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Nkx2.2 and Arx genetically interact to regulate pancreatic endocrine cell development and endocrine hormone expression

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Abstract

Nkx2.2 and Arx are essential pancreatic transcription factors. Nkx2.2 is necessary for the appropriate specification of the islet alpha, beta, PP and epsilon cell lineages, whereas Arx is required to form the correct ratio of alpha, beta, delta and PP cells. To begin to understand the cooperative functions of Nkx2.2 and Arx in the development of endocrine cell lineages, we generated progenitor cell-specific deletions of *Arx* on the *Nkx2.2* null background. The analysis of these mutants demonstrates that expansion of the ghrelin cell population in the *Nkx2.2* null pancreas is not dependent on Arx; however, Arx is necessary for the upregulation of *ghrelin* mRNA levels in Nkx2.2 mutant epsilon cells. Alternatively, in the absence of *Arx*, delta cell numbers are increased and Nkx2.2 becomes essential for the repression of *somatostatin* gene expression. Interestingly, the dysregulation of *ghrelin* and *somatostatin* expression in the Nkx2.2/Arx compound mutant ($Nkx2.2^{null};Arx^{\Delta panc}$) results in the appearance of ghrelin+/somatostatin+ co-expressing cells. These compound mutants also revealed a genetic interaction between Nkx2.2 and Arx in the regulation of the PP cell lineage; the PP cell population is reduced when *Nkx2.2* is deleted but is restored back to wildtype numbers in the $Nkx2.2^{null};Arx^{\Delta panc}$ mutant. Moreover, conditional deletion of *Arx* in specific pancreatic cell populations established that the functions of Arx are necessary in the Neurog3+ endocrine progenitors. Together, these experiments identify novel genetic interactions between Nkx2.2 and Arx within the endocrine progenitor cells that ensure the correct specification and regulation of endocrine hormone-producing cells.

Keywords

Nkx2.2; Arx; transcriptional regulation; endocrine cell fate; ghrelin; PP; somatostatin

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Introduction

At embryonic day (E) 9.5, the region of dorsal foregut endoderm fated to become pancreas evaginates from the gut tube creating the dorsal pancreatic bud. One day later, the ventral pancreatic domain is similarly produced from the ventral foregut endoderm (Jorgensen et al., 2007). The generation of these pancreatic anlagen requires signals from the surrounding tissues, which ultimately permit the patterning of all organs along the length of the primitive gut tube (Hebrok et al., 1998; Kim et al., 1997; Martin et al., 2005; Wells and Melton, 2000; Zorn and Wells, 2009). Cells within this pancreatic domain expressing the transcription factors Pdx1 and Ptf1a mark the pancreatic progenitor cells (Burlison et al., 2008; Kawaguchi et al., 2002; Offield et al., 1996). Once these progenitor cells are specified, a cascade of transcription factors together with signals from the mesenchyme (Ahlgren et al., 1997; Bhushan et al., 2001), peripheral nervous system (Lausier et al., 2010; Nekrep et al., 2008; Olerud et al., 2009; Plank et al., 2011) and developing vasculature (Yoshitomi and Zaret, 2004) are required to specify the three pancreatic lineages: endocrine, exocrine and ductal cells. Cells of the exocrine lineage will function in the production and secretion of digestive enzymes (Macdonald et al., 2010), while the ductal lineage will generate the epithelial cords that compose the ductal network, which runs throughout the pancreas and contains Neurogenin3+ (Neurog3) endocrine progenitor cells (Gradwohl, 2006; Villasenor et al., 2011). Activation of Neurog3, a pro-endocrine bHLH transcription factor, is necessary to specify the endocrine progenitors that will give rise to the five major endocrine subtypes located in the islets of Langerhans, including alpha, beta, delta, PP and epsilon cells, and expressing the hormones glucagon, insulin, somatostatin, pancreatic polypeptide and ghrelin, respectively (Gradwohl et al., 2000; Prado et al., 2004).

A major wave of endocrine differentiation occurs from E12.5 to E15.5, the stage of pancreas development termed the secondary transition (Pictet and Rutter, 1972). This differentiation and subsequent maturation of all hormone-producing endocrine cells is reliant upon the temporal and spatial activation and/or repression of a complex network of transcription factors (Pan and Wright, 2011). Two transcription factors necessary for specific endocrine cell lineage development are NK2 homeobox 2 (Nkx2.2) and Aristaless-related homeobox (Arx). The developing pancreas of the Nkx2.2 null embryo shows a complete absence of insulin-expressing beta cells, a ~90% reduction of glucagon-expressing alpha cells and a ~50% reduction in pancreatic polypeptide-expressing PP cells. In place of these cell populations there is an increase in ghrelin-expressing epsilon cells, although the small population of glucagon/ghrelin coexpressing cells that exist in wildtype pancreata is lost (Chao et al., 2007; Prado et al., 2004; Sussel et al., 1998). Similar to the Nkx2.2 null embryos, deletion of Arx results in a significant loss of alpha cells and the glucagon/ghrelin coexpressing cells, but unlike Nkx2.2 mutants, display an increase of the beta and delta cell populations (Collombat et al., 2003). Although Arx does not appear to be necessary for PP cell development, the misexpression of *Arx* in Pdx1- or Pax6-expressing cells results in an increase of alpha and PP cells (Collombat et al., 2007). The cumulative phenotypic analyses indicate that the primary commonality between the Nkx2.2 and Arx single mutants is the absence of the glucagon-expressing alpha cells, as well as the rare population of glucagon+/ghrelin+ cells (Chao et al., 2007; Heller et al., 2005).

Bihormonal cells have been described in several endocrine organs, including the developing pancreas and stomach. In particular, glucagon+/insulin+, ghrelin+/glucagon+ or ghrelin+/PP+ co-expressing islet cells have been identified in the embryonic pancreatic domain of the mouse, rat and human (De Krijger et al., 1992; Heller et al., 2005; Herrera, 2000; Lukinius et al., 1992; Wierup et al., 2004). In the developing stomach, gastrin and somatostatin co-expressing cells have also been observed (Larsson, 2000). Interestingly, while it is believed that mature gastrin-secreting G cells and somatostatin-secreting D cells derive from the

common bihormonal precursor cells in the gastric epithelium (Larsson, 2000; Larsson et al., 1996), the pancreatic glucagon+/insulin+ bihormonal cells do not appear to represent endocrine progenitors; cells expressing insulin do not derive from those previously expressing glucagon, and the reverse is also true (Herrera, 2000). Therefore, hormone coexpression may or may not indicate a lineage relationship between two endocrine subtypes. Similarly, the emergence of bihormonal cell populations in genetically mutant backgrounds could represent either the expansion of rare bipotential cells or single lineage populations with dysregulated hormone genes. Both situations are likely influenced by the presence or absence of specific transcription factors within each lineage. While mutations in single transcription factors can lead to dysregulated lineage decisions and/or hormone expression (Ahlgren et al., 1998; Collombat et al., 2003; Prado et al., 2004; Wang et al., 2008), it is also possible that specific combinations of transcription factors are required to regulate appropriate lineage decisions or hormone gene regulation.

Given the overlapping, and sometimes opposing, roles for Nkx2.2 and Arx in specifying endocrine lineages, we hypothesized Nkx2.2 and Arx genetically interact to regulate endocrine cell differentiation and hormone gene expression in the developing pancreas. In this study we identified Arx+/Nkx2.2+ co-expressing cells in the early pancreatic progenitor domain, and demonstrated that Arx is expressed in wildtype and Nkx2.2-deficient ghrelin+ cells. To investigate the requirement of Arx and Nkx2.2 in regulating islet cell fates, we deleted Arx in the pancreas progenitor cells (Pdx1-cre; (Hingorani et al., 2003)) or endocrine progenitor cells (Neurog3-cre; (Schonhoff et al., 2004)) in the Nkx2.2 null background. These compound mutants revealed a genetic interaction between Arx and Nkx2.2 in the development of the PP cell lineage. Moreover, simultaneous deletion of Arx and Nkx2.2 lead to the dysregulation of *ghrelin* and *somatostatin* gene expression. Arx-dependent *ghrelin* gene regulation and Nkx2.2- dependent *somatostatin* gene regulation were altered in delta and epsilon cells, respectively, resulting in the expansion of a ghrelin+/somatostatin+ co-expressing cell population. Taken together, these data indicate that Nkx2.2 and Arx genetically interact in the regulation of islet PP cell specification and endocrine hormone gene expression in the ghrelin- and somatostatin-expressing cell populations.

Materials and Methods

Mice

All mice were bred and maintained on an outbred Black Swiss background (NTac:NIHBS, Taconic), according to Columbia University IACUC approved protocols. All strains were previously generated. Cell-type specific *Arx* null mice were generated by intercrossing $Arx^{tm1Gldn}$ ($Arx^{flox/flox(or\ Y)}$; (Fulp et al., 2008)) and either *Tg(Ipf1-cre)1Tuv* (*Pdx1-cre;* (Hingorani et al., 2003)) or *Tg(Neurog3-cre)C1Able* (*Neurog3-cre*; (Schonhoff et al., 2004)) mice. $Arx^{flox/flox(or\ Y)}$*;Pdx1-cre* and $Arx^{flox/flox(or\ Y)}$*;Neurog3-cre* mice were viable and fertile. The *Pdx1-cre* deletes *Arx* in all pancreatic progenitor cells; however, the Pdx1 expression domain also includes the glandular stomach and the duodenum (Larsson et al., 1996; Offield et al., 1996). These mice were then crossed to $Nkx2\text{-}2^{tm1Jlr}$ knock-in mice (Sussel et al., 1998) to generate compound heterozygotes. Embryos were collected from timed matings between $Nkx2.2^{+/-}$;$Arx^{flox/flox(orY)}$;*Pdx1-cre* or $Nkx2.2^{+/-}$;$Arx^{flox/flox(or\ y)}$;*Neurog3-cre* mice The $Nkx2.2^{LacZ/+}$ knock-in reporter line was used for expression analysis; the homozygous $Nkx2.2^{LacZ/LacZ}$ mice are phenotypically identical to the Nkx2.2 null allele (Arnes and Sussel, in preparation). Noon on the day of appearance of a vaginal plug was considered embryonic day (E) 0.5. The experimental genotypes of wildtype, $Nkx2.2^{-/-}$ ($Nkx2.2^{null}$), $Arx^{flox/flox(or\ Y)}$*;Pdx1-cre* ($Arx^{\Delta panc}$), $Nkx2.2^{-/-}$;$Arx^{fiox/flox(or\ Y)}$;*pdx1-cre* ($Nkx2.2^{null}$;$Arx^{\Delta panc}$), $Arx^{flox/flox(or\ Y)}$*;Neurog3-cre* ($Arx^{\Delta endo}$), and $Nkx2.2^{-/-}$;$Arx^{flox/flox(or\ Y)}$*;Neurog3-cre* ($Nkx2.2^{null}$;$Arx^{\Delta endo}$) were studied. Litters were assessed at E12.5, E15.5, and postnatal day (P) 0. All embryo dissections were

carried out in cold PBS, using a dissecting microscope (Leica MZ8). Tail or yolk sac was removed from the embryo, digested with proteinase K, and DNA extracted for genotyping purposes. Genotyping was carried out with standard conditions and primers as previously described (Fulp et al., 2008; Hingorani et al., 2003; Schonhoff et al., 2004; Sussel et al., 1998).

Realtime PCR

Pancreas was dissected from each embryo and stored in RNAlater (Ambion) until RNA was extracted using the NucleoSpin RNAII Kit (Clonetech). Subsequently, cDNA was made with equal amounts of RNA for each sample and oligo(dT) (Superscript III Kit, Invitrogen, CA). Realtime PCR was performed using TaqMan gene expression assays (Applied biosystems) for *glucagon* (Mm00801712_m1), *ghrelin* (Mm00445450_m1), *somatostatin* (Mm00436671_m1), *insulin1* (Mm01950294_s1), *insulin2* (Mm00731595_gH), *pancreatic polypeptide* (Mm00435889_m1), *Neurod1* (Mm01280117_m1), *Pdx1* (Mm00435565_m1), *Nkx6.1* (Mm00436671_m1), *Hes1* (Mm00468601_m1), *Arx* (Mm00545903_m1), and *Brn4 (Pou3f4;* Mm00447171_s1). *Nkx2.2* and cyclophilinB, which was used as a control housekeeping gene, were also assayed using probe and primer sets previously described (Chao et al., 2007). A standard two-step realtime PCR program was used for all genes assessed, with an annealing temperature of 61°C and 40 cycles of amplification (CFX96 RealTime System C1000 Thermal Cycler, Biorad). All gene expression values were normalized to the internal control gene, *cyclophilinB*, and relative quantification was performed using a standard curve from embryonic age-matched wildtype cDNA. A standard two-tailed Student t-test was performed to determine significance.

Immunofluorescence

Immunofluorescence was performed according to standard protocols, on E12.5 and P0 whole embryos that were embedded in OCT, after fixation with 4% PFA and cryopreservation in 30% sucrose. Transverse frozen sections (8 μm) were cut and mounted on glass slides. Sections were stained with rabbit α-ghrelin (1:800; Phoenix Pharmaceuticals, CA), goat α-ghrelin (1:800; Santa Cruz), guinea pig α-glucagon (1:1000; Linco/Millipore, MA), guinea pig α-insulin (1:1000; Millipore), rabbit α-insulin (1:1000; Cell Signaling Technology), rabbit α-somatostatin (1:200; Phoenix Pharmaceuticals), rabbit α-pancreatic polypeptide (1:200; Zymed), rabbit α-amylase (1:1000; Sigma), goat α-carboxypeptidase A (1:800; R&D Systems), rabbit α-Arx (1:500; kind gift from Dr. Kanako Miyabayashi, Kyushu University, Japan; (Kitamura et al., 2002)), rabbit α-Pdx1 (1:1000; Millipore), guinea pig α-Pdx1 (1:1000; C. Wright, Vanderbilt University; supplied by BCBC), rabbit α-Sox9 (1:500; Santa Cruz), rabbit α-Neurog3 (1:500; BCBC Antibody Core), and chicken α-beta-galactosidase (1:250; Abcam). Donkey α-guinea pig-Cy3 or Cy5, α-rabbit-Cy2 or Cy3, α-chicken-Cy3, and α-goat Cy2 or Cy5 secondary antibodies were used (1:400, Jackson ImmunoResearch). DAPI (1:1000; Invitrogen) was applied for 30 minutes following secondary antibody incubation. Images were acquired on either a fluorescent (Leica DM5500) or confocal (Zeiss) microscope. Morphometric analysis was performed by immunostaining every 10^{th} section throughout each embryo (N=3 or 4 for each genotype). For quantification of individual hormone-expressing cells, cell number was assessed versus total pancreas as defined by amylase area, and calculated using ImagePro software.

Results

Arx expression is detected in *Nkx2.2^{null}* ghrelin cells

Despite the dramatic reduction of the glucagon-expressing alpha cell population in *Nkx2.2^{null}* pancreata, expression of the alpha cell factor, *Arx*, was not reduced (Chao et al.,

2007) (Figure 1A). To identify which cell populations expressed Arx in the Nkx2.2 mutant pancreas, Arx expression was examined by immunostaining in wildtype and *Nkx2.2*^{null} pancreas. In addition to the few remaining alpha cells that were positive for Arx (Figure 1B, D; arrows), Arx expression in the Nkx2.2^{null} pancreas was found in the ghrelin-expressing population (Figure 1C,E; arrows). Since the loss of Nkx2.2 leads to an increase in the number of Arx+ ghrelin cells, we hypothesized that Nkx2.2 and Arx genetically interact to control early cell specification events in the pancreas. Consistent with this idea, we determined that Nkx2.2 and Arx were co-expressed in the early E10.5 developing pancreatic domain in both the *Nkx2.2* heterozygote (*Nkx2.2*$^{LacZ/+}$) and *Nkx2.2* mutant (*Nkx2.2*$^{LacZ/LacZ}$) mice (Figure 1F-I; Arnes, Sussel, in preparation; see Materials and Methods). We determined that, while cells expressing either Arx or Nkx2.2 expressed glucagon in the early Pdx1+ pancreatic domain (Figure 1F-I, dotted boxes; Figure 1J-M), there was also a population of Arx+/Nkx2.2+ co-expressing cells that did not express hormone (Figure 1F-I, arrows).

To further investigate which cell populations normally produced Arx during early pancreas development, Arx expression was examined in conjunction with islet progenitor markers or islet hormones. As expected, Arx expression was detected in glucagon+ cells from E9.5 through E15.5 (Figure 2A-H, dotted boxes; Figure 1B,D; (Collombat et al., 2003); Supplemental Figure 1). However, a small subset of Pdx1+ progenitor cells in the pancreatic bud, which did not express glucagon, also expressed Arx as early as E9.5; the presence these Arx+/Pdx1+ cells persisted into the secondary transition at E15.5 (Figure 2A-H, arrows and inset panels). A small population of Neurog3+ cells also co-expressed Arx at the beginning of the secondary transition, E12.5 (Figure 2I), and these cells were located in or adjacent to the epithelial cords, which were defined by the expression of the transcription factor Sox9 (Figure 2J). Given the established role for Arx in regulating the formation of the glucagon+/ghrelin+ cell population (Heller et al., 2005), we examined wildtype E15.5 pancreas for glucagon, ghrelin and Arx and confirmed the expression of Arx in glucagon+/ghrelin+ cells (Figure 2K-N, arrows). Interestingly, similar to what was observed in the *Nkx2.2*^{null} pancreas, we also detected Arx expression in ghrelin-expressing single hormone+ cells (Figure 2K-N; dotted boxes identify a ghrelin+/Arx+ cell), as well as in a population of cells that did not express hormone (Figure 2K-N).

Arx is required for ghrelin expression but not ghrelin cell specification

Based on the observation that Arx was expressed in Nkx2.2-deficient and wildtype ghrelin single positive cells, and was also co-expressed with Nkx2.2 in the early pancreatic progenitors, *Arx* was removed in the Pdx1+ pancreatic progenitor cells in the *Nkx2.2*^{null} background to examine the role of Arx in ghrelin cell specification. Using immunofluorescence, the number of ghrelin cells was quantified and compared between the single mutant mice, *Nkx2.2*^{null} and *Arx*$^{flox/flox(or\ Y)}$*;Pdx1-cre* (from hereon referred to as *Arx*$^{\Delta panc}$), the compound double mutant, *Nkx2.2*$^{-/-}$*;Arx*$^{flox/flox(or\ Y)}$*;Pdx1-cre* (*Nkx2.2*^{null}*;Arx*$^{\Delta panc}$), and control littermates. The *Nkx2.2*^{null} and *Nkx2.2*^{null}*;Arx*$^{\Delta panc}$ mice displayed an increase in the number of ghrelin-expressing cells compared with wildtype, and there was no significant difference in ghrelin cell number between the *Nkx2.2*^{null} and *Nkx2.2*^{null}*;Arx*$^{\Delta panc}$ (Figure 3A; Supplemental Table 1). Using realtime PCR, we confirmed that *ghrelin* expression was unchanged in the P0 pancreas of the *Arx*$^{\Delta panc}$, and the *Nkx2.2*^{null} displayed the expected increase in *ghrelin* compared with wildtype (Figure 3B); however, surprisingly, a reduced level of *ghrelin* expression was detected in the *Nkx2.2*^{null}*;Arx*$^{\Delta panc}$ when compared with the *Nkx2.2*^{null} (Figure 3B). The observed reduction of *ghrelin* expression in the *Nkx2.2*^{null}*;Arx*$^{\Delta panc}$ compared with the *Nkx2.2*^{null}, without a corresponding reduction in ghrelin cell numbers was confirmed using immunofluorescence analysis, imaging all pancreas sections with identical exposure time. The difference in intensity of

ghrelin expression in equivalent numbers of cells was quantified using ImagePro Plus software and determined to be greater in the *Nkx2.2*^{null} (17637 arbitrary intensity units) compared with *Nkx2.2*^{null};*Arx*^{Apanc} (6703 arbitrary intensity units). Therefore as predicted, we did not detect a difference in ghrelin cell numbers between the *Nkx2.2*^{null} and *Nkx2.2*^{null};*Arx*$^{\Delta panc}$ (Figure 3C-F), but the expression of *ghrelin* per cell was greatly reduced in the *Nkx2.2*^{null};*Arx*$^{\Delta panc}$ compared with the *Nkx2.2*^{null} (Figure 3E-F, inset images). Taken together these results suggest that Arx is not necessary for ghrelin cell specification, but is essential in the regulation of *ghrelin* gene expression.

Nkx2.2 and Arx genetically interact to affect multiple endocrine lineages

Arx and Nkx2.2 are each known to affect the development of several endocrine lineages, therefore we investigated whether these two factors also function together in the development or specification of the other islet endocrine populations. The expected phenotype for glucagon-expressing alpha cells and insulin-expressing beta cells was recapitulated in the *Arx*$^{\Delta panc}$ *and Nkx2.2*^{null} mutants at P0 (Supplemental Figure 2). In the *Nkx2.2*^{null};*Arx*$^{A\Delta panc}$, the simultaneous deletion of *Arx* did not affect the absence of beta cells or loss of expression of the *insulin* genes (*Ins1, Ins2*), resulting from the loss of *Nkx2.2* (Figure 4 A-D; Supplemental Figure 2). Moreover, the transcription factors *Neurod1, Pdx1* and *Nkx6.1* were unaffected by the deletion of *Arx* alone, but displayed a decrease in expression when *Nkx2.2* was deleted either alone or in combination with *Arx* in the *Nkx2.2*^{null};*Arx*$^{\Delta panc}$ (Supplemental Figure 3). The expected loss of alpha cells in both the *Arx*$^{\Delta panc}$ and *Nkx2.2*^{null} was also observed in the *Nkx2.2*^{null};*Arx*$^{\Delta panc}$ (Figure 4E-H); however, when both *Arx* and *Nkx2.2* were deleted from the pancreas, *glucagon* (*Gcg*) expression showed an additive decrease. The augmented decrease was also observed at E15.5, but not earlier in development at E12.5 (Supplemental Figure), suggesting that Nkx2.2 and Arx function together during the secondary transition to regulate the major wave of alpha cell development and differentiation. The alpha cell factor *Pou3f4* (*Brn4*) was also decreased in all mutants, which mimicked the loss of *glucagon* expression and the observed reduction of alpha cells (Supplemental Figure 3).

Immunostaining analyses also showed that while PP cell numbers were reduced in the *Nkx2.2*^{null} pancreas, the PP cell population was restored to wildtype levels in the *Nkx2.2*^{null};*Arx*$^{\Delta panc}$ (Figure 4I-L, Q; Supplemental Table 1). *Pancreatic polypeptide* (*Ppy*) expression reflected the change in cell number, with the restoration of *Ppy* hormone expression to wildtype levels in the *Nkx2.2*^{null};*Arx*$^{\Delta panc}$ (Figure 4Q). These findings revealed an unexpected combined role for Nkx2.2 and Arx in the development of PP cells, and further suggests that these two lineage specification factors are likely to genetically interact to regulate the PP cell population.

The somatostatin-expressing delta cell population was previously shown to be increased in the *Arx* null mice (Collombat et al., 2005; Collombat et al., 2003), but was unaffected by the loss of *Nkx2.2* (Sussel et al., 1998). Consistent with these findings, the number of delta cells was increased in both the *Arx*$^{\Delta panc}$ and *Nkx2.2*^{null};*Arx*$^{\Delta panc}$, but there was no difference in total delta cell number between these two mutants (Figure 4A-D, R; Supplemental Table 1). Interestingly, realtime PCR analysis demonstrated not only an increase in *somatostatin* (*Sst*) expression in the *Arx*$^{\Delta panc}$ but also revealed a further increase in *Sst* in the *Nkx2.2*^{null} *Arx*$^{\Delta panc}$ at P0 that was significantly higher than in the *Arx*$^{\Delta panc}$ (Figure 4R). These data confirm that Arx, but not Nkx2.2, is necessary for delta cell development; however, the additional deletion of *Nkx2.2* in an *Arx*-deficient pancreas further alters *somatostatin* transcript expression.

Ghrelin+/Somatostatin+ co-expressing cells are identified in the *Nkx2.2^{null};Arx$^{\Delta panc}$*

The unexpected finding in the *Nkx2.2^{null};Arx$^{\Delta panc}$* that *ghrelin* was downregulated by the *Arx* deletion and *somatostatin* was upregulated by the *Nkx2.2* deletion (Figure 3 and 4), prompted us to further explore the potential interaction of Nkx2.2 and Arx in regulating the expression of these two hormones. Analysis of *ghrelin* expression at earlier embryonic stages demonstrated that the reduction in *ghrelin* expression in the *Nkx2.2^{null};Arx$^{\Delta panc}$* compared with *Nkx2.2^{null}* was observed as early as E12.5 and was consistent through gestation (Figure 5A). Interestingly, we also detected a precocious and significant upregulation of somatostatin expression in both the *Arx$^{\Delta panc}$* and *Nkx2.2^{null};Arx$^{\Delta panc}$* at E12.5; this increase in somatostatin expression was increased to significantly higher levels in the *Nkx2.2^{null};Arx$^{\Delta panc}$* at E15.5 compared with the *Arx$^{\Delta panc}$* (Figure 5B). Using immunofluorescence, we stained tissue at E12.5 for ghrelin and somatostatin and discovered an unexpected population of cells in the *Nkx2.2^{null};Arx$^{\Delta panc}$* that co-express ghrelin and somatostatin (Figure 5C-F); these co-expressing cells were also present at P0 (Figure 5G-J). While a small number of ghrelin+/somatostatin+ co-expressing cells were detected in the *Arx$^{\Delta panc}$* at each time point (Figure 5D,H), no such cells were found in the wildtype or *Nkx2.2^{null}* pancreata (Figure 5C,E,G,I) indicating that the deletion of both *Arx* and *Nkx2.2* results in a significant increase of these bihormonal cells.

Endocrine-specific deletion of *Arx* in the *Nkx2.2^{null}* background phenocopies the *Nkx2.2^{null};Arx$^{\Delta panc}$*

We demonstrated that Arx was expressed in the Pdx1+ pancreatic progenitors and in the Neurog3+ endocrine progenitors (Figure 2). To determine whether Arx function was required early within the pancreas progenitor population to influence the competency of the endocrine progenitor population or whether it is specifically required in the Neurog3+ endocrine progenitor population, we generated an endocrine-specific (*Neurog3-cre*) deletion of *Arx* in the *Nkx2.2* mutant background. Hormone expression was assessed by immunostaining and realtime PCR at P0 in *Nkx2.Z$^{-/-}$;Arx$^{flox/flox(or\ Y)}$;Neurog3-cre (Nkx2.2^{null};Arx$^{\Delta endo}$)* and associated wildtype, *Arx$^{flox/flox(or\ Y)}$;Neurog3-cre* (*Arx$^{\Delta endo}$*), and *Nkx2.2^{null}* littermates. Specifically, *glucagon* (*Gcg*) expression was decreased in all mutants, with an additive decrease in expression in the *Nkx2.2^{nuII};Arx$^{\Delta endo}$* compared with the single mutants (Figure 6A-D,Q). Interestingly, a significant decrease in *glucagon* expression was also observed between *Nkx2.2^{null}* and *Arx$^{\Delta endo}$*; this change was not observed between the *Nkx2.2^{null}* and *Arx$^{\Delta panc}$*. The increase in *ghrelin* (*Ghr*) expression in the *Nkx2.2^{null}* was also reduced when *Arx* was also deleted in the Neurog3+ cells (Figure 6E-H, Q). The expression changes of both *somatostatin* (*Sst*) (Figure 6I-L, Q) and *pancreatic polypeptide* (*Ppy*) (Figure 6M-P,Q) were identical to the *Arx$^{\Delta panc}$* and *Nkx2.2^{null};Arx$^{\Delta panc}$* mutants. Both *insulin1* (*Ins1*) and *insulin2* (*Ins2*) were absent in the *Nkx2.2^{null}* and *Nkx2.2^{null};Arx$^{\Delta endo}$*; the *Arx$^{\Delta endo}$* displayed a trend toward increased *insulin1* and unaltered *insulin2* expression (Supplemental Figure 4). Altogether, the changes in gene expression were consistent with the *Nkx2.2^{null};Arx$^{\Delta panc}$* mutant, suggesting that Arx functions within the Neurog3+ endocrine progenitor cells to affect the development and differentiation of hormone-expressing cells in the embryonic pancreas.

Discussion

The specification and differentiation of all hormone-expressing endocrine lineages in the pancreas relies on the temporal and spatial activation of a network of transcription factors. Using loss-of-function and gain-of-function mouse models, the contribution of individual transcription factors to these lineage decisions has been well described (Pan and Wright, 2011); however, the relationships among these transcription factors within each endocrine cell type have yet to be identified. In this study, using compound mutants of Nkx2.2 and

Arx, we uncover a previously unappreciated genetic interaction between these two transcription factors within the endocrine progenitors, which regulates endocrine cell differentiation and hormone gene expression.

The hallmark of the pancreatic phenotype in the Nkx2.2 mutant mouse is the aberrant increase in ghrelin-expressing cells in place of the beta, alpha and (a subset of) PP cells (Prado et al., 2004). Here, we have determined that Arx is not required for ghrelin cell specification, but functions in the Neurog3+ endocrine progenitor cells to regulate *ghrelin* gene expression in the absence of Nkx2.2. Interestingly, we performed *in silico* analysis examining the *ghrelin* promoter for Arx consensus binding motifs (TAATTA; (Fulp et al., 2008)) and identified two putative Arx binding sites upstream of the transcriptional start site that are conserved between mouse and rat (Supplemental Figure 5), which suggests that Arx may directly regulate *ghrelin*; however, the ability of Arx to activate *ghrelin* expression appears to depend on the absence of Nkx2.2.

Previous studies have identified a role for Arx in delta cell specification (Collombat et al., 2005; Collombat et al., 2003), and as expected we demonstrated that delta cells and *somatostatin* expression were increased when *Arx* was deleted in the Pdx1+ cells. We observed a similar outcome when *Arx* was removed in the Neurog3+ cells; these data indicate that Arx is functioning in the endocrine progenitors to regulate the delta cell fate. Interestingly, delta cell numbers do not change between the $Arx^{\Delta panc}$ and $Nkx2.2^{null};Arx^{\Delta panc}$ but *somatostatin* expression is significantly different. Moreover, ghrelin cell numbers do not change between the $Nkx2.2^{null}$ and $Nkx2.2^{null};Arx^{\Delta panc}$ but *ghrelin* expression is significantly altered. These data suggest that the population of ghrelin+/somatostatin+ co-expressing cells observed in the $Nkx2.2^{null};Arx^{\Delta panc}$ are mutant somatostatin+ cells that misexpress *ghrelin* and mutant ghrelin+ cells that misexpress *somatostatin*. Previous reports have identified that deletion of *Nkx2.2* directly affects the expression of *Neurod1* (Anderson et al., 2009a), which is expressed in all endocrine lineages except delta cells (Anderson et al., 2009b) and is a known repressor of the *somatostatin* gene (Itkin-Ansari et al., 2005). Therefore we hypothesize that the further increase in *somatostatin* expression observed in the $Nkx2.2^{null};Arx^{\Delta panc}$ compared with the $Arx^{\Delta panc}$ single mutant could be indirectly caused by the reduction of *Neurod1*, which is associated with the loss of *Nkx2.2*. Interestingly, a triple deletion of *Nkx2.2, Neurod1* ($Neurod1^{tm1Jle}$; (Miyata et al., 1999)) and *Arx* in the Pdx1+ cells ($Nkx2.2^{null};Neurod1^{null};Arx^{flox/flox};Pdx1\text{-}cre$) shows a very dramatic increase in *somatostatin* expression (n=2; data not shown), supporting the idea that an additive increase in *somatostatin* expression could be caused by the combined deletion of *Arx, Nkx2.2* and complete loss of *Neurod1*.

Our findings demonstrate that Nkx2.2 and Arx are required for ghrelin cell specification and *ghrelin* gene regulation, respectively. Previous studies have also indicated that Nkx2.2 is necessary for a subset of the PP cell lineage (Chao et al., 2007; Sussel et al., 1998) and spurious expression of *Arx* induces the PP cell fate; however, no specific alteration in the PP cell population was reported in the *Arx* null mouse (Collombat et al., 2005; Collombat et al., 2003). Therefore, it was quite unexpected that deletion of *Arx* in an $Nkx2.2^{null}$ background restored the PP cell population reduced in the $Nkx2.2^{null}$. While this suggests that Nkx2.2 and Arx genetically interact to regulate the PP cell lineage, it also indicates that additional transcription factors, possibly regulated by Arx, are involved in the PP lineage decision. Interestingly, the PP cell lineage is affected by the deletion of *Notch* in Pdx1-expressing cells (Apelqvist et al., 1999). The Arx single mutant displays a significant increase in the Notch effector *Hes1* and the compound mutant shows a trend toward increased *Hes1* expression (data not shown); therefore, it is possible that aberrant notch function as a result of *Arx* deletion contributes to the restoration of the PP cell lineage in the Nkx2.2;Arx compound mutant.

Similar to studies conducted in transcription factor compound mutants, including *Pax4;Pax6* (St-Onge et al., 1997), *Nkx6.1;Nkx6.2* (Henseleit et al., 2005), *Nkx2.2;Neurod1* (Chao et al., 2007), this study provides further evidence that a specific "transcription factor code" is required to fine tune both cell identify, and appropriate timing and location of gene products in pancreatic endocrine cells. In addition, the genetic interaction we uncovered between Nkx2.2 and Arx may also be necessary for cell fate decisions during the development of other organs, given that both transcription factors are expressed in the brain and central nervous system (Briscoe et al., 1999; Fulp et al., 2008; Poirier et al., 2004), and intestine ((Desai et al., 2008); May CL, unpublished observation). In particular, Nkx2.2 and Arx may cooperate to regulate the ghrelin cell lineage in the stomach, given that many ghrelin+ cells express Arx and Nkx2.2 in the adult stomach (Du and May, Arnes and Sussel, unpublished observation). Ultimately distilling the interactions of transcription factors within specific settings will provide great insight into how these factors function in the larger context of organ development.

Conclusion

Our study clearly demonstrates that Nkx2.2 and Arx genetically interact in the endocrine progenitor cells to regulate both pancreatic endocrine cell development and hormone gene expression. A proposed model illustrating how these two key transcription factors function in regulating endocrine lineages is summarized in Figure 7. Specifically, increased numbers of delta and ghrelin cells are detected in Arx and Nkx2.2 single mutants, respectively, and the combined deletion of these two transcription factors leads to an increase in mutant ghrelin+/somatostatin+ co-expressing cells in the compound mutant and restoration of the PP cell lineage.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Nkx2.2 and Arx cooperate to regulate pancreatic endocrine cell fates
- Removal of Arx in the pancreas of Nkx2.2 mutants results in restoration of pancreatic polypeptide-expressing PP cells
- Ghrelin+/somatostatin+ co-expressing cells are formed in the Nkx2.2/Arx compound mutant pancreas
- Transcriptional regulation of ghrelin and somatostatin gene expression by Arx and Nkx2.2, respectively
- Genetic interaction between Nkx2.2 and Arx occurs specifically in Neurog3+ endocrine progenitor cells

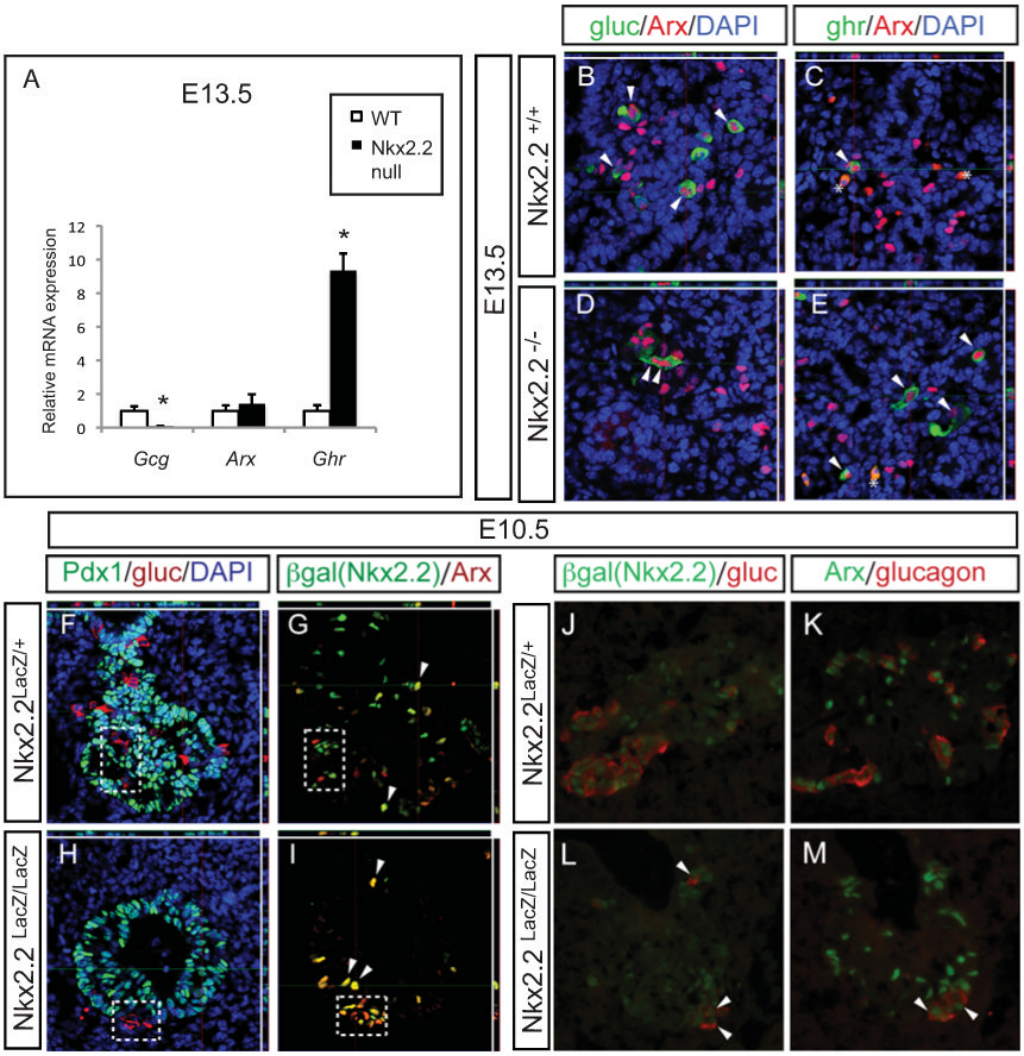

Figure 1. Arx is co-expressed in glucagon+, ghrelin+ and Nkx2.2+ cells

Quantitative realtime PCR demonstrates that *glucagon* expression is decreased and *ghrelin* expression is increased in the *Nkx2.2*^{null}, whereas there is no change in the expression of the alpha cell factor, *Arx* (A; * p>0.05). Using immunofluorescence and confocal imaging, both glucagon+ and ghrelin+ cells express Arx (arrows), in the wildtype and *Nkx2.2*^{null} pancreas at E13.5 (B-E; 48×). Using adjacent sections, Arx+/Nkx2.2(β-gal)+ coexpressing cells are observed in the E10.5 Pdx1+ pancreatic domain in both the Nkx2.2 heterozygote (*Nkx2.2*$^{LacZ/+}$) and Nkx2.2 mutant (*Nkx2.2*$^{LacZ/LacZ}$) (F-I, arrows; 40×). Dotted boxes encircle Arx+ or Nkx2.2 (β-gal)+ cells that are glucagon+. Direct costaining of Arx or Nkx2.2 with glucagon+ alpha cells at E10.5 (J-M; arrows denote the remaining glucagon+ cells in the null; 40×). In B-I, top and right rectangular panels represent a Z projection of 10 stack pictures at the level of intersection of the red/green crosshairs. In C and E, asterisks mark blood cell or apoptotic cell autofluorescence. DAPI marks all nuclei.

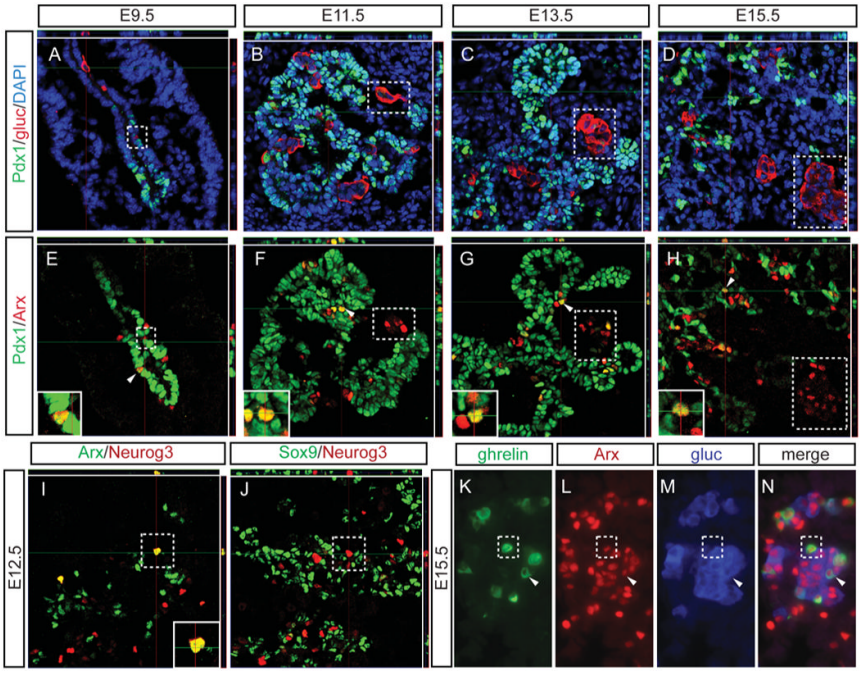

Figure 2. Arx expression during pancreas development

Sections are stained for Pdx1, to identify the pancreatic area, and glucagon to identify alpha cells (A-D; 40× confocal). Adjacent sections are stained for Pdx1 and Arx, and demonstrate Arx+ cells that are glucagon+ (dotted boxes) as well as Pdx1+/Arx+ coexpressing cells that do not express glucagon (arrows); Arx expression is detected in Pdx1+ progenitor cells from the beginning of pancreas development at E9.5 through the secondary transition at E15.5 (E-H, 40× confocal; insets 60×, confocal). Neurog3+ cells co-express Arx at the beginning of the secondary transition (E12.5), and these cells are located in the epithelial cords, defined by the expression of Sox9 (I-J; 40× confocal; inset 60×). In wildtype E15.5 tissue, Arx is expressed in ghrelin+ (dotted box) or glucagons+ single hormone+ cells, glucagon+/ghrelin + coexpressing cells (arrow), and cells that do not express either hormone (K-M; 40×). In A-J, top and right rectangular panels represent a Z projection of 10 stack pictures at the level of intersection of the red/green crosshairs. DAPI marks all nuclei.

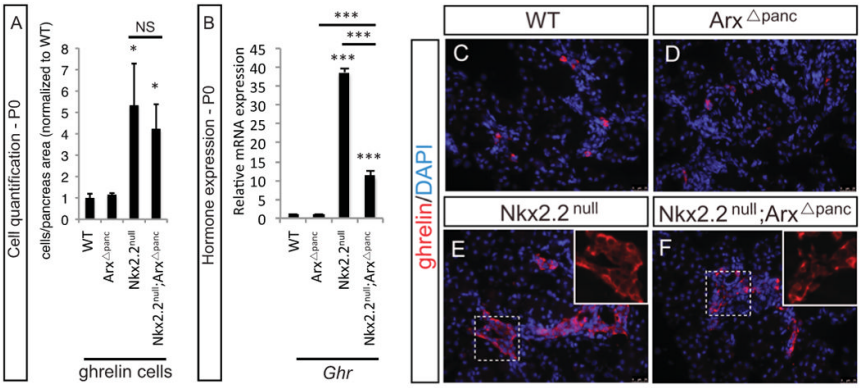

Figure 3. Expression of ghrelin in the *Nkx2.2^{null};Arx$^{\Delta panc}$* mutants

At P0, the number of ghrelin-expressing cells is unchanged between the wildtype (WT) and *Arx$^{\Delta panc}$*, whereas the number of cells is dramatically increased in the *Nkx2.2^{null}* and *Nkx2.2^{null};Arx$^{\Delta panc}$* (A). Realtime PCR was used to quantitate *ghrelin* expression at P0. Expression levels match that of cell numbers except for the *Nkx2.2^{null};Arx$^{\Delta panc}$*, where *ghrelin* expression was significantly decreased compared with the *Nkx2.2^{null}* (B). Representative images display ghrelin cells in the pancreas at P0. When imaged with identical exposure time, the intensity of ghrelin expression is decreased in the *Nkx2.2^{null};Arx$^{\Delta panc}$* compared with the *Nkx2.2^{null}* (C-F; 20×). Each inset image (E-F, 40×; DAPI has been removed) contains 23 ghrelin+ cells; and the intensity of ghrelin expression in these cells was quantified using ImagePro Plus software and determined to be greater in the *Nkx2.2^{null}* (17637 arbitrary intensity units) compared with *Nkx2.2^{null};Arx$^{\Delta panc}$* (6703 arbitrary intensity units). DAPI marks all nuclei.

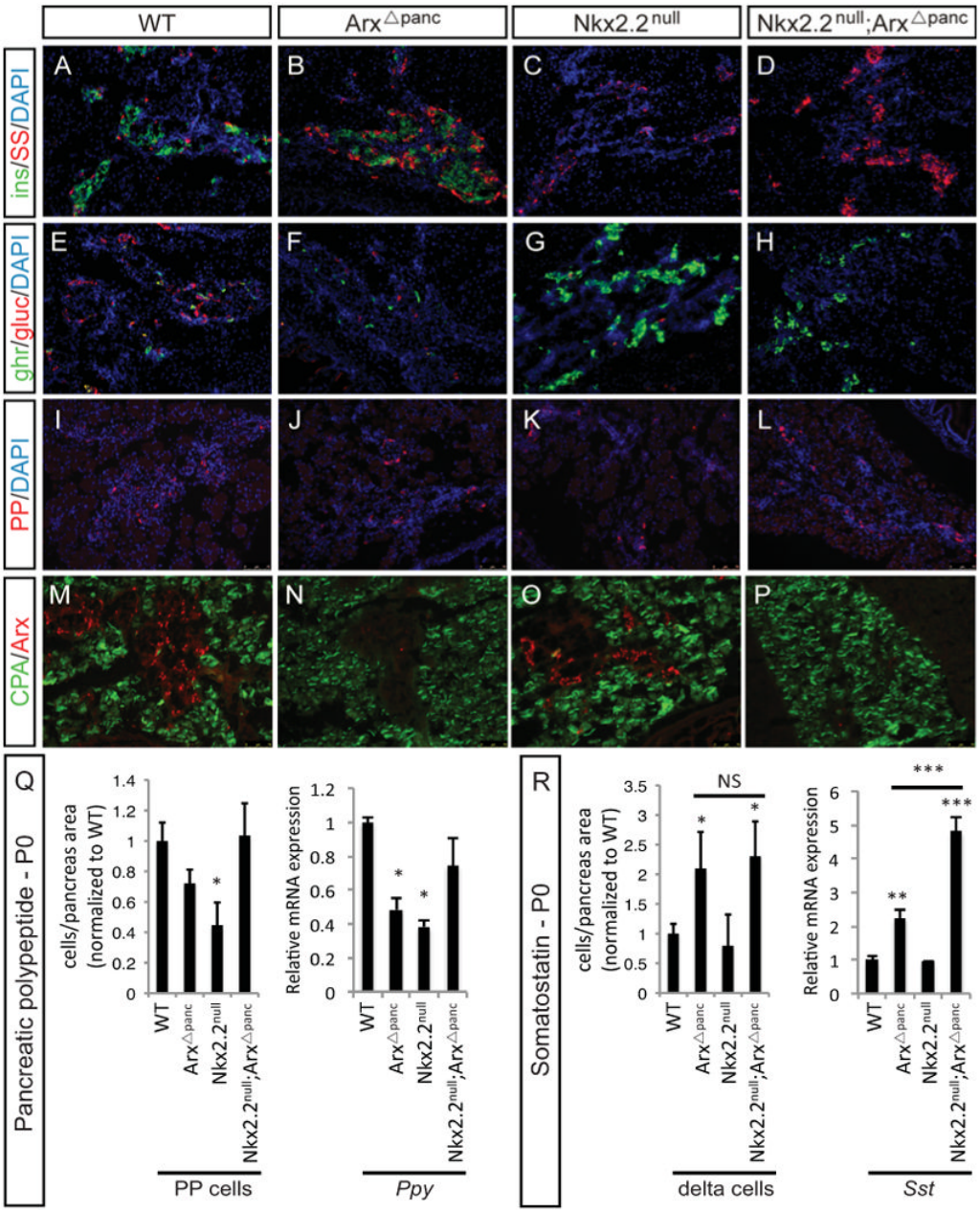

Figure 4. Pancreatic phenotype in the *Nkx2.2*^{null};*Arx*$^{\Delta panc}$ mutants at P0

Insulin-expressing beta cells are absent in the *Nkx2.2*^{null} and *Nkx2.2*^{null};*Arx*$^{\Delta panc}$ compared to wildtype (WT) and *Arx*$^{\Delta panc}$ (A-D). Somatostatin-expressing delta cells are increased in both the *Arx*$^{\Delta panc}$ and *Nkx2.2*^{null};*Arx*$^{\Delta panc}$, but unchanged in the *Nkx2.2*^{null} (A-D). Ghrelin-expressing cells are increased in both the *Nkx2.2*^{null} and *Nkx2.2*^{null};*Arx*$^{\Delta panc}$ compared to wildtype and *Arx*$^{\Delta panc}$ (E-H). Glucagon-expressing alpha cells are reduced in both single mutants and the *Nkx2.2*^{null};*Arx*$^{\Delta panc}$ compared to wildtype (E-H). Pancreatic polypeptide-expressing PP cells are reduced in the *Nkx2.2*^{null} and *Nkx2.2*^{null};*Arx*$^{\Delta panc}$(I-L). Arx expression was used to confirm deletion in the *Arx*$^{\Delta panc}$ single mutant and the *Nkx2.2*^{null};*Arx*$^{\Delta panc}$ compound mutant, compared with the wildtype and *Nkx2.2*^{null}, which maintain Arx expression; carboxypeptidase-A (CPA) marks the exocrine tissue (M-P). Quantitation of cell populations reveals that PP cells are reduced in the *Nkx2.2*^{null}, but this

reduction is restored to wildtype levels in the *Nkx2.2*^{null};*Arx*$^{\Delta panc}$ pancreas (Q). Using realtime PCR, the expression of *Ppy* is reduced in both the *Arx*$^{\Delta panc}$ and *Nkx2.2*^{null}, and *Ppy* expression is restored in the *Nkx2.2*^{null};*Arx*$^{\Delta panc}$ (Q). Somatostatin cells are increased in the *Arx*$^{\Delta panc}$, and this increase is maintained in the *Nkx2.2*^{null};*Arx*$^{\Delta panc}$ pancreas (R). Expression of *Sst* is increased in the *Arx*$^{\Delta panc}$, and *Sst* expression in the *Nkx2.2*^{null};*Arx*$^{\Delta panc}$ is further significantly increased compared with the *Arx*$^{\Delta panc}$ (R). Cell numbers quantified relative to total pancreas area and displayed normalized to wildtype. * $p>0.05$; ** $p>0.01$; *** $p>0.001$. DAPI marks all nuclei. All images are 20×.

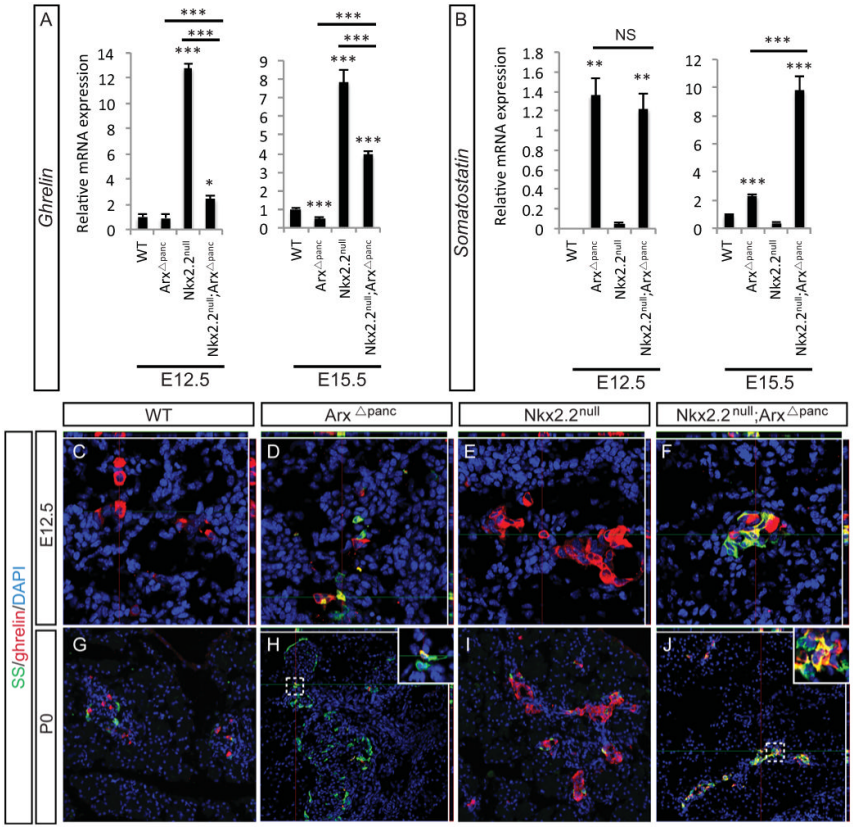

Figure 5. Ghrelin+/somatostatin+ co-expressing cells are present in the Nkx2.2;Arx compound mutant

At E12.5 and E15.5 *ghrelin* expression is reduced in the *Nkx2.2^{null};Arx$^{\Delta panc}$* compared to the *Nkx2.2^{null}* (A). *Somatostatin* expression is increased in both the *Arx$^{\Delta panc}$* and *Nkx2.2^{null};Arx$^{\Delta panc}$* at E12.5 and E15.5; the increase at E15.5 in the *Nkx2.2^{null};Arx$^{\Delta panc}$* is also further significantly upregulated compared with the *Arx$^{\Delta panc}$* (B). Using immunofluorescence and confocal microscopy, E12.5 (C-F; 48× confocal) and P0 (G-J, 20×; insets 48× confocal) pancreas sections were examined for ghrelin- and somatostatin-expressing cells. At both ages, precocious somatostatin cells are present in the *Arx$^{\Delta panc}$* (D, H) and increased ghrelin+ cells are present in the *Nkx2.2^{null}* (E, I). Whereas ghrelin+/somatostatin+ cells are rare or absent in the *Arx$^{\Delta panc}$* (H inset) or Nkx2.2^{null}, this population of co-expressing cells in greatly increased in the *Nkx2.2^{null};Arx$^{\Delta panc}$* (F, J, inset). In C-F, H and J, top and right rectangular panels represent a Z projection of 10 stack pictures at the level of intersection of the red/green crosshairs. DAPI marks all nuclei. * p>0.05; ** p>0.01; *** p>0.001

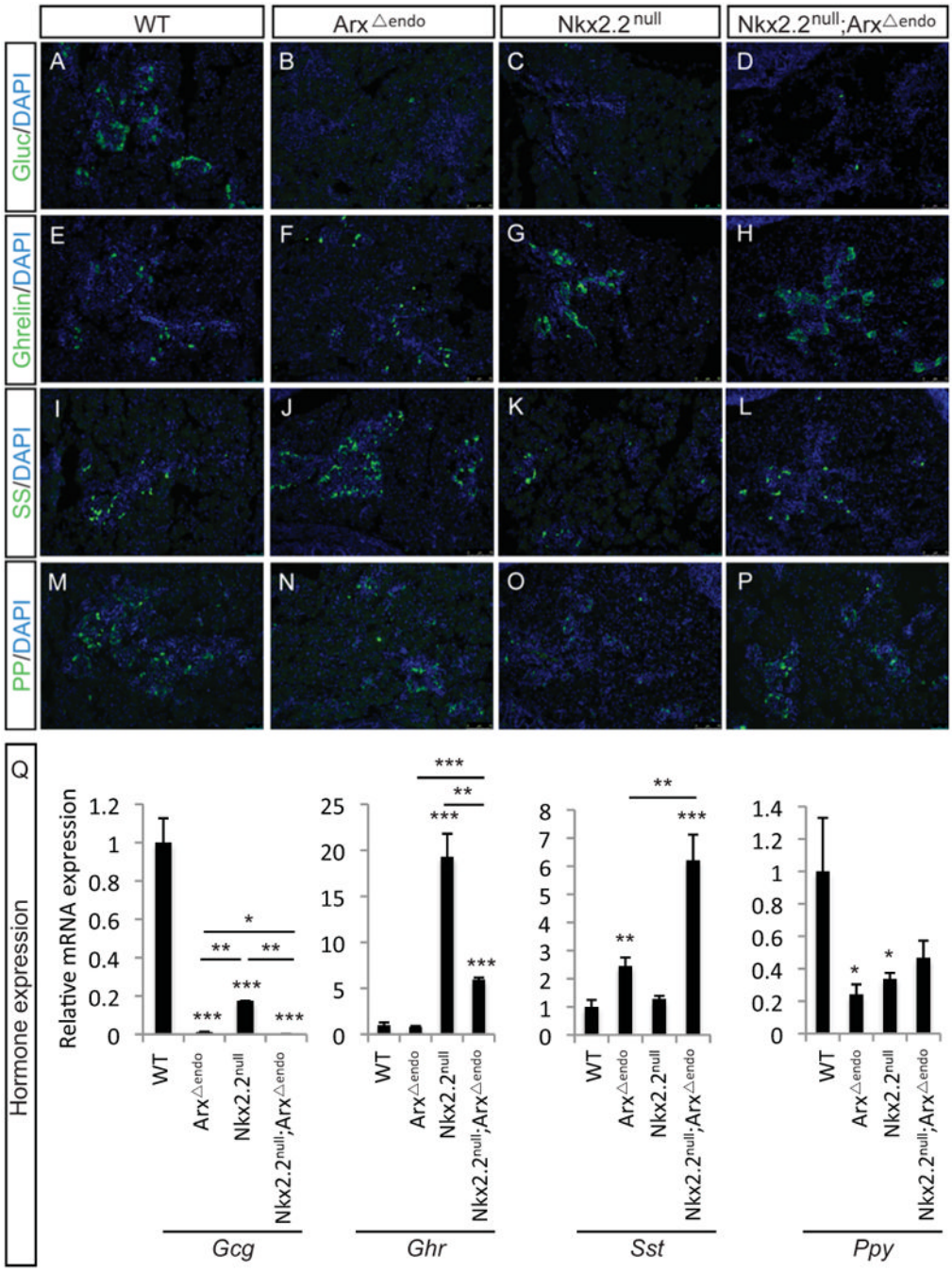

Figure 6. Phenotype of endocrine-specific Nkx2.2;Arx compound and single mutants at P0

The pancreatic phenotype at P0 for the *Nkx2.2*^{null}*;Arx*$^{flox/flox(or\ Y)}$*;Neurog3-cre* (*Nkx2.2*^{null}*;Arx*$^{\Delta endo}$) and associated wildtype (WT), *Arx*$^{flox/flox(or\ Y)}$*;Neurog3-cre* (*Arx*$^{\Delta endo}$), and *Nkx2.2*^{null} littermates. Using immunofluorescence analysis, glucagon cells are reduced in the single mutants and DKO (A-D). Ghrelin cells are unchanged in the *Arx*$^{\Delta endo}$ compared to wildtype, whereas ghrelin cells are increased in both the *Nkx2.2*^{null} and *Nkx2.2*^{null}*;Arx*$^{\Delta endo}$ (E-H). Somatostatin cells are increased in the *Arx*$^{\Delta endo}$ and *Nkx2.2*^{null}*;Arx*$^{\Delta endo}$ (M-P). PP cells are reduced in the *Nkx2.2*^{null}, but this reduction appears rescued in the *Nkx2.2*^{null}*;Arx*$^{\Delta endo}$ (M-P). Using realtime PCR the hormone expression profile was determined (Q). *Glucagon* (*Gcg*) expression is reduced in all mutants, with an

additive reduction in the *Nkx2.2*^{null};*Arx*$^{\Delta endo}$. *Somatostatin* (*Sst*) is increased in the *Arx*$^{\Delta endo}$ and further increased in the *Nkx2.2*^{null};*Arx*$^{\Delta endo}$. *Ghrelin* (*Ghr*) expression is increased in the *Nkx2.2*^{null} and *Nkx2.2*^{null};*Arx*$^{\Delta endo}$ compared to wildtype, but the expression in the *Nkx2.2*^{null};*Arx*$^{\Delta endo}$ is reduced compared with the *Nkx2.2*^{null}. *Pancreatic polypeptide* (*Ppy*) is reduced in both single mutants, and this expression is rescued in the *Nkx2.2*^{null};*Arx*$^{\Delta endo}$. Relative mRNA expression was normalized to the housekeeping gene, *cyclophilinB*. * p>0.05; ** p>0.01; *** p>0.001. DAPI marks all nuclei. All images are 20×.

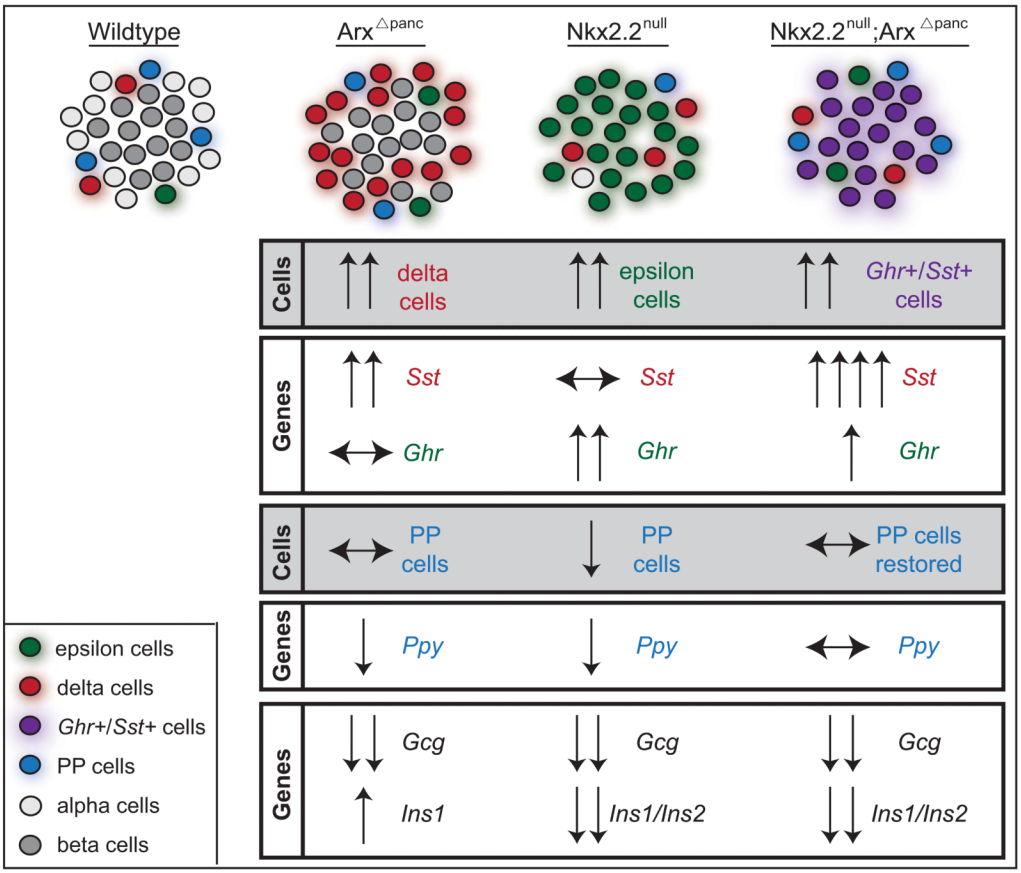

Figure 7. Proposed model for Nkx2.2 and Arx in specifying endocrine cell lineages

A summary of the novel changes to normal endocrine development when both Nkx2.2 and Arx are deleted in the embryonic pancreas. As expected, *somatostatin* (*Sst*) expression and delta cells are increased in the $Arx^{\Delta panc}$. Similarly, in the $Nkx2.2^{null}$ *ghrelin* (*Ghr*) expression and ghrelin-expressing cells are increased. However, when *Nkx2.2* and *Arx* are both absent in the pancreas, *Sst* expression is further increased and *Ghr* expression is reduced, but remains increased compared to wildtype. In addition, a mutant population of ghrelin+/somatostatin+ cells appears in the $Nkx2.2^{null};Arx^{\Delta panc}$. Most interestingly, PP cells and *pancreatic polypeptide* (*Ppy*) expression are reduced in the $Nkx2.2^{null}$, and both are restored in the $Nkx2.2^{null};Arx^{\Delta panc}$. As expected, *glucagon* (*Gcg*) expression and alpha cells are reduced in both the $Arx^{\Delta panc}$ and $Nkx2.2^{null}$; the $Nkx2.2^{null};Arx^{\Delta panc}$ compound mutant demonstrates the same phenotype. While *insulin1* expression is increased in the $Arx^{\Delta panc}$, *insulin1/insulin2* expression and beta cells are absent from both the $Nkx2.2^{null}$; the $Nkx2.2^{null};Arx^{\Delta panc}$. The expression changes observed in the $Arx^{\Delta endo}$ and $Nkx2.2^{null};Arx^{\Delta endo}$ mutant mimics those of the $Arx^{\Delta panc}$ and $Nkx2.2^{null};Arx^{\Delta panc}$ mutants. Grey boxes represent data from morphometric analyses; white boxes summarize data from gene expression analyses.