^{high} expressing cells that co-express with glucagon, as seen in *Nkx2.2^{lacZ/+}*. (f-j) By e15.5, beta-galactosidase is co-expressed with Ngn3 in the ductal epithelium and the endocrine hormones; beta-galactosidase is not detected in the amylase-expressing exocrine tissue. (k) Quantification of the hormone producing cells co-stained with beta-galactosidase in e15.5 *Nkx2.2^{lacZ/+}* and *Nkx2.2^{lacZ/lacZ}* embryos. (l) The number of beta-galactosidase positive cells is unchanged in e15.5 *Nkx2.2^{lacZ/lacZ}* compared to *Nkx2.2^{lacZ/+}*. White and black bars represent *Nkx2.2^{lacZ/+}* and *Nkx2.2^{lacZ/lacZ}* respectively. * p 0.05. Magnification 20X. Insets show higher magnification. DAPI staining was used to visualize the nuclei in a,c,f,h, and i.

Figure 5. mRNA expression of endocrine markers is altered in *Nkx2.2^{lacZ/-}* embryos
 (a) *lacZ* mRNA expression at different time points during pancreas development is unchanged in *Nkx2.2^{lacZ/-}* and *Nkx2.2^{lacZ/+}* embryos, suggesting that the number of *lacZ*-expressing cells is unchanged in *Nkx2.2^{lacZ/-}* and *Nkx2.2^{lacZ/+}* embryos. (b) *Nkx2.2* function is required for maximum expression of *Ngn3* at e12.5 and e15.5. White and black bars represent *Nkx2.2^{lacZ/+}* and *Nkx2.2^{lacZ/-}* respectively. * p 0.05. (c) Hormone mRNA quantification in e15.5 *Nkx2.2^{lacZ/-}* embryos compared to *Nkx2.2^{lacZ/+}*.

Figure 6. *Nkx2.2-lacZ* is expressed in the absence of *Ngn3*

(a–d) Co-immunostaining of beta-galactosidase and amylase, marker of acinar cells or DBA, marker of pancreatic ducts in *Ngn3^{TA/+}* (a, c) and *Ngn3^{TA/TA}* (b, d) e18.5 embryos.

Arrows in a' indicate the rare beta-galactosidase positive, *Nkx2.2*-expressing cells present in the ducts of wild type embryos. Arrowheads in b' indicate beta-galactosidase expression in amylase-expressing cells in *Ngn3* null mice. (e) *Nkx2.2* mRNA expression in wild type (white bar, n=3), *Ngn3^{TA/TA} Nkx2.2^{+/+}* (grey bar, n=5) and *Ngn3^{TA/TA}; Nkx2.2^{lacZ/+}* (black bar, n=5) e16.5 embryos. * p 0.05 vs wild type. There is no statistical difference (p=0.1544) in *Nkx2.2* expression between the *Ngn3^{TA/TA} Nkx2.2^{+/+}* and *Ngn3^{TA/TA}; Nkx2.2^{lacZ/+}* embryos (two-tailed student's t test). Magnification 20X. DAPI staining was used to visualize the nuclei in a, c, and d.

Figure 7. A subset of *Nkx2.2-lacZ* expressing neural progenitor cells fail to migrate out of the ventricular zone in the rostral spinal cord of *Nkx2.2^{lacZ/lacZ}* mice
 (a,d) At e10.5, beta-galactosidase-expressing cells migrate laterally out of the ventricular zone in both *Nkx2.2^{lacZ/+}* and *Nkx2.2^{lacZ/lacZ}* embryos. Immunofluorescent staining of Olig2 (red), which is normally repressed by *Nkx2.2*, shows co-expression of Olig2 and beta-galactosidase in the *Nkx2.2^{lacZ/lacZ}* embryos, confirming this embryo has lost *Nkx2.2* activity, despite the normal migration of progenitor cells. (b,e) At e12.5, beta-galactosidase cells migrate laterally out of the ventricular zone of the hindbrain in *Nkx2.2^{lacZ/+}* embryos, but not in *Nkx2.2^{lacZ/lacZ}* littermates. (c,f) By e15.5, a large number of beta-galactosidase cells continue to migrate away from the midline in the *Nkx2.2^{lacZ/+}* embryos, but little migration can be observed in the *Nkx2.2^{lacZ/lacZ}* embryos. DAPI staining was used to visualize the nuclei in all panels.