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DELETION OF RETINOIC ACID RECEPTOR β (RARβ) IMPAIRS PANCREATIC ENDOCRINE DIFFERENTIATION

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Abstract

All-trans retinoic acid (RA) signals via binding to retinoic acid receptors (RARs α, β, and γ). RA directly influences expression of Pdx1, a transcription factor essential for pancreatic development and β-cell maturation. In this study we follow the differentiation of cultured wild-type (WT) vs. RARβ knockout (KO) embryonic stem (ES) cells into pancreatic islet cells. We found that RARβ KO ES cells show greatly reduced expression of some important endocrine markers of differentiated islet cells, such as glucagon, islet amyloid polypeptide (Iapp), and insulin 1 (Ins1) relative to WT. We conclude that RARβ activity is essential for proper differentiation of ES cells to pancreatic endocrine cells.

Keywords

Retinoic acid; RARβ; Stem cell; Endocrine; Pancreas; Islet cells

Introduction[1]

In 2011 there were an estimated 366 million cases of diabetes worldwide, according to the International Diabetes Federation, and these cases are estimated to increase to 522 million by 2030 [1, 2]. In the U.S. there were 23.7 million diagnosed cases, with an estimated healthcare cost of $113 billion [2, 3]. Diabetes results when insulin production by pancreatic β-cells does not meet the metabolic demand of peripheral tissues such as liver, fat, and muscle [4]. A reduction in β-cell number and function leads to hyperglycemia in both type 1 and type 2 diabetes [4]. In type 1 diabetes, insulin-producing pancreatic β-cells lose self-tolerance and this gives rise to hyperglycemia [5]. Each year in the United States there are over 30,000 new cases of type I diabetes diagnosed [6]. Patients with type I diabetes can

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[1]*Abbreviations:* ES, embryonic stem; Gcg, glucagon; Iapp, islet amyloid polypeptide; Ins1, insulin 1; KO, knockout; Ngn3, Neurogenin3; RA, all-trans retinoic acid; RAR, retinoic acid receptor; RARE, retinoic acid response element; Sst, somatostatin; WT, wild-type.

control their blood glucose level with insulin supplements [7]. However, the differentiation of stem cells into pancreatic β-cells could be a long term, better solution [8, 9].

Mouse embryonic stem (ES) cells are pluripotent cells derived from the inner cell mass of blastocyst-stage (day 3.5) embryos [9, 10]. Upon LIF removal, ES cells spontaneously differentiate into all three primary embryonic germ layers: endoderm, mesoderm, and ectoderm [9]. Several research groups have shown that the directed differentiation of ES cells along the endocrine pathway can be achieved by using a wide range of growth/differentiation factors, including retinoic acid (RA) treatment [11–16].

The effects of RA on cells and tissues are known to occur through RA binding and activation of retinoic acid receptors (RARα, RARβ, and RARγ) and their isoforms [17, 18]. Each RAR has some specific functions and activates specific subsets of genes [19–21]. RA signaling is crucial for endocrine pancreatic development in Xenopus [22]. In addition, transgenic mice that express a dominant negative RARα403 mutant, used to ablate all retinoic acid-dependent processes *in vivo*, lack both dorsal and ventral pancreas, and die at the neonatal stage [23]. Impaired pancreatic islet function was also observed in vitamin A deficiency and repletion rodent models [24, 25]. Another study, focused on the role of CRABP1 and RBP4 in pancreatic differentiation, showed an increase in RARβ expression in early differentiation [10]. While previous studies showed that RARβ is expressed during pancreas development, little is known about the role of RARβ in normal islet maintenance and function in adult animals [26, 27].

The RARβ gene is frequently hypermethylated at CpG islands in human pancreatic adenocarcinoma [28] and this phenomenon could also be associated with other pathologies such as diabetes. We hypothesized that RARβ plays a key role in ES cell differentiation to pancreatic endocrine cells, and that this function of RARβ may be altered in pancreatic physiopathology. In this report, we measured the expression profiles of various pancreatic differentiation and retinoid signaling markers in WT and RARβKO ES cells. We show that the lack of all three isoforms of RARβ impairs the differentiation of cultured ES cells to pancreatic β-like cells.

Materials and Methods

Cell culture and isolation of RARβ homozygous ES cell lines

Murine J1 wild-type ES cells were cultured as described previously [29]. 129; C57BL/6 RARβ homozygous null mice were provided by Dr. Pierre Chambon (Strasbourg-Cedex, France) [27]. Mice were housed and treated according to appropriate WCMC IACUC guidelines. Blastocysts were harvested on day E3.5 and individually cultured in ES cell medium as previously described [29] to generate RARβ KO ES cells by homologous recombination. These RARβ KO ES cells were karyotyped and shown by Southern analysis to possess two RARβ KO alleles (not shown).

Pancreatic endocrine differentiation protocol

A slightly modified version of the established protocols published by Borowiak [13] and D'Amour [14] was used to carry out differentiation of hormone expressing endocrine cells from mouse ESCs. Prior to differentiation, ESCs were seeded at 5×10^5 on 30 mm gelatin-coated plates. After overnight culture, cells were exposed to 250 nM BIO-Acetoxime (EMD Bioscience, San Diego, CA) + 50 ng/ml activin A (R&D Systems, Minneapolis, MN) in Advanced RPMI (GIBCO, Grand Island, NY) supplemented with 1X L-Glu and 0.2% FBS (GIBCO) for 1 day, and then to activin A alone in the same media. Cells were then cultured for 4 days to induce endoderm differentiation. For pancreatic progenitor induction, the cells were transferred to 50 ng/ml FGF10 (R&D Systems), 7.5 μM cyclopamine (Calbiochem,

San Diego, CA) in DMEM supplemented with 1X L-Glu, 1X Pen/Strep, and 1X B27 (Invitrogen, Grand Island, NY) for 2 days. At day 7, cells were transferred to FGF10, cyclopamine, and 2 μM all-trans RA (Sigma, St. Louis, MO) in DMEM supplemented with 1X L-Glu, 1X Pen/Strep, and 1X B27 (Invitrogen) for 4 days. At day 11, cells were cultured in the presence of DMEM supplemented with 1X L-Glu, 1X Pen/Strep, and 1X B27 for 3 days. At day 14, CMRL (Invitrogen) medium was added and supplemented with 1X L-Glu, 1X Pen/Strep, 1X B27, 50 ng/ml IGF-1 (R&D Systems), 50 ng/ml HGF (R&D Systems), and, in some experiments, 10 mM nicotinamide (Sigma) for 3 more days. All stock compounds were made in either PBS or ethanol.

RT-PCR analysis

Various markers for endodermal (day 5), pancreatic progenitor (day 11), endocrine progenitor (day 14) and endocrine (day 17) differentiation were analyzed by semi-quantitative RT-PCR in J1 WT and RARβ KO ESCs. Specific primers used and amplification conditions are listed in Table 1. Total RNA extraction, semi-quantitative, and quantitative PCR reactions were performed as previously described [30]. Amplified PCR products were resolved on 1.5% agarose gels and visualized by staining with ethidium bromide. PCR bands were sequenced for verification of the correct amplicon. Quantitation was performed using ImageJ software (National Institutes of Health) from three experimental, independent biological repeats.

Indirect immunofluorescence

Immunofluorescence assays on cells and tissue sections were performed as previously described [31]. Briefly, differentiated samples were fixed using 4% (w/v) paraformaldehyde and membrane permeabilization (for cells only) was done with 0.3% (w/v) Triton-X 100 (Sigma). Unspecific sites were blocked using 2% BSA for 30 min prior to incubation with rabbit polyclonal anti–Pdx1 (Millipore, 06-1379, 1:1000), rabbit anti-C-Peptide (Cell Signaling, 4593, 1:500, Danvers, MA) and mouse monoclonal anti-Glucagon (Abcam, ab10988, 1:200) primary antibodies. Phalloidin-TRITC (Millipore, FAK100, 1:1000, Billerica, MA) was used to stain the actin stress fibers network (F-actin). Nuclei were stained using DAPI contained in Vectashield® mounting medium for fluorescence (Vector labs, Burlingame, CA). Quantitation of C-peptide positive stained cells and islet surface area was performed using NIS-Elements Advanced Research software (Nikon).

Microarray analysis

Total cellular RNA was isolated with Trizol reagent (Invitrogen), and concentration and integrity were assessed using Nanodrop® technology (Thermo Scientific). Preparation of cRNA, chip hybridization and scanning were carried out by the Microarray Core Facility at Weill Cornell Medical College (WCMC). The microarray analyses were performed following the Affymetrix Genechip expression analysis technical manual. The fragmented cRNA was hybridized to the microarray chips (MG-430.2, Cat. #900496, Affymetrix, CA, USA), which include over 45 000 transcripts representing 34 000 substantiated mouse genes. Data analysis was performed using Genespring v7.0 software (Agilent Technologies) as previously described [30].

Statistical analyses

All experiments were performed at least 3 times using independent biological triplicates. Results are presented as means ± SEM. All statistical tests were performed using GraphPad InStat software version 3.10. A p-value of ≤0.05 indicates statistical significance.

Results

Pancreatic differentiation and assessment of pancreatic markers in WT murine ES cells

We selected the endocrine differentiation protocol of D'Amour et al [14] because this protocol resulted in expression of later stage endocrine markers using human ES cells [14]. We modified the D'Amour et al protocol by replacing Wnt3a with BIO-acetoxime. Wnt3a has been documented as being important for mesendoderm specification and BIO-acetoxime acts in the same signaling pathway [32, 33]. Second, we included nicotinamide during the last stage of differentiation; various published protocols included this reagent because of its reported effectiveness in supporting pancreatic differentiation [34, 35] (Fig. 1A, B).

To characterize the differentiation of WT ES cells, we harvested cellular extracts at various time points (Fig. 1B) and assessed the mRNA levels of various differentiation markers by RT-PCR. LIF removal combined with BIO and Activin A caused a major decrease in transcripts of the stem cell markers Nanog [36] and Rex1 (Zfp42) [37] (Fig. 1B, lane 6) compared to the levels in ES cells cultured with LIF (Fig. 1B, lanes 1–4). Nanog and Rex1 transcript levels remained low for the remainder of the differentiation process (Fig. 1B, lanes 7–12). While we observed robust expression of transcripts for glucagon (Gcg), a functional marker of pancreatic α-cells (37), by day 14 (Fig. 1B, lane 8), somatostatin (Sst), a hormone secreted by δ-cells [38], was detectable as early as day 5 (Fig. 1B, lane 6). Insulin-1 (Ins1), a β-cell marker [38], was detected by day 11 of the differentiation process and its expression fluctuated depending on the presence of HGF, IGF1, or both factors together (Fig. 1B, lanes 9 to 11). The most consistent expression of all three pancreatic endocrine differentiation markers tested was observed in the presence of HGF, IGF1, and nicotinamide from days 14 to 17 (Fig. 1B, lane 12). As another control we cultured WT ES cells in absence of LIF for 17 days but with no other factors added; we observed a decrease in Nanog and Rex1 transcripts, but no induction of the differentiation markers tested (Fig. 1B, lane 5). For comparison, RNA from adult mouse pancreas is shown (Fig. 1B, right panel).

Thus, we demonstrated the differentiation of WT ES cells to endocrine cells capable of expressing pancreatic hormone-encoding genes. This differentiation protocol was then employed to investigate the role of RARβ at specific stages of pancreatic endocrine differentiation.

The Lack of RARβ delays Pdx1 expression during pancreatic endocrine differentiation of ES cells

We next subjected WT and RARβKO ES cells to the endocrine differentiation protocol described above. We confirmed the absence of RARβ transcripts in the RARβKO ES cells by RT-PCR (Fig. 2A). We detected an RA-associated increase in Cyp26a1 mRNA level in both WT and RARβKO ES cells, indicating that the RARβKO ES cells can still respond to RA via RARγ [39] (Fig. 2A) to activate Cyp26a1 transcriptionally.

We evaluated the expression profile of Pdx1, a master regulator of pancreatic development [40], in WT and RARβKO ES cells, in the presence or absence of RA treatment (Fig. 2B). RT-PCR experiments revealed lower levels of Pdx1 in RARβKO (55.2%, $p=0.047$) cells compared to WT in untreated conditions (Fig. 2B, C). Interestingly, RA treatment caused a remarkable increase in Pdx1 expression in both WT (~3.3-fold, $p=0.0012$) and RARβKO (~4.1-fold, $p\leq0.0001$) cells compared to untreated conditions (Fig. 2B, C). Microarray-based transcriptome analyses performed on WT and RARβKO ES cells showed an increase in RARγ gene expression in RARβ-null cells following RA treatment (Suppl. Table S1). Although we observed no statistically significant changes in RARα expression in RARβKO ES cells compared to WT, RT-PCR analysis confirmed the induction of $RAR\gamma_2$ expression in RA-treated RARβKO ES cells (Fig 2B). The absence of RARβ, combined with low

RARγ$_2$ expression, lead to reduced Pdx1 levels (Fig. 2C). Moreover, the RA-dependent RARγ$_2$ induction observed in RARβ-null cells (~5-fold, p=0.0162) correlates with a restoration of Pdx1 expression (Fig. 2C).

WT and RARβKO ES cells were differentiated into pancreatic endocrine cells, as described in Figure 1, and we performed indirect immunofluorescence staining at different time points to determine the Pdx1 protein expression profile (Fig. 2D). We detected Pdx1 protein in differentiating WT cells by day 5, and Pdx1 was detectable at all of the other differentiation stages tested (Fig. 2D). In contrast, Pdx1 protein was absent from nuclei of differentiating RARβKO cells at days 5 and 11, was only detected at day 14, and was only detected at a low level (Fig. 2D). These data indicate that the lack of RARβ results in a delay in the induction of Pdx1, which could potentially affect subsequent steps in endocrine specialization.

The Absence of RARβ expression impairs the pancreatic endocrine differentiation process

Decreased expression of pluripotency factors, including Nanog, in ES cells is required for proper ES cell differentiation [41]. By comparing Nanog transcript levels in WT and RARβKO ES cells, we observed the sustained expression of Nanog mRNA in RARβKO cells, while in WT Nanog mRNA levels declined over time (Fig. 3A). A similar phenomenon was also observed for other pluripotency factors, such as Pou5f1 and Sox2, in microarray experiments comparing WT and RARβKO ES cell transcriptomes (Suppl. Table S1 and Fig. S2). Furthermore, Neurogenin-3 (Ngn3), a master transcriptional regulator during the onset of pancreatic islet differentiation [40, 42, 43], displayed a transient induction pattern in WT but was not induced in RARβKO cells (Fig. 3A). The RARβ$_2$ isoform mRNA was expressed during this ES differentiation protocol in WT cells, but not in RARβ KO cells (Fig. 3A–C). Hypoxanthine phosphoribosyltransferase 1 (HPRT1) mRNA levels did not change during the ES differentiation, and thus this transcript was used as a loading control in RNA expression assays (Fig. 3A–C).

Like Ngn3, Paired-box 6 (Pax6) and Islet1 (Isl1) are two transcription factors which are expressed from the intermediate (“mid”) to the terminally differentiated (“late”) stages [43–46]. While we noted no differences in Pax6 expression between WT and RARβKO, we detected the delayed expression of Isl1 in RARβKO as compared to WT cells (day 14 versus day 11) (Fig. 3B).

We also analyzed the expression of different, functional, endocrine differentiation markers, such as glucagon (Gcg; α-cells), insulin-1 (Ins1; β-cells) and islet amyloid polypeptide (IAPP; β-cells) [14, 47, 48] in WT and RARβ KO cells (Fig. 3C). RARβ KO cells exhibited reduced expression of these three transcripts as compared to WT (Fig. 3C). By day 17 Gcg, Ins1, and Iapp transcripts, respectively, were higher by ~5-fold (p=0.04), ~120-fold (p=0.013), and ~7-fold (p=0.0002) in WT as compared to RARβKO (Fig. 3C).

Taken together, these data indicate that retinoid signaling through RARβ plays a central role in ES differentiation to pancreatic endocrine cells by regulating the expression of certain master genes at early and intermediate stages of the differentiation process. The absence of RARβ results in impaired expression of functional markers of islet cells.

Discussion

By using an ES cell-based directed differentiation system we demonstrate a crucial role for RARβ in proper pancreatic endocrine cell differentiation. The absence of RARβ leads to a decrease in terminal pancreatic differentiation and to a great reduction in expression of functional markers, such as insulin1 and glucagon. We also conclude that Pdx1 expression during the pancreatic differentiation process is delayed in the absence of RARβ (Fig. 2).

Pdx1 is a key transcription factor in the early determination of pancreatic progenitors and bud expansion [43, 44, 49, 50]. RA directly induces Pdx1 expression in WT ES cells [49]. Moreover, a putative retinoic acid response element (RARE) is located at ~3 kb upstream of the transcription start site of Pdx1 in F9 teratocarcinoma cells by ChIP-chip analyses (unpublished data). We speculate that RARα and RARβ together participate in the Pdx1 biphasic expression pattern during endocrine pancreatic differentiation, as reviewed by Soria [43]. However, the lack of RARβ would need to be compensated by RARγ, which is induced by RA treatment from day 8 through day 11 (Figure 1A and 2B, C). This would explain the delayed Pdx1 expression we observe in the RARβKO cells (Fig. 2D).

Pdx1 mis-expression was previously associated with severe β-cell dysfunction and increased cell death [51]. Accordingly, we show that the lack of RARβ causes a reduction in pancreatic β-cell terminal differentiation in this cell culture system, as assessed by markers such as Ins1 and Iapp (Fig. 3C). Recent findings by Dalgin et al. [52] in zebrafish also link RA signaling and endocrine cell fate. Our data suggest that the lack of RARβ also results in a decrease in α-cell differentiation, characterized by reduced expression of glucagon in our cell culture system (Fig. 3C).

Like Pdx1, the bHLH transcription factor Neurogenin3 (Ngn3) is a key protein in the commitment of endoderm cells to become pancreatic precursors [42, 44, 48]. Among the transcription factors involved in pancreas development, Ngn3 is the earliest to be expressed in the endocrine differentiation pathway [44, 53]. We found that the RARβKO ES cells displayed decreased levels of Ngn3 transcripts during pancreatic differentiation (Fig. 3A). Pax6 and Isl1 are two transcription factors that play roles in endocrine lineage specification after bud formation [46, 54]. Since Pax6 and Nkx6.1 transcript levels are not altered in the RARβKO cells (Fig. 3B and Fig. S1B) and Isl1 expression is only delayed (Fig. 3), we speculate that the absence of RA signaling through RARβ is not sufficient to abrogate completely endocrine differentiation, but may lead to significant defects in islet cell function. Moreover, the low, residual expression of Ngn3 mRNA detected in the RARβKO ES cells could account for the low levels of Gcg and Iapp mRNA observed in these cells (Fig. 3C). According to our observations, the directed-differentiation protocol used in our experiments gives raise to a mixed population of α, β, and δ-cells, considering the simultaneous detection of their respective markers Gcg, Ins1, and Sst (Figs. 1 and 3). Interestingly, the absence of RARβ in our model seems to specifically impair α and β-cell terminal differentiation, while Sst expression (δ-cell marker) is unchanged in RARβKO cells (Fig. S1A).

Another aspect of cell differentiation that is altered by the absence of RARβ is the pluripotency marker Nanog; Nanog expression persists in the mutant RARβ cells throughout the endocrine pancreatic differentiation protocol (Fig. 3A). Microarray analyses also highlighted the diminished capacity of RARβKO cells to repress other pluripotency genes, such as Pou5f1 and Sox2, upon RA treatment (Fig. S2). This was accompanied by the reduced induction of early pancreatic endoderm markers, such as Gata4, Gata6, and FoxA1 (Fig. S2) [44, 55]. Thus, our data suggest that RARβ, in addition to its role in Pdx1 transcriptional regulation, affects other aspects of ES cell differentiation in this cell culture system.

RARβ expression is known to depend on epigenetic regulation [56, 57]. Aberrant hypermethylation of the $RAR\beta_2$ promoter was reported in different pancreatic disorders, such as cancer, diabetes, and chronic pancreatitis [58–60]. Therefore, we suggest that the epigenetic silencing of RARβ, combined with partial vitamin A deficiency, could play a causal role in various diseases involving the pancreas, including diabetes and pancreatic adenocarcinoma.

Conclusions

The production of insulin secreting endocrine cells from ES cells using RA-based protocols is a promising tool for diabetic therapy. Our findings provide new insights into the role of RARβ in pancreatic endocrine differentiation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Retinoic Acid Receptor β controls aspects of differentiation
- Nanog mRNA remains high in RARβ−/− ES cells
- Pdx1 Expression is delayed and greatly reduced in RARβ−/− vs WT cells

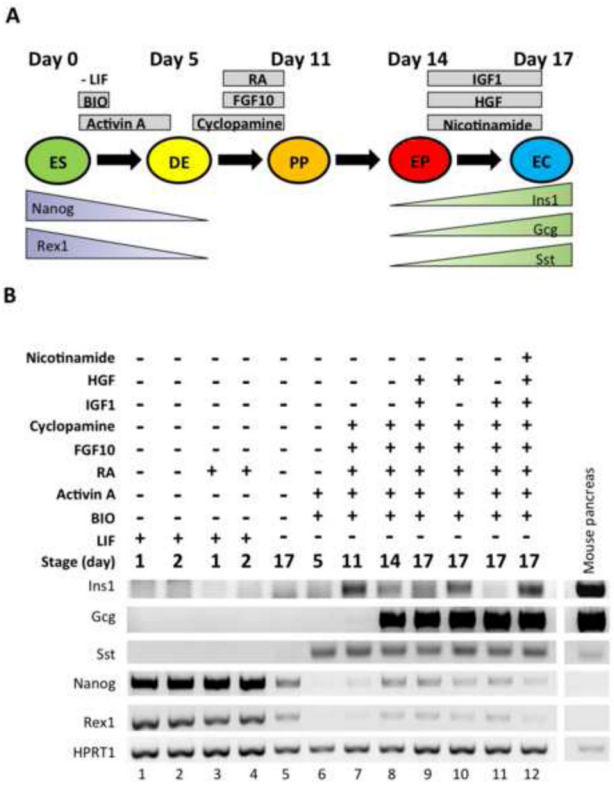

Figure 1. Differentiation of WT ES cells to pancreatic endocrine cells

(A) Schematic representation of the endocrine differentiation protocol (**14**). WT murine ES cells are treated with different growth factors to differentiate the cells into definitive endoderm (DE), pancreatic progenitor (PP), endocrine progenitor (EP), and endocrine cells (EC). (B) WT ES cells were subjected to the 17-day differentiation protocol. Each lane represents a different condition at a specific time point. RT-PCR analyses were performed to monitor the expression of pancreatic differentiation markers such as insulin-1 (Ins1), glucagon (Gcg), somatostatin (Sst), neurogenin-3 (Ngn3), Pdx1, and Sox17, as well as the ES cell markers Nanog and Rex1. HPRT1 was used as a loading control. Pancreas extracts from C57BL/6 WT mice were used as a positive control (far right lane). Sample lanes are labeled 1–12 at the bottom. This experiment was performed 7 times, starting with fresh cells, with similar results.

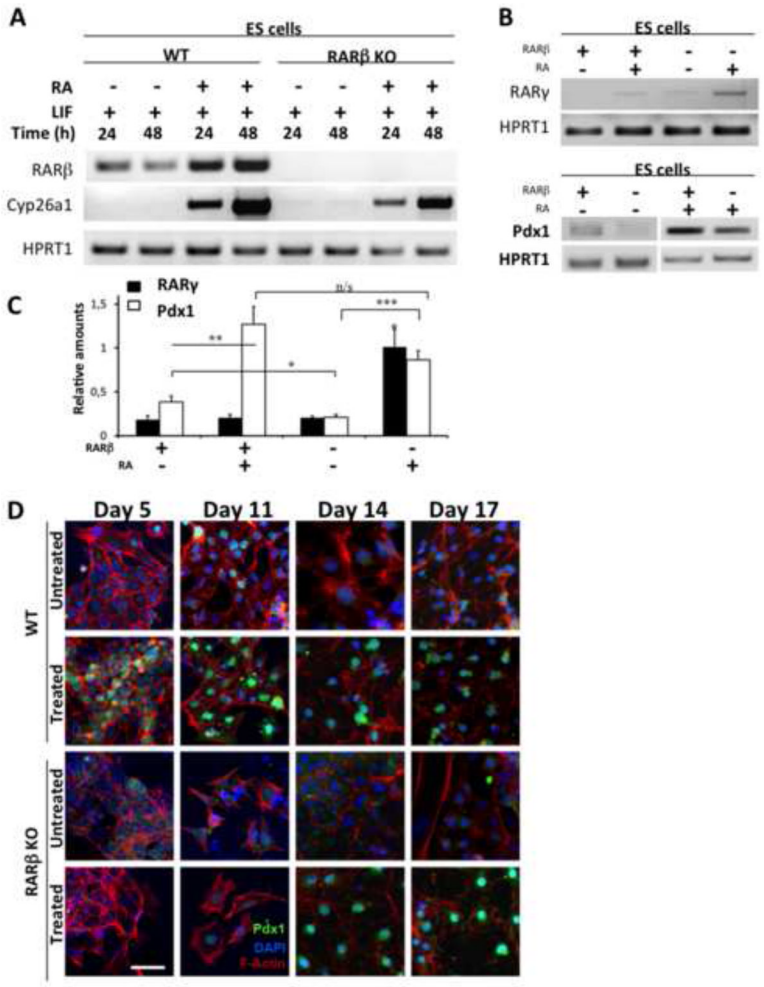

Figure 2. Impact of RARβ deletion on Pdx1 protein expression

(A) RT-PCR analysis confirming the lack of RARβ transcripts in RARβKO ES cells. Analysis of Cyp26a1, a RA-responsive gene, demonstrates the presence of RA signaling activity via other receptors in RARβKO cells. HPRT1 was used as a loading control. (B) Representative RT-PCR analysis of Pdx1 and RARγ$_2$ expression in WT and RARβ KO ES cells, control untreated (NT) and RA treated (1 μM, 48 h). (C) Relative amounts of Pdx1 and RARγ$_2$, normalized to HPRT1 levels, are shown in the histogram (n=3; *: p≤0.047; **: p=0.001; ***: p≤0.0001). (D) Indirect immunofluorescence staining for Pdx1 protein (green) in WT and RARβKO cells at 5, 11, 14, and 17 days in the absence (untreated) or in the presence (treated) of growth factors used in the differentiation protocol. Cells were counterstained using rhodamine-conjugated phalloidin, which binds to F-actin (red), and nuclei were stained with DAPI (blue) (Bars = 50 μm).

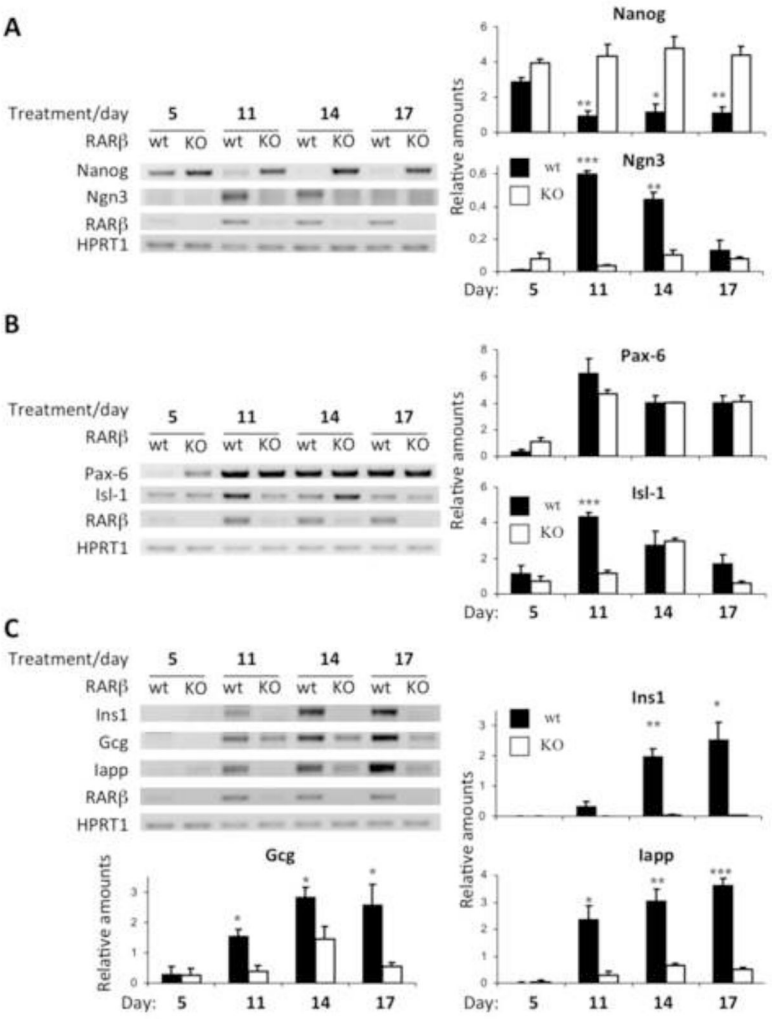

Figure 3. Expression of pancreatic differentiation markers in WT and RARβKO ES cells

Transcript expression analyses of (A) early, (B) mid, and (C) late stage endocrine pancreatic differentiation markers in WT and RARβKO ES cells. RT-PCR analyses of (A) Nanog, Ngn3, (B) Pax6, Isl1, and (C) Ins1, Gcg, and Iapp mRNA were performed in both cell lines at 5, 11, 14, and 17 days of the differentiation protocol (Fig. 1A). Relative levels, normalized to HPRT1 levels for each marker tested, are shown in histograms (n=3; *: $p \leq 0.05$; **: $p \leq 0.0079$; ***: $p \leq 0.0003$).

Table 1

Primer sequences used for RT-PCR

Primer	Application	Forward sequence (5′-3′)	Reverse sequence (5′-3′)	Product size (bp)
mIns1	RT-PCR	TAGTGACCAGCTATAATCAGAG	ACGCCAAGGTCTGAAGGTCC	289
mGcg	RT-PCR	CCGCCGTGCCCAAGATTTT	CCTGCGGCCGAGTTCCT	232
mSst*	RT-PCR	GAGCCCAACCAGACAGAGAA	GAAGTTCTTGCAGCCAGCTT	150
mNgn3*	RT-PCR	CTGCGCATAGCGGACCACAGCTTC	CTTCACAAGAAGTCTGAGAACACCAG	233
mRARβ	RT-PCR	GATCCTGGATTTCTACACCG	CACTGACGCCATAGTGGTA	248
mRARγ2	RT-PCR	ATGTACGACTGCATGGAATCGT	GATACAGTTTTTGTCACGGTGACAT	366
mNanog	RT-PCR	AAAGGATGAAGTGCAAGCGGTGG	CTGGCTTTGCCCTGACTTTAA	520
mRex1	RT-PCR	GAAAGCAGGATCGCCTCACTGTGC	CGATAAGACACCACAGTACACAC	641
mCyp26a1	RT-PCR	GAAACATTGCAGATGGTGCTTCAG	CGGCTGAAGGCCTGCATAATCAC	272
mPax6	RT-PCR	GCAACCCCCAGTCCCCAGTCAGA	AGTCCATTCCCGGGCTCCAGTTCA	399
mIsl1*	RT-PCR	CCCGGGGGCCACTATTTG	CGGGCACGCATCACGAA	397
mIapp*	RT-PCR	TGGGCTGTAGTTCCTGAAGC	GCACTTCCGTTTGTCCATCT	199
mPdx1	RT-PCR	CTTTCCCGTGGATGAAATCC	GTCAAGTTCAACATCACTGCC	205
mNkx6.1	RT-PCR	AGAGAGCACGCTTGGCCTATTC	GTCGTCAGAGTTCGGGTCCAG	215
HPRT1	RT-PCR	TGCTCGAGATGTGATGAAGG	TCCCCTGTTGACTGGTCATT	192

All primers for RT-PCR are designed around introns, except those marked with *.