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Role of COUP-TFI during Retinoic Acid-Induced Differentiation of P19 Cells to Endodermal Cells

Brandy S. Pickens¹, Bryan W. Teets¹, Kenneth J. Soprano², and Dianne Robert Soprano¹

¹Department of Biochemistry, Temple University School of Medicine, Philadelphia, PA 19140

²Department of Microbiology & Immunology, Temple University School of Medicine, Philadelphia, PA 19140

Abstract

Retinoic acid (RA) is a positive regulator of P19 cell differentiation. Silencing of pre-B cell leukemia transcription factors (PBXs) expression in P19 cells (AS cells) results in a failure of these cells to differentiate to endodermal cells upon RA treatment. Chicken Ovalbumin Upstream Promoter Transcription Factor I (COUP-TFI) is an orphan member of the steroid-thyroid hormone superfamily. RA treatment of wild type P19 cells results in a dramatic increase in the expression of COUP-TFI however COUP-TFI mRNA levels fail to be elevated upon RA treatment of AS cells indicating that PBX expression is required for elevation in COUP-TFI expression. To study the role of COUP-TFI during RA-dependent differentiation of P19 cells, AS cells that inducibly express various levels of COUP-TFI were prepared. Exogenous expression of COUP-TFI in AS cells, in a dose-dependent fashion, leads to growth inhibition, modest cell cycle disruption and early apoptosis. Furthermore, AS cells can overcome the blockage in RA-dependent differentiation to endodermal cells when either pharmacological levels of COUP-TFI are expressed or a combination of both the expression of physiological levels of COUP-TFI and RA treatment. Additionally, the mRNA level of several pluripotency associated genes including OCT-4, DAX-1 and SF-1 in the COUP-TFI expressing AS cells are reduced. Moreover, analysis of the expression of primary RA response genes indicates that COUP-TFI is involved in the regulatory modulation of the expression of at least two genes, CYP26A1 and HoxA1. These studies demonstrate that COUP-TFI functions as a physiologically relevant regulator during RA-mediated endodermal differentiation of P19 cells.

Keywords

COUP-TFI; COUP-TFII; retinoic acid; P19 cells

Introduction

Retinoids make up a diverse class of compounds including Vitamin A and its natural and synthetic derivatives. Retinoic acid (RA), the most biologically active naturally occurring retinoid, plays key roles in the regulation of a wide variety of physiological processes including embryonic development and cellular differentiation (Ross et al., 2000; Zile, 2001; Clagett-Dame and De Luca 2002). It exerts its effects at the level of regulation of target gene transcription by binding to ligand-inducible transcription factors belonging to the steroid/

thyroid hormone superfamily including retinoic acid receptors (RARs) and retinoid X receptors (RXRs) (Chambon, 1996).

Embryonal carcinoma (EC) cells such as P19 cells and embryonic stem (ES) cells are excellent model systems to study early events during embryonic development (Soprano, 2007). RA treatment of P19 cells grown as a monolayer results in formation of endodermal and mesodermal derivatives (Mummery et al., 1986) while RA treatment of cells grown as aggregates results in the formation of cells which resemble neurons, glia and fibroblast-like cells (Jones-Villeneuve et al., 1982). Previous work from our laboratory has shown that the mRNA and protein levels of Pre-B cell leukemia transcription factors (PBXs) are rapidly increased in P19 cells following RA treatment (Qin et al., 2004a). Furthermore, P19 cells that express an antisense PBX mRNA that greatly reduces the RA-dependent increase in PBX protein levels (AS cells) fail to differentiate to both endodermal and neuronal cells (Qin et al., 2004b). Since PBX proteins are members of the three-amino acid loop extension superclass of Homeobox proteins and function as important cofactors to enhance the DNA binding affinity and specificity of members of the Hox family of transcription factors (Burglin, 1997; Capellini et al., 2011), one of the goals of this work was to identify genes whose expression levels were regulated by PBX that are critical for RA-dependent differentiation of P19 cells to endodermal cells.

Chicken ovalbumin upstream promoter-transcription factors I and II (COUP-TFI and COUP-TFII) are orphan members of the steroid/thyroid hormone superfamily for which a ligand has yet to be identified (Tsai and Tsai, 1997). COUP-TFI and COUP-TFII are very closely related with an overall amino acid identity of 87% (Wang et al., 1991). COUP-TFI and COUP-TFII display overlapping, but distinct, patterns of expression in the three germ layers which is first detected on mouse embryonic day 7.5 (Pereira et al., 2000). COUP-TFI expression is high in the nervous system while COUP-TFII expression is high in the mesenchyme of internal organs (Pereira et al., 2000). Mammalian COUP-TFs play vital physiological roles during angiogenesis, neuronal development, organogenesis, cell fate determination, metabolic homeostasis and circadian rhythm (Pereira et al., 2000; Park et al., 2003; Pereira et al., 1999). COUP-TFI null mutant mice die perinatally and display defects in neurogenesis, axon guidance, and arborization (Qiu et al., 1997; Yamaguchi et al., 2004; Zhou et al., 1999; Zhou et al., 2001); whereas homozygous deletion of COUP-TFII is embryonic lethal at embryonic day 10 and results in defects in angiogenesis, vascular remodeling, diaphragmatic and stomach organogenesis, and fetal heart development (Pereira et al., 1999; You et al., 2005; Kurihara et al., 2007; Petit et al., 2007; Takamoto et al., 2005).

In P19 cells, COUP-TFI and COUP-TFII mRNA levels are elevated 24 to 48 hrs following RA treatment. Furthermore, the kinetics of the induction of COUP-TFI and COUP-TFII expression is inversely correlated with the kinetics of the down-regulation of the pluripotency gene OCT-4 (Ben-Shushan et al., 1995). While it has been demonstrated that RA treatment of P19 cells causes an increase in the expression of both COUP-TFI and COUP-TFII, the functional importance of these proteins during the endodermal cell differentiation cascade remains unclear.

In this report we demonstrate that the RA-dependent increase in the mRNA levels of both COUP-TFI and COUP-TFII is dependent upon an increase in PBX expression in P19 cells. To study the role of COUP-TFI in the absence of RA-dependent increase in PBX expression and endodermal differentiation, P19 AS cells lines that inducibly express COUP-TFI were prepared. Expression of COUP-TFI in these cells, in a dose dependent manner, led to growth inhibition, modest cell cycle disruption and early apoptosis. Furthermore, a pharmacological level of COUP-TFI expression alone was sufficient to induce endodermal differentiation while a physiological level of COUP-TFI expression resulted in differentiation to

endodermal cells only upon RA treatment. Taken together, these data demonstrate that COUP-TFI is a physiologically relevant regulator of RA-dependent differentiation of P19 cells to endodermal cells.

Materials and Methods

Cell Culture and Differentiation

P19 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 μ g/ml penicillin and 100 units/ml streptomycin. TO9 (vector control P19 cells) and AS2 cells (P19 cells that express antisense PBX RNA) (Qin et al., 2004b) were routinely passaged in complete DMEM supplemented with 400 μ g/ml zeocin (Invitrogen). For endodermal differentiation, 10^5 cells in complete DMEM/100-mm tissue culture dish were treated with 10^{-7} M all-trans RA for 4 days followed by an additional 3 days without RA.

Preparation of Inducible COUP-TFI Expression Cell Lines

AS2 cells that stably express the Tet-transactivator protein were prepared by transfecting AS2 cells with pTet-Off Advanced Vector DNA (Clontech, Mountain View, CA) as previously described (Teets et al., 2012). Cells were selected on complete DMEM supplemented with 400 μ g/ml zeocin, 800 μ g/ml G418 and 100 ng/ml doxycycline (Dox). Individual G418 resistant clones (ASTO clones) were screened for the expression of the Tet-transactivator protein by transfection with pTRE-Tight-Luc DNA (Clontech) followed by assay for firefly luciferase activity in the absence of Dox. ASTO clones which displayed high inducible expression of luciferase were also screened to assure that they failed to display RA-dependent differentiation by analyzing expression of SSEA-1, TROMA-I, and OCT-4 by immunofluorescence, and the lack of a RA-dependent increase in PBX expression by RT-PCR and Western blot. ASTO29 cells were used for all additional studies.

To prepare inducible COUP-TFI expression clones (ASTT clones), ASTO29 cells were transfected with pTRE-Tight Vector DNA (Clontech) containing the full length open reading frame of mouse COUP-TFI cDNA fused to the N-terminal V5-tag along with linear hygromycin DNA (Clontech). Cells were selected on complete DMEM supplemented with 400 μ g/ml zeocin, 800 μ g/ml G418, 300 μ g/ml hygromycin and 100 ng/ml Dox. Individual hygromycin resistant ASTT clones were screened for inducible expression of V5-COUP-TFI upon removal of Dox by In Cell Western using mouse anti-V5 (Invitrogen) as the primary antibody and donkey anti-mouse IRDye 800CW (LI-COR) for the secondary antibody. Clones that were positive by In Cell Western were examined by Western blot analysis and immunofluorescence to confirm the expression of V5-COUP-TFI and to determine the percent positive cells in the absence of Dox. In addition, ASTT grown in the presence of 100 ng/ml Dox (and do not express COUP-TFI) were also screened to assure that they failed to display RA-dependent differentiation by analyzing expression of SSEA-1, TROMA-I, and OCT-4 by immunofluorescence, and the lack of a RA-dependent increase in PBX expression by RT-PCR and Western blot

To induce expression of COUP-TFI expression, cells were plated in complete DMEM containing 10% Tet-free fetal bovine serum (Clontech) and the indicated concentrations of Dox ranging from 0 to 100 ng/ml. After both 4 hrs and 24 hrs the culture media were removed and replaced with the fresh culture media containing the same concentration of Dox. After the second media change (24 hrs following plating) when COUP-TFI expression was maximal for the indicated concentration of Dox, the cells were treated in the presence of the same concentration of Dox with either ethanol or 10^{-7} M RA for the indicated periods of time.

RNA Analysis

Total RNA was isolated using RNA-Bee™ B reagent (Tel-Test, Friendswood, TX). cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit from Applied Biosystems as described by the manufacturer. Standard PCR was performed using GoTaq DNA polymerase (Promega Inc., Madison, WI) as described by the manufacturer and quantitative real time PCR (qPCR) was performed using SYBR Green Master Mix (Fermentas, Thermo Fisher Scientific, Rockford, IL) according to the manufacturer's instructions essentially as previously described (Zhao et al., 2009; Vucetic et al., 2008; Teets et al., 2012). Primers purchased from Integrated DNA Technologies (IDT, Coralville, IA) are listed in Table S1. For qPCR analysis, changes in gene expression were calculated using the ddCT method for relative quantification of each target gene normalized to the endogenous GAPDH control. All primers used for qPCR yielded a dissociation curve with a single peak and a single PCR product of the appropriate size as determined by electrophoresis in an acrylamide gel.

Western Blot

Western blot analysis was performed essentially as previously described (Vucetic et al., 2008; Zhao et al., 2009). Primary antibodies used were mouse anti-PBX1,2,3,4 (Santa Cruz, Santa Cruz, CA, sc-28313), mouse anti-V5 (Invitrogen) and goat anti-GAPDH (Santa Cruz, sc-20357). Secondary antibodies used were donkey anti-mouse IRDye 800CW and donkey anti-goat IRDye 680CW purchased from LI-COR, Lincoln, NE. Images were captured and quantitated using the LI-COR Odyssey instrument and software. GAPDH levels were used as the loading control.

Immunocytochemistry

P19 cells were seeded on glass coverslips. At the end of the treatment period, cells were fixed by immersion of the coverslips in 3.7% formaldehyde at room temperature for 30 min followed by poration by immersion in 0.18% Triton X100 in PBS for 10 min. To minimize non-specific binding of antibodies, the coverslips were blocked using blocking buffer (1% BSA dissolved in PBS) for 10 min at room temperature. Coverslips were incubated at room temperature for 45 min with primary antibody solution (1 µg/ml antibody in blocking buffer) followed by 2 washes with PBS and 1 wash with blocking buffer. Primary antibodies were mouse anti-SSEA-1 (MC-480, Developmental Study Hybridoma Bank, University of Iowa, Iowa City, IA), rat anti-cytokeratin Endo-A (TROMA-I, Developmental Study Hybridoma Bank, University of Iowa), and rabbit anti-OCT-3/4 (Santa Cruz Biotechnology, sc-9081). The coverslips were then incubated for 30 min at room temperature in secondary antibody solution (1 µg/ml antibody in blocking buffer) while avoid exposure to light. Secondary antibodies were anti-rat-TRITC, anti-mouse-TRITC, anti-mouse-FITC, anti-rabbit-TRITC and anti-rabbit-FITC purchased from Santa Cruz Biotechnology. Prolong Gold with DAPI (Invitrogen) was used as the mounting solution. Slides were examined with an Olympus BX41 fluorescent microscope with filters for blue (DAPI), green (FITC) and red (TRITC) and an Olympus Digital Camera Spot-Xplorer with SPOT Advanced Software to capture and merge images.

Cell growth, cell cycle and apoptosis assays

Cell growth was determined by cell counting using a hemacytometer and trypan blue dye exclusion. The distribution of cells within the stages of the cell cycle was determined by propidium iodide (PI) staining followed by fluorescence-activated cell scan (FACS) flow cytometry. Briefly, cells were fixed by incubation in 1 ml of 100% ethanol at 4° C for 15 min followed by the addition of 10 ml of PBS and centrifugation at 1200g for 5 min at 4°C. The cell pellet was resuspended in 300 µl of PBS containing 0.1% NP-40 and 5 µg/ml

DNase-free RNase (Roche Applied Sciences, Indianapolis, IN) and incubated for 15 min at 25° C. After RNase digestion, 2 μ l of 3 μ M PI (Molecular Probes, Life Technologies, Grand Island, NY) was added to the solution while avoiding exposure to light. Apoptosis was assessed with PI and Annexin V staining followed by FACS analysis using the FITC Annexin V Apoptosis Detection Kit II (BD Pharmingen, Franklin Lakes, NJ) as described by the manufacturer. For both the cell cycle and apoptosis studies, cells were analyzed using a BD FACSCanto™ Flow Cytometer (Model #641548) running FACSDiva™ software version 1.0 (BD Biosciences, San Jose, CA).

Results

RA-induced increase in COUP-TFI and COUP-TFII expression is PBX dependent

Since an increase in PBX expression is required for RA-dependent differentiation of P19 cells to endodermal cells (Qin et al., 2004b), a microarray analysis was performed using wild type vector control P19 cells (TO) and PBX antisense expressing P19 cells (AS) to identify downstream targets of PBX during endodermal differentiation. Among the mRNAs whose level of expression were found to be elevated in TO cells, but not AS cells, upon RA treatment during endodermal differentiation were COUP-TFI and COUP-TFII. Figures 1A and 1B demonstrate that COUP-TFI and COUP-TFII expression is increased approximately 600-fold and 300-fold, respectively, in TO9 cells following treatment with RA for 3 days while AS2 cells displayed only a very slight increase in the level of these two mRNAs.

Two variants of COUP-TFII have been reported that are likely to arise due to the use of alternative promoters (Strausberg et al., 2002). Variant 1 is 414 amino acids in length while variant 2 is 281 amino acids. Variant 1 and variant 2 share a common C-terminal 267 amino acid sequence encoding the E region containing the ligand binding domain, dimerization interfaces and ligand-dependent transactivation region. Variant 2 has a short unique amino terminal region consisting of 14 amino acids and lacks the A/B regions and the DNA binding domain found in variant 1. Both variant 1 and variant 2 are expressed in P19 cells however RT-PCR analysis using primers specific to each variant demonstrates that the mRNA level of only COUP-TFII variant 1 was increased upon RA treatment of P19 cells (Figure 1C).

To study the effect of COUP-TFI expression in the absence of the RA-dependent increase in PBX levels in P19 cells, three independent inducible COUP-TFI expressing AS cell lines were prepared (Figure 2A). These cell lines express variable amounts of V5-COUP-TFI upon exposure to concentrations of Dox ranging from 0 to 100 ng/ml (ASTT clones) (Figures 2B). Comparison of the level of COUP-TFI expression in the ASTT cells treated with various concentrations of Dox with that of wild type P19 cells demonstrated that ASTT cells treated with 0.5 and 1.0 ng/ml Dox expressed a level of COUP-TFI comparable to that of wild type P19 cells treated with RA (physiological), while treatment with 0 ng/ml Dox resulted in a level of COUP-TFI expression that is approximately 60-fold greater than physiological (pharmacological) (Figure 2C). In addition, like AS cells, ASTT cells grown in the presence of 100 ng/ml Dox expressed a low level of COUP-TFI that was not elevated upon RA treatment (Figure 2C). Furthermore, exogenous COUP-TFI in ASTT cells independent of RA treatment is functional as indicated by its ability to increase the mRNA levels of two known COUP-TFI targets (Adam et al., 2000; Hall et al., 1995), vitronectin and PEPCK, (Figure 2D) however as expected exogenous COUP-TFI had no effect on PBX levels even upon RA treatment (Figure 2E). Finally, analysis of individual cells by immunofluorescence demonstrated that V5-COUP-TFI was localized in the nucleus in greater than 90% of the cells upon removal of Dox for 24 hr (data not shown). All studies were performed with each of the three ASTT clones; however, since similar results were obtained with cells from all three clones data is presented for ASTT154 cells.

Effect of COUP-TFI Expression on Growth of P19 cells

Figure 3A demonstrates that there is an inverse correlation between cell growth and the levels of exogenous COUP-TFI expression. Cell growth rate declines stepwise upon lowering the concentration of Dox (from 100 ng/ml to 0 ng/ml) over the period of 4 days with the greatest amount of growth inhibition occurring when exogenous COUP-TFI levels were expressed at a pharmacological level using 0 ng/ml Dox. In addition, cell growth is even further reduced at each level of COUP-TFI expression at each time point upon RA treatment. FACS analysis of propidium iodine stained cells demonstrated a modest increase in the number of cells in G0/G1 only in cells expressing a pharmacological level of COUP-TFI (0 ng/ml Dox) while no difference in the distribution of cells in the stages of the cell cycle was observed in cells expressing physiological levels of COUP-TFI (1 ng/ml Dox) (Figure 3B). Analysis of apoptosis using Annexin V staining followed by FACS analysis demonstrated that there is a dose dependent increase in the number of cells in early apoptosis to a maximum of 25% of the cells when expressing a pharmacological level of COUP-TFI (Figure 3C). Therefore, the reduction in the growth of P19 cells expressing pharmacological levels of COUP-TFI can be accounted for at least in part by accumulation of cells in G0/G1 and an increase in apoptosis.

Effect of COUP-TFI on RA-mediated Differentiation of P19 cells

To determine if COUP-TFI expression alone can cause endodermal differentiation in cells which are resistant to RA-mediated differentiation due to an absence in PBX induction (AS cells), we examined the expression of SSEA-1, OCT-4 and TROMA-I in ASTT cells by immunofluorescence. Expression of pharmacological levels of COUP-TFI in ASTT cells for 4 days resulted in differentiation to endodermal cells both with and without RA treatment as demonstrated by the loss of expression of both SSEA-1 and OCT-4 and the gain of expression of TROMA-I (Figure 4, 0 Dox). Furthermore, RA was required for the differentiation of ASTT cells expressing physiological levels of COUP-TFI for 4 days since these cells demonstrate the ability to gain TROMA-I expression, albeit at a lower level than wild type cells and ASTT expressing pharmacological levels of COUP-TFI, and to lose both SSEA-1 and OCT-4 expression only upon RA treatment (Figure 4, 0.5 and 1.0 Dox). As expected, ASTT cells in which COUP-TFI expression is suppressed failed to differentiate to endodermal cells upon RA treatment as previously demonstrated for AS cells (Figure 4, 100 Dox).

Effect of COUP-TFI on the Expression of Select Pluripotency and Differentiation Associated Genes

Since the expression of COUP-TFI in the absence of PBX expression resulted in differentiation to endodermal cells, we examined the expression of several genes known to be either induced or downregulated during RA-dependent differentiation of P19 cells (Figure 5). RT-qPCR analysis demonstrated that the mRNA levels of OCT-4, SF-1 and DAX-1 were unchanged when COUP-TFI is expressed at physiological levels (1.0 and 0.5 ng/ml Dox) and reduced in cells expressing a pharmacological level (0 ng/ml Dox) of COUP-TFI after 3 days of ethanol treatment. However, the mRNA levels of these three genes in cells expressing both physiological and pharmacological levels of COUP-TFI are substantially reduced upon RA treatment for 3 days. This is consistent with the immunofluorescence studies showing that ASTT cells expressing physiological levels of COUP-TFI differentiate to endodermal cells only upon RA treatment while cells expressing pharmacological levels of COUP-TFI differentiate to endodermal cells irrespective of RA treatment. Furthermore, these data demonstrate that COUP-TFI plays an important role in the negative regulation of certain key genes critical for pluripotency thereby highlighting the notion that COUP-TFI expression is a key regulator governing the loss of pluripotency.

On the other hand, in cells expressing both pharmacological and physiological levels of COUP-TFI, COUP-TFII mRNA levels are increased by a modest amount (approximately 50-fold and 120-fold after 3 days ethanol and RA treatment, respectively) when compared to the approximately 1000-fold increase in wild type TO9 cells treated with RA. This suggests that a high level of COUP-TFII expression may not be essential for differentiation or possibly COUP-TFI can compensate for COUP-TFII during endodermal differentiation of P19 cells due to functional redundancies between the two nuclear receptors.

Effect of COUP-TFI on the Expression of Primary RA-Responsive Genes

We also examined the effect of COUP-TFI on the expression of primary RA-response genes that have well established RAREs in their promoter and are known to have elevated expression levels within 3 to 6 hrs following RA treatment of P19 cells. Of the genes examined, three (HNF3 α , RAR β 2 and STRA4) exhibited similar increases in their mRNA levels following RA treatment of COUP-TFI expressing cells and empty vector cells (Figure 6 C-E). Surprisingly, CYP26A1 mRNA levels were not increased by RA treatment in the cells expressing both physiological and pharmacological levels of COUP-TFI (Figure 6B). This suggests that COUP-TFI has a repressive effect on CYP26A1 expression and that the high CYP26A1 mRNA levels observed in wild type P19 cells shortly after RA treatment may not be necessary for differentiation of P19 cells. Contrary to this finding, both pharmacological and physiological levels of COUP-TFI alone without RA treatment caused an increase in HoxA1 mRNA levels substantially above the baseline levels in control TO9 cells (Figure 2A). This suggests that HoxA1 expression can be regulated by COUP-TFI and may contribute to the endodermal differentiation of these cells. Finally, these findings suggest that there is a gene-dependent effect of COUP-TFI expression whereby COUP-TFI can cause different effects on the RA-regulated expression of different primary response genes.

Discussion

Prior studies from our laboratory have demonstrated that an increase in PBX expression in P19 cells is required for the RA-dependent reduction in the expression of several pluripotency genes (OCT-4, SF-1 and DAX-1) and differentiation of P19 cells to endodermal cells (Qin et al, 2004b; Teets et al., 2012). Furthermore, the PBX-dependent reduction in SF-1 but not DAX-1 expression is necessary for differentiation of P19 cells to endodermal cells (Teets et al., 2012). Here we show that the expression of two differentiation associated genes (COUP-TFI and COUP-TFII variant 1) is elevated upon RA treatment of P19 cells and that the increases in their mRNA levels are also PBX-dependent during endodermal differentiation. The overall goal of this work was to determine if expression of COUP-TFI can overcome the loss of PBX induction during RA-dependent differentiation of AS P19 cells. We found that in a dose-dependent fashion COUP-TFI expression leads to growth inhibition, modest cell cycle disruption and early apoptosis. Furthermore, AS cells can overcome their blockage in RA-dependent differentiation leading to endodermal cells when either pharmacological levels of COUP-TFI are expressed or with a combination of physiological levels of COUP-TFI and RA treatment. Finally, COUP-TFI is involved in the regulatory network governing the expression of at least two primary RA response target genes, CYP26A1 and HoxA1. Taken together, our studies demonstrate that elevation of COUP-TFI expression is a critical step in the RA-dependent differentiation cascade/pathway of P19 cells to endodermal cells.

Consistent with the findings of Ben-Shushan et al. (1995), COUP-TFI and COUP-TFII mRNA levels in P19 cells were found to be highly elevated upon RA treatment with the maximal increase after 72 hrs. In addition, only variant 1 of COUP-TFII was found to display this RA-dependent increase in mRNA levels. Furthermore, the increase in COUP-

TFI and COUP-TFII variant 1 mRNA levels is not a direct response to RA but rather requires the RA-dependent increase in PBX expression. Although our studies did not address whether PBX directly or indirectly regulates the mRNA levels of COUP-TFI and/or COUP-TFII variant 1, it is clear that these genes are downstream of PBX in the cascade of events initiated by RA leading to endodermal differentiation of P19 cells.

The hallmark of AS cells is their failure to induce PBX expression and their subsequent inability to differentiate upon RA treatment (Qin et al., 2004b). Our data strongly suggest a functional role for COUP-TFI during the differentiation cascade/pathway of P19 cells to endodermal cells. Immunofluorescence analysis of SSEA-1, OCT-4 and TROMA-1 expression along with RT-QPCR measurement of several pluripotency genes mRNA levels demonstrates that AS cells expressing a pharmacological level of COUP-TFI for 4 days differentiate to endodermal cells irrespective of RA treatment. On the other hand, RA is needed to induce differentiation when COUP-TFI is expressed at a physiological level. This suggests that AS cells expressing a physiological level of COUP-TFI still require a RA-regulated event(s) distinct from those associated with the elevation of PBX for differentiation. In contrast, cells expressing a pharmacological level of COUP-TFI either do not require this RA-regulated event (s) or high levels of COUP-TFI can replace the need for RA. Since active cell proliferation and cell differentiation are known to be opposing processes, it is possible that the more dramatic growth inhibition observed in cells expressing a pharmacological level of COUP-TFI contributes to the differentiation of these cells to endodermal cells in the absence of RA.

Consistent with the analysis of differentiation marker gene expression, the overall trend from the study of pluripotency gene expression (OCT-4, SF-1 and DAX-1) in AS cells expressing either physiological levels of COUP-TFI along with RA treatment and AS cells expressing only pharmacological levels of COUP-TFI is a reduction in their mRNA levels. These are not surprising findings since prior studies have demonstrated that (i) COUP-TFI can bind to regulatory regions of the SF-1 promoter and repress its transcriptional activity (Xing et al., 2002; Shibata et al., 2001), (ii) COUP-TFI can directly repress OCT-4 transcriptional activity (Ben Sushan et al., 1995), and (iii) COUP-TFI can repress the transcription of DAX-1 (Yu et al., 1998). Therefore, COUP-TFI is likely to participate as a transcriptional repressor of genes associated with pluripotency thereby facilitating differentiation by promoting escape from pluripotency.

Interestingly, only a very modest increase in COUP-TFII mRNA levels was observed in AS cells expressing COUP-TFI that differentiated to endodermal cells (approximately 50-fold compared with 1000-fold in wild type cells treated with RA). Since COUP-TFI and COUP-TFII genes show a remarkably high degree of homology and their expression pattern in tissues often overlaps (Qiu et al., 1994), it is likely that COUP-TFI can compensate at least in part for COUP-TFII during differentiation of P19 cells to primitive endoderm. Consistent with this is a recent study (Tang et al., 2010) that demonstrated using conditional knockout mice that COUP-TFI and COUP-TFII are functionally redundant in the developing eye.

Strikingly, the time required for the differentiation of AS cells expressing COUP-TFI appears to be unusually short. Differentiation as indicated by loss of SSEA-1 expression, gain of TROMA-1 expression, and morphological changes of cells was readily apparent after 4 days of COUP-TFI expression. However, in wild type P19 cells treated with RA, an increase in COUP-TFI expression is detected after 48 hrs and differentiation is apparent only after 5 to 7 days. This shortened COUP-TFI dependent timeframe for the differentiation of AS cells is consistent with the concept that there is an RA triggered cascade of events where both the primary RA response genes and PBX induction can be bypassed and this cascade can be entered at some intermediary point that is regulated by COUP-TFI.

In light of the observation that COUP-TFI expression in AS cells resulted in differentiation similar to that seen upon RA mediated differentiation of wild type P19 cells, we next determined if primary RA response gene expression was affected by COUP-TFI expression. Surprisingly we found that the expression of two RA-responsive genes (HoxA1 and CYP26A1) was altered with either an induced expression in the absence of RA treatment (HoxA1) or lack of RA-dependent increase (CYP26A1) in AS cells expressing COUP-TFI. These data suggest that COUP-TFI is able to either directly or indirectly regulate the expression of both HoxA1 and CYP26A1.

HoxA1 contains an RARE in its 3' enhancer region which has been demonstrated to be controlled by RA-dependent transcriptional regulation through RAR γ (Boylan et al., 1993). Additionally, HoxA1 has been implicated in the antiproliferative effect of RA on ES cells. Hoxa1^{-/-} cells are more resistant to the growth inhibitory effects of RA than Hoxa1^{+/-} which are more resistant than Hoxa1^{+/+} suggesting that the Hoxa1 protein plays a role in the RA-induced inhibition of growth in ES cells in a dose dependent manner (Martinez-Ceballos et al., 2005). On the other hand, expression of COUP-TFI in mouse ES cells caused the cells to be more resistant to the growth inhibitory effects of RA however the authors did not examine if HoxA1 levels were elevated in these cells (Zhuang et al., 2007). Strikingly, we found that expressing COUP-TFI at either physiological or pharmacological levels resulted in increased expression of HoxA1, with or without RA-treatment, and resulted in a subsequent reduction in the growth of the P19 cells. Although there was an apparent increase in growth inhibition upon expression of a pharmacologic level of COUP-TFI, there was no subsequent increase in HoxA1 expression. This is likely to be explained by the increased amount of cells expressing pharmacological levels of COUP-TFI that entered early apoptosis. Taken together this suggests that the COUP-TFI induced HoxA1 expression may contribute to the growth inhibition observed in these cells and in wild type P19 cells induced to differentiate by RA treatment.

COUP-TFI is well known to serve as a potent transcriptional repressor of genes and to efficiently antagonize transcriptional activation mediated by a number of nuclear receptors including RAR, RXR, PPAR, and ER (Cooney et al., 1993; Burbach et al., 1994; Klinge et al., 1997; Nakshatri and Bhat-Nakshatri, 1998) by competition for target response elements. Analysis of the promoter region of both mouse and human CYP26A1 using NUBISCAN (www.nubiscan.unibas.ch/) (Podvinec et al., 2002), demonstrates one fully conserved DR1 sequence which could potentially bind COUP-TFI. This DR1 lies about 25 base pairs downstream of the R2 RARE located approximately 2 kilobases upstream from the start site of transcription. Both the R2 RARE and the R1 RARE located within the proximal promoter of the CYP26A1 gene have been shown to work synergistically to direct a large increase in transcription upon RA treatment (Loudig et al., 2005). Deletion of either R1 or R2 results in a dramatic reduction in the level of RA dependent transcription however R2 RARE is more sensitive to low concentrations of RA. It is possible that COUP-TFI binds to this DR1 sequence adjacent to the R2 RARE and interferes with the functioning of the R2 RARE sequence resulting in a loss of RA-dependent increase in transcription.

In summary, we have demonstrated that the RA-dependent increase in PBX protein levels is required for the increase in COUP-TFI and COUP-TFII variant 1 expression in P19 cells induced to differentiate to endodermal cells. An increase in the level of expression of COUP-TFI appears critical for the RA-dependent differentiation of P19 cells to endodermal cells. Here, we report for the first time that physiological levels of COUP-TFI in cells lacking PBX cells combined with RA treatment induces their differentiation to endodermal cells while pharmacologic expression levels circumvents the need for RA treatment. In addition, two additional genes have been found to be regulated by COUP-TF, Hoxa1 expression was increased while the RA-dependent increase in Cyp26a1 was repressed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. PBX expression is required for the increase in COUP-TFI and COUP-TFII Variant 1 mRNA levels during RA-dependent differentiation of P19 cells to endodermal cells
 TO9 or AS2 cells were treated with 10^{-7} M RA or ethanol for 3 days. (A and B) Levels of COUP-TFI and COUP-TFII mRNA were quantitated by RT-qPCR analysis and normalized to GAPDH mRNA levels. Values are the Mean $\pm$ SD of triplicate samples. (C) Levels of COUP-TFII Variant 1I (V1) and Variant 2 (V2) mRNA were determined using primers specific to each variant by RT-PCR analysis followed by gel electrophoresis.

Figure 2. Preparation of ASTT cells inducibly expressing various levels of COUP-TFI
 (A) AS2 cells stably transfected with both pTet-Off-Advanced plasmid DNA and pTRE-V5-COUP-TFI plasmid DNA (ASTT clones) were grown in media containing 100 ng/ml Dox or 0 ng/ml Dox for 24 hrs. Levels of V5-tagged COUP-TFI and GAPDH were determined by Western blot using primary antibodies specific for V5 and GAPDH. (B) ASTT154 cells were grown in the presence of the indicated concentrations of Dox for 24 hrs. Levels of V5-tagged COUP-TFI and GAPDH were determined by Western blot. (C) ASTT154 cells and TO9 cells were grown in the presence of the indicated concentrations of Dox for 24 hrs. Cells were then treated with 10^{-7} M RA for an additional 72 hrs. Total RNA was isolated and the level of COUP-TFI mRNA was determined by RT-qPCR and normalized to GAPDH levels. The level of COUP-TFI mRNA in the TO9 cells was set to 1 and all other values shown for ASTT cells at varying concentrations of Dox were calculated relative to this sample. Values are indicated as Mean $\pm$ SD of triplicate samples. One way ANOVA, ***P<0.001, **P<0.01 and *P<0.05. (D) ASTT154 cells expressing various levels of COUP-TFI (0, 1.0 and 100 ng/ml Dox) were treated with ethanol or 10^{-7} M RA for 72 hrs. The levels of mRNA for vitronectin, PEPCK and GAPDH were determined by RT-PCR analysis followed by gel electrophoresis. (E) TO9 cells and ASTT154 cells expressing various levels of COUP-TFI (0, 0.5, 1.0 and 100 ng/ml Dox treatment) were treated with ethanol or 10^{-7} M RA for 72 hrs. The levels of PBX and GAPDH were determined by Western blot analysis.

Figure 3. Effect of COUP-TFI expression on the cell growth

(A) ASTT154 cells expressing various levels of COUP-TFI (0, 0.5, 1.0 and 100 ng/ml Dox treatment) were treated with ethanol or 10⁻⁷ M RA (Day 0) and the cell number determined on the indicated days. (B and C) ASTT154 cells expressing various levels of COUP-TFI (0, 1.0 and 100 ng/ml Dox treatment) were grown in the presence of the indicated concentrations of Dox for 96 hrs. The percentage of cells in each of the cell cycle phases were determined by FACS analysis with PI staining (B) and the percentage of cells in apoptosis was assayed by FACS analysis with Annexin V and PI staining (C). Values are the Mean $\pm$ SD of triplicates. One way ANOVA, ***P<0.001, **P<0.01 and *P<0.5.

Figure 4. Effect of COUP-TFI expression on RA-mediated endodermal differentiation
 ASTT154 cells expressing various levels of COUP-TFI (0, 0.5, 1.0 and 100 ng/ml Dox treatment) were treated with either ethanol or 10^{-7} M RA. After either 0 or 4 days of treatment, cells were fixed and then probed for OCT-4, SSEA-1 and TROMA-I expression by immunohistochemistry. DAPI was included as a nuclear stain. Scale bar equals 50 μ M.

Figure 5. Effect of COUP-TFI expression on the mRNA level of select pluripotency and differentiation associated genes during RA-dependent differentiation to endodermal cells TO9 cells and ASTT154 cells expressing various levels of COUP-TFI (0, 0.5, 1.0 and 100 ng/ml Dox treatment) were treated with either 10^{-7} M RA or ethanol. After 0 or 3 days treatment, total RNA was isolated and RT-qPCR analysis was performed using primers specific to (A) OCT-4, (B) DAX-1, (C) SF-1 and (D) COUP-TFII. The mRNA levels were normalized using the corresponding GAPDH mRNA level. All values for fold reductions/ inductions were calculated relative to mRNA expression of TO9 Day 0 sample, used as the calibrator set to 1. Mean $\pm$ SD of triplicate samples.

Figure 6. Effect of COUP-TFI expression on the mRNA level of select primary RA response genes during RA-dependent differentiation to endodermal cells
 TO9 cells and ASTT154 cells expressing various levels of COUP-TFI (0, 0.5, 1.0 and 100 ng/ml Dox treatment) were treated with either 10^{-7} M RA or ethanol. After 0 or 1 day treatment, total RNA was isolated and RT-qPCR analysis was performed using primers specific to (A) HoxA-1, (B) CYP26A1, (C) RARβ2, (D) STRA4, (E) HNF3α and (F) βactin. The mRNA levels were normalized using the corresponding GAPDH mRNA level. All values for fold inductions were calculated relative to mRNA expression of TO9 Day 0 sample, used as the calibrator set to 1. Mean $\pm$ SD of triplicate samples.