The nuclear hormone receptor family member NR5A2 controls aspects of multipotent progenitor cell-formation and acinar differentiation during pancreatic organogenesis

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ABSTRACT

The orphan nuclear receptor NR5A2 is necessary for the stem-like properties of the epiblast of the pre-gastrulation embryo and for cellular and physiological homeostasis of endoderm-derived organs postnatally. Using conditional gene inactivation, we show that Nr5a2 also plays crucial regulatory roles during organogenesis. During the formation of the pancreas, Nr5a2 is necessary for the expansion of the nascent pancreatic epithelium, for the subsequent formation of the multipotent progenitor cell (MPC) population that gives rise to pre-acinar cells and bipotent cells with ductal and islet endocrine potential, and for the formation and differentiation of acinar cells. At birth, the NR5A2-deficient pancreas has defects in all three epithelial tissues: a partial loss of endocrine cells, a disrupted ductal tree and a >90% deficit of acinar. The acinar defects are due to a combination of fewer MPCs, deficient allocation of those MPCs to pre-acinar fate, disruption of acinar morphogenesis and incomplete acinar cell differentiation. NR5A2 controls these developmental processes directly as well as through regulatory interactions with other pancreatic transcriptional regulators, including PTF1A, MYC, GATA4, FOXA2, RBPJL and MIST1 (BHLHA15). In particular, Nr5a2 and Ptf1a establish mutually reinforcing regulatory interactions and collaborate to control developmentally regulated pancreatic genes by binding to shared transcriptional regulatory regions. At the final stage of acinar cell development, the absence of NR5A2 affects the expression of Ptf1a and its acinar specific partner Rbpjl, so that the few acinar cells that form do not complete differentiation. Nr5a2 controls several temporally distinct stages of pancreatic development that involve regulatory mechanisms relevant to pancreatic oncogenesis and the maintenance of the exocrine phenotype.

KEY WORDS: Nr5a2, Pancreas, Acinar cell, Transcriptional control, Organogenesis, Mouse

INTRODUCTION

Pancreatic organogenesis proceeds through several crucial regulatory stages (Cleaver and MacDonald, 2010; Rieck et al., 2012): (1) specification and emergence of the pancreatic epithelial buds from the distal foregut endoderm; (2) expansion of an early pancreatic progenitor cell population and developmental commitment; (3) formation of a more restricted multipotent progenitor cell (MPC) population for endocrine, ductal and acinar tissues; (4) separation of committed pre-acinar cells at the tips of epithelial branches and a bipotent cell-population for islets and ducts in more central trunk domains; (5) separation of the endocrine and ductal programs, morphogenetic resolution of a precursor ductal plexus and completion of cell type-specific differentiation. This process creates three interrelated tissues: the islets of Langerhans (endocrine cells that produce polypeptide hormones, including insulin and glucagon), acini (exocrine cells that produce and secrete hydrolytic digestive enzymes) and ducts (exocrine cells that produce a bicarbonate flush and channel the acinar secretions to the intestine).

Much is known about the general roles of nearly thirty DNA-binding transcription factors and several intercellular signaling pathways required for proper pancreatic development (Cleaver and MacDonald, 2010; Pan and Wright, 2011; Serup, 2012). Yet, the molecular specifics of the regulatory transitions are incompletely understood – especially the formation of the multipotent cell population and the resolution of acinar precursor cells distinct from ductal and endocrine precursors. In this report, we show that NR5A2 is crucial for the proper formation of the multipotent cells and subsequently the acinar lineage.

NR5A2 (LRH1, FTF) is a member of the nuclear hormone receptor family (Becker-Andre et al., 1993) with diverse developmental and physiological functions (Lee and Moore, 2008; Fernandez-Marcos et al., 2011; Lazarus et al., 2012). Nr5a2 has been associated with breast, colon, ovarian and pancreatic cancers (Lazarus et al., 2012). NR5A2 and its paralog NR5A1/SF-1 bind as monomers to extended half-site DNA response elements. NR5A2 binds and can be activated by phospholipids (Lee et al., 2011); this interaction does not appear to be necessary for its basal transcriptional activity (Sabin et al., 2003). The presence of a functional ligand-binding pocket suggests the potential to derive drugs that alter pathological, physiological or developmental processes affected by NR5A2 (Lazarus et al., 2012). NR5A2 activity is modulated by phosphorylation and sumoylation (Lee et al., 2006; Venteclfe et al., 2010) and through interaction with co-activators (Xu et al., 2004; Sakai et al., 2006) and co-repressors (Goodwin et al., 2000; Suzuki et al., 2003). NR5A2 controls cell type-specific programs through direct transcriptional regulation of discrete subsets of target genes (Chen et al., 2008; Holmstrom et al., 2011; Chong et al., 2012).

During early development, Nr5a2 is expressed in the inner cell mass of the blastocyst and in the epiblast (Paré et al., 2004; Gu et al., 2005). NR5A2 can substitute for Oct4 (POU5F1 – Mouse Genome Informatics) to induce stem cell-like pluripotency in adult fibroblast cells (Heng et al., 2010). The absence of NR5A2 causes the loss of Oct4 expression in the epiblast and possible loss of pluripotency (Gu et al., 2005; Wagner et al., 2010), leading to the...
disruption of the primitive streak and failure of gastrulation (Labelle-Dumais et al., 2006). NR5A2 is normally present in the foregut endoderm at embryonic day 7.5 (E7.5) (Paré et al., 2001) and its expression continues in the intestine, liver and pancreas during endodermal development (Rausa et al., 1999; Annicotte et al., 2003). NR5A2 can activate hepatic genes in cell transfection experiments (Paré et al., 2001, 2004), but its role in developmental regulation of these genes and liver development is unproven, and Nr5a2 inactivation during early hepatic development has no overt affect (Lee et al., 2008). The results of our study demonstrate a pleiotropic role for Nr5a2 in pancreatic organogenesis.

In the adult pancreas, NR5A2 collaborates with PTF1A to control exocrine genes (Holmstrom et al., 2011). PTF1A is a class B bHLH transcription factor restricted to the pancreas, selected regions of the neural tube, retina and the cerebellum. The active form of PTF1A is an atypical trimeric containing a class A common bHLH protein (Roux et al., 1989; Rose et al., 2001) and an RBP protein, either RBPJ or RBPJL (Obata et al., 2001; Beres et al., 2006; Hori et al., 2008). The trimeric complex containing all three DNA-binding subunits is referred to as PTF1. Disruption of the complex eliminates transcriptional activity (Beres et al., 2006; Masui et al., 2007). The collaboration between NR5A2 and PTF1A requires the complete PTF1 complex (Holmstrom et al., 2011), and we show that this collaboration also occurs during fetal development.

In this report we demonstrate that Nr5a2 is crucial during fetal organogenesis for the formation of the pancreas. A previous study of pancreas-specific Nr5a2 inactivation late in fetal development detected only modest prenatal defects, the primary impact of which was the destabilization of the acinar phenotype post weaning (von Figura et al., 2014). Our analyses of two independent schemes of gene inactivation at the very onset of pancreatic development show that Nr5a2 controls the expansion of the nascent pancreatic epithelium, the proper formation of the MPC population that gives rise to pre-acinar cells and bipotent cells with ductal and islet endocrine potential, and the proper formation and differentiation of pre-acinar cells. This might be attributed to a reinforcing regulatory triad of Nr5a2, Ptf1a and Rbpf1 that controls additional pancreatic regulatory genes, including Foxa2, Gata4 and Myc, as well as direct effectors of morphogenesis and cell type-specific differentiation.

RESULTS

Nr5a2 expression increases in the epithelial ‘tip’ MPC population

We first placed NR5A2 in the context of known functional, stage-specific markers of pancreatic development. During the early stage at embryonic day 10.5 (E10.5), NR5A2 is restricted to regions of the endoderm, including the newly emergent dorsal and ventral pancreatic epithelia and the adjacent gall bladder primordium (Fig. 1A-E). The expression patterns of Nr5a2 and Pdx1, a key regulator of the early progenitor cells (Jonsson et al., 1994; Offield et al., 1996; Gu et al., 2002), overlap between the posterior foregut and the anterior midgut endoderm. The PDX1 domain comprises the ventral and dorsal pancreatic epithelia and the intervening intestinal endoderm extending posteriorly (Fig. 1A’), and overlap with the posterior foregut and the anterior midgut endoderm. The PDX1 domain comprises the ventral and dorsal pancreatic epithelia and the intervening intestinal endoderm extending posteriorly (Fig. 1A’). NR5A2 overlaps with PDX1 in these regions and extends caudally along the PDX1-deficient intestinal endoderm (Fig. 1A’). NR5A2 is co-localized with SOX9 (Fig. 1C’), a functional marker of progenitor cells within the epithelium (Kopp et al., 2011).

When multipotent ‘tip’ domains begin to form at E11.5, NR5A2 is present throughout much of the epithelium (Fig. 1). NR5A2 is often co-localized with CPA1 and HNF1B/TCF2, two markers of pancreatic MPCs at this stage (Zhou et al., 2007; Solar et al., 2009). Cells with high NR5A2 levels (NR5A2HI) are largely restricted to the peripheral layer of CPA1HI epithelial cells in contact with the surrounding mesenchyme (Fig. 1F). Hnf1b-expressing cells are preferentially in the interior, with about half containing NR5A2 (Fig. 1G). The levels of NR5A2 and HNF1B are inversely related: only 11% of the cells with high HNF1B levels are also high for NR5A2, and only 6% of the cells with high NR5A2 levels are also high for HNF1B (data not shown). NR5A2 is excluded from the differentiated first wave endocrine cells (Fig. 1H), as well as from all later appearing endocrine cells. The increased NR5A2 levels in the surface layer of epithelial cells coincide with the formation of the tip MPC population, which is also marked by the presence of MYC (Fig. 1H).

At E12.5, an early branching epithelium is decorated by small protrusions, the tips of which have CPA1, SOX9 and NR5A2 (Fig. 2), as well as low HNF1B and high PTF1A levels (e.g. see Fig. 7), consistent with the attributes of either MPCs or newly committed pre-acinar cells (Zhou et al., 2007; Pan et al., 2013). As the protrusions mature, the MPC population is depleted and the tips progress to acini. During the process of tip maturation (E13.5 and later), NR5A2 levels remain high along with CPA1 (Fig. 2A’-E’) and PTF1A, whereas SOX9 levels diminish (Fig. 2F-J). By contrast, NR5A2 levels are low in the trunk regions of epithelial tubules expressing transcription factors that promote precursor status (i.e. high SOX9; Fig. 2F-J) or suppress differentiation (i.e. HES1; Fig. 2M) (Kopinke et al., 2011). Hybridization in situ confirmed a higher level of Nr5a2 expression in pre-acini than in tubules (supplementary material Fig. S1B). NR5A2 levels remain high in tip cells during the transition from multipotency to preacinar unipotency.

NR5A2 is high in MPC-depleted tips at E15.5, which now form differentiating acini (Fig. 2K, PTF1A; Fig. 2N, MUC1; and

Fig. 1. Nr5a2 expression is dynamic during bud morphogenesis.

(A-E) Immunofluorescence localization in ventral and dorsal buds of NR5A2 (A-D, red), PDX1 (A, B, green) and SOX9 (C, D, green) at E10.5. (A’-D’) merged images. p, pancreas; gb, gall bladder. Green arrows, posterior boundary of PDX1; red arrows, anterior boundary of NR5A2. (E) Summary of NR5A2 and PDX1 distributions at E10.5. bd, bile duct; st, stomach; dp/vp, dorsal/ventral pancreas; d, duodenum. (F-H) Colocalization of NR5A2 with CPA1 (F), HNF1B (G) and MYC (H) in dorsal epithelium at E11.5. Arrowheads, high NR5A2 levels in the periphery, where CPA1 and MYC are co-localized and HNF1B is largely excluded. Dashed line in H, first wave endocrine cell cluster is devoid of NR5A2. Scale bars: 50 μm.
NR5A2 is retained in cells of large and intercalated ducts as well as centroacinar cells, and is decreased in differentiated acinar cells at E18.5 (Fig. 2E,J) and adults (supplementary material Fig. S2). Yet, Nr5a2 mRNA remains an abundant transcription factor mRNA (MacDonald et al., 2010).

This developmental expression pattern for Nr5a2 was corroborated with an independently derived antibody directed against a non-overlapping region of NR5A2 (see supplementary material methods). The presence of NR5A2 in the HNF1B+ and CPA1+ cells that precede tip MPCs, its superinduction in MPCs, retention in pre-acinar, acinar and ductal cells, and its exclusion from islet cells and their immediate (NGN3+) precursors are all consistent with specific roles for NR5A2 in the formation of MPCs and subsequently in acinar development.

Developmental defects in NR5A2-deficient pancreas

To assess these potential developmental roles, we examined mouse embryos homozygous for a floxed allele of Nr5a2 (Lee et al., 2008) and heterozygous for Cre replacement of the coding sequence of the Ptf1a locus (Kawaguchi et al., 2002) (Ptf1a+/cre; Nr5a2 flox/flox, hereafter named Nr5a2ΔP). Gene inactivation and depletion of NR5A2 protein were nearly complete by E12.5 (supplementary material Fig. S3). Note that the loss of immunoreactivity confirms the specificity of the anti-NR5A2 antibody. At this stage, the mutant epithelium was smaller than normal, due to fewer proliferating cells (Fig. 3A); infrequent apoptosis was unchanged in the mutant. The number of Pdx1-expressing progenitor cells was reduced by one-third, and that of Ptf1a-expressing cells by half (Fig. 3B). Myc is crucial for pancreatic epithelial cell replication at this stage (Nakhai et al., 2008; Bonal et al., 2009), and the total number of MYC+ cells in the NR5A2-depleted pancreas decreased by 75% (Fig. 3B), and of PTF1A-MYC co-positive cells by 60% (Fig. 3C). Moreover, the level of MYC in individual cells was skewed to lower levels (Fig. 3D). These observations indicate that continued proliferation driven by MYC requires the presence of NR5A2.

By E18.5, the NR5A2-depleted pancreas has recognizable gastric, splenic and duodenal lobes, but the tissue is thin and nearly devoid of acini, which normally provide its bulk (Fig. 4A,E). Acinar tissue was less than 10% of normal levels (Fig. 4Q). In contrast to normal tissue, the NR5A2-deficient pancreas was a convoluted, anastomotic ductal epithelium with closely apposed endocrine cell clusters, and the few acinar structures were immature and restricted to the periphery (Fig. 4B,F).

Fig. 2. Expression of Nr5a2 during development. (A–F) By E12.5, NR5A2 levels are high in epithelial tips (arrows) that contain CPA1+ MPCs and pre-acinar cells (A), and low in cells of SOX9+ tubules (F). (A–D) Arrows, CPA1 and high NR5A2 levels; arrowheads, low NR5A2 levels in interior tubules. (E) Arrows, acinar nuclei with decreased NR5A2 levels; arrowhead, false staining of blood vessels at this stage. (A–E) Higher magnification image of the conversion of CPA1+ epithelial tips to nascent acini. (E15.5, most NR5A2HI cells lack SOX9. Arrows: high SOX9, low NR5A2 cells. (arrowhead). (F–J) NR5A2 begins to segregate in tips at E13.5. At E14.5 and are low in acinar, intercalated duct (dashed lines) and centroacinar cells (Fig. 4B,F). (K–P) E15.5 expression. (K) Cells with NR5A2HI co-express HNF1B+ cells of the tubules (arrows). (L) NR5A2 is coincident with the ducts, often at the entrance to acini, and in mesenchymal cells. HES1+ HNF1B+ cells of the tubules (arrows). (M) HES1 is in some cells (arrows) of the tubules, often at the entrance to acini, and in mesenchymal cells. HES1+ cells are NR5A2LO. (N) MUC1 marks ducts (NR5A2LO) and acini (NR5A2HI). (O) High NGN3 cells are without NR5A2 (e.g. arrow). (P) Endocrine clusters with synaptophysin (Syp; asterisks) lack NR5A2. d, duct. Scale bars: A–E, 20 µm; F–P, 50 µm.

Fig. 3. Effects of Nr5a2 inactivation on growth and differentiation. (A) Rate of cell proliferation was measured by the fraction of PDX1+ cells that incorporate BrdU. (B) Total numbers of epithelial cells per pancreas with PDX1, PTF1A and MYC. (C) Total number of PTF1A-MYC co-positive cells decreased by two-thirds. (D) The fraction of cells and intensity of MYC immunofluorescence decreased in mutant pancreas. Error bars indicate s.e.m.; Student’s t-test, unpaired. (E) The fraction of CPA1+ pre-acinar cells that acquire MIST1 is lower in the mutant at E13.5. (F) RNA-seq results for Nr5a2ΔP mRNA levels compared with normal levels at E13.5.
fewer β-cells and a lower ratio of β- to α-cells (1.6 versus 0.7 for normal; Fig. 4R-T), consistent with fewer endocrine cells born from MPCs during the secondary transition. Thus, depletion of NR5A2 by E12.5 affects the development of islet, ductal and acinar tissues, but limits the formation and differentiation of acinar cells preferentially.

Mice born with an NR5A2-deficient pancreas survived, but their postnatal growth was stunted; seven-month-old male mice were approximately 26% smaller (Student’s t-test, unpaired; \( P=0.004 \)). The adult islets have normal architecture, but individual endocrine cells, principally glucagon expressing, were scattered throughout the parenchyma (supplementary material Fig. S5). Adult mice had normal fasting glucose levels and tolerance to a glucose challenge (data not shown). Acinar mass recovered to approximately 30% (25-33%) of normal levels in adult mice. The acinar tissue was predominantly poorly branched elongated tubes rather than blind-ended acini and domains of fibrosis with fat replacement, exocrine cell apoptosis and unusual hybrid acinar/ductal structures reminiscent of acinar-ductal metaplastic transitions (supplementary material Fig. S5).

The loss of NR5A2 prevents normal acinar formation
To discover the developmental processes controlled by Nr5a2, we analyzed defects at key developmental transitions.

Normal acinar formation
The formation of acini begins with the evacuation of cells within the evaginating epithelial tips, so that by E14.5 most nascent acini have short immature ductule (Fig. 5A). At E15.5, cellular growth appears to force the pre-acinar cap over the terminus of the intercalated duct. Centroacinar cells (Fig. 5A) are often detected deep in the acinar lumen and form continuous cell-cell contacts (Fig. 5A, 18.5, arrow) and absence of centroacinar cells by E18.5.
Morphogenetic defects
Acinar defects of Nr5a2ΔP epithelia are first detected at E12.5: fewer proacinar tips occur and clearance of cells within the tips has not yet begun (Fig. 5). Subsequently, substantially fewer pre-acini form, and mature slower and less completely than normal. Evacuation of interior cells of multilayered tips is incomplete at E15.5, two days later than normal (Fig. 5B, yellow arrowheads), and acinar over-capping of the terminal intercalated duct has not occurred. Acinar caps do extend over the ductules at E18.5; however, the termini of ducts are malformed, with multiple cell layers and no apparent lumen (Fig. 5B, 18.5, yellow arrow). Acinar morphology is immature: more and smaller cells per acinus, fewer secretory granules and lower levels of secretory enzyme mRNAs (see below), although apical-basal polarity is maintained. The larger number of cells per acinus and the disorganized acinar:ductal junctions suggest a defect in the branching process that would otherwise divide the acini into smaller units during growth.

Effects on acinar developmental gene expression
Analyses of the E13.5 mRNA populations from normal and NR5A2-depleted pancreas by RNA-Seq identified 317 differentially expressed genes (false discovery rate, fdr <0.05) with mRNA levels that differed 1.6-fold or more (Fig. 6A; supplementary material Table S2). In total, 225 genes had lower and 92 had higher mRNA levels than normal. Eighty-five percent of the genes with decreased mRNA levels were linked to genomic regions of NR5A2 binding, with the presence of an NR5A2 consensus-binding sequence at 42% of those bound sites. Consensus-binding sequences for FOXA and GATA factors were also enriched at the NR5A2-bound sites. By contrast, only 67% of the genes with apparent increased expression were associated with NR5A2 binding, none had an NR5A2 consensus-binding sequence and no binding sequence motifs were enriched. Therefore, the upregulated genes are probably not controlled directly by NR5A2; instead, their expression appears to increase because the next developmental stage dependent on NR5A2 is not properly initiated and mRNAs for functions of early organogenesis persist (Fig. 6B).

The most prominent attribute of the Nr5a2ΔP mutants was the arrest of acinar cell development, which was due to curtailed expression of regulators of the transition to mature acinar cells and proteins characteristic of acinar differentiation or mature acinar cells (supplementary material Table S2). For example, mRNAs for 21 acinar secretory proteins, including 13 digestive pro-enzymes, decreased 1.6-fold or more. We re-measured 11 of these mRNAs by qRT-PCR measurement of the mRNAs for selected genes. Levels are relative to normal E13.5 or E17.5, with s.d. shown; Student’s t-test, unpaired. (D,E) Co-localized binding of NR5A2, PTF1A and RBPJL at positions of active chromatin marks (H3K4me2 and RNApo2) in E17.5 chromatin for examples of genes for a secretory digestive enzyme (CPA1) and an acinar transcription factor (GATA4) affected by NR5A2 loss. Gray bars indicate NR5A2 binding overlapping with PTF1A, RBPJL, H3K4me2 and RNApo2. NR5A2 consensus-binding sequences (see Fig. 8D) near peak centers are indicated. Boxes enclose gene regions that direct expression of a transgenic reporter selectively to pancreas (Cpa1, 1.3 kb) (Meredith et al., 2013) and to pancreas, stomach and duodenum (Gata4, 0.9 kb) (Rojas et al., 2009).
qRT-PCR and confirmed that their levels were 40 to >99% lower than normal at E13.5 (Fig. 6C). Consistent with continued defects during acinar development, the relative levels of the mRNAs for these proteins were even lower at E17.5 (Fig. 6C). NR5A2 is normally bound to regulatory regions of these genes at this stage (Fig. 6D; supplementary material Fig. S6; data not shown). For example, the locus for CPA1, a prominent secretory carboxypeptidase, has three sites of NR5A2 binding in a 1.2-kb promoter-proximal region. These sites have functional marks of active transcriptional control regions: co-localization of other acinar specific factors (PTF1A and RBPJL) and enrichment of H3K4me2 and RNApol2. This 1.2-kb region is sufficient to direct pancreatic acinar-specific expression in transgenic mouse embryos (Meredith et al., 2013).

Suppressed acinar development is consistent with decreased expression of the known pancreatic regulators Ptf1a, Myc, Gata4, Rbpjl and Mist1 (Bhlha15 – Mouse Genome Informatics) (Fig. 6C). Other transcriptional regulators present selectively at high levels in mature acinar cells (MacDonald et al., 2010) were also reduced 50% or more: Spdef, Bhlhe40/Dec1, Nr0b2, Nurr1, Cebpδ, Ovo2 and Phox2b. NR5A2 is bound to probable regulatory regions of these genes at E17.5. For example, Gata4 is required for proper acinar differentiation (Xuan et al., 2012), is decreased 2.5-3.0-fold at E13.5 and E17.5 in the Nr5a2 mutant and has six sites with high NR5A2 occupancy, all of which have co-localized PTF1A, RBPJL, H3K4me2 and RNApol2 (Fig. 6E). The region with co-localized peaks at ~80 kb was active selectively in the pancreas when tested in transgenic animals (Rojas et al., 2009). Most of these regulatory genes are known to affect the embryonic development of other organ systems, and therefore are also likely to be direct targets of NR5A2 that control aspects of pancreatic development.

Defects in the transition of MPCs to committed pre-acinar cells

To identify potential causes of the disrupted acinar development, we focused on transcriptional regulators affected by NR5A2-depletion (Fig. 6C). At the onset of acinar development, fewer cells in the mutant epithelial tips expressed Ptf1a and Myc (Fig. 3B and Fig. 7A). Myc mRNA levels were reduced by half (Fig. 6C), consistent with fewer replicating acinar cells. MYC is crucial for the proliferation of MPC and acinar cells, but not for other pancreatic cell types (Nakhai et al., 2008; Bonal et al., 2009). NR5A2 is present at an NR5A2 consensus-binding sequence in the Myc locus during development and in adulthood. Decreased expression of additional key cell cycle control genes associated with chromatin-bound NR5A2 (Cdk1, Cdkn1a/p21, Plk2; supplementary material Table S2 and Fig. S7F-H) further indicates that cell proliferation depends directly on NR5A2.

To measure the progression from MPCs to newly committed acinar cells requires markers that distinguish these two cell types. MPCs are identified by the presence of PTF1A, CPA1 and high levels of MYC (Fig. 7A, ‘tip’ markers) and the absence of amylase (Zhou et al., 2007). However, cells recently committed to acinar fate are expected to have the same marker set until the appearance of amylase, which is a later, post-acinar commitment event. Distinguishing the two states is particularly problematic for E12.5 epithelial tips, which are expected to have the same marker set until the appearance of amylase, which is a later, post-acinar commitment event. Distinguishing the two states is particularly problematic for E12.5 epithelial tips, which are expected to have a mixed population of MPCs and committed pre-acinar cells. From their coupled analysis of stage-identifying markers and lineage tracing of Ptf1a-expressing cells, Pan and colleagues developed a solid foundation for monitoring the transition from multipotency to proacinar unipotency (Pan et al., 2013). SOX9, HNF1B and NKX6.1...
are present in MPCs during normal development and become excluded from the acinar lineage beginning at about E13.5 (Solar et al., 2009; Schaffer et al., 2010; Kopp et al., 2011). These three transcription factors linger in the tip cells of NRS5A2-depleted pancreas (Fig. 7A,C), and their mRNA levels do not decline at E13.5 (Fig. 3F). For example, 16% of CPA1+ cells in normal E13.5 pancreas have NKS6.1, whereas 87% of CPA1+ cells retain NKS6.1 in the NRS5A2-depleted pancreas.

MIST1/BHHLA15 is an early acinar specific marker (Pin et al., 2001). At E12.5, none of the CPA1+ (0/610) progenitor cells in normal pancreatic endothelium had detectable MIST1 levels (Fig. 7B); one day later, 90% did (Fig. 3E). By contrast, only 27% of the CPA1+ cells in NRS5A2-deficient pancreas were MIST1+ at E13.5. Moreover, the level of MIST1 in individual cells was much reduced (Fig. 7B), and this reduction might contribute to the incomplete acinar differentiation of later stages. The appearance of amylase was similarly delayed. The persistence of markers shared by MPCs and trunk cells (SOX9, HNF1B and NKS6.1) and the reduction of the markers MIST1 and amylase indicate that, in addition to fewer MPCs, their progression to acinar development is slowed by the absence of NRS5A2.

The loss of MPCs in Ptf1a+/Cre,Nr5a2flox/flox embryos was incomplete, probably due to delayed expression and action of Cre expressed from the Ptf1a locus. To address this issue, we examinedNr5a2flox/flox embryos bearing a Pdx1-Cre transgene (Gu et al., 2002) that is active earlier in the pancreatic endoderm than Ptf1a (Burlison et al., 2008). Pdx1-Cre;Nr5a2flox/flox embryos had a much greater disruption of the MPC population, and consequently of the formation of fewer acini and β-cells (supplementary material Fig. S8), with the exception of domains of normal acinar tissue composed of NRS5A2+ cells that escaped Nr5a2 inactivation (data not shown).

The PTF1 complex drives Nr5a2 expression in the pancreas

To examine the regulation of Nr5a2, we searched for and analyzed the transcriptional control regions active in the developing pancreas. Ptf1a appears to control the expression of Nr5a2. The level of NRS5A2 protein and the number of Nr5a2-expressing cells were greatly diminished in PTF1A-deficient embryonic pancreas (supplementary material Fig. S9). To determine whether Ptf1a controls Nr5a2 transcription directly, we examined the binding of PTF1A and RBPJL to chromatin from embryonic and adult pancreas. The results of ChIP-seq experiments identified four sites at the Nr5a2 locus with overlapping binding of PTF1A and RBPJL, indicating the presence of the PTF1-L complex (Fig. 8A). Two of the bound sites are within a 1.2-kb conserved upstream region (CUR) common to birds as well as mammals, and the sites themselves are highly conserved (Fig. 8F). The distal site conforms to the PTF1 consensus-binding sequence, and quantitative measure by conventional ChIP-PCR confirmed the presence of PTF1A on this site (Fig. 8E). RNApol2 and nucleosomess containing the active H3K4me2 chromatin mark are also present.

To determine whether the 1.2-kb CUR is enhancer-like and dependent on PTF1, we generated transgenes bearing the CUR linked to a minimal promoter with a lacZ reporter gene. Transgenic expression at E17.5 was specific to the acinar cells in the pancreas with high penetrance (18 of 27 transgenic embryos) (Fig. 8G). The transgene was not active in other organs in which Nr5a2 is present, such as liver and ovary, but endogenous β-galactosidase in the intestine precludes the detection of low transgenic expression. Thus, the CUR is sufficient for selective transcription to the pancreas.

The dependency of CUR activity on PTF1 binding was demonstrated by results with two altered transgenic constructs. First, disrupting the distal PTF1-binding site of the CUR by base changes within the TC-box prevents binding of the trimeric PTF1 complex as well as of an Rbp subunit alone, and virtually eliminated transgenic expression: β-galactosidase was detected in only a few scattered cells in only one of 13 independent transgenic embryos (Fig. 8G, C-tmut). Second, altering the distal PTF1-binding site by inserting a half-turn of DNA between the E-box and the TC-box also nearly eliminated the activity of the CUR (Fig. 8G, 1.5 turn). This is a particularly rigorous test, because binding by the trimeric PTF1 complex requires paired E- and TC-boxes with unit DNA helical turn spacing (Berets et al., 2006; Thompson et al., 2012). Thus, the activity of the CUR depends on the distal PTF1-binding site, which in turn requires the binding of the complete PTF1 complex, and not on the binding of the bHLH subcomplex or an RBP subunit independently.

Nr5a2, Ptf1a and Rbpjl compose an interacting regulatory triad

Nr5a2 inactivation diminished the expression of Ptf1a and Rbpjl at E13.5 by 93% and 62%, respectively, by 94% and 37% (Fig. 6C) at E17.5 and by 51% for Ptf1a after induced gene inactivation in adult pancreas (Holmstrom et al., 2011). To determine whether these effects could be due to direct regulation by NRS5A2, we examined the binding of NRS5A2 to Ptf1a and Rbpjl loci at E17.5 and adulthood, when PTF1A and RBPJL are restricted to differentiating and differentiated acinar cells. NRS5A2 was bound to four and two sites of the Ptf1a and Rbpjl loci, respectively, in E17.5 chromatin (Fig. 8B,C). Each site contains a sequence that closely conforms to the NRS5A2-binding consensus. The major NRS5A2-bound site in Rbpjl is within the 1.4-kb enhancer/promoter region shown to be active in the pancreas of transgenic mice (Masui et al., 2007), and adjacent to a bound PTF1 complex necessary for the enhancer/promoter activity (Fig. 8C). Similarly, the 2.3-kb Ptf1a autoregulatory enhancer has NRS5A2 bound in close proximity to one of the two PTF1-bound sites necessary for its activity. The extensive regulatory interactions and mutually dependent expression among these three developmental control genes suggest that they form a mutually reinforcing triad that drives acinar development.

DISCUSSION

The spatiotemporal expression pattern of Nr5a2 and the consequences of the Nr5a2 inactivation at different developmental stages indicate that Nr5a2 has at least three temporally distinct functions during pancreatic organogenesis (supplementary material Fig. S10). Nr5a2 is important for the formation and maintenance of the MPCs of the secondary transition, for allocation of MPCs to the acinar lineage and for expansion and differentiation of pre-acinar cells.

Depletion of NRS5A2 during the MPC-stage using Ptf1aCre decreased the number of cells bearing MPC markers, and the development of all three tissue types that derive from the MPC population was affected. Prenatal insulin content and β-cell mass were reduced by two-thirds, acinar mass by more than nine-tenths and branching of the ductal epithelium was mismanaged and unfinished. Additional developmental defects from NRS5A2 depletion were restricted to acinar tissue. Fewer MPCs were allocated to the acinar lineage compared with the endocrine lineage. Because MYC is crucial for the proliferation of differentiating acinar cells but not of endocrine or ductal cells (Nakhai et al., 2008; Bonal et al., 2009), expansion of the pre-acinar cell population was selectively curtailed
by the loss of Nr5a2-dependent Myc expression. Moreover, the differentiation of the few pre-acinar cells that formed was delayed and incomplete. Thus, the more pronounced mutant phenotype of acinar tissue than for endocrine or ductal tissues is due to these cumulative effects on acinar development.

Earlier inactivation of Nr5a2, prior to the tip MPC stage, by Pdx1-Creearly (Gu et al., 2002) rather than by Ptf1aCre, caused more severe and balanced developmental defects of exocrine and endocrine tissues. Growth of the early bud epithelium, dependent on MPCs, was more acutely curtailed. With the exception of small regions that escaped NR5A2 depletion due to the incomplete action of Pdx1-Creearly, the deleterious effects on the formation of both acinar and β-cells were much greater than with Ptf1aCre-mediated inactivation.

By contrast, late Nr5a2 inactivation using PdxCrelate had much less affect on development than through Ptf1aCre, although some impairment of acinar terminal development was evident (von Figura et al., 2014). The PdxCrelate transgene is effective after the MPC stage, because β-galactosidase-labeled ductal cells have been rarely observed in PdxCre;R26R mice (Heiser et al., 2006). The effects of NR5A2-depletion at the post-MPC stage appear restricted to the completion of acinar differentiation and are consistent with the need for maintenance of acinar cell identity (Holmstrom et al., 2011; Flandez et al., 2014; von Figura et al., 2014).

MPC and pre-acinar cell expansion

The emergence of the MPC population around the periphery and in the tips of the expanding epithelium corresponds to the onset of the ‘secondary developmental transition’ (Pictet and Rutter, 1972; Pictet et al., 1972). The secondary transition initiates the resolution of acinar and bipotent duct/endocrine lineages from the MPCs, extensive epithelial growth and branching emanating from epithelial ‘tips’, induction and delamination of islet cell precursors (principally pre-β-cells) and formation of committed pre-acinar

Fig. 8. The Nr5a2 CUR is a pancreas-specific enhancer dependent on PTF1 binding. (A-C) ChIP-Seq analyses with chromatin from E17.5 and adult pancreas identify sites bound by NR5A2, PTF1A and RBPjl loci. ARE is the PTF1a autoregulatory enhancer; E/P is the Rbpjl enhancer-promoter region. Other markings are similar to Fig. 6D,E. (D) Consensus NR5A2-binding sequences from E17.5 (this study), adult pancreas (Holmstrom et al., 2011), adult liver (Chong et al., 2012) and mouse ES cells (Heng et al., 2010). (F) Phylogenetic conservation of the 5’ flanking region of Nr5a2 using BLAT (Kent, 2002). The two sites bound by PTF1A and RBPjl (open rectangles) contain conserved E- and TC-boxes spaced one DNA turn apart. The distal site conforms precisely to the PTF1-binding consensus, and PTF1A binding was confirmed by quantitative measurement by ChIP-PCR (E). PTF1A was not enriched at three loci (Arx, Ngn3/Neurog3, Meox2) active in non-exocrine compartments of the pancreas and without PTF1-binding elements. Error bars are s.d.; Student’s t-test, unpaired. (G) The CUR directs pancreas-specific expression of a lacZ reporter in E17.5 embryos that is dependent on an intact distal PTF1-binding site. w.t., unaltered CUR. Expression was detected in 18 of 27 founder transgenic embryos and uniform throughout the pancreas in nine embryos. Inset, staining of the nuclear β-galactosidase in acinar (a), but not in endocrine (e), ductal or connective tissue cells in an eosin-stained section from the pancreas. TC-mut, mutation of the TC-box disrupts transgene expression. Inset, detail from the single embryo that had only a few pancreatic cells that stained for β-galactosidase. Insert, 1.5 turn, insertion of 5 bp between the E- and TC-boxes also disrupts transgene expression. Inset, scattered cells stained in four of 11 transgenic embryos. Dorsal and ventral lobes of pancreas are outlined (dashed lines).
cells – all of which are descendants of the modified MPCs (Zhou et al., 2007; Pan et al., 2013). A better understanding of the factors that regulate the secondary transition will inform strategies to produce and expand equivalent MPCs from embryonic or induced pluripotent stem cells, and to direct the resolution in vitro toward endocrine β-cells over acinar and ductal cells.

Our results demonstrate that inactivation of Nr5a2 disrupts the secondary transition by limiting the formation and expansion of MPCs and pre-acinar cells. NR5A2 directly sustains Myc expression, and the loss of MYC in NR5A2-deficient pancreatic epithelium is coincident with the onset of hypotrophy. Developmental defects that accrue from inactivation of Nr5a2 are very similar to those of Myc inactivation (Nakhai et al., 2008; Bonal et al., 2009). Depletion of Myc reduces cell replication, expansion of the MPC epithelium and NGN3+ endocrine precursor cells by E12.5. At birth, islet tissue and insulin content appear reduced to one-half, ductal arborization is poor and acinar tissue is severely hypoplastic, with Ptf1a and Mist1 mRNA levels less than half of normal levels. The few remaining acinar cells are smaller, less differentiated and the total amount of amylase is 7% of normal amounts (Nakhai et al., 2008; Bonal et al., 2009).

Myc is also a target of Wnt/β-catenin signaling (He et al., 1998), and the effect of inactivating Ctnnb1 in the early pancreas largely phenocopies that of Myc inactivation (Murtaugh et al., 2005; Wells et al., 2007). Remarkably, the parallel with Nr5a2 inactivation extends further: inactivation of Ctnnb1 by the late-expressing version of Pdx1-Cre had little effect on acinar development (Dessimoz et al., 2005), just as Pdx-CreloxP for Nr5a2 inactivation (von Figura et al., 2014). Thus, for all three gene deficiencies, early inactivation reduces the acinar compartment preferentially, and the pancreatic gland at birth is principally a complex ductal structure with associated islets and with sparse, poorly developed acini. It is probable that NR5A2 and β-catenin cooperate to maintain Myc expression during the MPC phase of development. In some instances, NR5A2 replaces the LEF/TCF factors as the DNA-binding component for Wnt-directed β-catenin gene regulation (Fernandez-Marcos et al., 2011). A similar regulatory relationship might sustain MPC and acinar cell replication during pancreatic development.

**Cross- and collaborative regulation among Nr5a2, Ptf1a and Rbpjl during development**

NR5A2 binds and regulates the known pancreas-restricted developmental regulators Gata4, Foxa2, Mist1, Ptf1a and Rbpjl, as well as Myc. The regions of these genes bound by NR5A2 are active pancreas-specific transcriptional enhancers in transgenic mice (Masui et al., 2007, 2008; Rojas et al., 2009; Meredith et al., 2013) (Mei Jiang, T.G.D., G.H.S. and R.J.M., unpublished). The colocalization of PTF1A and RBPJL at many of these same genomic sites reflects frequent collaboration between NR5A2 and the PTF1 complex, and co-immunoprecipitation experiments indicate that NR5A2 and PTF1A can reside in the same complex (Holmstrom et al., 2011).

Moreover, NR5A2, PTF1A and RBPJL bind each others pancreatic enhancers, and the PTF1A and RBPJL binding sites are necessary for the activity of the enhancers in transgenic fetal pancreas (Fig. 8; Masui et al., 2007, 2008). Our results indicate that this binding is important during development, because Ptf1a and Rbpjl expression decreases dramatically after Nr5a2 inactivation, and, conversely, Nr5a2 expression is lost upon Ptf1a inactivation. Thompson and colleagues have shown that PTF1A also resides on the Nr5a2 enhancer region that we identified in this study in chromatin from an immortalized acinar cell line that has a gene expression profile consistent with embryonic progenitor cell character (Thompson et al., 2012). Thus, it appears that the regulatory interactions persist throughout pancreatic development. Nr5a2 and Ptf1a are crucial at several developmental stages, so that each must change which sets of genes they control as development progresses. It will be informative to discern the roles they play in changing the developmental gene regulatory network during pancreatic development and how their collaboration is preserved for different sets of genes at progressive developmental stages. A summary of the gene regulatory interactions derived from this study is provided in supplementary material Fig. S11.

Greater insight into the nature, formation and expansion of pancreatic MPCs could be applied to improving the yield of β-like cells from pluripotent cells in vitro. In this regard, NR5A2 might have a special utility: its functional ligand-binding pocket offers the opportunity for natural (Lee et al., 2011) or synthetic compound agonists to enhance NR5A2-driven formation of the MPCs, then suppress NR5A2 activity with inverse agonists (Busby et al., 2010) to bias endocrine cell formation by suppressing acinar cell fate.

**MATERIALS AND METHODS**

**Genetically modified mouse lines**

Ptf1aCre mice have the protein-coding region of Ptf1a replaced by that of Cre recombinase, containing an N-terminal nuclear localization signal (Kawaguchi et al., 2002). Pdx1-CreTA mice have the protein-coding region of Pdx1 replaced with that of TAoff (Holland et al., 2002). Nr5a2−ΔloxP mice have the Nr5a2 exon 5, which codes for the second zinc-finger of the DNA-binding domain, flanked by lox-P sites (Lee et al., 2008). Nr5a2 conditional knockout mice (Nr5a2ΔAP) are homozygous for the floxed allele of Nr5a2 and heterozygous for Cre recombinase replacement of the Ptf1a gene. Pdx1-CreERTm mice contain the Cre recombinase gene driven by the mouse 5.5-kb Pdx1 promoter (Gu et al., 2002). Details of the cloning and transgenic analysis of Nr5a2 regulatory regions are provided in the supplementary material methods. University of Texas Southwestern Institutional Animal Care and Use Committee approved all of the animal experiments.

**Chromatin immunoprecipitation studies and transcriptome analyses**

For this study, we performed ChIP-Seq of E17.5 pancreatic chromatin for NR5A2, H3K4me2 and RNA polymerase II. Preparation of mouse pancreatic chromatin, immunoprecipitation, quantification, preparation of Illumina sequencing libraries and their analysis were performed as described previously (Meredith et al., 2013), with details and modifications described in the supplementary material methods.

Comprehensive analysis of the mRNA population in E13.5 normal or Nr5a2ΔAP pancreas was performed by RNA-Seq, using total RNA pooled from three normal pancreases or pooled from three Nr5a2ΔAP pancreases. RNA-Seq analysis of the E17.5 pancreas has been reported previously (Masui et al., 2010; Meredith et al., 2013). Differentially expressed genes were identified using edgeR (McCarthy et al., 2012).

**Dissections, tissue preparation and immunostaining**

Histology and immunohistology were performed as described previously (Masui et al., 2007, 2008). Details and procedural modifications are in the supplementary material methods; primary and secondary antibodies are listed in supplementary material Table S1.

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Competing interests

The authors declare no competing financial interests.

Author contributions

M.A.H., G.H.S. and R.J.M. designed the experiments. M.A.H., G.H.S., C.Q.H., T.G.D., T.M., Y.-L.M. and J.X. performed the experiments and analyzed the data. M.A.H., G.H.S. and R.J.M. prepared the figures, wrote and revised the manuscript.

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Supplementary material

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