Published in final edited form as:

Differentiation. 2017 ; 98: 55–61. doi:10.1016/j.diff.2017.11.002.

RAR β 2-dependent signaling represses neuronal differentiation in mouse ES cells

Sri L. Kona¹, Amita Shrestha¹, Xiaoping Yi¹, Serenthia Joseph¹, Humberto Munoz Barona², and Eduardo Martinez-Ceballos^{1,*}

¹Department of Biological Sciences and Chemistry, Southern University and A&M College, Baton Rouge, LA 70813, USA

²Department of Physics and Mathematics, Southern University and A&M College, Baton Rouge, LA 70813, USA

Abstract

Embryonic Stem (ES) cells are pluripotent cells that can be induced to differentiate into cells of all three lineages: mesoderm, endoderm, and ectoderm. In culture, ES cells can be differentiated into mature neurons by treatment with Retinoic Acid (RA) and this effect is mediated mainly through the activation of the RA nuclear receptors (RAR α , β , and γ), and their isoforms. However, little is known about the role played by specific RAR types on ES cell differentiation. Here, we found that treatment of ES cells with AC55649, an RAR β 2 agonist, increased endodermal marker gene expression. On the other hand, we found that the inhibition of RAR β with 5 μ M LE135, together with RA treatment, increased the efficiency of mouse ES cell differentiation into neurons by more than 4-fold as compared to cells treated with RA only. Finally, we performed proteomic analyses on ES cells treated with RA vs RA plus AC55649 in order to identify the signaling pathways activated by the RAR β agonist. Our proteomic analyses using antibody microarrays indicated that proteins such as p38 and AKT were upregulated in cells treated with RA plus the agonist, as compared to cells treated with RA alone. Our results indicate that RAR β may function as a repressor of neuronal differentiation through the activation of major cell signaling pathways, and that the pharmacological inhibition of this nuclear receptor may constitute a novel method to increase the efficiency of ES to neuronal differentiation in culture.

Keywords

Hoxa1; RAR beta; Embryonic stem cells; neuronal differentiation; retinoic acid

*Author to whom correspondence should be addressed. Eduardo_martinez@subr.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1. Introduction

Mouse Embryonic Stem (ES) cells are pluripotent in nature with a potential to differentiate along any of the three germ layers: ectoderm, mesoderm, or endoderm (Murry and Keller, 2008; Weitzer, 2008). In culture, neuronal differentiation of ES cells can be achieved by treatment with Retinoic Acid (RA), a Vitamin A derivative (Martinez-Ceballos and Gudas, 2008). Although ES neuronal differentiation can occur in both monolayer (2D) and tri-dimensional (3D) culture conditions, a great percentage of endodermal cells are obtained and neuronal differentiation is impaired when differentiation is attempted using 2D cultures (Gerrard et al., 2005). In this regard, we previously reported that overexpression of the endodermal master gene *Sox17* prevented ES cell differentiation into neurons even in the presence of RA (Martinez Ceballos and Gudas, 2008). Thus, these observations suggest that unrestricted endodermal gene expression can repress neuronal differentiation.

In cells, RA exerts its effects by entering the nucleus and binding to the Retinoic Acid Receptors or RARs, of which there are three types, RAR α , β , and γ , and their isoforms (reviewed by Chambon, 1996). Because the RA-bound receptors can form homodimers, or heterodimers with the Retinoid X Receptors (RXRs), it is believed that each RAR has some specific function and activates specific subsets of genes (Gudas, 2012). For instance, gene disruption experiments demonstrated that RAR γ is required for the RA-induced expression of *Hoxa1*, a gene whose protein product is member of the homeobox (Hox) family of transcription factors (TFs) (Boylan et al., 1993; Boylan et al., 1995; Akin and Nazarali, 2005; Al Tanoury et al., 2014) and is required for the neuronal differentiation of mouse ES cells in vitro (Martinez-Ceballos and Gudas, 2008). Conversely, it has been reported that RA can induce endodermal gene expression in F9 teratocarcinoma cells acting mainly through RAR β 2 (Zhuang et al., 2003), and that the lack of RAR β impairs the differentiation of cultured ES cells along the endodermal lineage (Pérez et al., 2013). While these studies indicate that activation of RAR β induces endodermal cell fates, little is known about the effect of this TF on the neuronal differentiation of ES cells. In the present study, we examine the effect of RAR β 2 activation on embryonic stem cell differentiation by employing AC55649 (AC, an RAR β 2 agonist) and/or LE135 (LE, an RAR β antagonist) on 2D or 3D mouse ES cell cultures.

We observed that AC, in combination with RA, upregulated the mRNA levels of various markers of endodermal differentiation. Inhibition of RAR β 2, on the other hand, was found to increase the efficiency of neuronal differentiation in RA-treated ES cells. To investigate the cell signaling pathways involved on the transduction of the RAR β 2 signal, and to increase our understanding on the molecular mechanism responsible for the RAR β 2-dependent induction of endodermal gene expression, we performed antibody microarray analyses on 3D ES cell cultures treated with RA versus RA plus AC. These experiments indicated that the PI3K/AKT, MAPK/ERK and p38/MAPK cell signaling pathways were significantly activated by the combined treatment of RA plus AC55649 (RA/AC) as compared to RA alone. Our study using pharmacological modulators of RAR β provides a new insight into the molecular mechanism of action of this nuclear receptor. This knowledge may result in novel approaches for a more efficient generation of neurons from ES cells that can be used for cell replacement therapies or to treat neurodegenerative diseases.

2. Materials and Methods

2.1. Cell Culture and Drug Treatment

Mouse J1 Wild-type ES cells (American Type Culture Collection or ATCC) were used in this study. Undifferentiated ES cells were maintained on gelatin-coated dishes in ES medium (Dulbecco's Modified Eagle medium supplemented with 10% ES qualified fetal calf serum (Atlanta Biologicals) as previously described (Addae et al., 2012). In addition, 1×10^3 U/ml leukemia inhibitory factor (LIF, Esgro, Millipore) was added to the medium. To decrease spontaneous differentiation, ES cells were passaged every 48 hours for up to 3 passages before plating for 2D or 3D experiments. AC55649 and LE135 were dissolved in dimethyl sulfoxide (DMSO) at 100 mM and these stock solutions were stored at -80°C until further use.

2.1.1. Monolayer cultures (2D)—ES cells were treated 24 hours after plating with 1 μM RA, 5 μM AC55649, and 5 μM LE135, alone or in distinct combinations. Control cells were treated with DMSO (vehicle). Expression of *RARB*, and various endodermal markers (i.e., *Foxa2*, *Afp*, *Gata6*, and *Sox17*) was examined by quantitative PCR (qPCR) on cells treated with drugs for 48 hours. To determine the effect of pharmacological inhibition of $\text{RAR}\beta 2$ by LE on neuronal induction, monolayer ES cells were treated with 1 μM RA, 5 μM LE, or RA plus LE (RA/LE). Control cells were treated with vehicle only. Cells were cultured for 8 days in Neurobasal medium plus drugs, with medium changes every two days. Cells were fixed and the expression of the neuronal marker Beta Tubulin III was examined by immunofluorescence.

2.1.2. Generation of cell aggregates (3D)—Aggregates, or Embryoid Bodies (EBs), were generated by plating 3×10^6 cells onto 100 mm low attachment plates (Corning, Corning, NY) in ES medium without LIF, as previously described (Martinez-Ceballos and Gudas, 2008). The aggregates were cultured in ES medium for 6 day and the medium was changed every two days. Drug treatments were done on day 4 with RA (5 μM), RA (5 μM) plus LE (5 μM), or vehicle-only (DMSO). On day 6, the EBs were harvested and fixed for immunofluorescence analyses as previously described (Martinez-Ceballos and Gudas, 2008). In separate experiments, EBs were treated at day 4 with RA (5 μM), AC (5 μM), RA (5 μM) plus AC (5 μM), or vehicle-only. Samples were harvested after two days of treatment for either antibody microarray experiments or Western blot assays (see below).

2.2. qPCR analyses

For quantitative PCR assays, total RNA from cell samples was extracted using TRIzol reagent (Thermo Fisher Scientific Life Sciences), according to standard procedures. The concentration of RNA was determined using a NanoDrop (Thermo Scientific). Real-time PCR with SYBR green detection was performed using a QuantStudio 3 thermocycler (Applied Biosystems) and the following primers: *Rplp0* (internal control), forward 5'-AGAACAACCCAGCTCTGGAGAAA-3', reverse 5'-ACACCCTCCAGAAAGCGAGAGT-3' (Martinez-Ceballos et al., 2005); *Foxa2*, forward 5'-CGGCCAGCGAGTTAAAGTAT-3', reverse 5'-TCATGTTGCTCACGGAAGAG-3'; *Afp*, forward 5'-AGCTGACAACAAGGGGAGTG-3', reverse 5'-

TTAATAATGGTTGTTGCCTGGA-3'; Gata6, forward 5'-
 ACCATCACCCGACCTACTCG-3', reverse 5'-CGACAGGTCCCTCCAACAGGT-3';
 Sox17, forward 5'-GGAATCCAACCAGCCCACTG-3', reverse 5'-
 GGACACCACGGAGGAAATGG-3'; The sequence of the *Rarb* primers is available upon
 request. Reactions were run in triplicate in three independent experiments. Expression data
 were normalized to the geometric mean of housekeeping gene *Rplp0* to control the
 variability in expression levels and were analyzed using the standard 2^{-CT} method.

2.3. Immunofluorescence analysis

Cells were fixed in 4% formalin for 15 min, followed by permeabilization for 20 min in 0.1% Triton X-100. Samples were blocked with goat or horse serum and incubated with the appropriate primary antibodies for 1 hour. The primary antibody used was rabbit anti- β -Tubulin III (PRB-435P; Covance, Berkeley, CA). The primary antibody dilution was 1:1000. Secondary antibodies included goat anti-rabbit AlexaFluor 488 used for analysis of 3D cultures and goat anti-rabbit AlexaFluor 594 for 2D cultures. Immunostained cells were examined using an Olympus Fluoview FV10i microscope (Olympus, Center Valley, PA). The percentage of β -Tubulin III-positive cells from triplicate experiments was determined by counting the number of cells with signal from anti- β -Tubulin III staining relative to the number of cells with nuclear DAPI blue fluorescence.

2.4. Antibody microarray analysis

The Cell Signaling Panorama antibody microarrays were purchased from Sigma. These microarrays contain 224 different antibodies spotted in duplicate on nitrocellulose coated glass slides. EBs were treated at day 4 of culture with RA versus RA plus AC. After two days of treatment, protein extracts were collected, labelled with Cy3 and Cy5, respectively, and hybridized to the arrays according to the manufacturer's instructions. Image acquisition and analysis was performed using an AlphaScan microarray scanner (Alpha Innotech Corporation, USA) and ArrayVision™ Version 8.0 (Imaging Research Inc., Ontario, Canada) or ScanAlyze (Stanford University, Stanford, CA) software packages. Background subtracted data were normalized to the median of a set of housekeeping genes using BRB-ArrayTools (NCI, Bethesda, MD). The Cy5/Cy3 signal ratio was calculated using MS Excel.

2.5. Western blotting

Western blot analysis was performed as previously described (Yadavilli and Muganda, 2004). The blots were probed with anti-GAPDH (SAB2108266, 1:500, Sigma-Aldrich), anti-phospho-p38 MAPK (44-684G, 1:1000, Invitrogen/Thermo Fisher Scientific), and anti-phospho-PI3K (4292, 1:1000, Cell Signaling Technology). Antigen levels were detected by utilizing a chemi-luminescent substrate (Kirkegaard & Perry Laboratories) and a Fluorichem 8000 Chemifluorimager (Alpha Innotech). Quantitation of the bands was performed by densitometry tracing using ImageJ and/or the AlphaEase™ software.

2.6. Cell viability and Statistical analyses

For these experiments, day 4 EBs were treated with RA, LE, RA/AC, RA/AC/LE, or vehicle only (Control) for 48 hours. After harvesting, EBs were trypsinized and determination of

cell viability was carried out using the trypan blue exclusion method using a Cellometer (Nexcelom Bioscience, Lawrence, MA, USA). Statistical analyses were performed using One-way Anova with Tucker post-test.

3. Results

3.1. RAR β 2 activation induces endodermal gene expression

Neuronal differentiation of cultured ES cells can be promoted by RA. In previous work, we found that expression of *Hoxa1*, a direct RA target, is required for the neuronal differentiation of mouse ES cells in vitro, and that *Hoxa1* function is repressed by endodermal gene expression (Martinez-Ceballos and Gudas, 2008). Because it has been previously reported that RA can induce endodermal gene expression in F9 teratocarcinoma cells acting mainly through the Retinoic Acid Receptor beta 2 (RAR β 2) (Zhuang et al., 2003), we first sought to investigate the effect of pharmacological RAR β 2 activation on endodermal gene expression in mouse ES cells. For this purpose, Wt ES cells were treated with combinations of RA, AC, and LE as described in Materials and Methods. After treatment, samples were examined by quantitative PCR for the expression of *RARB* and various endodermal markers. Treatment of cells with RA resulted in upregulation of all the genes examined, as compared to control cells. In most cases, treatment with a combination of RA plus AC dramatically increased endodermal gene expression as compared to that obtained with RA treatment alone (Fig. 1, panels a, b, d, and e). In general, treatment of cells with a combination of RA, AC, and LE restored endodermal gene expression to those levels seen in RA-treated samples. Thus, these results suggest that RAR β 2 activation promotes the endodermal differentiation of ES cells.

3.2. Inhibition of RAR β activity increases neuronal differentiation

The neuronal differentiation of ES cells can be carried out using both monolayer (2D) and tri-dimensional (3D) culture conditions. However, when differentiation is attempted in monolayer cultures, a great percentage of endodermal cells expressing *Bmp4* are obtained and neuronal differentiation is impaired (Gerrard et al., 2005). To solve this problem, researchers have employed Noggin, a Bmp antagonist, which was found to decrease endodermal commitment and increase neuronal differentiation (Gerrard et al., 2005). Thus, these observations suggest that unrestricted endodermal gene expression can repress neuronal differentiation. Because we observed that RAR β 2 activation by RA/AC resulted in upregulation of endodermal gene expression, and because we previously reported that upregulation of *Sox17* in ES cells impairs neuronal differentiation (Martinez-Ceballos and Gudas, 2008), we hypothesized that the pharmacological inhibition of RAR β 2 would improve the differentiation of mouse ES cells into neurons. To test this hypothesis, ES cells were cultured as monolayers on chamber slides until a 70% confluence was achieved. Next, the ES medium was replaced with Neurobasal medium and cells were treated with RA (1 μ M), LE (5 μ M), or RA plus LE. Control cells were treated with vehicle only. Cells were cultured for 8 days in Neurobasal medium plus drugs, with medium changes every two days. Immunofluorescence analyses for the neuronal marker β -Tubulin III indicated little to non-neuronal differentiation in control cells (Fig. 2a). Conversely, significant neuronal differentiation was observed in RA treated cells. Neurons were also observed in cells treated

with LE only, albeit the levels of differentiations were lower than in RA-treated cells. When cells were treated with 1 μ M RA plus 5 μ M LE (RA/LE), a three to four-fold increase in the percentage of cells expressing β -Tubulin III was obtained as compared to that obtained with RA only (Fig. 2b). When β -Tubulin III levels were examined by Western blot, only a 1.5-fold induction of this neuronal marker was observed in RA/LE versus RA samples, which suggests that the antibody used detects background β -Tubulin III levels in all cells when used for Western assays. Thus, our results suggest that endodermal differentiation after RA treatment of mouse ES cells depends on RAR β signaling and its inhibition increases the neuronal differentiation efficiency of ES cells cultured as monolayers.

3.3. Effect of RAR β inhibition in 3D cultures

The neuronal differentiation of mouse embryonic stem cells has been widely reported to occur more efficiently in 3D versus 2D cultures (Bibel et al., 2004; Martinez-Ceballos and Gudas, 2008, Weitzer, 2008). One commonly-used method for ES to neuronal differentiation involves the formation of cell aggregates or embryoid bodies (EBs) grown in the presence of RA (Weitzer, 2008). To determine the effect of LE on tri-dimensional ES cell cultures, we employed a previously-reported protocol for the differentiation of ES cells grown as EBs that requires treatment with 5 μ M RA (Bibel et al., 2004; Martinez-Ceballos and Gudas, 2008). Briefly, cells were grown in low-attachment plates for 8 days and medium plus/minus drugs was replaced every two days. Drugs (5 μ M RA plus/minus 5 μ M LE) were added at days 4 and 6 of culture. EBs were harvested at day 8 and the expression of B-Tubulin III was examined by immunofluorescence.

Expression of B-Tubulin III was absent in Control untreated cells (Fig. 3). Conversely, expression of this neuronal marker was clearly detected in cells treated with RA. In cells treated with RA plus LE, a dramatic increase in B-Tubulin III expression was observed as compared to cells treated with RA only (Fig. 3), which indicates that inhibition of RAR β action can increase the efficiency of ES to neuronal differentiation in the presence of RA under 3D conditions (by about 2.5-fold as compared to RA only; Fig. 3b).

3.4. Identification of cell signaling pathways activated by RAR β

Because a number of cell signaling pathways have been shown to be involved in endodermal differentiation, we next sought to identify the signaling pathways activated by RAR β 2 by performing antibody microarray analyses on EBs using Sigma's Cell Signaling Panorama Antibody Arrays. For these studies, cell aggregates were treated at day 4 of culture with RA plus AC versus RA only for 24 hours. Data analysis indicated that 31 (out of 224) proteins represented in the array were increased (18 proteins), or decreased (13 proteins), by 2-fold or more in cell treated with RA plus AC versus RA only (Table I, fold changes shown after log2 transformation).

The results obtained are shown in Fig. 4a as a bar graph where positive Log2 ratio of means indicates protein upregulation by RA/AC as compared to RA-treated samples, and negative values indicate downregulation. Following examination with the STRING online program to identify protein-protein interactions (Szklarczyk et al., 2014), our results from the antibody array analyses suggested a strong involvement of the PI3K/AKT, MAPK/ERK and p38/

MAPK pathways on the activation of RAR β 2 by AC (Fig. 4b). To verify the activation of the p38/MAPK and PI3K/AKT pathways, Western blot analyses were performed using antibodies directed against the phosphorylated forms of p38 and PI3K (Fig. 4c). We found that the levels of both p-p38 and p-PI3K were significantly increased by RA/AC as compared to RA-treated samples (about 1.8 and 1.5-fold, respectively; Fig. 4d).

3.5. Effect of drug treatments on cell viability

The observation from our Antibody microarray results, which indicate that several proteins that play a role in apoptosis are upregulated by RA/AC, prompted us to investigate the effect that the drugs (and drug combinations) used in this work have on cell viability. For this purpose, cells were grown as aggregates as described in Materials and Methods and the percentage of live cells was determined by the Trypan-exclusion method using a Cellometer cell counter. As shown in Figure 5, no significant differences in cell viability were observed between treated samples and control, except for samples treated with LE (about 15% decrease in viability with respect to control) and RA/AC/LE (about 10% decrease).

4. Discussion

Treatment of ES cells with RA is a common method to generate neurons in culture (Martinez-Ceballos and Gudas, 2008). Upon entering cells, RA travels to the nucleus and binds its nuclear receptors (RAR α , β , or γ) and their isoforms (Gutierrez-Mazariegos et al., 2013). Because RA can bind and activate any of the three RAR types, major efforts have been made to elucidate the role played by each receptor on the differentiation of cells along distinct fates. Research using F9 teratocarcinoma cells demonstrated that RAR β 2 is required for the RA-induced activation of endodermal gene expression (Zhuang et al., 2003). Similarly, gene inactivation experiments indicated that RA induces the neuronal differentiation of mouse ES cells acting through the RAR γ 2 subtype (Al Tanoury et al., 2014; for a review see Rochette-Egly, 2015). Previously, we reported that *Hoxa1*, an RAR γ 2 target gene, is required for the neuronal differentiation of mouse ES cells (Martinez-Ceballos and Gudas, 2008). Because we hypothesized that RAR β and RAR γ play opposing roles during the differentiation of mouse ES cells, here we first sought to investigate the effect of the pharmacological activation of RAR β 2 on the induction of endodermal gene expression. Our qPCR results demonstrated that treatment of ES cells with AC plus RA resulted in the upregulation of several markers for endodermal differentiation, including markers for primitive endoderm (*Gata-6*) and markers for definitive endoderm (*Foxa2* and *Sox17*) (Fig. 1). A second marker for primitive endoderm, the alpha-Fetoprotein gene or *AFP*, was induced by RA but not by RA/AC (Fig. 1c), which suggests that this gene is downregulated by RAR β . Because AC shows more than 100-fold selectivity for RAR β 2 over RAR α , RAR γ and RXR α , RXR β and RXR γ (Lund et al., 2005), our experiments using AC suggest that the endodermal differentiation of mouse ES cells requires activation of RAR β and results in repression of neuronal differentiation in both monolayer and 3D cultures. This observation is in line with our previous work using *Hoxa1*-null mouse ES cells that have impaired neurogenesis and differentiate along the endodermal lineage upon RA treatment (Martinez-Ceballos et al., 2005; Martinez-Ceballos and Gudas, 2008), which suggests the

presence of a negative feedback loop between the Hoxa1 and the RAR β signaling pathways in mouse ES cells.

In attempts to further verify the negative role of RAR β on neuronal differentiation, we monitored the effects of LE plus/minus RA on 2D and 3D ES cell cultures. Under both culture conditions, co-treatment of cells with RA plus LE resulted in a higher efficiency of neuronal differentiation as compared to cells treated with RA only. Although a high number of cells did not express the neuronal marker β -Tubulin-III after RA-LE treatment, our experiments represent an initial step on the generation of highly pure neuronal cultures for future therapeutic uses.

As a number of major cell signaling pathways have been shown to be involved in endodermal differentiation, we then hypothesized that the observed effect of AC in ES cells may involve the activation of major cell signaling pathways. Furthermore, major cell signaling pathways have been shown to mediate some of the effects of RA in ES and human cancer cells (Frédéric et al., 2002; Pettersson et al., 2004; Kogai et al., 2008; reviewed by Niles, 2004). Our antibody microarray analyses indicated that cells treated with a combination of RA plus AC expressed members of the p38/MAPK and PI3K/AKT pathways in a differential manner as compared to cells treated with RA only, which suggests their involvement on the induction of ES to endodermal differentiation by RAR β 2. This idea is consistent with reports describing the involvement of the p38 and PI3K cell signaling pathways on the commitment of endodermal lineages either during embryonic carcinoma or ES cell differentiation in culture (Smedberg et al., 2002; Villegas et al., 2013; Yap et al., 2014; also, see Binetruy et al., 2007 for a review). Thus, our findings provide evidence of a role played by the p38 and PI3K cell signaling pathways during the RA-induced differentiation of mouse ES cells along an endodermal lineage. Our antibody microarray analyses also indicated that several proteins involved in apoptosis were upregulated in cells treated with RA plus AC as compared to RA-treated cells. However, assays employing trypan blue demonstrated that RA/AC does not significantly decrease cell viability as compared to RA in 3D cultured cells. Thus, the increase in apoptotic marker expression observed in our antibody microarray analysis may not result in significant cell death.

In conclusion, our experiments support a dual role for RAR β on ES cell differentiation: positive on endodermal differentiation, and negative on neuronal differentiation. Our research also establishes the p38/MAPK and PI3K/AKT cell signaling pathways as important transducers of the RAR β signal. As RA has been shown to suppress cell growth and induce apoptosis in several human cancers (for reviews see Tang and Gudas, 2011, and Chen et al., 2014), its effect is hindered in cancers that present downregulation of RAR β due to epigenetic changes (Widschwendter et al., 1997; Nakayama et al., 2001; reviewed by Xu, 2007). Therefore, it would be interesting to examine the effect of pharmacological activators of p38 and/or PI3K on RA-resistant cancer cells as an attempt to bypass RAR β activation and stop cancer cell growth. In conclusion, we have identified a role for RAR β as a mayor endodermal inducer and as a repressor of neuronal differentiation in mouse ES cells. Our results suggest that this effect of RAR occurs through the activation of major Cell Signaling pathways such as the p38/MAPK and PI3K/AKT pathways.

References

1. Addae C, Yi X, Gernapudi R, Cheng H, Musto A, Martinez-Ceballos E. All-trans-retinoid acid induces the differentiation of encapsulated mouse embryonic stem cells into GABAergic neurons. *Differentiation*. 2012; 83(5):233–241. [PubMed: 22466603]
2. Akin ZN, Nazarali AJ. Hox genes and their candidate downstream targets in the developing central nervous system. *Cellular and molecular neurobiology*. 2005; 25(3–4):697–741. [PubMed: 16075387]
3. Al Tanoury Z, Gaouar S, Piskunov A, Ye T, Urban S, Jost B, ... Rochette-Egly C. Phosphorylation of the retinoic acid receptor RAR γ 2 is crucial for the neuronal differentiation of mouse embryonic stem cells. *J Cell Sci*. 2014; 127(9):2095–2105. [PubMed: 24569880]
4. Bibel M, Richter J, Schrenk K, Tucker KL, Staiger V, Korte M, ... Barde YA. Differentiation of mouse embryonic stem cells into a defined neuronal lineage. *Nature neuroscience*. 2004; 7(9):1003–1009. [PubMed: 15332090]
5. Binetruy B, Heasley L, Bost F, Caron L, Aouadi M. Concise Review: Regulation of Embryonic Stem Cell Lineage Commitment by Mitogen-Activated Protein Kinases. *Stem Cells*. 2007; 25(5): 1090–1095. [PubMed: 17218395]
6. Boylan JF, Lohnes D, Taneja R, Chambon P, Gudas LJ. Loss of retinoic acid receptor gamma function in F9 cells by gene disruption results in aberrant Hoxa-1 expression and differentiation upon retinoic acid treatment. *Proceedings of the National Academy of Sciences*. 1993; 90(20): 9601–9605.
7. Boylan JF, Lufkin T, Achkar CC, Taneja R, Chambon P, Gudas LJ. Targeted disruption of retinoic acid receptor alpha (RAR alpha) and RAR gamma results in receptor-specific alterations in retinoic acid-mediated differentiation and retinoic acid metabolism. *Molecular and Cellular Biology*. 1995; 15(2):843–851. [PubMed: 7823950]
8. Chambon P. A decade of molecular biology of retinoic acid receptors. *The FASEB Journal*. 1996; 10(9):940–954. [PubMed: 8801176]
9. Chen MC, Hsu SL, Lin H, Yang TY. Retinoic acid and cancer treatment. *Biomedicine*. 2014; 4(4)
10. Frédéric BOST, Caron L, Marchetti I, Christian DANI, Le Marchand-Brustel Y, Binétruy B. Retinoic acid activation of the ERK pathway is required for embryonic stem cell commitment into the adipocyte lineage. *Biochemical Journal*. 2002; 361(3):621–627. [PubMed: 11802792]
11. Gerrard L, Rodgers L, Cui W. Differentiation of human embryonic stem cells to neural lineages in adherent culture by blocking bone morphogenetic protein signaling. *Stem Cells*. 2005; 23(9): 1234–1241. [PubMed: 16002783]
12. Gudas, LJ. *Seminars in cell & developmental biology*. Vol. 24. Academic Press; 2013. Retinoids induce stem cell differentiation via epigenetic changes; p. 701-705.
13. Gutierrez-Mazariegos, J., Schubert, M., Laudet, V. *The Biochemistry of Retinoic Acid Receptors I: Structure, Activation, and Function at the Molecular Level*. Springer; Netherlands: 2014. Evolution of retinoic acid receptors and retinoic acid signaling; p. 55-73.
14. Kogai T, Ohashi E, Jacobs MS, Sajid-Crockett S, Fisher ML, Kanamoto Y, Brent GA. Retinoic acid stimulation of the sodium/iodide symporter in MCF-7 breast cancer cells is mediated by the insulin growth factor-I/phosphatidylinositol 3-kinase and p38 mitogen-activated protein kinase signaling pathways. *The Journal of Clinical Endocrinology & Metabolism*. 2008; 93(5):1884–1892. [PubMed: 18319322]
15. Lund BW, Piu F, Gauthier NK, Eeg A, Currier E, Sherbukhin V, et al. Discovery of a potent, orally available, and isoform-selective retinoic acid beta2 receptor agonist. *J Med Chem*. 2005; 48(24): 7517–9. [PubMed: 16302793]
16. Martinez-Ceballos E, Chambon P, Gudas LJ. Differences in gene expression between wild type and Hoxa1 knockout embryonic stem cells after retinoic acid treatment or leukemia inhibitory factor (LIF) removal. *Journal of Biological Chemistry*. 2005; 280(16):16484–16498. [PubMed: 15722554]
17. Martinez-Ceballos E, Gudas LJ. Hoxa1 is required for the retinoic acid-induced differentiation of embryonic stem cells into neurons. *Journal of neuroscience research*. 2008; 86(13):2809–2819. [PubMed: 18512762]

18. Murry CE, Keller G. Differentiation of embryonic stem cells to clinically relevant populations: lessons from embryonic development. *Cell*. 2008; 132(4):661–680. [PubMed: 18295582]
19. Nakayama T, Watanabe M, Yamanaka M, Hirokawa Y, Suzuki H, Ito H, ... Shiraishi T. The Role of Epigenetic Modifications in Retinoic Acid Receptor [beta] 2 Gene Expression in Human Prostate Cancers. *Laboratory Investigation*. 2001; 81(7):1049. [PubMed: 11454993]
20. Niles RM. Signaling pathways in retinoid chemoprevention and treatment of cancer. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*. 2004; 555(1):97–105. [PubMed: 15476853]
21. Pérez RJ, Benoit YD, Gudas LJ. Deletion of retinoic acid receptor β (RAR β) impairs pancreatic endocrine differentiation. *Experimental cell research*. 2013; 319(14):2196–2204. [PubMed: 23756134]
22. Pettersson F, Couture MC, Hanna N, Miller WH. Enhanced retinoid-induced apoptosis of MDA-MB-231 breast cancer cells by PKC inhibitors involves activation of ERK. *Oncogene*. 2004; 23(42):7053–7066. [PubMed: 15273718]
23. Rochette-Egly C. Retinoic acid signaling and mouse embryonic stem cell differentiation: Cross talk between genomic and non-genomic effects of RA. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids*. 2015; 1851(1):66–75. [PubMed: 24768681]
24. Szklarczyk D, Franceschini A, Wyder S, Forslund K, Heller D, Huerta-Cepas J, ... Kuhn M. STRING v10: protein–protein interaction networks, integrated over the tree of life. *Nucleic acids research*. 2014; gku1003.
25. Smedberg JL, Smith ER, Capo-chichi CD, Frolov A, Yang DH, Godwin AK, Xu XX. Ras/MAPK pathway confers basement membrane dependence upon endoderm differentiation of embryonic carcinoma cells. *Journal of Biological Chemistry*. 2002; 277(43):40911–40918. [PubMed: 12145292]
26. Tang XH, Gudas LJ. Retinoids, retinoic acid receptors, and cancer. *Annual Review of Pathology: Mechanisms of Disease*. 2011; 6:345–364.
27. Villegas SN, Rothová M, Barrios-Llerena ME, Pulina M, Hadjantonakis AK, Le Bihan T, ... Brickman JM. PI3K/Akt1 signalling specifies foregut precursors by generating regionalized extracellular matrix. *Elife*. 2013; 2:e00806. [PubMed: 24368729]
28. Weitzer, W. *Stem Cells*. Springer; Berlin Heidelberg: 2008. Embryonic stem cell-derived embryoid bodies: an in vitro model of eutherian pregastrulation development and early gastrulation; p. 21–51.
29. Widschwendter M, Berger J, Daxenbichler G, Müller-Holzner E, Widschwendter A, Mayr A, ... Zeimet AG. Loss of retinoic acid receptor β expression in breast cancer and morphologically normal adjacent tissue but not in the normal breast tissue distant from the cancer. *Cancer research*. 1997; 57(19):4158–4161. [PubMed: 9331065]
30. Yadavilli S, Muganda PM. Diepoxybutane induces caspase and p53-mediated apoptosis in human lymphoblasts. *Toxicology and applied pharmacology*. 2004; 195(2):154–165. [PubMed: 14998682]
31. Yap C, Goh HN, Familiar M, Rathjen PD, Rathjen J. The formation of proximal and distal definitive endoderm populations in culture requires p38 MAPK activity. *J Cell Sci*. 2014; 127(10): 2204–2216. [PubMed: 24481813]
32. Xu XC. Tumor-suppressive activity of retinoic acid receptor- β in cancer. *Cancer letters*. 2007; 253(1):14–24. [PubMed: 17188427]
33. Zhuang Y, Faria TN, Chambon P, Gudas LJ. Identification and Characterization of Retinoic Acid Receptor β 2 Target Genes in F9 Teratocarcinoma *Molecular cancer research*. 2003; 1(8):619–630.

Fig. 1. RAR β induces the activation of endodermal gene expression

Real Time PCR (qPCR) analysis of the effect of RAR β 2 activation on RAR beta and endodermal gene expression. The RAR β 2 agonist, AC, in combination with RA upregulated *RAR β* (panel a) as well as markers for endodermal differentiation: *Foxa2* (b), *Gata6* (d), and *Sox17* (e). However, the expression of *Afp*, an endodermal precursor marker, was repressed by AC (c). The effect of AC plus RA (RA/AC) on the induction of *Foxa2*, *Gata6*, and *Sox17* was abolished by LE, the RAR β antagonist. Relative mRNA levels to *Rplp0* (an ubiquitous gene used as loading control) are shown.

Fig. 2. Immunofluorescence analysis of RA-induced neuronal differentiation in the presence of LE135

(a) Monolayer cultures were treated with vehicle only (Control), 5 μ M LE135 (LE), 1 μ M RA, or 1 μ M RA + 5 μ M LE135 (RA/LE). (b) Percentage of cells expressing the neuronal marker β -Tubulin III. Treatment of cells with RA plus LE resulted in about a 3.5-fold increase of neuronal differentiation as compared to cells treated with RA only. (c) Analysis of β -Tubulin III by Western blot. (d) Quantitation of Western blot analyses. * $P < 0.05$; ** $P < 0.01$, *** $P < 0.001$.

Fig. 3. Effect of LE135 on ES neuronal differentiation in 3D cultures

EB (3D) cultures were treated with vehicle only, 5 μ M RA, or 5 μ M RA plus 5 μ M LE (RA/LE). β -Tubulin III expression was examined by immunofluorescence. (a) Treatment of EBs with RA/LE was found to increase the expression of the neuronal marker as compared to treatment with RA only. Little or no β -Tubulin III expression was detected in control EBs. Representative sections are shown from triplicate experiments. (b) As it was the case for monolayer cultures, treatment of EBs with RA plus LE resulted in an increase of the percentage of cells that express the neuronal marker as compared to cells treated with RA only (about 2.5-fold). *** P = 0.001.

Fig. 4. Antibody microarray analysis

(a) Ratio distribution of differentially expressed proteins in ES cells treated with RA/AC compared with RA-treated samples. Of the 224 proteins represented in the array, 18 were upregulated ($\log_2$ ratio > 1) and 13 were downregulated ($\log_2$ ratio < -1). (b) STRING analysis of protein interactions. Nodes in red tones represent upregulated proteins and green tones represent downregulated proteins. Edges represent action types: blue = binding, red = inhibition, pink = postranslational modification, yellow = transcriptional regulation. (c) Representative immunoblot of p-p38 and p-PI3K expression in EB samples treated with vehicle (control), RA, AC, or RA/AC. (d) Relative protein levels measured by Western blot and normalized to GAPDH levels. ** $P < 0.01$; *** $P < 0.001$.

Fig. 5. Cell viability

Effect of drug treatments on cell viability determined by trypan blue exclusion. Only cells that contained LE showed a significant decrease in cell viability with respect to controls (Ctl.). LE: about 15% decrease in viability. RA/AC/LE: about 10% decrease. * $P < 0.05$; *** $P < 0.001$.

Table I

Analysis of protein expression in cells treated with AC55649 plus RA versus RA alone Mouse names are shown in parentheses. Log2 values are shown for Ratio of Means.

Protein	Area	Ratio of Means (cy5/cy3)	P value
Cdc27	Cell Cycle	4.183	0.007
Calnexin (Canx)	Ca Associated	3.887	2.3E-04
HDAC2 (Hdac2)	Nuclear	3.842	0.005
ERK5 (Mapk7)	Signal Transduction	3.731	0.017
Amyloid Precursor Prot (App) c-term.	Neurobiology	3.438	1.5E-04
p120CTN (Ctnd1)	Neurobiology	3.336	0.008
MAPK activated prot. k2 (Mapkapk2)	Signal Transduction	3.296	6.6E-04
Caspase 3 (Casp3)	Apoptosis	3.137	0.002
PAR4 (Pawr)	Apoptosis	3.118	0.003
MAP Kinase; Erk1 (Mapk3)	Signal Transduction	2.943	5.0E-05
Phosphoserine	Signal Transduction	2.808	0.008
PKB/AKT (Akt1)	Signal Transduction	2.704	1.9E-04
Cofilin (Cfl1)	Neurobiology	2.662	0.001
SMAC/DIABLO (Diablo)	Apoptosis	2.466	0.001
Caspase3 active	Apoptosis	2.390	0.003
Estrogen Receptor (Esr)	Signal Transduction	2.380	0.010
p38 MAPK activated (Mapk14)	Signal Transduction	1.881	0.003
p38 MAPK	Signal Transduction	1.867	0.011
p21 (Cdkn1a)	Cell cycle	-1.000	0.005
pHistone H3-pSer10	Nuclear	-1.220	0.001
cdk7	Cell Cycle	-1.223	1.9E-15
pan Cytokeratin	Cytoskeleton	-1.275	0.009
Cdk6	Cell Cycle	-1.397	0.002
Cystatin A (Csta)	Apoptosis	-1.598	0.008
Caspase 11 (Casp11)	Apoptosis	-1.629	8.1E-04
Caspase 7 (Casp7)	Apoptosis	-1.779	0.002
Cyclin D3 (Cnd3)	Cell Cycle	-1.853	0.002
Caspase 6 (Casp6)	Apoptosis	-1.938	1.1E-04
AP-1/cJUN (Jun)	Nuclear	-2.591	3.9E-04
cytokeratin peptide 4	Cytoskeleton	-3.109	0.003
Cyclin A (poly) (Ccna)	Nuclear	-3.137	3.1E-04