The simultaneous loss of *Arx* and *Pax4* genes promotes a somatostatin-producing cell fate specification at the expense of the α- and β-cell lineages in the mouse endocrine pancreas

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Summary

The specification of the different mouse pancreatic endocrine subtypes is determined by the concerted activities of transcription factors. However, the molecular mechanisms regulating endocrine fate allocation remain unclear. In the present study, we uncover the molecular consequences of the simultaneous depletion of Arx and Pax4 activity during pancreas development. Our findings reveal a so far unrecognized essential role of the paired-box-encoding *Pax4* gene. Specifically, in the combined absence of *Arx* and *Pax4*, an early-onset loss of mature α- and β-cells occurs in the endocrine pancreas, concomitantly with a virtually exclusive generation of somatostatin-producing cells. Furthermore, despite normal development of the PP-cells in the double-mutant embryos, an atypical expression of the pancreatic polypeptide (PP) hormone was observed in somatostatin-labelled cells after birth. Additional characterizations indicate that such an expression of PP was related to the onset of feeding, thereby unravelling an epigenetic control. Finally, our data provide evidence that both Arx and Pax4 act as transcriptional repressors that control the expression level of one another, thereby mediating proper endocrine fate allocation.

Key words: Endocrine pancreas development, Arx, Pax4, Mouse, Hyperglycaemia, Fate specification

Introduction

The pancreas plays a key role in the maintenance of nutritional homeostasis through the regulated synthesis and secretion of enzymes and hormones by exocrine and endocrine cells, respectively. The islets of Langerhans represent the functional units of the endocrine pancreas, and are classically thought to consist of four cell types, α-, β-, δ- and PP-cells, producing the hormones glucagon, insulin, somatostatin and pancreatic polypeptide (PP), respectively (Adrian et al., 1978; Csaba and Dournaud, 2001; Roncoroni et al., 1983). During development, the first morphological indications of pancreatic formation are observed at around embryonic day 9.5 (E9.5), as two evaginations of the primitive gut endoderm at the foregut/midgut junction. These protrusions, or pancreatic buds, grow, branch, differentiate and eventually merge to form the definitive pancreas (Edlund, 2002). At the same time, endocrine development is initiated, resulting in the emergence of early glucagon-producing cells (Herrera et al., 1991; Teitelman et al., 1993; Upchurch et al., 1994). One day later, scattered insulin-producing cells appear, most of which also secrete glucagon (Teitelman et al., 1993). At E14.5, a peak of endocrine cell genesis results in the development of numerous mature insulin- and glucagon-expressing cells, followed, one day later, by the appearance of the first somatostatin-producing δ-cells (Pictet et al., 1972). Finally, shortly before birth, PP-expressing cells differentiate and endocrine cells begin to form well-organized islets of Langerhans.

In recent years, major advances have been made towards a better understanding of the molecular mechanisms controlling endocrine cell genesis. One of the first determinants controlling the endocrine specification program was found to be the activation of the bHLH factor neurogenin 3 (Ngn3; Neurog3 – Mouse Genome Informatics) in the mouse E9 pancreatic epithelium (Apelqvist et al., 1999; Gu et al., 2002; Jensen et al., 2000; Schwitzgebel et al., 2000; Sommer et al., 1996). Notably, *Ngn3*-deficient mice fail to develop any hormone-producing endocrine cells (Gradwohl et al., 2000), whereas the misexpression of *Ngn3* in *Pdx1* (*Ipf1* – Mouse Genome Informatics) expression domains results in the differentiation of most of the pancreas into endocrine cells (Apelqvist et al., 1999; Schwitzgebel et al., 2000). Following Ngn3 activation, several downstream factors participate in endocrine subtype specification. These include the homeodomain-containing proteins Nkx2.2, Nkx6.1, Pax4 and Pdx1 (Ahlgren et al., 1998; Mansouri et al., 1999; Sander et al., 2000; Smith et al., 1999; Sosa-Pineda et al., 1997; Sussel et al., 1998). Once this fate is

established, additional transcription factors such as Isl1, Pax6 and Pdx1 act to maintain specified islet cells (Ahlgren et al., 1997; Guz et al., 1995; Jonsson et al., 1994; Offield et al., 1996; Sander et al., 1997; St-Onge et al., 1997).

Recently, the involvement of an additional homeobox-containing gene localized on the X chromosome, *Arx*, was demonstrated in the α-cell specification process (Collombat et al., 2003). Mice deficient for *Arx* lack mature α-cells, whereas the numbers of β- and δ-cells are proportionally increased so that the total islet cell content is unaltered. Such phenotypic changes are opposite to those observed in *Pax4*-deficient mice (Sosa-Pineda et al., 1997), and it was suggested that, during the early stages of endocrine development, a mutual inhibition operates between Arx and Pax4 to allocate endocrine fate. These findings suggest that, early during islet cell specification, endocrine progenitors are confronted with the choice of becoming precursors of either β-/δ-cells or α-cells, the alternative cell fate being promoted by Pax4 and Arx, respectively. To gain further insights into the genetic program controlling the genesis of the different endocrine subtypes, we generated mice deficient for both *Arx* and *Pax4* genes. We found that these animals died perinatally, after having developed a severe hyperglycaemia. Immunohistochemical analysis of *Arx/Pax4* mutant pancreas revealed an absence both of α- and β-cells. Strikingly, a dramatic increase in the number of somatostatin-producing cells was observed, whereas the total number of endocrine cells remained unchanged. Further studies during embryogenesis suggested that the lack of both *Arx* and *Pax4* provokes an early-onset virtually exclusive generation of somatostatin-expressing cells at the expense of the α- and β-cell lineages. Equally striking was the observation that, in *Arx/Pax4* mutants, production of PP occurs in somatostatin-expressing cells only following feeding onset, unravelling an epigenetic control. We provide evidence that Arx and Pax4 inhibit transcription of one another by direct interaction with their respective promoter regions in order to achieve proper endocrine cell allocation. Finally, our study suggests an unrecognized essential role for Pax4 in β-cell fate specification.

Materials and methods

Generation of double mutant animals

Compound *Arx* and *Pax4* heterozygous mice were generated by crossing *Arx*$^{+/-}$ females (Collombat et al., 2003) with *Pax4*$^{+/-}$ males (Sosa-Pineda et al., 1997) and maintained in a NMRI background. Genotyping was performed as described previously (Collombat et al., 2003; Sosa-Pineda et al., 1997). Double heterozygous females were further bred with *Pax4*$^{+/-}$ males and the genotypes of their progeny characterized.

Immunohistochemistry

Tissues were fixed in 4% paraformaldehyde overnight at 4°C, embedded in paraffin wax and 6-μm sections were applied to slides. These sections were assayed as described previously (Collombat et al., 2003). The primary antibodies used were: mouse monoclonal anti-insulin, anti-glucagon (1/1000, Sigma), anti-somatostatin (1/100, Promega), anti-Ghrelin (1/1000, kindly provided by C. Tomasetto), anti-CA812 (undiluted), anti-Ngn3 (1/500); guinea pig anti-insulin, anti-glucagon (1/1000, Sigma), rabbit anti-somatostatin (1/600, Dako), anti-PP (1/200, Dako), anti-*Nkx6.1* (1/3000), anti-*Nkx2.2* (1/1000, kindly provided by T. Jessell), anti-*Pax6* (1/500, kindly provided by S. Saule), anti-Arx (1/1000), and anti-CART (1/1000). The secondary antibodies (1/1000, Molecular Probes) used for immunofluorescence were: 594-Alexa anti-mouse, 488-Alexa anti-mouse, 594-Alexa anti-rabbit, 488-Alexa anti-rabbit, 594-Alexa anti-guinea pig; and 488-Alexa anti-guinea pig. Pictures were processed using confocal microscopy.

Glucose levels

Glucose levels (mg/dl) were determined with the One Touch Glucose monitoring kit (Johnson & Johnson) using 15 μl of peripheral blood. Blood glucose levels are represented as an average±s.e.m.

β-galactosidase staining

Whole-embryos were isolated at E10.5 and the yolk sac saved for DNA preparation and genotyping. After fixing in 4% paraformaldehyde, embryos were washed in PBS and stained in 4 mM $K3[Fe(CN)_6]$, 4 mM $K4[Fe(CN)_6]$, 0.02% NP-40, 0.01% Na-deoxycholate, 5 mM EGTA, 2 mM $MgCl_2$ and 0.4 mg/ml 5-bromo-4-chloro-3-inodolyl-D-galactopyranoside.

Sequence processing

Sequence comparisons were performed online using the Vista program (http://genome.lbl.gov/vista/index.shtml). The search for *P4BS* was performed using the consensus sequence from Fujitani et al. (Fujitani et al., 1999) with a program of our own conception (available upon request). Twenty-seven potential candidate sites were thereby obtained.

Plasmid construction

The full-length cDNA clones for the mouse *Arx* and *Pax4* genes were subcloned into the pBluescript KSII vector (Stratagen) for in vitro translation, and, in the case of *Pax4*, into a modified pCDNA vector (Invitrogen) containing an intron and a HA epitope (kindly provided by R. Lührman). The Pax4- or Arx-responsive luciferase (Luc) reporter constructs were created by cloning five copies of *P4BS* or *ArBS*, respectively, into the *Xho*I site present in the T81-Luc vector (kindly provided by S. Nordeen).

Cell culture and transfection

The β-cell-derived βTC 13 T cells, COS cells, and the α-cell-derived αTC 1.9 cells were grown in DMEM medium supplemented with 10% heat-inactivated foetal calf serum (FCS), penicillin and streptomycin. Twenty-four hours before transfection experiments, the cells were replated in 100-mm-diameter plates (approximately 3×10^6 cells/plate). Transfection experiments were performed using the Fugene 6 transfection reagent (Roche), according to the manufacturer's instructions.

Electrophoretic mobility shift assay

Complementary single-stranded oligonucleotides (IBA-Göttingen) were incubated in a medium containing 10 mM Tris-HCl, 5 mM $MgCl_2$ and 100 mM NaCl, and then denatured at 80°C for 5 minutes in a waterbath. Annealing was performed after switching off the waterbath and leaving the mixture cool down overnight. The resulting double-stranded oligonucleotides were end-labelled with the T4 polynucleotide kinase and [γ-^{32}P]ATP. The Pax4 and Arx proteins, generated by in vitro translation using the TNT reticulocyte lysate transcription/translation kit (Promega) according to the manufacturer's instructions, were incubated with the labelled probe and processed as described in Fujitani et al. (Fujitani et al., 1999). For competition studies, a 10-, 100-, or 200-fold molar excess of unlabeled oligonucleotide competitor was added together with the probe.

South-western blot

The in vitro-translated Arx protein was manually spotted onto nylon membranes that were incubated in 5% non-fat dry milk in 10 mM

Hepes (pH 8.0) for 1 hour at room temperature. The membranes then were incubated overnight in binding buffer [10 mM Hepes (pH 8.0), 50 mM NaCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol, 0.25% non-fat dry milk] containing 1×10^5 cpm of ^{32}P-labeled DNA per ml. After extensive washes in binding buffer containing 0.3 M NaCl, the membranes were exposed to X-ray film.

Chromatin immunoprecipitation (ChIP) assay

Embryonic tissues or cells transfected with a vector encoding HA-tagged Pax4 were sonificated and treated as described (Spieler et al., 2004). The following primers were used: 5′-TGAGCACCACGTTTTAAAATATACACATAGTC-3′ with 5′-GTAGTGGGTACTGACCTAGACAGACATGTCATC-3′ for the amplification of the *P4BS*-containing 200-bp fragment; and 5′-GACAAAGTCTCACTAGGTAACGCAGCCCAGCC-3′ with 5′-TAAAGATATATCATAGGGGCGGGGCTGGTCGA-3′ as negative control (2.2 kb downstream of the 9.7 kb *Nhe*I-*Eco*RV fragment (Fig. 5A). The 5′-AGTCTGGCGCGTTGGGCAGAGGGCTGAGTGACTGA-3′ and 5′-ATAGACAGCTGCCTGAGGGTGTACGTAGGGGTGTT-3′ were used for the amplification of the *ArBS*-containing 256-bp fragment and 5′-TTGAACTTGGTATAACAATAATCCTTAGGTAGCGA-3′ with 5′-ATTTACGTGATTGTTTTGGAATTGCACACGTTAGG-3′ was used as a negative control (3.5 kb upstream of the *Pax4* enhancer, see Fig. 5F).

Reporter assay

The luciferase constructions (2 μg each) were co-transfected together with over-expression vectors into appropriate cells. A co-transfected *lacZ* reporter (1 μg) was used to normalize transfection efficiency.

Results

Arx/Pax4 double-deficient mice display severe hyperglycaemia prior to death

To gain further insight into the genetic interactions underlying Arx and Pax4 functions during endocrine pancreas formation, we generated mutant mice with a double loss of function of these transcription factors. It is important to note that *Arx* is located on the X chromosome (Blair et al., 2002) in the progeny of $Arx^{+/-}$ females (Collombat et al., 2003) that were first crossed with $Pax4^{+/-}$ males (Sosa-Pineda et al., 1997). Compound heterozygous *Arx* and *Pax4* females were born normally and subsequently bred with heterozygous *Pax4* males to generate male Arx^{-} $Pax4^{-/-}$ double mutants. The resulting 5.3% allelic frequency of compound *Arx/Pax4* mutant animals (n=306) indicates that the lack of functional *Arx* and *Pax4* alleles did not result in embryonic lethality.

Arx/Pax4 double-mutant mice appeared to be indistinguishable from their littermates at birth. However, phenotypic differences arose within the first day postpartum: despite normal feeding, as evidenced by the presence of milk in the stomach; double-deficient animals rapidly developed growth retardation and died around postnatal day 2 (P2). To determine whether the observed lethality was related to an endocrine pancreatic dysfunction, blood glucose levels were measured for all the different genotypes of the offspring of $Arx^{+/-}$ $Pax4^{+/-}$::$Pax4^{+/-}$ crosses. Twenty-four hours after birth, blood glucose levels were normal in all littermates (Table 1). Differences first became apparent at P2 and were amplified shortly before death: *Arx*- and *Pax4*-single-deficient mice displayed severe and lethal hypo- and hyperglycaemia, respectively (Table 1). Strikingly, unlike their *Arx* single mutant counterparts, Arx^{-} $Pax4^{+/-}$ mice did not die at P2, but survived until P8-P12, with an initially mild hypoglycaemia that progressively became more severe (Table 1 and data not shown). Importantly, the animals lacking both *Arx* and *Pax4* genes died around P1-P2 exhibiting an acute hyperglycaemia that contrasted with glucose levels of age-matched wild-type and single or double heterozygous *Arx/Pax4* animals.

Combined Arx/Pax4 deficiency results in the loss of mature insulin- and glucagon-expressing cells concomitantly with an increase in somatostatin-/PP-producing cells

To examine potential pancreatic defects following the loss of one or more *Arx* and/or *Pax4* alleles, islets were assayed for the presence of the four endocrine cell types using immunohistochemistry (Fig. 1). We did not observe any obvious differences between controls and *Arx*/*Pax4* double-heterozygous islets (Fig. 1A-R). However, *Arx-/Pax4*-deficient mice were found to be completely lacking glucagon-producing cells (Fig. 1D,V), and we detected only a few scattered insulin-expressing cells in the endocrine pancreas (Fig. 1A-C,S-U). Strikingly, a dramatic increase in the somatostatin-producing cell population was observed in these animals (Fig. 1E,W). Likewise, the number of PP-cells was drastically higher in the double-knockout pancreas (Fig. 1F,X). Co-localization experiments permitted us to demonstrate clearly that somatostatin and PP hormones were co-expressed in most cells (see below). To extend our data, we performed a quantitative analysis: independent P2 pancreata (estimated to be of the same size) from the offspring of $Arx^{+/-}$ $Pax4^{+/-}$::$Pax^{+/-}$ crosses were sectioned and the numbers of hormone-immunoreactive cells were determined in every tenth section. The results obtained, by comparing the average

Table 1. Glucose level determination in the offspring of $Arx^{+/-}$ $Pax4^{+/-}$::$Pax4^{+/-}$ crossed animals

Genotype	P24h (mg/dl)	P48h (mg/dl)	PSBD (mg/dl)	Life expectancy
Wild type	89±11	81±17	85±12	Normal
$Arx^{+/-}$	87±8	72±10	78±17	Normal
$Pax4^{+/-}$	84±15	86±14	91±16	Normal
Arx^{-}	82±7	45±18*	<10*	P1-P2
$Pax4^{-/-}$	80±17	126±26*	242±18*	P1-P2
$Arx^{+/-}$ $Pax4^{+/-}$	86±12	88±17	84±13	Normal
Arx^{-} $Pax4^{+/-}$	79±14	59±19	11±5*	P8-P12
$Arx^{+/-}$ $Pax4^{-/-}$	81±11	134±17*	201±32*	P1-P2
Arx^{-} $Pax4^{-/-}$	89±18	152±23*	289±29*	P1-P2

P24h, 24 hours after birth; P48h, 48 hours after birth; PSBD, shortly before death.
Values indicate means±s.e.m. (n>7).
*Statistically significant differences between glucose levels compared with wild-type animals. Student's t-test (P<0.01).

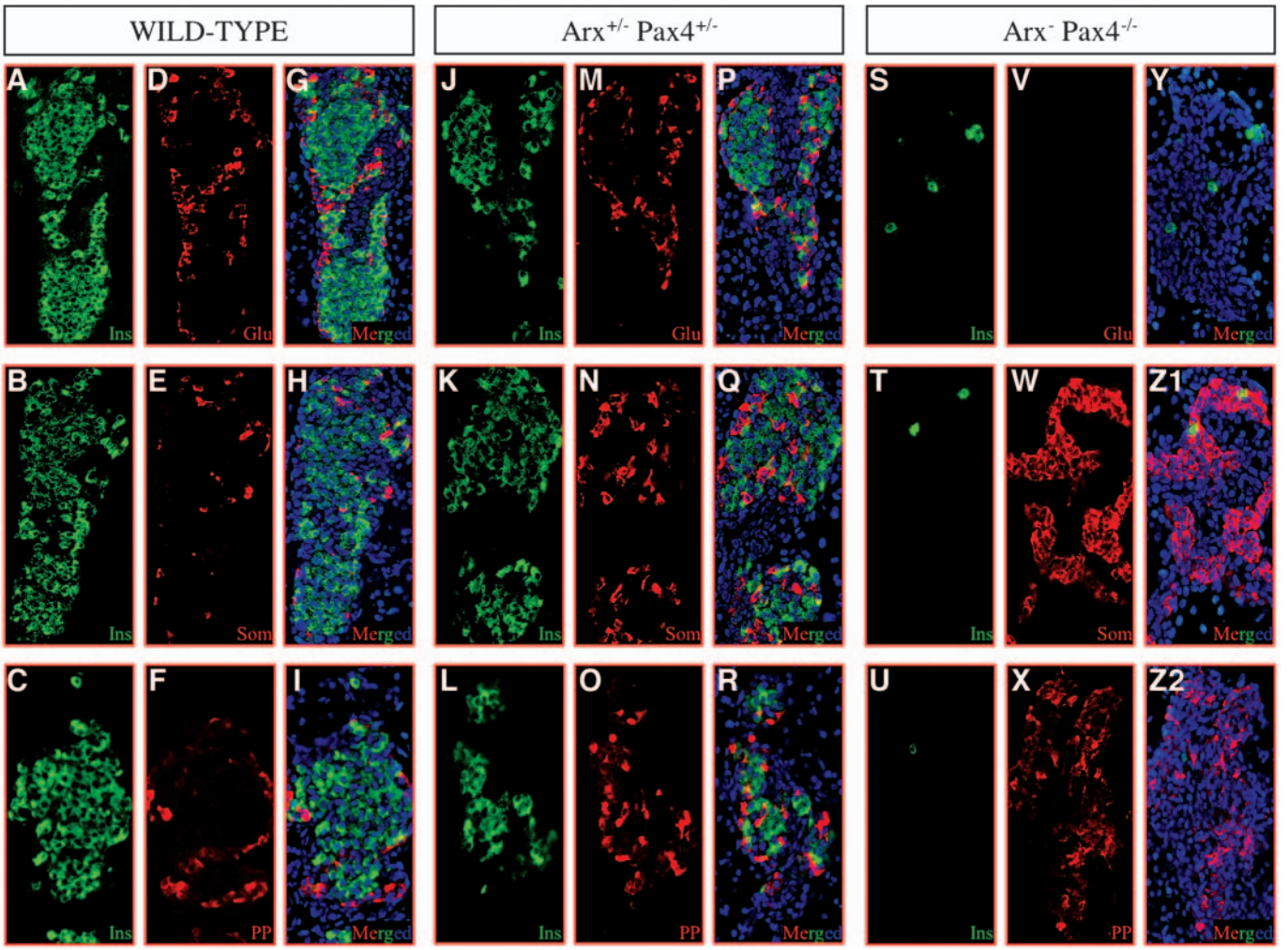


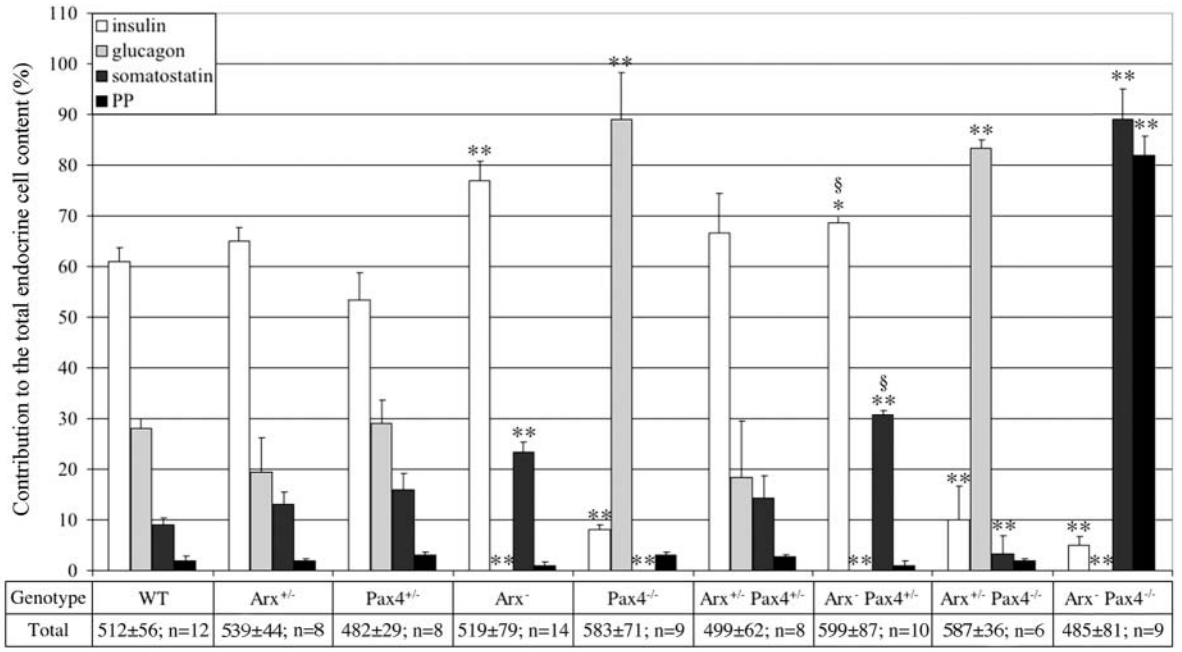
Fig. 1. Increase in the population of somatostatin- or PP-producing cells at the expense of insulin- and glucagon-expressing cells in *Arx*/*Pax4* double-mutant pancreas. Sections of P2 mice were examined for pancreatic hormones in wild-type (A-I), *Arx*/*Pax4* double-heterozygous (J-R) and *Arx*/*Pax4* double-deficient (S-Z2) animals by co-immunofluorescence. Islets were stained with antibodies directed against insulin (A-C,J-L,S-U), and antibodies recognizing either glucagon- (D,M,V), somatostatin- (E,N,W) or PP-producing cells (F,O,X), and sections were counter-stained with DAPI (merged in G-I,P-R,Y-Z2). Note the loss of the insulin- (A-C,S-U) and glucagon- (D,V) producing cell population, and the dramatic increase of the somatostatin- (E,W) or PP- (F,X) expressing cell numbers in the double mutants. The simultaneous lack of a single *Arx* and *Pax4* allele does not provoke any significant endocrine alteration when compared with wild-type animals (A-I,J-R).

counts reported with the total endocrine population (Fig. 2), confirmed a complete loss of glucagon-producing cells in *Arx*/*Pax4* double-mutant mice and a drastic reduction (89%) in the number of insulin-expressing cells. By contrast, the mean numbers of somatostatin- and PP-producing cells were dramatically increased in these animals (8.9- and 8.1-fold, respectively), when compared with their wild-type littermates. Importantly, the total endocrine cell content was statistically unaltered in all the genotypes analyzed (Fig. 2, bottom), suggesting that the missing hormone-producing cells in a particular genotype are replaced by cells of the alternative phenotype. It should also be underlined that the loss of a single *Pax4* allele in islets of *Arx*$^{-}$ animals was associated with a significant decrease in the number of insulin-positive cells and a simultaneous rise in the number of somatostatin-labelled cells. All together, our data suggest a *Pax4* haploinsufficient phenotype that becomes apparent only in *Arx* mutants. Furthermore, these findings show that the combined absence of *Pax4* and *Arx* results in the loss of β- and α-cell endocrine subtypes concomitantly with a striking increase in somatostatin- and PP-producing cells, with no modification of the total content of endocrine cells.

Deletion of *Arx* and *Pax4* induces a somatostatin-producing cell fate at the expense of the α- and β-cell lineages

In order to determine whether the lack of both *Arx* and *Pax4* genes affected the early development of the pancreas, we examined endocrine cell genesis at E12. Our data demonstrate that early endocrine cell number was unaltered in all of the genotypes analyzed (Fig. 3A,B, data not shown), thus providing further evidence that neither Arx nor Pax4 is required for the generation of these cells.

As development proceeds, a peak of endocrine cell genesis occurs, at about E14.5, leading to the formation of cells contributing to the definitive islet of Langerhans. When



Genotype	WT	Arx$^{+/-}$	Pax4$^{+/-}$	Arx^{-}	Pax4$^{-/-}$	Arx$^{+/-}$ Pax4$^{+/-}$	Arx^{-} Pax4$^{+/-}$	Arx$^{+/-}$ Pax4$^{-/-}$	Arx^{-} Pax4$^{-/-}$
Total	512±56; n=12	539±44; n=8	482±29; n=8	519±79; n=14	583±71; n=9	499±62; n=8	599±87; n=10	587±36; n=6	485±81; n=9

Fig. 2. Quantification of the phenotypic changes in hormone-producing cell populations following the mono- or bi-allelic loss of *Arx* and/or *Pax4*. P2-independent pancreata estimated to be of the same size were serially sectioned. *n*, number of pancreata analyzed for each genotype. Every tenth section was stained as indicated and the numbers of positive cells were counted and compared with the total islet cell content (estimated on adjacent sections using a mixture of antibodies raised against the different endocrine hormones). Data are shown as percentage±s.e.m. of hormone-positive cells contributing to the total endocrine population. On average, the lack of one *Arx* and/or *Pax4* allele does not alter the endocrine cell content. Overall, the islet-cell alterations observed in *Arx*$^{-}$ and *Arx*$^{-}$*Pax4*$^{+/-}$, or in *Pax4*$^{-/-}$ and *Pax4*$^{-/-}$ *Arx*$^{+/-}$, appear to be similar. However, the loss of a single *Pax4* allele in *Arx*$^{-}$ *Pax4*$^{+/-}$ animals results in a significant reduction of the β-cell content together with an increase of the δ-cell population when compared with *Arx* mutants. Note, in mice depleted in Arx and Pax4, the loss of the insulin- and glucagon-expressing cell populations, and the substantial increase in the numbers of somatostatin- or PP-producing cells. It should be underlined that the total endocrine cell content is not statistically modified in all of the genotypes analyzed. Multiple comparisons of the data obtained for each endocrine population in each genotype were processed with a single-factor ANOVA coupled to Newman-Keuls test using the wild-type genotype as a reference (**P*<0.05, ***P*<0.01). A similar study was performed comparing subtype-specific cell numbers between *Arx*$^{-}$*Pax4*$^{+/-}$ mice and *Arx* mutants (§*P*<0.05).

quantitatively assayed at E15, *Arx*- and *Pax4*-deficient pancreata were found to be already entirely lacking glucagon-expressing cells (Fig. 3C,D), whereas only ten percent of the normal number of insulin-labelled cells were present (Fig. 3E-J). The number of cells positive for the pro-endocrine marker *Ngn3* was unchanged (Fig. 3C-F). At E18, a massive increase of the somatostatin-marked cell population (+720%) was clearly apparent (Fig. 3G,H,K,L), although these cells arose at the proper developmental stages (data not shown). Strikingly, the number of PP-cells was found to be normal at this stage (Fig. 3I-L). The results of co-immunodetection experiments showed that somatostatin-producing cells did not express the PP hormone, and vice versa (Fig. 3K-L). Taken together, these data provide evidence that *Pax4* and *Arx* are not required for the specification and differentiation of any of the islet endocrine cell types at early developmental stages. However, from E15 onwards, in the absence of both genes, there is an early-onset loss of mature α- and β-cells, and a proportionate increase of the somatostatin-producing cell population, while the number of PP-cells remains unchanged.

To further decipher the molecular alterations associated with *Arx* and *Pax4* deficiencies, we performed a quantitative immunohistochemical analysis of the known endocrine cell-specific markers, including transcription factors involved in endocrine pancreas specification. Every tenth section of the pancreas analyzed was assayed using antisera against the β-cell markers Nkx6.1 and Glut2, the ε- and α-cell-restricted peptide ghrelin (Prado et al., 2004) (P.C., unpublished), or the α-, β- and PP-specific factor Nkx2.2, in combination with antisera raised against insulin, somatostatin or PP hormones. The simultaneous lack of *Arx* and *Pax4* was found to result in a substantial decrease in the expression of all these factors: only the few scattered remaining insulin-positive cells were found to express Nkx6.1 (Fig. 3M,N) and Glut2 (Fig. 3O,P). Interestingly, the number of ghrelin-labelled cells was also found to be strongly reduced. Because ghrelin^{+}glucagon^{-} ε-cells were still detectable (Prado et al., 2004) (P.C., unpublished; Fig. 3Q-R), this result suggests that it was the ghrelin^{+}glucagon^{+} α-cells that were missing. Similarly, Nkx2.2 was found to be restricted to PP-cells and the residual insulin-producing cells (Fig. 3S,T; data not shown), whereas δ-cells were devoid of *Nkx2.2* expression (Fig. 3U,V). The δ-cell-specific markers, CART and CA812 (Jensen et al., 1999; Contreas et al., 1992) (P.S., unpublished), were also analyzed and were found to be uniformly expressed in somatostatin-producing cells of Arx^{-}Pax4$^{-/-}$ pancreas (data not shown). Similarly, the study of numerous additional transcription factors known to act in the genesis of the endocrine pancreas,

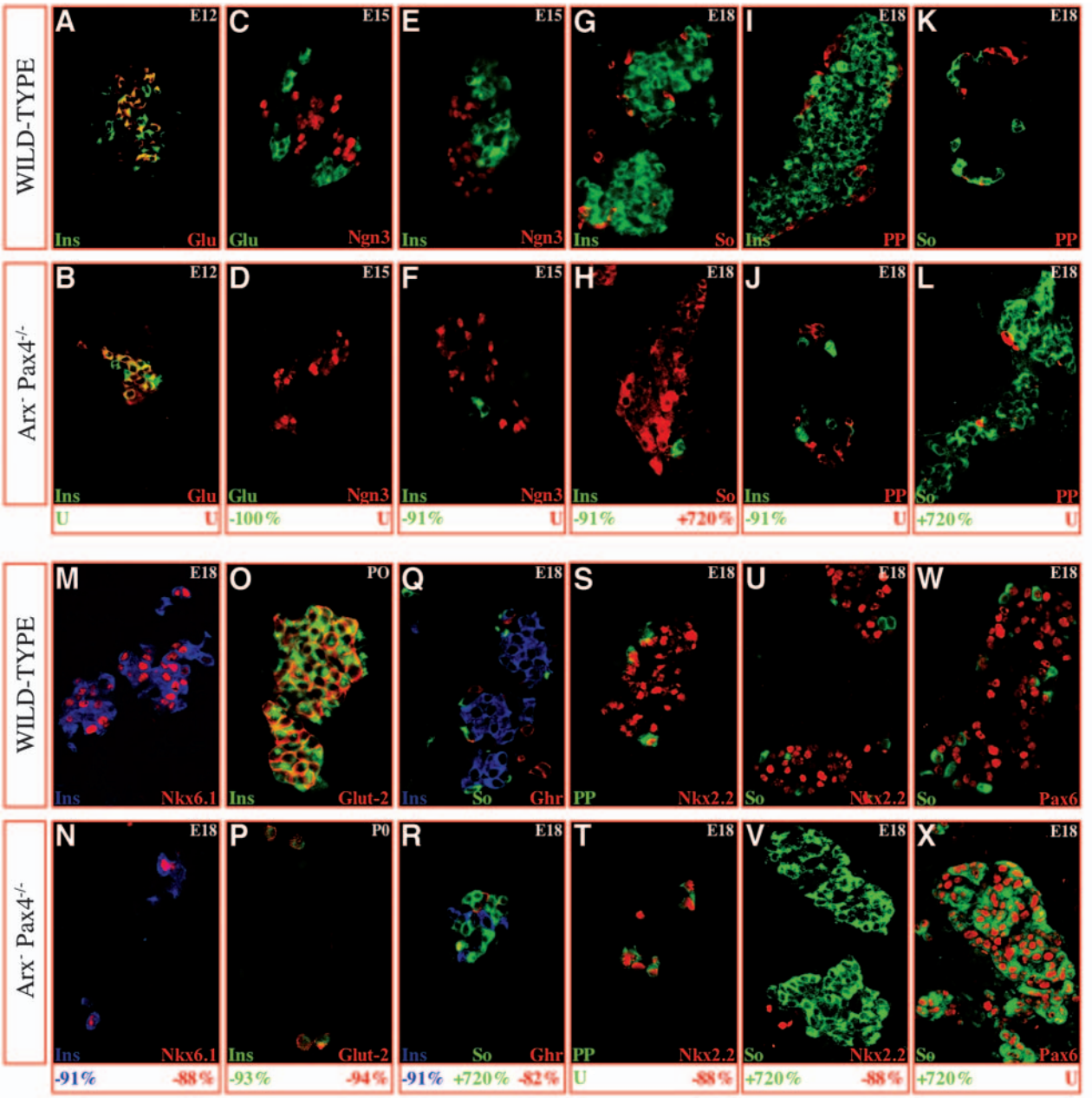


Fig. 3. Lack of α- and β-cell fate specification resulting in promotion of a somatostatin-producing cell destiny in *Arx*/*Pax4* double mutant embryos. Co-staining of E12 (A,B), E15 (C-F), E18 (G-N,Q-X) and P0 (O,P) pancreas. The genotypes examined and the different antibody combinations used are indicated. A quantification of the endocrine modifications between the two genotypes, estimated using Student's *t*-test, is provided in percent under each set of pictures ($n \geqslant 3$, $P<0.05$; U, unchanged). (A,B) At E12, no alteration in the hormone-expressing cell numbers could be detected between wild type and double mutants. (C-L) A loss of mature glucagon- (C,D) and insulin- (C-J) expressing cells, and a dramatic increase in the somatostatin-producing cell content (G,H,K,L) is already obvious as soon as cells begin to express hormone; the number of $Ngn3^{+}$ cells is not modified (C-F). Importantly, in embryos, the number of PP-expressing cells is not altered by Arx/Pax4 co-depletion (I-L). The supernumerary somatostatin-producing cells found in the double mutants do not express the PP hormone at E18 (K,L). (M-X) In *Arx*- and *Pax4*-deficient pancreas, the expression of the β-cell-specific transcription factors Nkx6.1 and Glut2, is dramatically reduced (N,P; compare with wild type in M,O). Interestingly, the number of ghrelin-expressing cells is also reduced (R, compare with Q) and these cells produce neither somatostatin nor insulin. (S,T) Similarly, the expression of Nkx2.2 appears to be severely diminished in the double mutants; it is found in the remaining PP-cells, but is excluded from the somatostatin-producing cells (U,V). Finally, Pax6 labelling can be seen in all endocrine cells in the double mutants (W,X).

such as Ngn3, Isl1, HB9 (Hlxb9 – Mouse Genome Informatics), Neurod and Pax6, indicated that they were all present and appropriately localized in double mutant animals (Fig. 3W,X; data not shown). In summary, through a thorough immunohistological analysis, we conclude that, despite the extensive alterations that follow the loss of *Arx* and *Pax4*, the

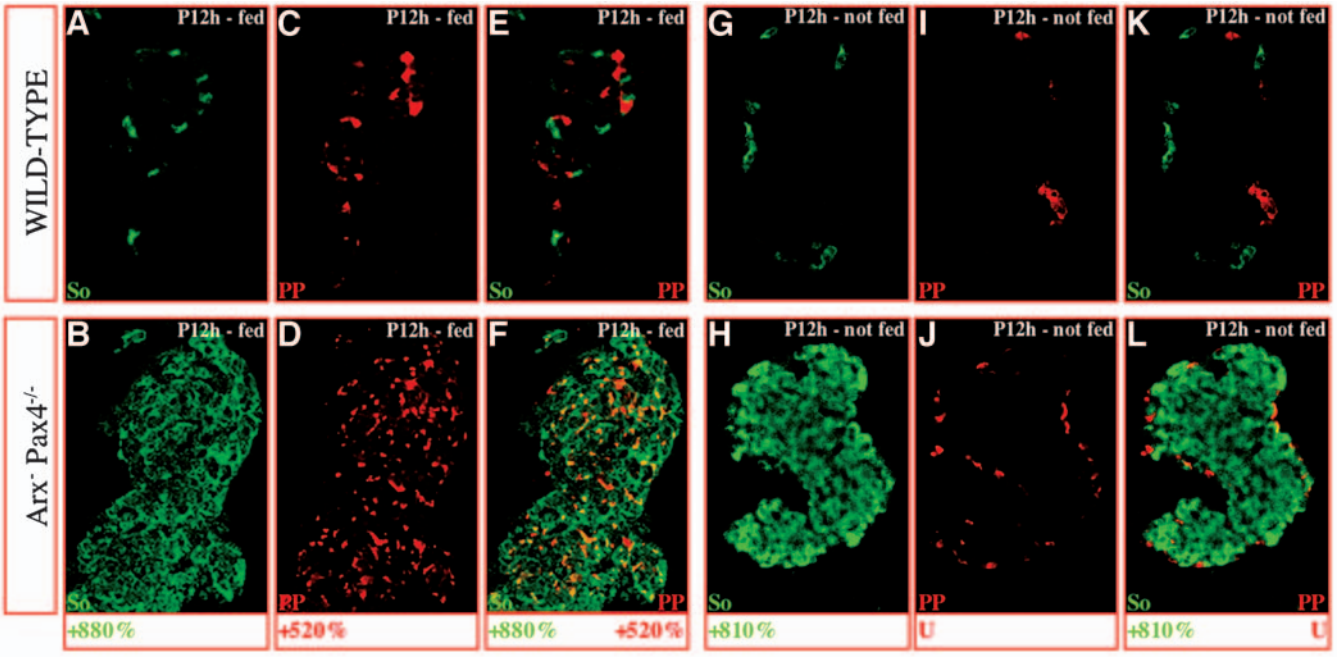


Fig. 4. Expression of the PP hormone in somatostatin-producing cells in *Arx*$^{-}$/*Pax4*$^{-/-}$ animals after the onset of feeding. Pancreas was isolated 12 hours after birth either from normally fed pups (P12h–fed; A-F) or from starved animal separated from their mother after delivery (P12h–not fed; G-L). Every tenth section of wild-type and *Arx*/*Pax4* double mutant pancreas (indicated on the left side) was labelled with anti-somatostatin (A,B,G,H) and anti-PP (C,D,I,J) antibodies, and the number of marked cells was quantified; merged pictures of the different staining are presented in E,F and K,L. A quantification of the endocrine modifications between the two genotypes, estimated using Student's *t*-test, is provided in percent under each set of pictures ($n \geq 3$, $P < 0.05$; U, unchanged). In normally fed P12h *Arx*/*Pax4* double mutants, note the more than fivefold increase in PP production by the excess somatostatin-producing cells, when compared with wild type (E,F). Importantly, in starved animals, the content in PP-marked cells was found to be normal when compared with controls (K,L).

endocrine cells that remain express a normal complement of transcription factors during pancreas morphogenesis.

The results obtained for the PP-producing cell population were intriguing: from E18 until birth (Fig. 3K,L and data not shown), *Arx*/*Pax4* mutant pancreas exhibited the normal content of PP-producing cells, whereas at P2 most of the somatostatin-producing cells found in this genotype co-expressed PP (see below). As the PP hormone is not normally expressed in δ-cells, these findings suggest that postnatal metabolic events might promote such a PP production in the absence of functional *Arx* and *Pax4* alleles. To confirm this assumption, we isolated pancreatic tissues 12 hours postpartum (P12h), from pups that had been either fed or starved after birth, and assayed them for the presence of somatostatin and PP. Our results demonstrate that, at P12h, the supernumerary somatostatin-labelled cells massively synthesized PP in *Arx*/*Pax4* double mutant animals fed normally (Fig. 4A-F). This production of PP resulted in a more than five-fold increase in the total number of PP-labelled cells, the number of somatostatin^{-} PP^{+} cells remaining statistically unchanged when compared with wild-type animals (data not shown). Importantly, the starved double mutants exhibited normal numbers of PP-expressing cells when compared with controls (Fig. 4G-L). Together, these results further demonstrate that in embryos lacking *Arx* and *Pax4*, although the total endocrine population is unchanged, α- and β-cells fail to appear and are replaced by somatostatin-producing cells, the PP-cells developing in normal numbers. We therefore conclude that, in the absence of *Arx* and *Pax4*, the somatostatin-expressing cell fate is favoured at the expense of the α- and β-cell lineages. After birth, the excess somatostatin-producing cells initiate an expression of the PP hormone that depends on feeding.

Characterization of the molecular mechanisms underlying Arx and Pax4 functions

It was previously suggested that the levels of both Arx and Pax4 are regulated through a mutually inhibitory cross-regulatory circuit that controls the transcriptional state of both genes during endocrine pancreas specification (Collombat et al., 2003). To shed light on the molecular mechanisms regulating the levels of *Arx* or *Pax4* transcripts, we analysed whether the mutual inhibition of *Arx* and *Pax4* is achieved by direct interaction of each factor with the promoter of the other. We initially determined the ability of each protein to bind to the promoter region of the gene encoding the other factor. We first looked for potential Pax4 binding sites within the *Arx* locus. A search for conserved DNA motifs within the *Arx* locus of different species revealed a high degree of similarity between the different organisms tested, but also uncovered two highly conserved domains within a region 3′ of the *Arx* coding sequence (Fig. 5A, shown in red within the alignment). To test the function of this particular region, transgenic animals were generated using a DNA construct containing a 9.7-kb *Nhe*I-*Eco*RV region downstream of the *Arx* locus together with the β-galactosidase gene (*lacZ*) driven by a minimal promoter (Fig. 5A). The analysis of β-galactosidase activity in four different founders by X-gal staining, revealed a similar pancreatic expression pattern that corresponded to that of the endogenous

Arx gene (Fig. 5B, arrowhead). Specifically, this 9.7-kb fragment perfectly recapitulated the *Arx* expression pattern throughout pancreas genesis (P.C., unpublished) (Collombat et al., 2003). Assuming that such a highly conserved DNA domain might correspond to the interaction site of the Pax4 factor, we undertook a search for potential Pax4 binding sites, using the consensus sequence reported previously by Fujitani et al. (Fujitani et al., 1999). By means of electrophoretic mobility shift assays (EMSA; Fig. 5C), we established that the Pax4 protein efficiently bound, in a dose-dependent manner, the 5′-taggaatcggaagaaactcgtggtgactagagtcgat-3′ sequence (Pax4 binding site – *P4BS*) located 14.2-kb downstream of the *Arx* translation stop site. This result was confirmed by Chromatin ImmunoPrecipitation (ChIP) assay: owing to the lack of suitable anti-Pax4 antibody, the immunoprecipitation was performed after transfection of a hemagglutinin-tagged Pax4-encoding vector into a Pax4^{+} TC1.9 β-cell line, using an anti-hemagglutinin antibody (Fig. 5D). Our data indicate that Pax4 specifically interacts with the *Arx* enhancer domain through the motif hereby characterized.

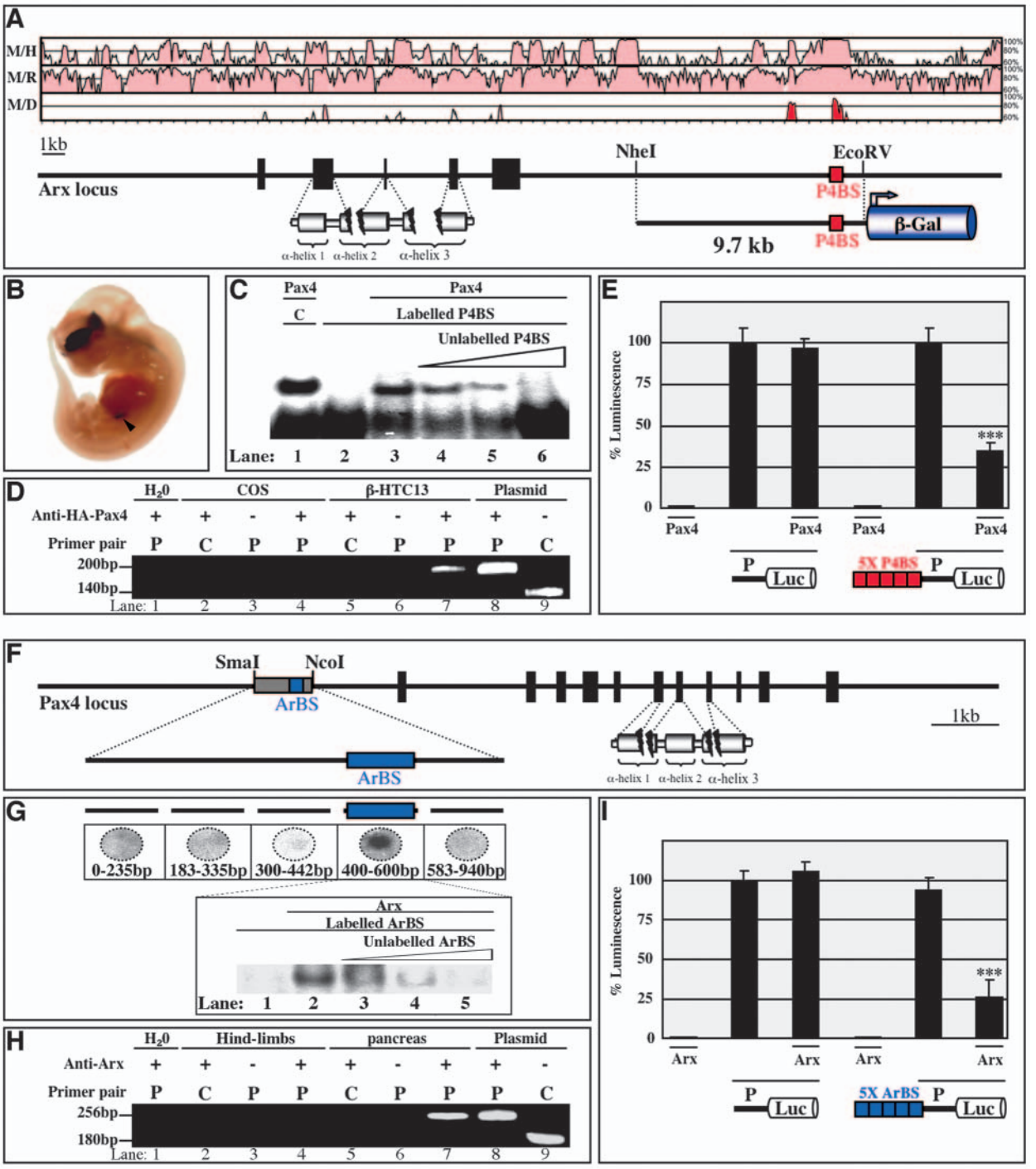


Fig. 5. See next page for legend.

In a second set of experiments, the presence of potential Arx binding sites (*ArBS*) in the *Pax4* promoter was investigated. Although Arx target sequences were unknown, we hypothesized that the Arx protein might specifically interact with the highly conserved 0.9-kb *Sma*I-*Nco*I *Pax4* enhancer region (Fig. 5F) reported by Brink et al. (Brink et al., 2001). Accordingly, five overlapping 0.2-kb DNA probes derived from this 0.9-kb sequence were radioactively end-labelled and incubated with Arx protein that had been previously spotted onto a nylon membrane (South-western analysis). Subsequent autoradiography revealed that the Arx protein preferentially interacted with the domain lying 400 to 600 bp within the 0.9-kb *Pax4* enhancer region (*ArBS*; Fig. 5G). Using EMSA, such a domain was found to be preferentially and dose-dependently bound by Arx. ChIP analysis using E14 pancreas confirmed the interaction of Arx with this 200-bp DNA fragment present within the *Pax4* promoter (Fig. 5H).

Fig. 5. Arx and Pax4 mutually inhibit the transcription of one another through a direct interaction. (A) A sequence analysis of a 50 kb region centered on the *Arx* locus was performed, comparing *Mus musculus* with *Homo sapiens* (H/M), *Rattus norvegicus* (M/R) or *Danio rerio* (M/D). Despite an overall high similarity, comparison with the zebrafish sequence reveals the presence of only a few highly homologous zones, in the homeobox region and also in a domain located 11 to 14 kb downstream of the *Arx* gene (highlighted in red in the alignment). No obvious similarity was found upstream of the *Arx* gene. The mouse *Arx* locus is represented underneath the result of the alignment (exons are shown as black rectangles), as are the three typical α-helices of the homeodomain. The *Nhe*I-*Eco*RV 9.7 kb region encompassing the 3′ conserved region was further used for the generation of transgenic animals using the β-galactosidase reporter gene *lacZ*. (B) β-Gal staining of such animals demonstrates a recapitulation of the *Arx* expression pattern at E10.5 (see Collombat et al., 2003). The pattern of labelling in the pancreas corresponds perfectly to endogenous *Arx* expression (arrowhead; P.C., unpublished). (C) A search for Pax4-binding sites (*P4BS*) within the 9.7 kb region demonstrated that a labelled 37 bp DNA fragment, located 8.5 kb downstream of the *Nhe*I site (red rectangle in A), can efficiently interact with Pax4, as evidenced by EMSA analysis (lane 3). A competition assay with increasing concentrations of unlabelled *P4BS* further demonstrates the specificity of the interaction (lanes 4-6). Lane 1 shows the Pax4-binding-site consensus used as control (C). (D) The specificity of the interaction Pax4-*P4BS* was validated by ChIP analysis. No binding was detected in COS-cells (lanes 2-4), whereas, in β-cells (lanes 5-7), PCR and sequencing reactions confirmed that Pax4 binds to *P4BS* (lane 7; P, *P4BS*-specific primers; C, control primers). (E) A reporter assay study indicates that Pax4 interacts with *P4BS* and acts as a transcriptional inhibitor. (F) Representation of the *Pax4* locus, as well as the encoded homeodomain and the *Sma*I-*Nco*I 0.9 kb *Pax4* enhancer region. (G) This 0.9 kb region was subdivided into five overlapping domains that were tested for recognition by the Arx protein. The 400 to 600 bp region (*ArBS*) can interact with Arx (blue rectangle in F,G). By means of EMSA and competition assay, Arx was found to preferentially interact with *ArBS* (lanes 2 and 3-5, respectively). (H) Using ChIP assay on isolated pancreas, an Arx-*ArBS* interaction is observed in E14 pancreas (lanes 5-7) but not in hind limb (lanes 2-4). (I) A reporter assay analysis suggests that Arx can bind to *ArBS* and acts as a transcription inhibitor. P, minimal promoter; Luc, luciferase gene. Results are presented as mean of luciferase activity ±s.e.m. Statistically significant differences of reporter activity, comparing cells transfected with the indicated reporters alone or with a Pax4-encoding vector (n=3), were estimated using Student's t-test (***P<0.001).

To validate these data, expression constructs were generated for *Arx* and *Pax4*. Each construct was introduced, together with a luciferase reporter gene, into the Arx^+ α- or $Pax4^+$ β-derived cell lines (TC1.9- and HC 13 T-cells, respectively), the luciferase reporter constructs containing five copies of the respective binding sites characterized above. Our results demonstrate that both Pax4 and Arx efficiently repress basal reporter activity, 2.7-fold and 3.8-fold, respectively (Fig. 5E,I). Taken together, these results suggest that Arx directly interacts with the *Pax4* enhancer domain thereby antagonizing *Pax4* transcription and, as a consequence, promoting the α-cell fate. Similarly, Pax4 appears to act early during endocrine cell genesis to favour the β-/δ-cell fate, at the expense of the α-cell destiny, through a direct inhibition of *Arx* transcription.

Discussion

A previous study revealed the importance of mutually antagonistic activities exerted by Pax4 and Arx transcription factors for proper islet cell genesis (Collombat et al., 2003): Pax4 was shown to favour β-/δ-cell fate at the expense of α-cell specification, whereas Arx promoted α-cell fate and repressed the β-/δ-cell lineage. These observations raised a question about the fate of endocrine progenitors in the absence of both genes. We therefore generated mice deficient for both genes. Our study demonstrates that *Arx/Pax4* double-deficient animals are born, but that they rapidly develop a severe hyperglycaemia and die at P2. Importantly, closer analysis of the pancreas revealed an early-onset loss of the mature α- and β-cell populations, concomitantly with a dramatic increase in the number of somatostatin-producing cells, which, also and inappropriately, secreted PP, but only once feeding had been initiated. Our analysis reveals that Arx and Pax4 undergo a direct interaction, mediating transcriptional inhibition. Finally, a so far unrecognized role of Pax4 in β-cell fate is suggested.

Arx and *Pax4* are required for the proper differential genesis of endocrine cells

Endocrine pancreas morphogenesis is associated with the early emergence of cells that often co-express glucagon and insulin. Despite an early expression of both *Pax4* and *Arx* genes in the pancreatic primordium (Collombat et al., 2003; Sosa-Pineda et al., 1997), it has been shown that neither gene is necessary for the formation of these early cells. The phenotypic defects observed in *Arx/Pax4* double-knockout mice corroborate these findings, and rule out the possibility of redundant activity between Arx and Pax4 in this respect. Specifically, we demonstrate that, during endocrine development, two distinct populations of insulin- and glucagon-producing cells arise successively; the early population is unaffected by the deficiency of *Arx* and/or *Pax4*, whereas the latter one corresponds to mature β- and α-cells whose correct development depends on the concerted activities of both factors. Accordingly, in *Arx/Pax4* double mutants, an early-onset loss of mature α- and β-cells is observed, concomitantly with an increase in somatostatin-expressing cell numbers. Along the same line, all the developmental markers associated with β- and α-cell lineages, including Nkx6.1, Glut2, Pdx1, Nkx2.2 and ghrelin, are missing or are found dramatically reduced. Interestingly, the supernumerary somatostatin-producing cells do not ectopically express any of these genes

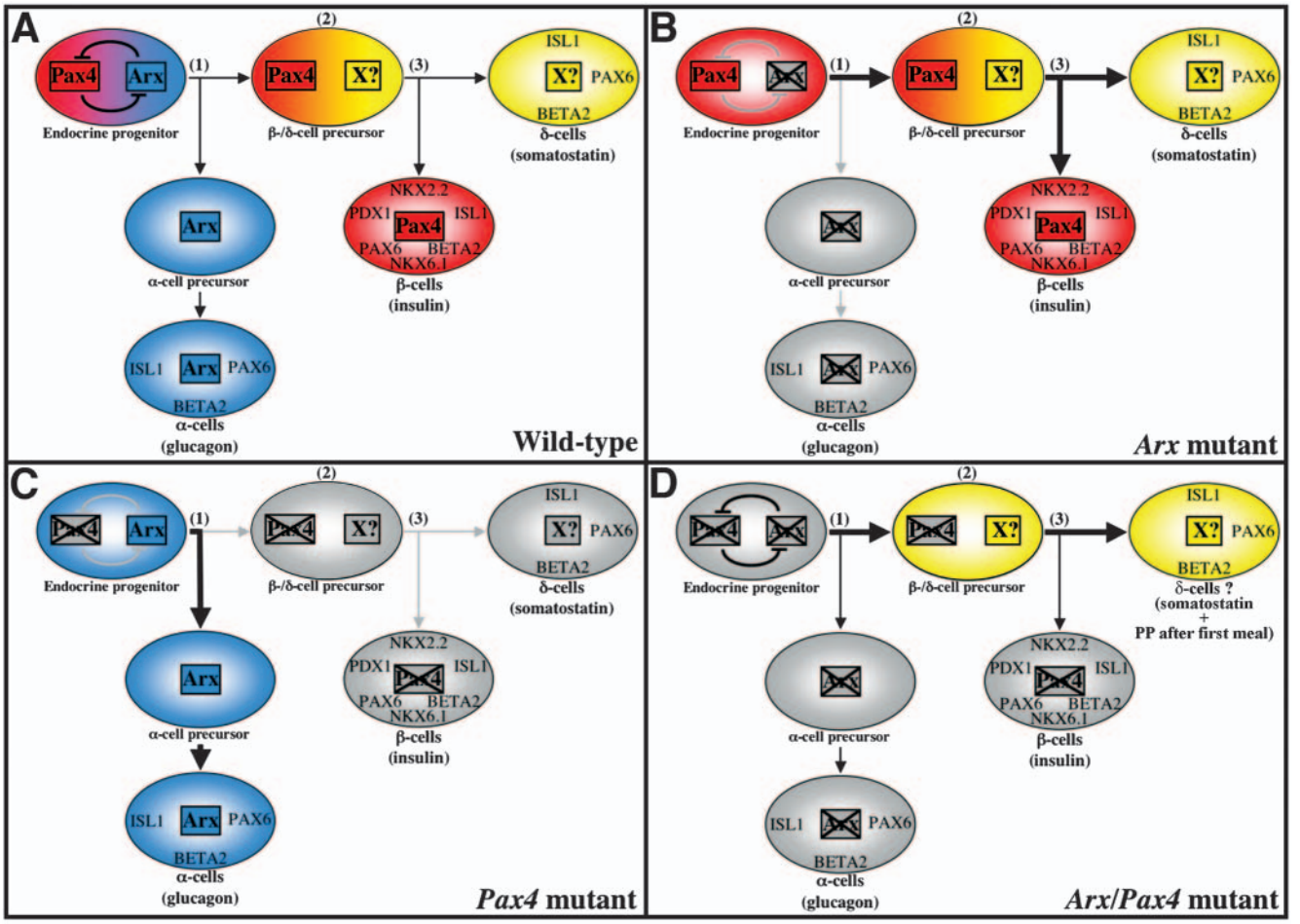


Fig. 6. Model of endocrine subtype specification during pancreatic development. (A) An endocrine precursor cell initially expresses both Arx and Pax4, most probably in an inactive form. In a first round of competitive fate allocation, an unknown factor determines which factor will predominate: if it is Arx, the α-cell fate will be specified (with Arx inhibiting *Pax4* expression), whereas Pax4 will induce β-/δ-cell lineages through the inhibition of *Arx* transcription (1). In the case of Pax4 prevalence, the resulting β-/δ-cell precursor is poised to undergo a second round of fate allocation (2). In this second event, Pax4 seemingly induces the β-cell fate at the expense of the δ-cell lineage. A hypothetical 'factor X' is envisioned to have an opposite function, promoting the δ-cell fate to the detriment of β-cell specification (3). (B-D) Fate changes in the case of *Arx* (B), *Pax4* (C), or combined *Arx/Pax4* (D) deficiency. See main text for details. For the purpose of simplification, exocrine cell and PP-cell development are not represented.

but are positive for the δ-cell specific markers CART and CA812. These data suggest that these cells do not share any characteristics with normal α- or β-cells, but rather express a δ-cell-specific complement of transcription factors. However, further analysis and lineage tracing experiments would be required to fully characterize the identity of such somatostatin-expressing cells, and to prove whether they correspond to δ-cells or not.

In *Arx/Pax4* mutants, PP-cell genesis is normal but most somatostatin-expressing cells begin, atypically, to produce PP after birth. We demonstrate that this rapid expression of PP is triggered by feeding after birth, thereby indicating that neither the lack of *Arx* nor that of *Pax4* alters PP-cell specification. Rather, it seems that the absence of both, combined with the onset of feeding, induces somatostatin-producing cells to synthesize PP. The shift after birth from an embryonic metabolism mainly based on lipids to a carbohydrate-centred metabolism in hyperglycaemic animals lacking both insulin and glucagon seems to promote such PP production. However, the detailed characterization of the mechanisms involved is still under investigation.

Another interesting finding was that new-born $Arx^{-}/Pax4^{+/-}$ animals survived until P8-P12, with a mild hypoglycaemia that became more severe with time. This result was unexpected because *Arx* mutant mice die at P2 and *Pax4* heterozygous mice do not exhibit any obvious endocrine alteration. However, the significant increase in δ-cell number and the proportional reduction in β-cell content observed in $Arx^{-}/Pax4^{+/-}$ animals when compared with *Arx* mutants reveals a dose-dependent requirement of Pax4 for β-cell fate specification at the expense of δ-cell destiny. In addition, the extended life expectancy suggests that the decrease in insulin-expressing cells, and/or the increase in somatostatin-producing cells, might attenuate the hypoglycaemia observed in *Arx* mutants, possibly due to an increased secretion of somatostatin, a known inhibitor of insulin secretion (Strowski et al., 2000). It is also important to notice that the loss of a single *Arx* allele does not affect the content in the different endocrine cell subtypes, as compared

to wild-type animals, suggesting that *Arx* might escape the X-inactivation processes. However, further work would be required to validate these statements.

Our present data, together with results obtained previously (Collombat et al., 2003), lead us to conclude that Arx is required for the acquisition of α-cell fate, whereas Pax4 is necessary for β-cell destiny; the simultaneous loss of these factors results in an alternative outcome in which cells presenting most of the characteristics of δ-cells develop. Finally, a *Pax4* haploinsufficiency phenotype can be recognized in an *Arx*-deficient background.

Arx and Pax4 interact through direct mutual transcriptional inhibition

The previous findings that both Arx and Pax4 transcription factors are initially co-expressed and that one predominates to promote a particular islet subtype fate (Collombat et al., 2003) prompted us to investigate the detailed mechanisms involved in this phenomenon. Thus, through a study combining transgenic, EMSA, ChIP and reporter assay approaches, we have characterized Arx and Pax4 binding sites and have provided evidence that Arx inhibits *Pax4* transcription by interacting with the *Pax4* enhancer domain, whereas Pax4 antagonizes *Arx* transcription by binding to a 3′ *Arx* enhancer region. The finding that both Arx and Pax4 can behave as transcriptional repressors is supported by in vitro studies or analyses performed in *C. elegans* (Fujitani et al., 1999; Seufert et al., 2004; Smith et al., 1999). However, the detailed mechanisms regulating the prevalence of one factor over the other remain to be elucidated.

Together with previous data obtained from *Arx* and *Pax4* single mutant phenotypes, our results support the model depicted in Fig. 6A. During early pancreas morphogenesis, endocrine precursor cells express both Arx and Pax4. As each protein directly inhibits transcription of the gene of the other, the initial co-expression may reflect a production of inactive precursors. Next, the selective activation of Arx or Pax4 will promote an α-cell specification or a β-/δ-cell fate, respectively (Fig. 6A, 1). The mechanisms involved are unclear but it is likely that an as yet undiscovered molecule selectively induces, in a concentration-dependent manner, the expression of *Arx* or *Pax4*. The factor thus induced will directly inhibit the other at the transcriptional level, thereby further reinforcing its dominance: if it is Arx, *Pax4* transcription is repressed and the α-cell fate is favoured, whereas Pax4 activation induces a β-/δ-cell fate through the inhibition of *Arx* expression (Fig. 6A, 1). All our data point to an early requirement for Arx in α-cell genesis, as, in *Arx* and *Arx*/*Pax4* mutant pancreas, the β- and/or δ-cell lineages are promoted (Fig. 6B and 6D, respectively). Importantly, in contrast to what was previously assumed (Collombat et al., 2003; Sosa-Pineda et al., 1997), Pax4 does not appear to be necessary for δ-cell genesis; rather, it seems that it acts only early during pancreatic islet specification to inhibit *Arx*, thereby specifying a β-/δ-cell fate. Indeed, the depletion in Pax4 results in a failure of β-/δ-cell specification, as Arx activity promotes an α-cell fate (Fig. 6C). However, in *Arx*/*Pax4* double mutants, single-hormone somatostatin-producing δ-cells develop normally concomitantly with an excess of cells presenting most of the characteristics of δ-cells (Fig. 6D). From these data, it seems that, in addition to its repressive action on *Arx*, Pax4 induces a β-cell fate at the expense of the δ-cell lineage. The loss of a single *Pax4* allele in *Arx*-deficient pancreas, resulting in a significant decrease in the number of β-cells and a simultaneous increase in the δ-cell content, is in agreement with this notion. It seems likely therefore that a third player ('factor X') is required to specify the δ-cell fate at the expense of β-cell formation (Fig. 6A, 2,3). At later developmental stages, an additional transcription factor, Pax6, is believed to act in the terminal differentiation of the endocrine cell subtypes (Ashery-Padan et al., 2004). Despite these advances towards an understanding of the differential genesis of endocrine cells, numerous questions remain. (1) Which factors determine whether Arx or Pax4 will prevail during the early steps of islet cell specification? (2) What is the identity of 'factor X'? (3) What mechanisms underlie the transition from endocrine progenitors to β-/δ-cell precursors? The study of the proteins interacting with Arx and Pax4 should provide a greater insight into the processes leading to the selection of a particular factor at the expense of the other (question 1). Likewise, a scrutiny of the transcriptome of the mutant analyzed in this study should enable the δ-cell genetic determinants to be characterized (question 2), thus shedding light on the mechanisms and molecules implicated in the selection of a particular endocrine cell fate (question 3).

In summary, our analysis establishes the requirement of Arx and Pax4 at multiple stages of α-, β- and δ-cell specification. Our findings uncover an essential role for Pax4 in β-cell specification at the expense of the δ-cell lineage, and are consistent with a model in which fate allocation occurs through repressive interactions between transcription factors.

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