Published in final edited form as:

J Cell Physiol. 2012 April ; 227(4): 1501–1511. doi:10.1002/jcp.22866.

Role of SF-1 and DAX-1 during Differentiation of P19 Cells by Retinoic Acid

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 critical for embryonic development and cellular differentiation. Previous work in our laboratory has shown that blocking the RA-dependent increase in pre- β cell leukemia transcription factors (PBX) mRNA and protein levels in P19 cells prevents endodermal and neuronal differentiation. Dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1 (DAX-1) and steroidogenic factor (SF-1) were found by microarray analysis to be regulated by PBX in P19 cells. To determine the roles of DAX-1 and SF-1 during RA-dependent differentiation, P19 cells that inducibly express either FLAG-DAX-1 or FLAG-SF-1 were prepared. Unexpectedly, overexpression of DAX-1 had no effect on the RA-induced differentiation of P19 cells to either endodermal or neuronal cells. However, SF-1 overexpression prevented the RA-dependent loss of OCT-4, DAX-1 and the increase in COUP-TFI, COUP-TFII and ETS-1 mRNA levels during the commitment stages of both endodermal and neuronal differentiation. Surprisingly, continued expression of SF-1 for seven days caused the RA-independent loss of OCT-4 protein and RA-dependent loss of SSEA-1 expression. Despite the loss of well characterized pluripotency markers, these cells did not terminally differentiate into either endodermal or neuronal cells. Instead, the cells gained the expression of many steroidogenic enzymes with a pattern consistent with adrenal cells. Finally, we found evidence for a feedback loop in which PBX reduces SF-1 mRNA levels while continued SF-1 expression blocks the RA-dependent increase in PBX levels. Taken together, these data demonstrate that SF-1 plays a dynamic role during the differentiation of P19 cells and potentially during early embryogenesis.

Keywords

SF-1; DAX-1; OCT-4; retinoic acid; P19 cells

Introduction

Retinoic acid (RA) is critical for embryonic development and cellular differentiation (Clagett-Dame and De Luca, 2002; Zile, 2001; Ross et al., 2000). Both pluripotent embryonal carcinoma (EC) cells and embryonic stem (ES) cells can be induced to differentiate *in vitro* along a variety of pathways upon treatment with RA (Soprano et al., 2007). RA functions by binding to ligand-inducible transcription factors belonging to the steroid/thyroid hormone receptor superfamily (RAR α , RAR β , RAR γ , RXR α , RXR β and RXR γ) that activate and repress the transcription of downstream target genes (Chambon,

1996). The mRNA and protein levels of a large number of genes in EC and ES cells have been demonstrated to be both directly and indirectly modulated by RA during differentiation along a number of different pathways (Soprano et al., 2007).

Pre- β cell leukemia transcription factors (PBX) are members of the three-amino acid loop extension superclass of Homeobox proteins (Burglin, 1997; Capellini et al., 2011). These proteins are essential for multiple developmental processes by functioning as cofactors to enhance the DNA binding affinity and specificity of members of the Hox family of transcription factors. Prior studies from our laboratory demonstrated that PBX mRNA and protein levels were elevated in P19 cells upon RA treatment (Qin et al., 2004a). Using P19 cells that express an antisense PBX mRNA that greatly reduces the RA-dependent increase in PBX protein levels (AS cells), we demonstrated that the RA-dependent increase in PBX proteins was necessary for differentiation to both endodermal and neuronal cells (Qin et al., 2004b). Therefore, one of the goals of this work was to identify genes whose expression levels were regulated by PBX during differentiation of P19 cells to endodermal and neuronal cells.

Steroidogenic factor 1 (SF-1/NR5A-1) and dosage sensitive sex-reversal, adrenal hypoplasia congenital locus on the X-chromosome, gene 1 (DAX-1/NR0B1) are two orphan members of the nuclear receptor superfamily (Kohler and Achermann, 2010; Lalli and Alonso, 2010). In adult mice and embryos beginning on gestation day 9, the expression of SF-1 and DAX-1 is restricted to tissues involved in steroid hormone production (adrenal cortex, testis Leydig and ovarian theca cells) and reproduction (testis Sertoli and ovarian granulosa cells, pituitary gonadotropes and ventromedial hypothalamic neurons) (Ikeda et al., 1994; Swain et al., 1996). Both SF-1 and DAX-1 are critical factors for adrenal and gonadal development since mutations in the *Sf-1* or *Dax-1* genes result in serious developmental defects associated with these organs. SF-1 is an important activator of a large number of enzymes critical for steroidogenesis while DAX-1 is a negative regulator of steroidogenesis. More recently SF-1 and DAX-1 have been found to be expressed in early preimplantation embryos along with EC and ES cells (Mullen et al., 2007; Clipsham et al., 2004; Gu et al., 2005). In addition, SF-1 can substitute for OCT-4 during reprogramming of murine somatic cells to pluripotent cells (iPS cells) (Heng et al., 2010). Among the known SF-1 target genes (Hoivik et al., 2010; Schimmer and White, 2010), only DAX-1 was also expressed in embryos prior to gestation day 9. These data suggest that SF-1 and DAX-1 may have a unique function(s) in early embryos different from their previously understood roles associated with steroidogenesis and that they could be instrumental during early embryogenesis.

In this report, DAX-1, OCT-4 and SF-1 were found to be regulated by PBX in P19 cells treated with RA. To study the role of SF-1 and DAX-1 during differentiation of P19 cells, cell lines that inducibly express these proteins were prepared. Overexpression of SF-1, but not DAX-1, was found to block the RA-induced differentiation of P19 cells into endodermal and neuronal cells. Interestingly, overexpression of SF-1 in P19 cells for an extended period of time resulted in the loss of pluripotency and the expression of a battery of steroidogenic enzymes consistent with adrenal-like cells. Finally, overexpression of SF-1 inhibited the RA-dependent increase in PBX protein levels in P19 cells. These data demonstrate that SF-1 plays a dynamic role during the differentiation of P19 cells and potentially a larger role during early embryogenesis than previously described.

Materials and Methods

Cell Culture and Differentiation

P19 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 μ g/ml penicillin and

100 units/ml streptomycin. TO3 (vector control P19 cells) and AS2 cells (P19 cells that express antisense PBX RNA) (Qin et al., 2004b) were routinely passed in complete DMEM supplemented with 200 µg/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. For neuronal differentiation, 9×10^5 cells in 9 ml of complete DMEM were added to 100 mm dishes coated with a 1X agarose/DMEM base and treated with 10^{-7} M all-trans RA for 4 days to form aggregates. After 4 days, the aggregates were trypsinized and plated onto tissue culture dishes for an additional 3 days in complete DMEM without RA.

Preparation of Inducible DAX-1 and SF-1 Expression Cell Lines

TO3 cells that stably express the Tet-transactivator protein were prepared by transfecting TO3 cells with pTet-Off Advanced Vector DNA (Clontech). Cells were selected on complete DMEM supplemented with 200 µg/ml zeocin, 800 µg/ml G418 and 100 ng/ml doxycycline (Dox). Individual G418 resistant clones (TTO 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. TTO clones which displayed high inducible expression of luciferase were also screened to assure RA-dependent differentiation by analyzing expression of SSEA-1, TROMA-1, TUJ1, and OCT-4 by immunofluorescence. TTO7 cells were used for all additional studies.

To prepare DAX-1 and SF-1 expression clones, TTO7 cells were transfected with pTRE-TIGHT Vector DNA (Clontech) containing either FLAG-DAX-1 cDNA or FLAG-SF-1 cDNA, respectively, along with linear hygromycin DNA (Clontech). Cells were selected on complete DMEM supplemented with 200 µg/ml zeocin, 800 µg/ml G418, 300 µg/ml hygromycin and 100 ng/ml Dox. Individual hygromycin resistant clones (TTD and TTS) were screened for inducible expression of FLAG-DAX-1 or FLAG-SF-1, respectively, upon removal of Dox by In Cell Western using mouse anti-FLAG M2 (Sigma, F1804) 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 FLAG-DAX-1 or FLAG-SF-1 and to determine the percent positive cells in the absence of Dox.

RNA Analysis

Total RNA was isolated using RNeasyTM B reagent (Qiagen). 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) as described by the manufacturer and quantitative real time PCR (qPCR) was performed using SYBR Green Master Mix (Fermentas) according to the manufacturer's instructions and an ABI Model 7500 Instrument as previously described (Zhao et al., 2009; Vucetic et al., 2008). Primers purchased from Integrated DNA Technologies (IDT) 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 rabbit anti-OCT-3/4 (Santa Cruz, sc-9081), mouse anti-PBX1,2,3,4 (Santa Cruz, sc-28313), mouse anti-FLAG M2 (Sigma, F1804) and goat anti-GAPDH (Santa Cruz, sc-20357). Secondary antibodies used were

donkey anti-rabbit IRDye 800CW, donkey anti-mouse IRDye 800CW, and donkey anti-goat IRDye 680CW purchased from LI-COR. Images were captured and quantitated using the LI-COR Odyssey instrument and software. GAPDH levels were used as the loading control.

Immunohistochemistry

P19 cells were plated 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 used were mouse anti-SSEA-1 (MC-480, Developmental Study Hybridoma Bank, University of Iowa), rat anti-cytokeratin Endo-A (TROMA-1, Developmental Study Hybridoma Bank, University of Iowa), mouse anti-FLAG M2 (Sigma, F1804), rabbit anti-OCT-3/4 (Santa Cruz Biotechnology, sc-9081), and rabbit anti-neuronal class III β-tubulin (TUJ1) (Covance, MRB-435P). The coverslips were then incubated for 30 min at room temperature in secondary antibody solution (1 µg/ml antibody in blocking buffer). Secondary antibodies used were anti-rat-TRITC, anti-mouse-TRITC, anti-mouse-FITC, anti-rabbit-TRITC and anti-rabbit-FITC purchased from Santa Cruz Biotechnology. In cases where two antibodies were raised in mice, coverslips were incubated with goat anti-mouse Fab2 Fluorescein conjugated fragments (Pierce) diluted 1:50 with PBS containing 1% BSA for 20 min between the incubation with the two mouse primary antibodies. 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.

Results

Expression of PBX-regulated genes during RA-induced commitment to endodermal and neuronal cells

Prior studies from our laboratory using TO (vector control) and AS (PBX antisense expressing) clones of P19 cells demonstrated that the RA-dependent increase in PBX protein levels is necessary for P19 cells to differentiate to both endodermal and neuronal cells (Qin et al., 2004b). To identify PBX-regulated genes during endodermal and neuronal differentiation, mRNA expression patterns in TO3 and AS2 cells treated for 72 hrs with RA were examined by microarray analysis. Among the RNAs whose levels of expression were found to be reduced in TO3 cells but not AS2 cells upon RA treatment during both endodermal and neuronal differentiation were OCT-4, DAX-1 and SF-1. Figure 1 contains a time course analysis of the levels of mRNA for OCT-4, DAX-1 and SF-1 in TO3 and AS2 cells induced to differentiate to endodermal and neuronal cells by RA treatment. By 48 hrs after RA treatment the levels of each of these mRNA were dramatically reduced in TO3 cells however they were unaffected in cells which have greatly reduced levels of PBX (AS2). Interestingly, the level of DAX-1 mRNA was consistently elevated approximately 5-fold in the AS2 cells treated with RA. These data demonstrate that the loss of OCT-4, DAX-1 and SF-1 expression requires the RA-dependent increase in PBX protein levels. Furthermore, DAX-1 and SF-1 in addition to the well known pluripotency factor OCT-4 (Nichols et al., 1998; Ovitt and Scholer, 1998) may be important regulators of the loss of pluripotency and gain of differentiation specific gene expression in P19 cells.

Role of DAX-1 during RA-induced differentiation of P19 cells to endodermal and neuronal cells

To study the effect of continued DAX-1 expression during RA-dependent differentiation of P19 cells, stable cell clones that inducibly express FLAG-DAX-1 upon removal of Dox (TTD clones) were prepared (Figure 2A). Analysis of individual TTD28 and TTD92 cells by immunofluorescence demonstrated that FLAG-DAX-1 protein was localized principally in the nucleus with a low level of protein in the cytoplasm similar to the findings of Holter et al., (2002) in greater than 90% of the cells upon removal of Dox for 24 hr (data not shown). All studies were performed with both TTD28 and TTD92 cells. Since similar results were obtained with cells from both clones. Data are presented for TTD28 cells.

We first examined the effect of inducible expression of DAX-1 on the mRNA levels of several genes known to be regulated during RA-dependent differentiation of P19 cells. Surprisingly, continued expression of DAX-1 did not block the loss in the mRNA levels of two pluripotency genes, OCT-4 and SF-1, upon treatment with RA in cells induced to differentiate to both endodermal and neuronal cells (Figure 2B). In addition, continued DAX-1 expression did not affect the RA-dependent increase in the mRNA levels of COUP-TFI, COUP-TFII and ETS-1 during both endodermal and neuronal differentiation along with PAX6 during neuronal differentiation (Figure 2C). Similar fold inductions in the mRNA levels of all genes in the DAX-1 expressing cells were observed except that COUP-TFII mRNA levels were increased to a lesser extent upon RA treatment during endodermal differentiation and PAX6 mRNA levels were increased to a greater extent upon RA treatment during neuronal differentiation.

Furthermore, examination of SSEA-1, OCT-4, TROMA-1 and TUJ1 expression seven days after RA treatment demonstrated that overexpression of DAX-1 did not affect the ability of these cells to differentiate to either endodermal cells or neuronal cells. DAX-1 expressing cells lost the expression of two markers for pluripotency (SSEA-1 and OCT-4) (Figure 3 A and B) and gained the expression of the endodermal specific marker TROMA-1 (Figure 3A) and the neuronal specific marker TUJ1 (Figure 3B). Taken together these data demonstrate that the RA-dependent loss of DAX-1 expression is not a critical step during the differentiation of P19 cells to endodermal and neuronal cells, and that DAX-1 alone is unable to maintain a pluripotent state in these cells.

Role of SF-1 during RA-induced differentiation of P19 cells to endodermal and neuronal cells

To study the effects of sustained SF-1 expression during RA-dependent differentiation of P19 cells, stable cell clones that inducibly express FLAG-SF-1 upon removal of Dox (TTS clones) were prepared (Figure 4A). The level of expression of FLAG-SF-1 protein in cells grown in media containing 0.4 ng/ml Dox was approximately 10% of that found in cells grown in the complete absence of Dox (No Dox). Analysis of individual TTS52, TTS64 and TTS63 cells which express a low, medium and high level FLAG-SF-1 protein demonstrated that the protein 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 TTS clones. Similar results were obtained with cells from all three clones therefore data is presented for TTS52 cells.

We first examined the effect of inducible expression of SF-1 on the mRNA level of several genes known to be regulated during RA-dependent differentiation of P19 cells. Continued expression of SF-1 blocked the RA-dependent loss in the mRNA levels of three pluripotency genes, OCT-4, DAX-1 and endogenous SF-1, in a dose-dependent fashion in cells induced to differentiate to both endodermal and neuronal cells with the exception of endogenous

SF-1 mRNA levels during neuronal differentiation (Figure 4C). In addition, continued SF-1 expression also blocked in a dose-dependent manner the RA-dependent increase in mRNA levels of COUP-TFI, COUP-TFII and ETS-1 during both endodermal and neuronal differentiation. On the other hand, continued SF-1 expression had no effect on the RA-induced increase in PAX6 mRNA levels during neuronal differentiation (Figure 4D). Since continued expression of SF-1 caused a lack of response in the expression of several pluripotency- and differentiation-associated genes upon treatment with RA, we next determined the mRNA level of two primary RA response genes, RAR β 2 and CYP26A1, to insure that these cells are capable of responding to RA. RA treatment increased the mRNA level of these two genes to a similar extent in both cells with and without SF-1 overexpression (Figure 4B) demonstrating that the SF-1 overexpressing cells are fully capable of responding to RA treatment.

Since we observed such dramatic changes in the expression of several pluripotency- and differentiation-associated genes upon RA treatment for three days in cells that overexpress SF-1, we next examined the ability of these cells to terminally differentiate to endodermal and neuronal cells by examining SSEA-1, OCT-4, TROMA-1 and TUJ1 expression after seven days of RA treatment. Since some cells (greater number in the ethanol compared with the RA treated group) grown in media containing either 0.4 ng/ml Dox or No Dox lost FLAG-SF-1 expression by day 7, we chose to focus only on those cells which retained FLAG-SF-1 expression for the full seven days. Similar findings were found using cells that were grown in media containing both 0.4 ng/ml Dox and No Dox therefore the results with No Dox are shown. Both SSEA-1 protein and OCT-4 protein expression were lost in cells with or without SF-1 expression induced to differentiate by RA treatment to both endodermal and neuronal cells (Figure 5A and B). Interestingly, there appeared to be a greatly reduced level or loss of detectable OCT-4 protein in SF-1 expressing cells treated only with ethanol suggesting that elevated expression of SF-1 for seven days is sufficient to reduce OCT-4 expression (Figures 5A and B). Examination of 250 FLAG-SF-1 positive cells plated as monolayers (endodermal) and aggregates (neuronal) resulted in only 18 or 14 OCT-4 positive cells, respectively, in the ethanol treatment group and 3 or 0 OCT-4 positive cells, respectively, in the RA treatment group. However, although sustained SF-1 expression and RA treatment caused the cells to lose SSEA-1 and OCT-4 expression by day 7, the cells failed to gain expression of two markers of differentiation, TROMA-1 and TUJ1 (Figures 5A and B). Out of 250 FLAG-SF-1 positive cells there were only 2 TROMA-1 positive cells in the RA treatment group induced to differentiate to endodermal cells and no TUJ1 positive cells in the RA treatment group induced to differentiate to neuronal cells. Taken together these data demonstrate that SF-1 expression leads to a RA-independent loss of OCT-4 expression and a RA-dependent loss of SSEA-1 protein expression indicating that the cells are no longer pluripotent. However, they fail to differentiate to either endodermal or neuronal cells.

Expression of SF-1 in P19 cells blocks the RA-induced increase in PBX protein during both endodermal and neuronal differentiation

We also examined the effect of sustained SF-1 expression on the levels of PBX proteins during both endodermal and neuronal differentiation. Similar to the report of Qin et al., 2004a, there was a large increase in PBX protein levels in cells that do not express SF-1 (100 ng/ml Dox) upon RA treatment for both 1 day and 3 days during both endodermal and neuronal differentiation (Figure 6A and B). Unexpectedly, PBX protein levels were substantially lower in the 0.4ng/ml Dox and No Dox treated cells induced to differentiate to both endodermal and neuronal cells. Strikingly this attenuation of the increase in PBX protein levels in response to RA treatment correlated with increasing amounts of FLAG-SF-1 expression.

Since Qin et al. (2004a) had previously demonstrated that the increase in PBX protein levels upon RA treatment involved both a small increase in PBX isoform mRNA levels and an increase in the stability of PBX proteins; we examined mRNA levels of each of the PBX isoforms after 24 hrs treatment with RA. A similar fold increase in PBX1, PBX2, PBX3 and PBX4 mRNA levels was found in the cells treated with RA both in the presence and absence of Dox treatment (Figure 6C) suggesting that SF-1 overexpression interferes with the RA-dependent stabilization of PBX protein levels.

SF-1 Induces P19 cells to Differentiate into Adrenal-Like Cells

Since sustained expression of SF-1 and RA treatment resulted in a loss of both SSEA-1 and OCT-4 expression without a gain in the expression of markers of endodermal or neuronal cells, we hypothesized based on the reports of Yazawa et al. (2006 and 2011) that SF-1 overexpression in P19 cells results in differentiation to steroidogenic cells. Our results also suggested that aggregation of P19 cells facilitated the RA-independent loss of OCT-4 (data not shown); therefore we chose to examine the effect of sustained SF-1 expression in cells grown as aggregates by examining the mRNA levels of known steroidogenesis-related genes (Yazawa et al., 2006; Yazawa et al., 2011). We found that, with the exception of HSD3b6, none of the mRNAs for steroidogenic enzymes were detected in either TO3 or AS2 cells upon conditions that promote neuronal differentiation (Figure 7A). In contrast, overexpression of SF-1 induced P19 cells to express the mRNA for STAR, p450scc and p450c17 in an RA-independent manner (Figure 7B). In addition, cAMP treatment of the ETOH treated cells increased the mRNA levels of STAR, p450c17, p45011b1, and HSD17b3 on day six; however this increase was transient as there was a decrease in the mRNA level of all the steroidogenic enzymes after an additional four days. Although the mRNA level of some of the genes was increased in an RA-independent fashion, the mRNA levels of each of the genes were greater when the aggregates were grown in the presence of RA. Unlike the ETOH treated cells, the RA-treated cells continued to express the mRNAs of steroidogenic enzymes at a high level after ten days. The only exception to this was p450c17 mRNA. One possibility for the reduction in p450c17 mRNA levels is that the newly formed steroidogenic-like cells were continuing to mature between days six and ten as adult murine adrenal cells do not express p450c17 (Yazawa et al., 2011). Taken together, this suggests that elevated SF-1 expression and RA treatment causes P19 cells to adopt an adrenal-like cell fate.

Discussion

This work began with the goal of identifying PBX-regulated genes important for the regulation of differentiation of P19 cells to endodermal and neuronal cells following RA treatment. Genes were identified by examining patterns of expression in AS2 cells that fail to elevate PBX levels following RA treatment compared with that of wild type P19 by microarray analysis. The mRNA levels of three genes (DAX-1, OCT-4 and SF-1) were found to be greatly reduced in wild type cells following RA treatment while their mRNA levels were unchanged in AS2 cells demonstrating that the RA-dependent reduction in the expression of DAX-1, OCT-4 and SF-1 is directly or indirectly regulated by PBX. OCT-4 is well known to be critical for the maintenance of pluripotency (Nichols et al., 1998; Ovitt and Scholer, 1998) while the role of SF-1 and DAX-1 in this process is less well understood. We then asked whether the overexpression of DAX-1 and/or SF-1 in wild type P19 cells blocks the downstream changes in gene expression upon RA treatment that leads to differentiation to either endodermal or neuronal cells. We found that SF-1, but not DAX-1, blocked the RA-induced differentiation of P19 cells into endodermal and neuronal cells. Interestingly, overexpression of SF-1 in P19 cells for an extended period of time resulted in the loss of pluripotency and the expression of a battery of steroidogenic enzymes consistent with

adrenal-like cells. Finally, overexpression of SF-1 inhibited the RA-dependent increase in PBX protein levels in P19 cells.

DAX-1 is expressed in preimplantation embryos and has been suggested to be important for self renewal in mouse ES cells (Clipsham et al., 2004; Loh et al., 2006; Wang et al., 2006; Kim et al., 2008). The level of DAX-1 in mouse ES cells grown in the presence LIF appears to be critical since both an increase and decrease in DAX-1 expression has been reported to cause cell differentiation (Niakkan et al., 2006; Sun et al., 2009; Khalfallah et al., 2009). Furthermore, complete loss of DAX-1 in mouse ES cells is lethal (Yu et al., 1998). Similar to mouse ES cells (Clipsham et al., 2004; Khalfallah et al., 2009), we found that DAX-1 mRNA levels are greatly reduced upon treatment of P19 cells with RA during the commitment stages of both endodermal and neuronal differentiation. Interestingly, in AS2 cells that fail to differentiate, DAX-1 mRNA levels were increased upon RA treatment. However, unlike mouse ES cells, we report that inducible overexpression of DAX-1 in P19 cells did not cause either spontaneous cell differentiation or a block in the differentiation pathway leading to endodermal and neuronal cells upon RA treatment. While DAX-1 may be critical for self renewal in mouse ES cells grown in the presence of LIF, our findings are consistent with the suggestion that DAX-1 does not function as an essential pluripotency factor (Lalli and Alonso; 2010). For example, in human ES cells DAX-1 expression is very low and it is inconsistently modulated during their differentiation (Xie et al., 2009). Furthermore, conventional methods of preparing DAX-1 knockout mice have failed while DAX-1 loss of function mutations in humans results in only developmental defects in the adrenal gland (Lalli and Alonso; 2010). Therefore, DAX-1 may not be an essential universal pluripotency factor but rather may be a more specialized protein that functions in conjunction with more *bona fide* pluripotency factors in repressing the expression of at least a subgroup of downstream differentiation specific genes in specific circumstances.

In contrast to our findings with DAX-1, overexpression of SF-1 in P19 cells provided protection from the RA-induced loss of expression of pluripotency-associated genes and the gain of expression of several differentiation-associated genes during the commitment stages of both neuronal and endodermal differentiation. Furthermore, after seven days of SF-1 overexpression and RA treatment the cells failed to gain markers of terminal endodermal (TROMA-1) or neuronal (TUJ1) differentiation. Surprisingly, although these SF-1 overexpressing cells did not differentiate to endodermal or neuronal cells, they lost the expression of markers of pluripotency (SSEA-1 and OCT-4) and the cells displayed a more vacuolated appearance suggesting that the cells had differentiated to another cell type. Analysis of the mRNA levels of a battery of steroidogenic enzymes demonstrated that the SF-1 overexpressing P19 cells had differentiated into steroidogenic cells resembling adrenal cells. Interestingly, overexpression of SF-1 alone in P19 cells resulted in the transient expression of several steroidogenic enzyme mRNAs however SF-1 overexpression in combination with RA treatment resulted in a more robust expression of a larger number of steroidogenic enzyme mRNAs.

Previous studies have reported that overexpression of SF-1 or the highly related NR5A family member liver specific receptor homologue-1 (LRH-1) in mouse mesenchymal stem cells results in the production of steroidogenic cells (Yazawa et al., 2006; Yazawa et al., 2009; Yazawa et al., 2011; Miyamoto et al., 2011). In mouse ES cells, steroidogenic cells were produced only when the cells were first differentiated to mesenchymal stem cells by growth on collagen IV-coated dishes and RA treatment prior to inducing the expression of SF-1 (Yazawa et al., 2011). However, P19 cells differentiated to steroidogenic cells upon induction of SF-1 expression and RA treatment while SF-1 overexpression in the highly related EC cell line, F9, did not result in the gain of expression of enzymes responsible for steroid production (Yazawa et al., 2006). One of the possibilities for the inability of both ES

and F9 cells to differentiate into steroid producing cells upon overexpression of SF-1 is that they are derived from cells at an earlier stage of development than mesenchymal stem cells and P19 cells.

Finally, we also found that the levels of SF-1 and PBX expression are inversely related. AS2 cells that fail to increase PBX levels upon RA treatment do not display a reduction in SF-1 mRNA levels. On the other hand, overexpression of SF-1 in P19 cells inhibits in a dose-dependent manner the RA-dependent increase in PBX protein levels in cells grown as both monolayers and aggregates. We previously reported that RA treatment of P19 cells causes an increase in PBX protein levels by modestly increasing PBX mRNA levels and stabilizing PBX proteins (Qin et al., 2004a). Since SF-1 overexpression did not alter the RA-dependent increase in the mRNA levels of all PBX isoforms, it would appear that SF-1 can affect the half-life of PBX proteins. Thus, at least in P19 cells, there is a feedback loop in which the expression levels of PBX and SF-1 are inversely regulated.

In summary, we have demonstrated that the RA-dependent increase in PBX protein levels is required for the decrease in OCT-4, SF-1 and DAX-1 expression in P19 cells induced to differentiate to both endodermal and neuronal cells. A reduction in the level of expression of SF-1, but not DAX-1, appears critical for the RA-dependent differentiation of P19 cells to endodermal and neuronal cells. In addition, we report for the first time that overexpression of SF-1 in P19 cells combined with RA treatment induces their differentiation to adrenal-like cells. Therefore, we propose that in addition to being a model cell system for the study of endodermal and neuronal differentiation, P19 cells can also serve as a model cell line for the study of adrenal cell differentiation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Mr. Zhenping Zhang and Ms. Dorret Garner for their expert technical assistance. This work was supported by a grant from the National Institutes of Health (DK070650) (DRS) and the Pennsylvania Department of Health (D.R.S.). The Pennsylvania Department of Health specifically disclaims responsibilities for any analyses, interpretations, and conclusions.

Literature Cited

- Burglin TR. Analysis of TALE superclass homeobox genes (MEIS, PBC, KNOX, Iroquois, TGIF) reveals a novel domain conserved between plants and animals. *Nucleic Acids Res.* 1997; 25:4173–4180. [PubMed: 9336443]
- Capellini TD, Zappavigna V, Selleri L. Pbx homeodomain proteins: TALEnted regulators of limb patterning and outgrowth. *Dev Dyn.* 2011; 240:1063–1086. [PubMed: 21416555]
- Chambon P. A decade of molecular biology of retinoic acid receptors. *FASEB J.* 1996; 10:940–954. [PubMed: 8801176]
- Clagett-Dame M, De Luca HF. The role of vitamin A in mammalian reproduction and embryonic development. *Annu Rev Nutr.* 2002; 22:347–381. [PubMed: 12055350]
- Clipsham R, Niakan KN, McCabe ERB. Nr0b1 and its network partners are expressed in murine embryos prior to steroidogenic axis and organogenesis. *Gene Expression Patterns.* 2004; 4:3–14. [PubMed: 14678822]
- Gu P, Goodwin B, Chung AC, Xu X, Wheeler DA, Price RR, Galardi C, Peng L, Latour AM, Koller BH, Gossen J, Klierer SA, Cooney AJ. Orphan nuclear receptor LRH-1 is required to maintain Oct4 expression at the epiblast stage of embryonic development. *Mol Cell Biol.* 2005; 25:3492–3505. [PubMed: 15831456]

- Heng JD, Feng B, Han J, Jiang J, Kraus P, Ng JH, Orlov YL, Huss M, Yang L, Lufkin T, Lim B, Ng HH. The nuclear receptor Nr5a2 can replace Oct4 in the reprogramming of murine somatic cells to pluripotent cells. *Cell Stem Cell*. 2010; 6:167–174. [PubMed: 20096661]
- Hoivik EA, Lewis AE, Aumo L, Bakke M. Molecular aspects of steroidogenic factor 1(SF-1). *Mol Cell Endocrinol*. 2010; 315:27–39. [PubMed: 19616058]
- Holter E, Kotaja N, Makela S, Strauss L, Kietz S, Janne OA, Gustafsson JA, Palvimo JJ, Treuter E. Inhibition of androgen receptor (AR) function by the reproductive orphan nuclear receptor DAX-1. *Mol Endocrinol*. 2002; 16:515–528. [PubMed: 11875111]
- Ikeda Y, Shen WH, Ingraham HA, Parker KL. Developmental expression of mouse steroidogenic factor-1, an essential regulator of the steroid hydroxylase gene expression. *Mol Endocrinol*. 1994; 8:654–662. [PubMed: 8058073]
- Khalfallah O, Rouleau M, Barbry P, Bardoni B, Lalli E. Dax-1 knockdown in mouse embryonic stem cells induces loss of pluripotency and multilineage differentiation. *Stem Cells*. 2009; 27:1529–1537. [PubMed: 19530134]
- Kim J, Chu J, Shen X, Wang J, Orkin SH. An extended transcriptional network for pluripotency of embryonic stem cells. *Cell*. 2008; 132:1049–1061. [PubMed: 18358816]
- Kohler B, Achermann JC. Update – Steroidogenic factor 1 (SF-1, NR5A1). *Minerva Endocrinol*. 2010; 35:73–85.
- Lalli E, Alonso J. Targeting DAX-1 in embryonic stem cells and cancer. *Expert Opin Ther Targets*. 2010; 14:169–177. [PubMed: 20055716]
- Loh YH, Wu Q, Chew JL, Vega VB, Zhang W, Chen X, Bourque G, George J, Leong B, Liu J, Wong KY, Sung KW, Lee CW, Zhao XD, Chiu KP, Lipovich L, Kuznetsov VA, Robson P, Stanton LW, Wei CL, Ruan Y, Lim B, Ng HH. The Oct4 and Nanog transcription network regulates pluripotency in mouse embryonic stem cells. *Nat Genet*. 2006; 38:431–440. [PubMed: 16518401]
- Miyamoto K, Yazawa T, Mizutani T, Imamichi Y, Kawabe S, Kanno M, Matsumura T, Ju Y, Umezawa A. Stem cell differentiation into steroidogenic cell lineages by NR5A family. *Mol Cell Endocrinol*. 2011; 336:123–126. [PubMed: 21134412]
- Mullen EM, Gu P, Cooney AJ. Nuclear receptors in regulation of mouse ES cell pluripotency and differentiation. *PPAR Res* 2007. 2007 Article ID 61563, 10 pages.
- Niakan KK, Davis EC, Clipsham RC, Jiang M, Dehart DB, Sulik KK, McCabe ERB. Novel role for the orphan nuclear receptor Dax1 in embryogenesis, different from steroidogenesis. *Mol Genet Metab*. 2006; 88:261–271. [PubMed: 16466956]
- Nichols J, Zevnik B, Anastasiadis K, Niwa H, Klewe-Nebenius D, Chambers I, Scholer H, Smith A. Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. *Cell*. 1998; 95:379–391. [PubMed: 9814708]
- Ovitt CE, Scholer HR. The molecular biology of Oct-4 in the early mouse embryo. *Mol Hum Reprod*. 1998; 4:1021–1031. [PubMed: 9835353]
- Qin P, Haberbush JM, Soprano KJ, Soprano DR. Retinoic acid regulates the expression of PBX1, PBX2, and PBX3 in P19 cells both transcriptionally and post-transcriptionally. *J Cell Biochem*. 2004a; 92:147–163. [PubMed: 15095411]
- Qin P, Haberbush JM, Zhang Z, Soprano KJ, Soprano DR. Pre-B cell leukemia transcription factor (PBX) proteins are important mediators for retinoic acid-dependent endodermal and neuronal differentiation of mouse embryonal carcinoma P19 cells. *J Biol Chem*. 2004b; 279:16263–16271. [PubMed: 14742427]
- Ross SA, McCaffrey PJ, Drager UC, De Luca LM. Retinoids in embryonal development. *Physiol Rev*. 2000; 80:1021–1054. [PubMed: 10893430]
- Schimmer BP, White PC. Minireview: Steroidogenic factor 1: Its roles differentiation, development and disease. *Mol Endocrinol*. 2010; 24:1322–1337. [PubMed: 20203099]
- Soprano DR, Teets BW, Soprano KJ. Role of retinoic acid in the differentiation of embryonal carcinoma and embryonic stem cells. *Vitamins and Hormones*. 2007; 75:69–95. [PubMed: 17368312]
- Sun C, Nakatake Y, Akagi T, Ura H, Matsuda T, Nishiyama A, Koide H, Ko MSH, Niwa H, Yokota T. Dax1 binds to Oct3/4 and inhibits its transcriptional activity in embryonic stem cells. *Mol Cell Biol*. 2009; 29:4574–4583. [PubMed: 19528230]

- Swain A, Zanaria E, Hacker A, Lovell-Badge R, Camerino G. Mouse Dax1 expression is consistent with a role in sex determination as well as in adrenal and hypothalamus function. *Nat Genet.* 1996; 12:404–409. [PubMed: 8630494]
- Vucetic Z, Zhang Z, Zhao J, Wang F, Soprano KJ, Soprano DR. Acinus-S' represses retinoic acid receptor (RAR)-regulated gene expression through interaction with the B domains of RARs. *Mol Cell Biol.* 2008; 28:2549–2558. [PubMed: 18250153]
- Wang J, Rao S, Chu J, Shen X, Levasseur DN, Theunissen TW, Orkin SH. A protein interaction network for pluripotency of embryonic stem cells. *Nature.* 2006; 444:364–368. [PubMed: 17093407]
- Xie CQ, Jeong Y, Fu M, Bookout AL, Garcia-Barrio MT, Sun T, Kim BH, Xie Y, Root S, Zhang J, Xu RH, Chen YE, Mangelsdorf DJ. Expression profiling of nuclear receptors in human and mouse embryonic stem cells. *Mol Endocrinol.* 2009; 23:724–733. [PubMed: 19196830]
- Yazawa T, Mizutani T, Yamada K, Kawata H, Sekiguchi T, Yoshino M, Kajitani T, Shou Z, Umezawa A, Miyamoto K. Differentiation of adult stem cells derived from bone marrow stroma into Leydig or adrenocortical cells. *Endocrinology.* 2006; 147:4104–4111. [PubMed: 16728492]
- Yazawa T, Inanoka Y, Mizutani T, Kuribayashi M, Umezawa A, Miyamoto K. Liver receptor homolog-1 regulates the transcription of steroidogenic enzymes and induces the differentiation of mesenchymal stem cells into steroidogenic cells. *Endocrinology.* 2009; 150:3885–3893. [PubMed: 19359379]
- Yazawa T, Kawabe S, Inaoka R, Mizutani T, Imamichi Y, Ju Y, Yamazaki Y, Usami Y, Kuribayashi M, Umezawa A, Miyamoto K. Differentiation of mesenchymal stem cells into steroidogenic cells using steroidogenic factor-1 and liver receptor homolog-1. *Mol Cell Endocrinol.* 2011; 336:127–132. [PubMed: 21129436]
- Yu RN, Ito M, Saunders TL, Camper SA, Jameson JL. Role of Ahch in gonadal development and gametogenesis. *Nat. Genet.* 1998; 20:353–357. [PubMed: 9843206]
- Zhao J, Zhang Z, Vucetic Z, Soprano KJ, Soprano DR. HACE1: A novel repressor of RAR transcriptional activity. *J Cell Biochem.* 2009; 107:482–493. [PubMed: 19350571]
- Zile M. Function of vitamin A in vertebrate embryonic development. *J Nutr.* 2001; 131:705–708. [PubMed: 11238746]

Figure 1. PBX is required for the reduction in the expression of several pluripotency-specific genes during RA-dependent differentiation to endodermal and neuronal cells

TO3 and AS2 cells were plated as monolayer (Endo) or as aggregates (Neuro) and treated with ETOH or RA for the indicated number of days. RNA was isolated and RT-qPCR was performed using primers specific to OCT-4, DAX-1 and SF-1. For each sample the mRNA level was normalized using the corresponding GAPDH mRNA level. The Day 0 value was set to 1 for each gene. ♦- TO3 cells treated with ETOH; ◇- TO3 cells treated with RA; ■- AS2 cells treated with ETOH; □- AS2 cells treated with RA. Values are Mean $\pm$ SD of triplicate samples.

Figure 2. The effect of DAX-1 on the mRNA level of select pluripotency- and differentiation-associated genes during RA-dependent differentiation to endodermal and neuronal cells

A. Protein lysates were prepared from individual TTD clones grown as monolayers for 24 hrs in media containing either 100 ng/ml Dox (+) or 0 ng/ml Dox (-). Western blot analysis was performed using primary antibodies to Flag (DAX-1), OCT-4 and GAPDH. B. and C. TTD28 cells were plated as monolayers (Endo) or as aggregate (Neuro) in media containing either 100 ng/ml Dox (Dox) or 0 ng/ml (No Dox) and treated with ETOH (E3) or 10^{-7} M RA (R3) for 72 hrs. RNA was isolated and RT-qPCR was performed using primers specific to the pluripotency-associated genes OCT-4, and SF-1 (B); and the differentiation-associated genes COUP-TFI, COUP-TFII, ETS-1 and PAX6 (C). For each sample the mRNA level was

normalized using the corresponding GAPDH mRNA level. The Day 0 with 100 ng/ml Dox (D0) value for each gene was set to 1. Values are the Mean $\pm$ SD of triplicate samples.

Figure 3. Expression of DAX-1 in P19 cells does not affect the ability of RA to cause terminal differentiation to endodermal (A) and neuronal (B) cells

TTD28 cells were induced to differentiate to endodermal (A) and neuronal (B) cells by treatment with RA as described in the methods section in media containing either 100 ng/ml Dox (Dox) or 0 ng/ml (No Dox). After seven days, cells were probed by immunofluorescence for Flag (DAX-1) (A and B), OCT-4 (A and B), SSEA-1 (A and B), TROMA-1 (A) and TUJ1 (B) expression. DAPI was included as a nuclear stain. Scale bar equals 20 μ m.

Figure 4. The effect of SF-1 overexpression on the mRNA level of select pluripotency- and differentiation-associated genes during RA-dependent differentiation to endodermal and neuronal cells

A. Protein lysates were prepared from individual TTS clones grown as monolayers for 24 hrs in media containing either 100 ng/ml Dox (+) or 0 ng/ml Dox (–) and for TTS52 cells grown for 24 hrs in media containing 100 ng/ml, 0.4 ng/ml and 0 ng/ml Dox. Western blot analysis was performed using primary antibodies to Flag (SF-1), OCT-4 and GAPDH. B. TTS52 cells were grown as monolayers in media containing 100 ng/ml Dox or 0 ng/ml (No Dox) for 24 hrs in the presence of ETOH (E1) or 10^{-7} M RA (R1). RNA was isolated and the mRNA levels of $RAR\beta_2$ and CYP26A1 were determined by RT-qPCR. For each sample the mRNA level was normalized using the corresponding GAPDH mRNA level. The E1 100 ng/ml Dox value for each gene was set to 1. Values are the Mean $\pm$ SD of triplicate samples. C. and D. TTS52 cells were plated as monolayers (Endo) or as aggregates (Neuro) in media containing either 100 ng/ml Dox, 0.4 ng/ml Dox or 0 ng/ml and treated with ETOH (E3) or 10^{-7} M RA (R3) for 72 hrs. RNA was isolated and RT-qPCR was performed using primers specific to the pluripotency-associated genes OCT-4, DAX-1 and endogenous SF-1 (C); and the differentiation-associated genes COUP-TFI, COUP-TFII, ETS-1 and PAX6 (D). For each sample the mRNA level was normalized using the corresponding GAPDH mRNA level. The Day 0 with 100 ng/ml Dox (D0) value for each gene was set to 1. Values are the Mean $\pm$ SD of triplicate samples.

Figure 5. Expression of SF-1 in P19 cells blocks RA-induced terminal differentiation to endodermal (A) and neuronal (B) cells
 TTS52 cells were induced to differentiate to endodermal (A) and neuronal (B) cells by treatment with RA as described in the methods section in media containing either 100 ng/ml Dox (Dox) or 0 ng/ml (No Dox). After seven days, cells were probed by immunofluorescence for Flag (SF-1) (A and B), OCT-4 (A and B), SSEA-1 (A and B), TROMA-1 (A) and TUJ1 (B) expression. DAPI was included as a nuclear stain. Scale bar equals 20 μ m.

Figure 6. Expression of SF-1 in P19 cells reduces the level of RA-induced increase in PBX protein levels

A. and B. TTS52 cells were plated as monolayers (Endodermal) (A and B) or as aggregate (Neuronal) (A) in media containing 100 ng/ml Dox, 0.4 ng/ml Dox or 0 ng/ml Dox and treated with ETOH (E3) or 10^{-7} M RA (R3) for 72 hrs (A); or ETOH (E1) or RA (R1) for 24 hrs (B). Total cell lysates were prepared and the level of PBX was determined by Western blot analysis. Values were normalized using GAPDH and the Day 0 (D0) value was set to 1 for each Dox concentration. C. TTS52 cells were plated as monolayers in media containing either 100 ng/ml Dox (Dox) or 0 ng/ml (No Dox) and treated with ETOH or 10^{-7} M RA for 24 hrs. Total RNA was isolated and RT-qPCR was performed using primers specific to each of the 4 PBX isoforms. For each sample the mRNA level was normalized using the corresponding GAPDH mRNA level. The 100 ng/ml Dox value for each gene was set to 1. Values are the Mean $\pm$ SD of triplicate samples.

Figure 7. Overexpression of SF-1 induces P19 cells to differentiate into adrenal-like cells
(A). T03 or AS2 cells were plated as aggregates and treated with ETOH (E) or 10⁻⁷ M RA (R). After 4 days RNA was isolated from a portion of the cells (4E and 4R) and the remainder of the cells were trypsinized, replated as monolayer and grown for an additional 2 days before isolation of RNA (6E and 6R). B. TTS52 cells were plated as aggregates in the presence of 100 ng/ml Dox (+) or 0 ng/ml Dox (--) and treated with ETOH or 10⁻⁷ RA. After 4 days RNA was isolated from a portion of the cells (4) and the remainder of the cells were trypsinized, replated as monolayer and grown for an additional 2 days or 6 days with (6c and 10c) and without (6 and 10) treatment with 10⁻³ M dibutyl cAMP.