



Published in final edited form as:

Development. 2007 February ; 134(3): 515–523. doi:10.1242/dev.02763.

Nkx2.2-repressor activity is sufficient to specify α -cells and a small number of β -cells in the pancreatic islet

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Abstract

The homeodomain protein Nkx2.2 (Nkx2-2) is a key regulator of pancreatic islet cell specification in mice; Nkx2.2 is essential for the differentiation of all insulin-producing β -cells and of the majority of glucagon-producing α -cells, and, in its absence, these cell types are converted to a ghrelin cell fate. To understand the molecular functions of Nkx2.2 that regulate these early cell-fate decisions during pancreatic islet development, we created Nkx2.2-dominant-derivative transgenic mice. In the absence of endogenous Nkx2.2, the Nkx2.2–Engrailed-repressor derivative is sufficient to fully rescue glucagon-producing α -cells and to partially rescue insulin-producing β -cells. Interestingly, the insulin-positive cells that do form in the rescued mice do not express the mature β -cell markers MafA or Glut2 (Slc2a2), suggesting that additional activator functions of Nkx2.2 are required for β -cell maturation. To explore the mechanism by which Nkx2.2 functions as a repressor in the islet, we assessed the pancreatic expression of the Groucho co-repressors, Grg1, Grg2, Grg3 and Grg4 (Tle1-Tle4), which have been shown to interact with and modulate Nkx2.2 function. We determined that Grg3 is highly expressed in the embryonic pancreas in a pattern similar to Nkx2.2. Furthermore, we show that Grg3 physically interacts with Nkx2.2 through its TN domain. These studies suggest that Nkx2.2 functions predominantly as a transcriptional repressor during specification of endocrine cell types in the pancreas.

Keywords

Nkx2.2; Transcriptional repression; islet; β -cells; α -cells; Mouse

INTRODUCTION

The pancreatic islet consists of five endocrine cell types – α , β , δ , PP and ϵ – each secreting a specific peptide hormone: glucagon, insulin, somatostatin, PP and ghrelin, respectively. Considerable progress has been made recently in understanding the transcription factor network regulating the development of the pancreatic islet (Habener et al., 2005; Servitja and Ferrer, 2004). In particular, the hierarchy of transcription factors functioning during the different stages of islet cell specification is being elucidated: Ptf1a and Pdx1 are the earliest-known factors in the pancreatic progenitors, and Ngn3 (also known as Neurog3 – Mouse Genome Informatics) is essential for further specifying the endocrine progenitors (Gradwohl et al., 2000; Jonsson et al., 1994; Kawaguchi et al., 2002; Matsuoka et al., 2004; Offield et al., 1996). Subsequently, the upregulation of Pdx1 and Nkx6.1 (a β -cell-specific factor; also known

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as Nkx6-1 – Mouse Genome Informatics), and the activation of MafA, directs the maturation of the insulin-producing β -cells (Henseleit et al., 2005; Jonsson et al., 1994; Matsuoka et al., 2004; Sander et al., 2000). One of the key regulators of the early cell-fate decisions in the islet is the homeodomain transcription factor Nkx2.2 (also known as Nkx2-2 – Mouse Genome Informatics). Nkx2.2 is expressed at the onset of pancreatic epithelium formation, and becomes restricted to mature α -, β - and PP cells (Sussel et al., 1998). Nkx2.2 has been identified as a crucial regulator for islet cell differentiation; in Nkx2.2-homozygous-null embryos, all β -cells and most α -cells are replaced by ghrelin-positive cells (Prado et al., 2004).

Although Nkx2.2 is known to be essential for proper islet cell development, little is known about its specific molecular functions or early direct targets that specify cell-fate decisions. At the transcriptional level, Nkx2.2 has been shown to have several molecular activities. In vitro studies have implicated Nkx2.2 in the direct regulation of *MafA*, of the mouse insulin genes and of *Nkx2.2* itself (Cissell et al., 2003; Raum et al., 2006; Watada et al., 2003). These studies have suggested that Nkx2.2 may act as an activator through the *Nkx2.2* and *MafA* promoters, but as a repressor through the Nkx2.2 consensus site in the *insulin* promoter. Watada et al. (Watada et al., 2000) have also identified a strong activation domain in the C-terminus of Nkx2.2; however, full-length Nkx2.2 had weak repression activity in the β TC3-cell line. In the spinal cord, where Nkx2.2 is crucial for the specification of ventral neural-progenitor cell fates, ectopic expression of a Nkx2.2-dominant repressor fusion protein (Nkx2.2hd-EnR) mimics the ability of endogenous Nkx2.2 to repress Pax6-positive neuronal progenitors. Furthermore, this repression activity is dependent on the interaction with the Groucho co-repressor, Grg4 (also known as Tle4 – Mouse Genome Informatics) (Muhr et al., 2001). Finally, it has been demonstrated that the Nkx2.2 *Drosophila* ortholog Vnd differentially regulates its targets depending on the presence of different cofactors (Yu et al., 2005). Together, these data suggest that Nkx2.2 may have complex regulatory activities during pancreatic development.

In this study, we test the ability of the Nkx2.2-dominant-activator or -repressor derivatives characterized by Muhr et al. (Muhr et al., 2001) to substitute for endogenous Nkx2.2 in the developing mouse islet. The Nkx2.2-repressor can partially rescue the Nkx2.2-null phenotype in the pancreas. In neonatal islets, we see a full recovery of glucagon-expressing cells and a corresponding decrease in ghrelin-positive cells. In addition, the Nkx2.2-repressor is able to restore a small population of insulin-producing cells; however, these cells appear to be incompletely differentiated and do not express the mature β -cell markers MafA and Slc2a2 (previously known as Glut2, and hereafter referred to as Glut2). It appears that more complex Nkx2.2 functions may be required for the full maturation and expansion of the β -cell population. Finally, we show that the Grg3 (also known as Tle3 – Mouse Genome Informatics) co-repressor is specifically expressed in the developing pancreatic epithelium and that it interacts with Nkx2.2. From these studies, we conclude that Nkx2.2 primarily functions as a transcriptional repressor during the specification of all α -cells and a subset of insulin-producing cells.

MATERIALS AND METHODS

Mice

Mice were maintained on a Swiss black (Taconic) background and were housed in the center for comparative medicine (CCM) at the University of Colorado Health Sciences Center. The mice were fed standard rodent chow and all animal procedures were performed according to UCHSC Institutional Review Board-approved protocols.

Generation of Nkx2.2 derivative transgenic mice

A 1200 bp *Cla*I fragment encoding the Nkx2.2 homeodomain (hd) and *Drosophila* Engrailed (En) repressor domain fusion protein (Muhr et al., 2001) was inserted into the *pPdx1-Eco*RI promoter vector, which includes the 4.5 kb Pdx1 regulatory region and a β -globin splice cassette (Norgaard et al., 2003). *Nhe*I sites were added, by PCR, to DNA fragments encoding the Nkx2.2 homeodomain and herpes simplex virus strong activator VP16 or the Nkx2.2-myc tagged fusion proteins (Muhr et al., 2001), and the fragments were each cloned into the *pPdx1-Eco*RI promoter vector. The *pPdx1:Nkx2.2hdEnR*, *pPdx1:Nkx2.2hdVP16* and *pPdx1:Nkx2.2myc* plasmids were linearized with *Sac*II, *Not*I and *Dra*III, respectively, for pronuclear injection (UCHSC UCCC Transgenic/KO Core Facility).

Immunohistochemistry

Tissue was fixed for 3 hours or overnight in 4% paraformaldehyde, and were then either cryopreserved or were fixed in 10% buffered formalin and paraffin embedded. Immunofluorescence was performed on cryopreserved tissue or paraffin-embedded tissue (Glut2). Primary antibodies include: rabbit α -amylase (Sigma; 1:1000), rabbit α -glucagon (Phoenix Pharmaceuticals, Belmont, CA; 1:200), guinea pig α -glucagon (Linco Research; 1:3000), guinea pig α -insulin (Linco; 1:1000), rabbit α -ghrelin (Phoenix; 1:500), rabbit α -Nkx6.1 (Beta cell biology consortium; 1:800), rabbit α -Pdx1 (Chemicon; 1:1000), rabbit α -Glut2 (a gift from B. Thorens, Lausanne, Switzerland; 1:100) and rabbit α -MafA (Bethyl Laboratory; 1:1000). Secondary antibodies include: Alexa-fluor 488, 594 or 305 (Molecular Probes, Eugene, OR; 1:800). For immunohistochemistry, biotinconjugated secondary antibodies were used (Jackson ImmunoResearch; 1:400) with the Vectastain ABC and DAB kits (Vector Laboratories). Images were obtained with a Leica DM5000 microscope, an Evolution MP color camera and ImagePro software from Media Cybernetics. Confocal images were obtained with a Zeiss LSM 510 Meta microscope.

In situ hybridization

RNA in situ hybridization was performed as previously described (Prado et al., 2004) using antisense riboprobes transcribed from linearized plasmids. Riboprobes were generated for mouse *Grg3* and *Nkx2.2* from full-length cDNA. A riboprobe-targeting mRNA encoding the *Drosophila* EnR repression domain was generated from the pCS2:EnR plasmid and a riboprobe to *Nkx2.2hdVP16* was generated from TOPO:Nkx2.2hdVP16 cDNA.

Quantitative real-time PCR

Total RNA was extracted from embryonic day (E)17.5 pancreatic tissue and prepared using a Qiagen RNeasy kit. cDNAs were prepared with oligo(dT) primers and Superscript III (Invitrogen). Real-time PCR was performed using Taqman probes (ABI Assays on Demand) for *insulin2*, *glucagon*, *ghrelin* and 18S ribosomal RNA (rRNA) or *Gapdh* on the ABI 7000. Taqman probes and primers were designed for *2.2hdEnR* (FAM-CAGGGCCCCGGGCGA, forward: 5'-GGCCGAGAAAGGTATGAATTCGA-3'; reverse: 5'-GGGCTGCAGCGATCCT-3') and *2.2hdVP16* (FAM-ACGGCCGACTTCGAGTTTGAGC, forward: 5'-TACGGCGCTCTGG AT-3', reverse: 5'-CGTACTGCTCAATTCCA-3') transgenes and *Nkx2.2* (FAM-CCATTGACTCTGCCCATCGCTTCT, forward: 5'-CCTCCCCGAGTGGCAGAT-3', reverse: 5'-GAGTTCTATCCTCTCC AAAAGTTCAAA-3'). Each gene was normalized to 18S rRNA or *Gapdh*. PCR products were quantified with ABI prism software.

Co-immunoprecipitation assays

PANC1 cells (ATCC) were transfected with pcDNA3:Grg3 and pcDNA3:Nkx2.2 or pcDNA3:Nkx2.2 Δ TN using FuGene 6 (Roche) following the manufacturer's instructions.

Cells were harvested 48 hours post-transfection and lysates collected. Lysate total protein (500 µg) was immunoprecipitated with proteinG-sepharose beads (Sigma) overnight with 1 µg α-Grg3 antibody (rabbit polyclonal, Santa Cruz Biotechnology) or 1 µg α-Nkx2.2 antibody (rabbit polyclonal, Santa Cruz Biotechnology). Proteins bound to the beads were eluted by boiling, resolved by SDS-PAGE and western blotted ($n=3$ experiments). The blots were probed with α-Nkx2.2 antibody, 1:100 (mouse monoclonal, DSHB), followed by horseradish peroxidase-conjugated α-mouse secondary antibody and developed using chemiluminescence (Pierce).

Luciferase-reporter assay

PANC1 and βTC3-cells were transiently co-transfected with the pFOxLuc1-7xNk2pr1 Nkx2.2-dependent luciferase reporter (Watada et al., 2000) and the pcDNA3 empty vector, pcDNA3:Nkx2.2-activator or pcDNA3:Nkx2.2-repressor expression plasmids. Each experiment was performed in triplicate. The transfected cells were harvested at 48 hours and assayed for luciferase activity (Promega; $n=3$ experiments).

Morphometry and statistics

The total area of insulin- or glucagon-positive cells and the total pancreatic area were measured using standard morphometric analysis with ImagePro software on every tenth section (10 µm each) for wild-type ($n=2$ for each time point) and Nkx2.2^{-/-};2.2hdEnR transgene embryos ($n=2$ for each time point) at E15.5 and E18.5. The total area of hormone-positive cells was normalized to the total pancreatic area. All values are expressed as the mean±s.e.m. Statistical analysis was carried out with a two-tailed Student's unpaired *t*-test. Results were considered significant when $P<0.05$.

RESULTS

Expression of Nkx2.2-derivative transgenes in the developing pancreas

To test the molecular mechanism of Nkx2.2 in the pancreatic islet, we used two previously characterized Nkx2.2-dominant derivatives: the Nkx2.2 DNA-binding domain fused to the *Drosophila* Engrailed repression domain (repressor; Nkx2.2hdEnR) and the Nkx2.2 DNA-binding domain fused to the VP16 activation domain from the herpes simplex virus (activator; Nkx2.2hdVP16) (Fig. 1A) (Muhr et al., 2001). In addition, we used a control construct containing the Nkx2.2 homeodomain alone (Nkx2.2hdCon) to ensure that the homeodomain does not function as a dominant negative by competing with endogenous Nkx2.2 (Muhr et al., 2001). Muhr et al. (Muhr et al., 2001) previously demonstrated the ability of these fusion proteins to correctly regulate transcription in Cos7 cells. To verify that the Nkx2.2 derivatives function in the pancreas, we tested the transcriptional activity of Nkx2.2hdVP16 and Nkx2.2hdEnR on a Nkx2.2-responsive luciferase reporter plasmid, pFOxLuc1-7xNk2pr1 (Watada et al., 2000), in βTC3- and PANC1-cell lines. Similar to the report by Muhr et al. (Muhr et al., 2001), Nkx2.2hdVP16 activates and Nkx2.2hdEnR represses the reporter gene, as expected (Fig. 1B), indicating that the Nkx2.2 derivatives are functional in pancreatic cell lines.

To drive expression of these dominant derivatives in the embryonic pancreas, the constructs were each placed under the control of the 4.5 kb *Pdx1*-promoter fragment, which has been shown to faithfully recapitulate the endogenous expression of *Pdx1* (Gannon et al., 2001; Stoffers et al., 1999). The *Pdx1* promoter was chosen because it has well-characterized regulatory regions, is commonly used to drive transgene expression in the developing pancreas and, unlike *insulin* and *Nkx2.2* itself (Cissell et al., 2003; Watada et al., 2003), is not regulated by Nkx2.2. Although Nkx2.2 has been shown to bind the *Pdx1* promoter (Van Velkinburgh et al., 2005), Nkx2.2 does not appear to regulate *Pdx1* gene transcription in vivo; *Pdx1* mRNA

levels are unaltered in *Nkx2.2*-null mice. Furthermore, the expression pattern of *Pdx1* is similar to *Nkx2.2*: it is first expressed in the pancreatic epithelium at embryonic day (E)8.5 and continues to be expressed throughout pancreatic development, predominantly in β -cells (Guz et al., 1995). *Nkx2.2* is also expressed throughout the pancreatic epithelium, beginning at E9.5, and later becomes restricted to mature α - and β -cells (Sussel et al., 1998).

For each of the *Nkx2.2* derivatives, we established two stable transgenic-mouse lines (*Nkx2.2hdEnR*: 7414 and 7660; *Nkx2.2hdVP16*: 7318 and 7319) that appropriately express the respective transgenes in the developing and adult pancreas (Fig. 2 and data not shown). Because antibodies are not available to detect either of the transgenes in tissue samples, we performed RNA in situ hybridization for *Nkx2.2hdVP16* and *Nkx2.2hdEnR* mRNAs to characterize the expression of the transgenes throughout pancreatic development in comparison to *Nkx2.2*. In all lines, the transgenes were expressed in the pancreatic epithelium at E10.5 and continued to be expressed in the pancreatic epithelium throughout development (Fig. 2). At E18.5, robust expression of each transgene was detected in the endocrine tissue and correlated well with *Nkx2.2* expression (Fig. 2). We occasionally detected low levels of transgene mRNA in a few acinar cells, which was expected because the *Pdx1* promoter has been shown to have low levels of activity in exocrine tissue (Gannon et al., 2001). We did not detect any exocrine phenotype in these mice. Interestingly, both lines of *Nkx2.2hdEnR* mice expressed the transgene at levels considerably lower than endogenous *Nkx2.2* when quantified by real-time PCR (Fig. 2C). Expression of the transgene in line 7414 was approximately 50% of endogenous *Nkx2.2* levels, whereas line 7660 expressed lower levels of the transgene. Expression of the transgene in line 7660 gradually decreased with each mouse generation, suggesting that transgene loss or silencing was occurring. The *Nkx2.2hdVP16* lines also did not express at supraphysiological levels, but did vary significantly in their expression of the transgene: line 7318 expressed at approximately 70% of the level of endogenous *Nkx2.2*, whereas line 7319 transgene expression was over twofold higher than endogenous *Nkx2.2* (Fig. 2C). The relatively normal or low expression of the transgenes somewhat alleviates concerns of off-target effects that can arise from over-expressing a protein or protein derivative. Consistent with this, transgenic mice from each of these lines and the *Nkx2.2hdCon* lines were fertile and lived to a normal lifespan, suggesting that these transgenes were not detrimental to islet development and did not adversely affect endocrine hormone expression. In particular, the higher-expressing 7319 line of activator mice remained normoglycemic throughout its lifespan.

The *Nkx2.2*-repressor protein can partially rescue the *Nkx2.2*-null pancreatic phenotype

To assess the precise molecular activity of *Nkx2.2* in the developing pancreas in vivo, we tested the ability of *Nkx2.2hdEnR* and *Nkx2.2hdVP16* to functionally substitute for endogenous *Nkx2.2*. We crossed both lines for each of the three transgenes into the *Nkx2.2*-null background and analyzed the phenotype at E18.5. The observed results were similar between each pair of lines. In *Nkx2.2*-null embryos expressing the *Nkx2.2hdEnR* transgene, significant recovery of glucagon-positive cells (98% of wild type) and a partial recovery of insulin-positive cells (18% of wild type; Fig. 3C,E,N) was observed, as well as a correlated decrease in ghrelin-positive cells (Fig. 3D,F). To obtain a more precise assessment of the degree of rescue, we quantified the expression of the *insulin2*, *glucagon* and *ghrelin* genes in *Nkx2.2^{-/-};Nkx2.2hdEnR* embryos using quantitative real-time PCR. *Glucagon* mRNA was expressed at levels comparable to wild-type embryos (Fig. 3K); however, *insulin2* mRNA was only recovered to approximately 10% of wild-type levels (Fig. 3L). Additionally, *ghrelin* mRNA expression was reduced by approximately 70% compared to *Nkx2.2*-null embryos, but it was still expressed at higher levels than in wild-type embryos (Fig. 3M). Rescue could also be detected as early as E12.5 (glucagon, Fig. 4B) or E13.5 (insulin, Fig. 5B), and at E15.5 (glucagon and insulin; data not shown).

By contrast, the Nkx2.2hdVP16 transgene, as well as the Nkx2.2hdCon transgene did not rescue islet cell differentiation in the absence of endogenous Nkx2.2: insulin and glucagon were absent or reduced, respectively (compare Fig. 3G,I with 3C), and ghrelin expression remained high throughout the islet (compare Fig. 3H,J with 3D). We were unable to observe rescue with either Nkx2.2hdVP16 line, including line 7319, which expressed the Nkx2.2-activator transgene at twice the level of the endogenous Nkx2.2 protein (Fig. 2C). Because we were unable to measure Nkx2.2-activator-transgene-protein production in these mice with the available antibodies, it remains possible that Nkx2.2hdVP16 protein is not produced at adequate levels to achieve rescue. However, the Nkx2.2-repressor does appear sufficient to rescue the glucagon cells and a small subset of insulin-producing cells, suggesting that the functions of the Nkx2.2 activator may not be required at these early time points. Furthermore, the Nkx2.2hdVP16 fusion protein is competent to activate transcription in pancreatic cell lines (Fig. 1B), indicating that the Nkx2.2-activator fusion protein is functional.

The Nkx2.2-repressor protein rescues mature α -cells and immature β -cells

Glucagon-positive cells appear to be rescued to levels similar to wild-type embryos in Nkx2.2^{-/-};Nkx2.2hdEnR embryos. We further characterized the integrity of the rescued α -cell population by testing for co-expression with other islet hormones. Glucagon and ghrelin double-positive cells are normally found in a small percentage of islet cells throughout development of the pancreas; however, this population is lost in Nkx2.2-null mice (Heller et al., 2005; Prado et al., 2004). In Nkx2.2^{-/-};Nkx2.2hdEnR embryos, the glucagon and ghrelin co-expressing population was also rescued; both the α -cells and glucagon/ghrelin double-positive population were expressed in numbers and ratios similar to wild-type islets (Fig. 4A-D). We also observed no evidence that the rescued glucagon-producing population abnormally co-expressed insulin, somatostatin or PP (Fig. 3E and data not shown), suggesting that the rescued cells are normal glucagon-producing α -cells. Furthermore, all glucagon-positive cells co-expressed Pax6, a characteristic α -cell transcription factor (Fig. 4E,F).

Although the Nkx2.2-repressor fully rescued α -cells in Nkx2.2-null embryos, the number of rescued insulin-producing β -cells was low and these mice died shortly after birth. There are a number of possibilities for this partial rescue of insulin-producing cells, including the timing and level of transgene expression, or a requirement for additional activation functions of Nkx2.2 in the β -cell lineage. To begin to understand the incomplete rescue of insulin-positive cells, we assessed the molecular features and maturation status of the insulin-positive cells that were maintained in the rescued animals. In the normal developing pancreas, insulin-producing cells are present as early as E9.5 (Teitelman et al., 1993). Several studies have proposed the existence of a precursor population of cells at the earliest stages of development that co-express insulin and glucagon (Pang et al., 1994; Teitelman et al., 1993; Upchurch et al., 1994). It does not appear that the rescued insulin-positive cells in Nkx2.2^{-/-}; Nkx2.2hdEnR mice represent this double-hormone-expressing population, because we were unable to detect any glucagon and insulin double-positive cells at any stage of development (Fig. 3E and data not shown). The rescued insulin-producing cells also never abnormally co-expressed somatostatin or PP (data not shown). We also assessed for ghrelin and insulin co-expression to ensure that we were not merely rescuing *insulin* gene transcription in the ghrelin cells; we did not observe the co-expression of insulin and ghrelin in Nkx2.2^{-/-}; Nkx2.2hdEnR mice (Fig. 5C,D).

It has been postulated that, in wild-type mice, there are both early insulin-positive, and glucagon and insulin double-positive populations that do not give rise to the mature β -cells of the islet (Herrera, 2000; Larsson, 1998). After E13.5, during the secondary transition, a distinct lineage of endocrine cells forms that give rise to the mature islet cell populations. The majority of β -cells are formed during this stage of islet development and proceed to differentiate in a stepwise manner. In Nkx2.2-null mice, no insulin-producing cell population could be detected during

pancreatic islet development. In *Nkx2.2*^{-/-}; *Nkx2.2*hdEnR embryos, only approximately 18% of the normal numbers of insulin-producing cells were rescued. This population of rescued cells may correspond to the earliest terminal insulin-positive cells that form prior to E13.5, to an immature β -cell population that fails to undergo complete differentiation and expansion, or they may represent reduced numbers of fully differentiated β -cells. The earliest (terminal) insulin cells often co-express glucagon and are believed not to express Pdx1 and Nkx6.1 (Kim and MacDonald, 2002). The majority of the precursor cells that give rise to β -cells express Pdx1 and Nkx6.1 (Kim and MacDonald, 2002), and recent studies have indicated that MafA and Glut2 become expressed later in the β -cell differentiation process (Kim and MacDonald, 2002; Matsuoka et al., 2004). To assess the differentiation state of the rescued β -cell population, we tested for the expression of Pdx1, Nkx6.1, MafA and Glut2. At E18.5, Pdx1 and Nkx6.1 were expressed at normal levels in the insulin-producing cells that were rescued by the *Nkx2.2*-repressor; however, the majority of these cells (>90%) remained MafA negative (Fig. 6). Furthermore, the rescued β -cells did not express Glut2, which normally becomes activated in β -cells after they have initiated terminal differentiation (Boj et al., 2001) (Fig. 6G-J). This would suggest that the repressor activity of *Nkx2.2* is sufficient to specify the immature Pdx1- and Nkx6.1-expressing β -cell population that forms during the secondary transition, but is not adequate to promote the β -cell differentiation process; additional *Nkx2.2* activities may be required to complete the maturation and expansion process. Alternatively, a higher threshold of *Nkx2.2*-repressor activity may be required to initiate further steps of the β -cell process.

Groucho3 is expressed in the developing pancreas and interacts with Nkx2.2

In the spinal cord, *Nkx2.2* functions as a transcriptional repressor and this function is mediated through an interaction with the Groucho co-repressor *Grg4* (Muhr et al., 2001). Because *Nkx2.2* appears to function as a repressor in the developing islet, we wanted to determine whether *Nkx2.2* also interacts with Groucho co-repressors in the developing pancreas. We assessed the expression profiles of the four full-length mouse Groucho co-repressors (*Grg1*, *Grg2*, *Grg3* and *Grg4*; also collectively known as Tle1-Tle4) in the developing pancreas. Although *Grg4* interacts with *Nkx2.2* in the spinal cord, we were unable to detect its expression in the developing pancreatic epithelium (data not shown). By contrast, *Grg1* and *Grg2* mRNAs are expressed at low levels throughout the pancreatic epithelium, while *Grg3* mRNA is expressed robustly throughout the pancreatic epithelium at E12.5 (Fig. 7A; data not shown). As pancreatic development proceeds, *Grg3* mRNA becomes restricted to the endocrine compartment (Fig. 7F) and, throughout pancreas development, is expressed in a manner similar to *Nkx2.2*, suggesting that it may be the interacting Groucho partner in the pancreas that is needed to mediate the repressive activities of *Nkx2.2*. Antibodies against *Nkx2.2* and *Grg3* that work well for immunolabeling of the embryonic pancreas were not available to us; however, RNA in situ analysis of *Grg3* and *Nkx2.2* expression on adjacent tissue sections shows a high correlation between these two genes; at E12.5, both genes are expressed throughout the pancreatic epithelium (Fig. 7A,B) and, by E16.5, both genes are expressed within the same cell clusters (Fig. 7C,D). Using combined RNA in situ hybridization and immunohistochemistry, we also demonstrated that, similar to *Nkx2.2*, *Grg3*-positive cells co-express glucagon, but do not overlap with amylase-expressing cells (Fig. 7E,F). These expression studies demonstrate that, although *Grg4* appears to be the binding partner of *Nkx2.2* in the spinal cord, *Grg3* is the more likely Groucho family member to modulate *Nkx2.2* function in the pancreatic islet.

To test for an interaction between *Nkx2.2* and *Grg3*, we performed co-immunoprecipitation assays with transfected cDNA into the pancreatic cell line PANC1 and demonstrated that full-length *Nkx2.2* and *Grg3* form a complex (Fig. 7G, lane 5). The interaction between *Nkx2.2* and *Grg4* was reported to be through the TN domain in *Nkx2.2* (Muhr et al., 2001) – a ten amino acid domain similar to the Engrailed eh1 domain, which binds to *Drosophila* Groucho

(Jimenez et al., 1997;Tolkunova et al., 1998). When we deleted the TN domain from Nkx2.2 in these assays, the interaction between Nkx2.2 and Grg3 was abolished (Fig. 7G, lane 6). These results suggest that Nkx2.2 interacts with unique Groucho co-repressors in different cell types to influence its transcriptional activity during development.

DISCUSSION

In this study, we have determined that, in the absence of endogenous Nkx2.2, the repressor activity of Nkx2.2 is sufficient to restore the specification of glucagon-producing α -cells and a small population of immature insulin-producing β -cells. For this analysis, we expressed the previously characterized Nkx2.2hd derivatives (Muhr et al., 2001) in the pancreas using *Pdx1*-promoter regulatory elements. We acknowledge that there are caveats associated with this experimental system, including the use of the *Pdx1* promoter, which is expressed in similar, but not identical, expression domains as Nkx2.2. In particular, Pdx1 is expressed at an earlier developmental time point in the pancreatic epithelium and, unlike Nkx2.2, becomes downregulated in the α -cell population. However, the fact that we were able to partially restore islet cell differentiation with these *Pdx1*:Nkx2.2hdEnR derivatives suggests that they have sufficient normal functions to replace some endogenous Nkx2.2 activities. The full rescue of the α -cell population is particularly intriguing, precisely because Pdx1 expression is normally downregulated in α -cells, where endogenous Nkx2.2 expression would normally be maintained. This would suggest that the repressor activity of Nkx2.2 is required early in the Pdx1-positive progenitor population to initiate the complete specification and differentiation program of α -cells; maintenance of Nkx2.2-repressor function in the glucagon-producing α -cells does not appear to be necessary for the full differentiation of these cells. The concomitant reduction in ghrelin-expressing cell numbers also indicates that expression of the Nkx2.2-repressor in Pdx1-positive precursors is sufficient to regulate these particular cell-fate decisions.

In contrast to the glucagon-producing α -cell population, only a small number of insulin-producing cells are rescued by the Nkx2.2-repressor transgene. Interestingly, the rescued insulin-producing cells persist through birth, however, they fail to activate MafA, the only transcription factor known to be expressed exclusively in the differentiated β -cell population that forms after E13.5 (Matsuoka et al., 2004). Furthermore, Glut2 is not expressed on the surface of these cells, indicating that the rescued β -cells are non-functional. Based on marker analysis, we favor the hypothesis that the Nkx2.2-repressor rescues an immature β -cell that is unable to complete the differentiation process; however, it is difficult to rule out that the rescued insulin-positive cells do not represent the earliest 'terminal' insulin-expressing population (Herrera, 2000). Currently, there are no markers that exclusively localize to the postulated first wave of insulin-producing cells and, therefore, we cannot fully resolve this issue. In support of our hypothesis, in the Nkx2.2-repressor-rescued mice, double-positive cells expressing both glucagon and insulin were not observed. These cells are thought to be characteristic of this early population (Herrera, 2000). Furthermore, insulin-positive cells that are specified express Pdx1 and Nkx6.1, markers, which are thought to be absent in the early hormone-positive population (Kim and MacDonald, 2002).

The inability to express MafA in the rescued insulin-producing cells suggests that there may be a requirement for additional Nkx2.2-activator functions in the β -cell-maturation process (Fig. 8). This would be consistent with the recent finding that Nkx2.2 binds to the promoter of *MafA* and is important for the activation of *MafA* in β -cells, both in vitro and in vivo (Raum et al., 2006). Alternatively, the partial rescue of the β -cell phenotype may reflect the low level of transgene expression in these cells or the inability of the truncated Nkx2.2 protein to regulate all of its downstream targets. We attempted to address this issue by assessing the degree of rescue that could be achieved by a *Pdx1*:Nkx2.2 full-length transgene; however, the

introduction of this transgene into a wild-type background caused perinatal lethality and we were unable to establish stable transgenic lines (Grasch and L.S., unpublished data). These results are consistent with our inability to establish more highly expressed Nkx2.2-derivative lines and suggest that tightly regulated levels of Nkx2.2 protein are crucial for appropriate islet cell development. We are pursuing these studies using the Tet-On system to establish inducible Nkx2.2 full-length and derivative transgenic lines.

Interestingly, we did not observe rescue with the Nkx2.2-activator transgenic lines. Although we detected *Nkx2.2hdVP16* mRNA throughout the pancreatic epithelium, it is possible that the lack of rescue is due to insufficient amounts of the Nkx2.2-activator fusion protein in these mice. This is not likely to be the case in the α -cell population because Nkx2.2-repressor activity is sufficient to fully rescue α -cells; however, it does remain possible that Nkx2.2-activator activities are required in β -cell formation and maturation. In addition, because we are unable to regulate the timing of Nkx2.2-activator expression in these studies, we cannot rule out the possibility that Nkx2.2-activator activity will be involved in the continued maturation of the β -cell population once the β -cells have been initially specified. Because many transcription factors are known to have dual activities and because evidence exists for Nkx2.2 regulating the transcriptional activation of several important β -cell genes, including *Mafa*, it is likely that Nkx2.2 will be found to have complex regulatory activities in the different islet cell types and at different stages of development. Importantly, this study reveals that the repressor activity of Nkx2.2 is sufficient for the correct specification of α -cells, and it is likely that repressor and activator activities will be required to promote β -cell differentiation.

This study also resulted in the unexpected finding that Grg3, and not Grg4, is the Groucho-family member expressed in the pancreatic islet. Previous studies have demonstrated that Nkx2.2 interacts with Grg4 to mediate its repressor activity in ventral neuron specification (Muhr et al., 2001). Because of the conservation of the Grg proteins, it is not surprising that we also demonstrated an interaction between Nkx2.2 and Grg3 in the pancreas. However, the different Grg factors are not identical at the protein level, and the expression of distinct family members in the different cell types may contribute to the differential gene regulation that must occur to specify cell-type-specific gene expression. Further exploration of these unique protein interactions and the resulting downstream regulatory consequences in different tissues will help to elucidate the complex regulatory pathways that allow overlapping sets of transcription factors to specify unique cell types.

In summary, this study highlights the importance of understanding the distinct molecular functions of a transcriptional regulator, such as Nkx2.2, and how its different biological activities influence cell-fate lineages. Furthermore, it emphasizes the need to elucidate the protein modifications and protein-protein interactions that modulate the molecular activities of a transcription factor at different stages of development. We have discovered that the repressor activities of Nkx2.2 are sufficient for the differentiation of one islet cell type, the α -cells; however, more-complex activities are required to generate a fully functional β -cell. These results have serious implications for the manipulation of cell-fate choices, both in vivo and in vitro, and for advancing our ability to engineer pancreatic islet cells from alternative sources for therapeutic purposes.

Acknowledgments

We thank Johan Ericson for the Nkx2.2hd-fusion constructs; Hans Clevers for mouse Grg3 cDNA; Jan Jensen for the Pdx1-promoter plasmid; Bernard Thorens for the Glut2 antibody; and Michael German for the pFoxLuc1-7xNk2pr1 plasmid. Technical assistance was provided by Sunshine Daddario and Tanya McNeal. We thank Lee Niswander and members of the Sussel laboratory for critical reading of the manuscript. This research was supported by an ADA Career Development Award, NIH NIDDK (U19 DK061248) and JDRF RRG to L.S., and an NIH Predoctoral Training Award

in Molecular Biology (T32-GM08730) to M.D. Support was provided by the UCHSC Cancer Center Transgenic Core and the BDC DERC (NIH P30 DK57516).

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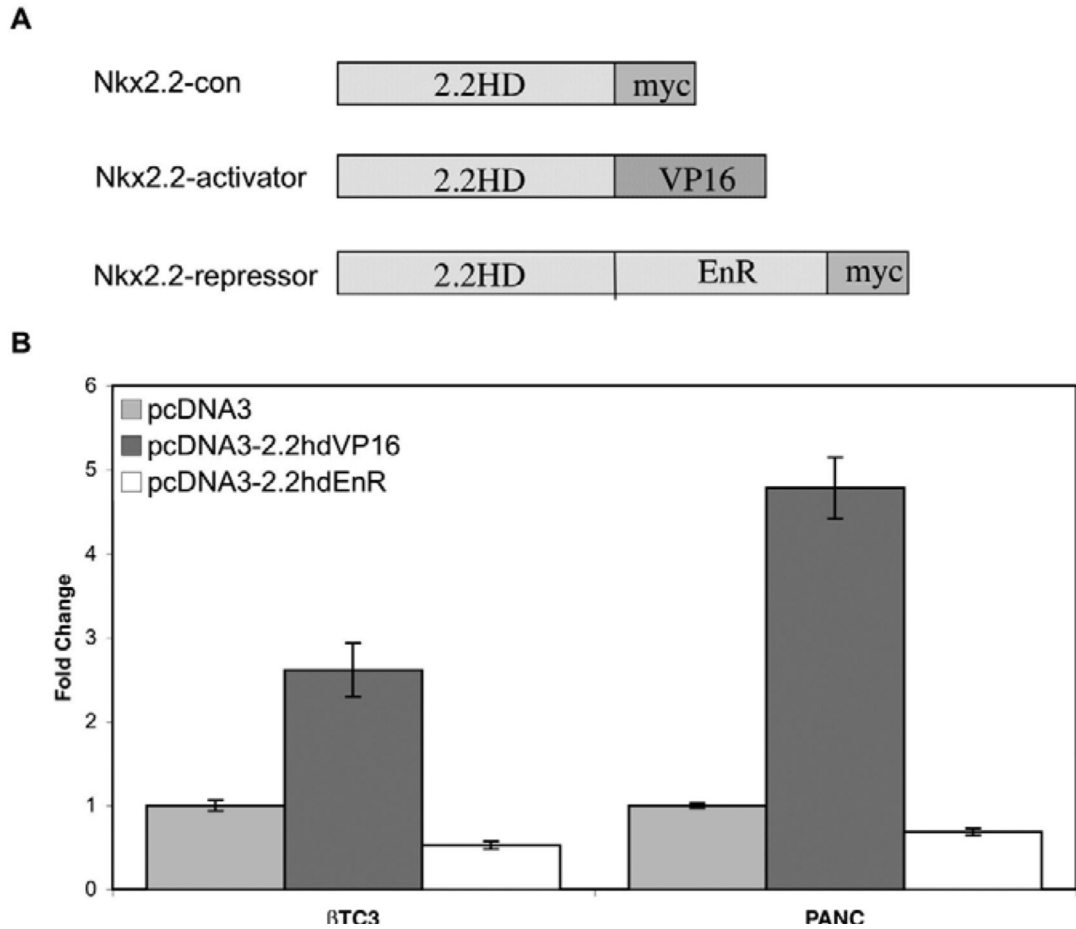


Fig. 1. Nkx2.2 derivatives are active in pancreatic cell lines

(A) Schematic of Nkx2.2-dominant-derivative transgenes. (B) The Nkx2.2-dependent luciferase reporter (pFoxLuc1-7xNk2prl) was co-transfected with pcDNA3 alone (light grey), pcDNA3-2.2hdVP16 (dark grey) or pcDNA3-2.2hdEnR (white) expression plasmids in β TC3- or PANC cells, and is activated with Nkx2.2hdVP16. Error bars represent the standard error (s.e.m.) of three independent experiments. 2.2HD, Nkx2.2 homeodomain; myc, myc epitope tag; VP16, VP16 activation domain from herpes simplex virus; EnR, Engrailed repressor domain.

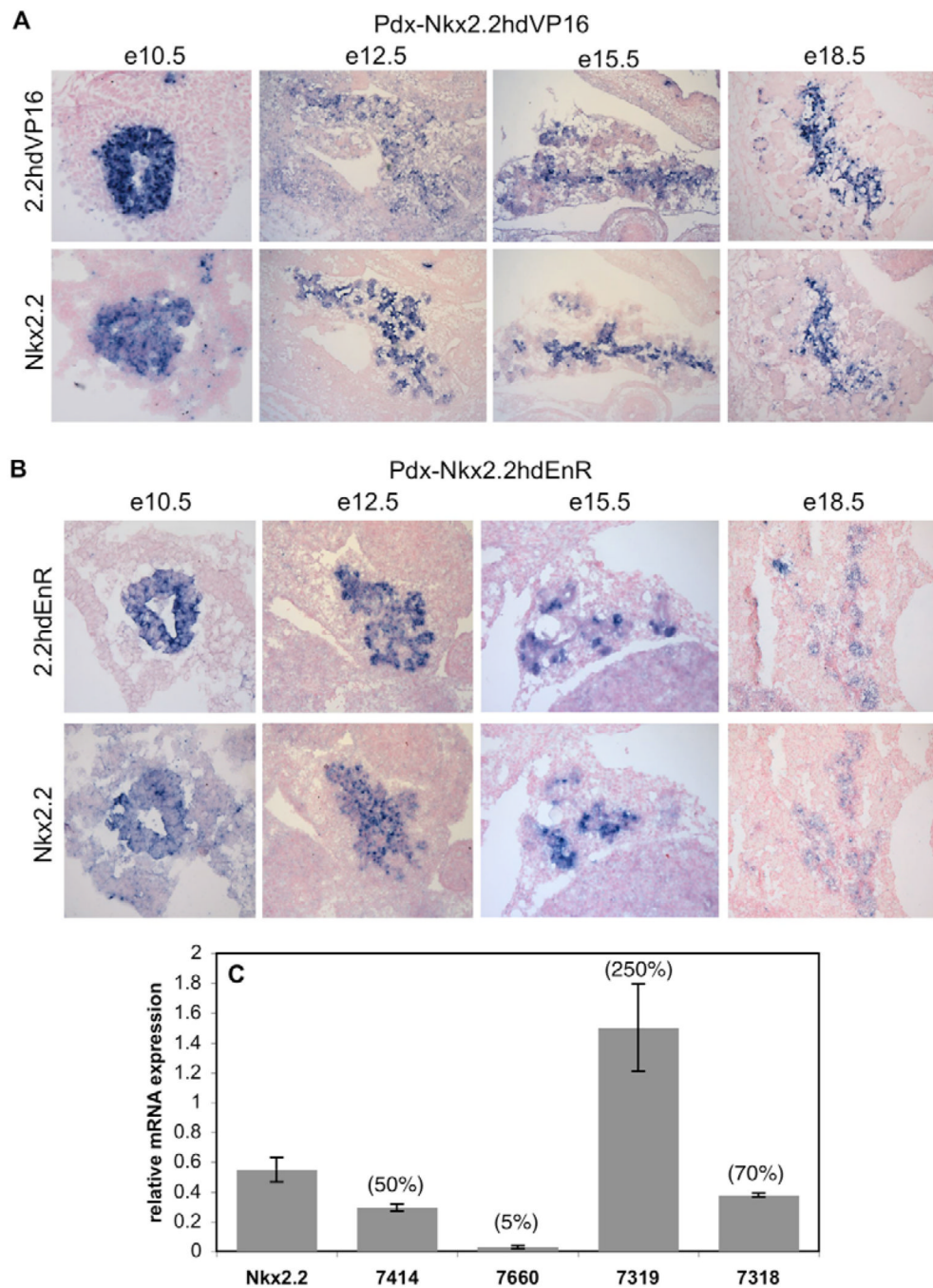


Fig. 2. Nkx2.2-derivative transgenes are expressed in patterns similar to endogenous Nkx2.2 when expressed under the control of the Pdx1 promoter

(A) *Nkx2.2hdVP16* mRNA is expressed throughout the pancreatic epithelium at E10.5 (L7319), E12.5 (L7318) and E15.5 (L7318), and is restricted to endocrine cells at E18.5 (L7319). (B) *Nkx2.2hdEnR* mRNA is detected in the pancreatic epithelium at E10.5, E12.5 and E15.5, and strong expression is detected in the endocrine tissue at E18.5 (all L7414). Both transgenes are expressed in a manner similar to *Nkx2.2* throughout development; however, a small amount of transgene expression is detectable in acinar cells. (C) Real-time PCR quantification of Nkx2.2-repressor and Nkx2.2-activator transgene expression compared to endogenous Nkx2.2 expression. Percentages indicate percent transgene expression relative to endogenous Nkx2.2.

Nkx2.2hdVP16: line 7319 ($n=5$), line 7318 ($n=4$); Nkx2.2hdEnR: line 7414 ($n=4$), line 7660 ($n=3$).

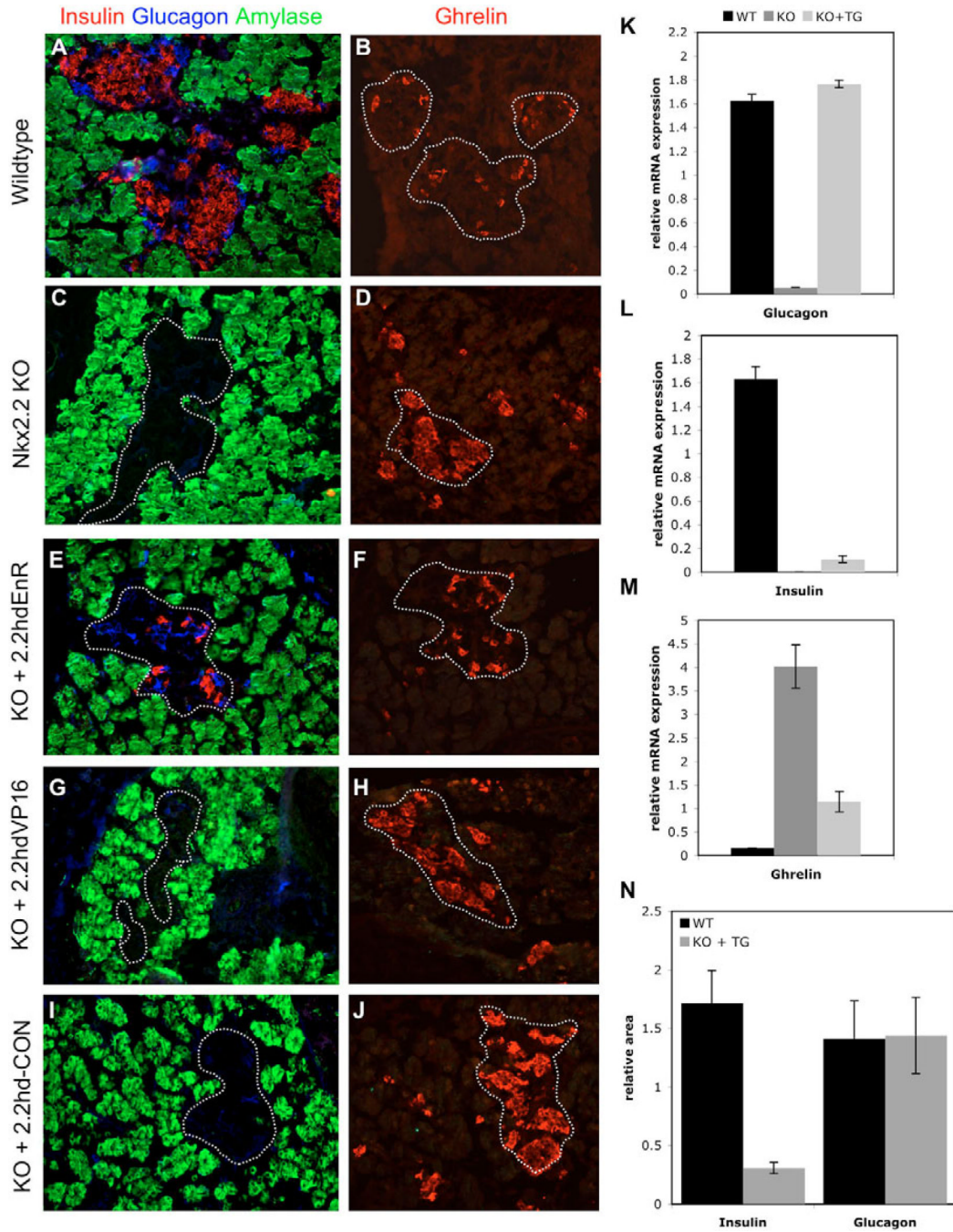


Fig. 3. The Nkx2.2-repressor transgene partially rescues the Nkx2.2-null pancreas phenotype (A-J) Immunofluorescence staining for insulin (red), glucagon (blue) and amylase (green) (A,C,E,G,I), or ghrelin (red; B,D,F,H,J) on E18.5 pancreata for wild-type (A,B), Nkx2.2-null (C,D), Nkx2.2^{-/-};Nkx2.2hdEnR (Line 7414; E,F), Nkx2.2^{-/-};Nkx2.2hdVP16 (Line 7318; G,H) and Nkx2.2^{-/-}; Nkx2.2hdCon (Line 5635; I,J) embryos (20× magnification) demonstrates that the Nkx2.2-repressor transgene can restore glucagon- and insulin-positive cells. (K-M) Quantitative PCR for glucagon (K), insulin (L) and ghrelin (M) on E16.5 pancreatic RNA isolated from wild-type (black bars), Nkx2.2^{-/-} (dark grey bars) and Nkx2.2^{-/-}; Nkx2.2hdEnR (light grey bars) embryos. (N) Summary of cell counting data demonstrates that insulin-positive

cell numbers are restored to approximately 20% of wild-type levels and glucagon-positive cell numbers are restored to wild-type levels (E18.5, $n=3$).

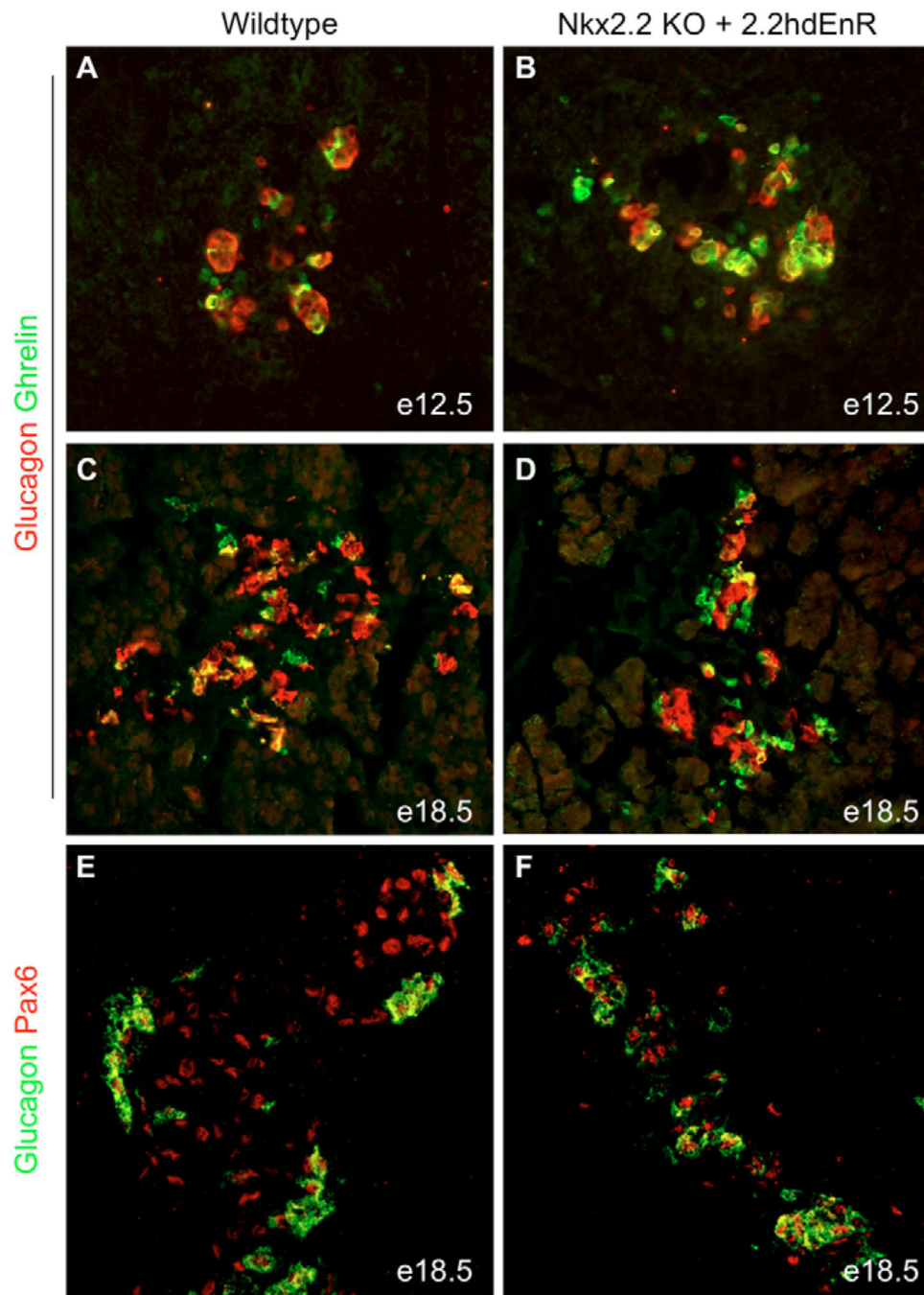


Fig. 4. Rescued glucagon-producing cells have normal α -cell characteristics
 (A-D) Immunofluorescence staining for glucagon (red) and ghrelin (green) at E12.5 and E18.5 on wild-type (A,C) and $Nkx2.2^{-/-};Nkx2.2hdEnR$ (B,D) pancreata. There are a small number of glucagon-ghrelin co-staining cells (yellow) in wild-type and rescued pancreata (20 \times magnification). (E,F) Glucagon-positive cells (green) co-express the α -cell marker Pax6 (red) in wild-type (E) and rescued (F) islets at E18.5. (40 \times magnification.)

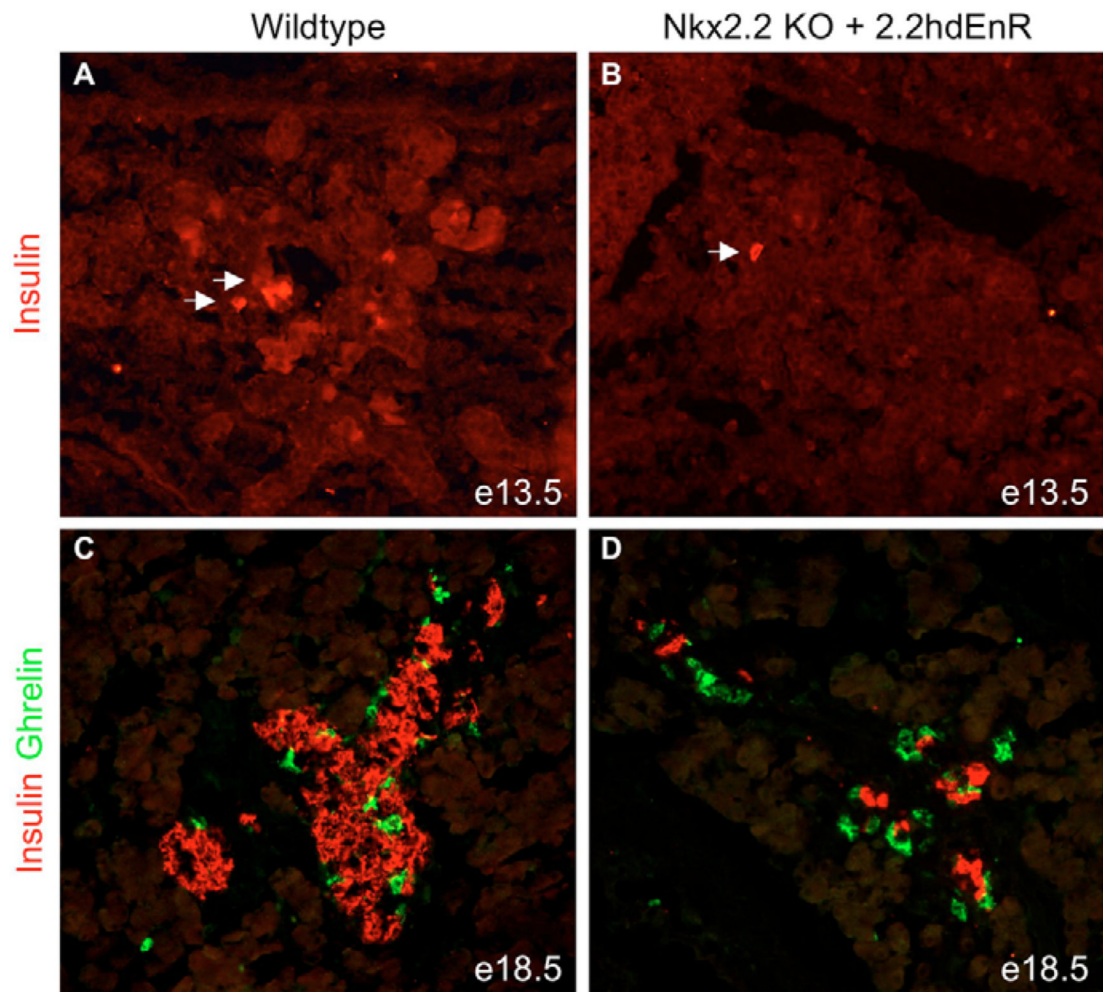


Fig. 5. Rescued insulin-positive cells are present by E13.5 and do not abnormally co-express the hormone ghrelin
(**A,B**) Insulin-positive (red, arrows) cells are found at E13.5 in $Nkx2.2^{-/-};Nkx2.2hdEnR$ embryos ($n=4$ embryos each). (**C,D**) Insulin (red) is not co-expressed with ghrelin (green) at E18.5 in either wild-type (C) or $Nkx2.2^{-/-};2.2hdEnR$ (D) embryos. (20 \times magnification.)

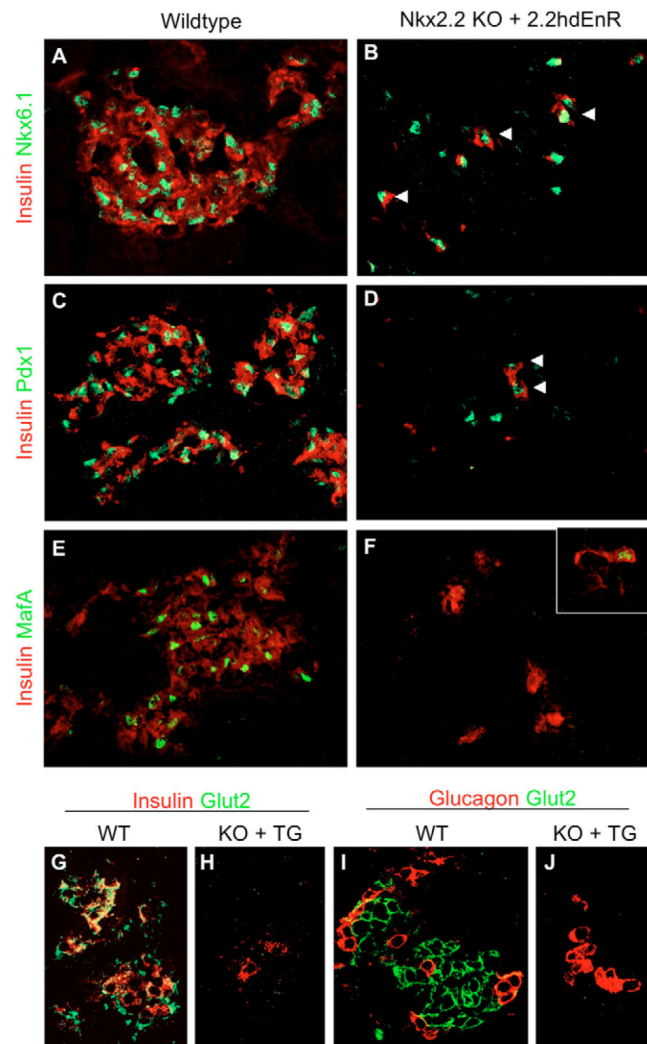


Fig. 6. Immature β -cells are restored in Nkx2.2-null embryos carrying the Nkx2.2-repressor transgene

Confocal images of immunofluorescence staining on E18.5 wild-type or Nkx2.2^{-/-}; Nkx2.2hdEnR islets. (A-D) Co-expression of insulin (red) with Nkx6.1 (green; A,B) or Pdx1 (green; C,D). (E-H) MafA and Glut2 (green) are co-expressed with insulin-positive (red) cells in wild-type (E,G) islets, but neither is co-expressed with rescued insulin-positive cells (F,H). Inset in F shows a rare insulin-positive cell co-expressing MafA. (I,J) Glucagon (red) and Glut2 (green) immunostaining. No Glut2-positive cells are found in Nkx2.2^{-/-}; 2.2hdEnR islets (H,J; 40 \times magnification).

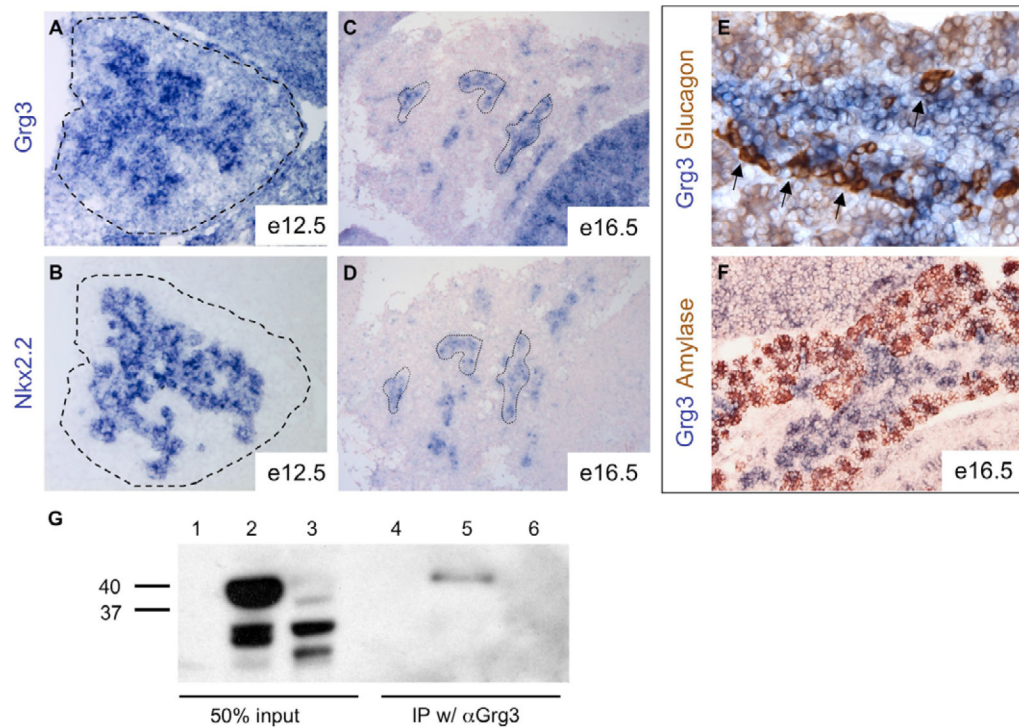


Fig. 7. Nkx2.2 interacts with Grg3 in the embryonic pancreas

(A-D) RNA in situ hybridization for *Grg3* at E12.5 (A) and E16.5 (C,E,F), and for *Nkx2.2* at E12.5 (B) and E16.5 (D). Adjacent sections demonstrate similar expression patterns throughout pancreas development. In C and D, outlines represent selected overlapping expression areas. (E,F) Combined RNA in situ hybridization and immunohistochemistry on E16.5 pancreata demonstrate overlapping expressions between *Grg3* (blue) and glucagon (brown; arrows in E), and distinct expression domains between *Grg3* (blue) and amylase (brown; F). (G) Co-immunoprecipitation for Nkx2.2 and Grg3: PANC cell lysates were incubated with Grg3 antibody and were western blotted with antibodies against Nkx2.2. Lanes 1 and 4: vector-only control; lanes 2 and 5: Nkx2.2 transfected with Grg3; lanes 3 and 6: Nkx2.2 Δ TN transfected with Grg3. Lanes 1-3 are input samples and lanes 4-6 are Grg3 pull-down samples. Nkx2.2 is co-immunoprecipitated with Grg3 (lane 5).

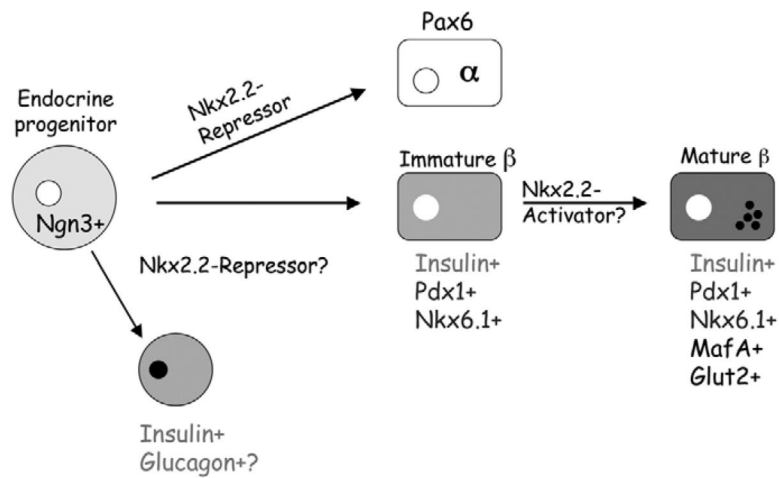


Fig. 8. A model of Nkx2.2 activity in the developing pancreas

Nkx2.2 functions as a repressor to specify and differentiate the glucagon-producing α -cell population. Nkx2.2 also functions as a repressor to specify the early insulin-positive population and/or the immature Pdx1^{low}, Nkx6.1⁺, MafA⁻, Glut2⁻ insulin-producing β -cells, but this activity is not sufficient to produce a fully differentiated mature Pdx1^{high}, Nkx6.1⁺, MafA⁺, Glut2⁺ β -cell. We propose that Nkx2.2-activator function will be required for this later differentiation step.