EXPERIMENTAL CELL RESEARCH 319 (2013) 2196–2204

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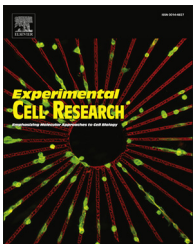

Research Article

Deletion of retinoic acid receptor β (RARβ) impairs pancreatic endocrine differentiation

Ronald J. Pérez[a,1], *Yannick D. Benoit*[a,1], *Lorraine J. Gudas*[a,b,*]

[a]*Pharmacology Department, Weill Medical College of Cornell University, New York, NY 10065, USA*
[b]*Department of Medicine, Weill Medical College of Cornell University, New York, NY 10065, USA*

ARTICLE INFORMATION

Article Chronology:
Received 12 March 2013
Received in revised form
30 May 2013
Accepted 31 May 2013
Available online 10 June 2013

Keywords:
Retinoic acid
RARβ
Stem cell
Endocrine
Pancreas
Islet cells

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 beta-cell (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.

Introduction

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 United States 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 the 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 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

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

*Corresponding author at: Department of Pharmacology, Weill Cornell Medical College, New York, NY 10065, USA. Fax: +1 212 746 8835.
E-mail address: ljgudas@med.cornell.edu (L.J. Gudas).

[1] These authors contributed equally to this research.

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http://dx.doi.org/10.1016/j.yexcr.2013.05.032

[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 1× 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 1× 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 1× L-Glu, 1× Pen/Strep, and 1× B27 (Invitrogen) for 4 days. At day 11, cells were cultured in the presence of DMEM supplemented with 1× L-Glu, 1× Pen/Strep, and 1× B27 for 3 days. At day 14, CMRL (Invitrogen) medium was added and supplemented with 1× L-Glu, 1× Pen/Strep, 1× 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)

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
mRARγ_2	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 *.