Retinoic acid causes cell growth arrest and an increase in p27 in F9 wild type but not in F9 retinoic acid receptor β2 knockout cells

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Abstract

We have previously shown that an F9 teratocarcinoma retinoic acid receptor β2 (RARβ2) knockout cell line exhibits no growth arrest in response to all-trans-retinoic acid (RA), whereas F9 wild type (Wt), F9 RARα−/−, and F9 RARγ−/− cell lines do growth arrest in response to RA. To examine the role of RARβ2 in growth inhibition, we analyzed the cell cycle regulatory proteins affected by RA in F9 Wt and F9 RARβ2−/− cells. Flow microfluorimetry analyses revealed that RA treatment of F9 Wt cells greatly increased the percentage of cells in the G1/G0 phase of the cell cycle. In contrast, RA did not alter the cell cycle distribution profile of RARβ2−/− cells. In F9 Wt cells, cyclin D1, D3, and cyclin E protein levels decreased, while cyclin D2 and p27 levels increased after RA treatment. Compared to the F9 Wt cells, the F9 RARβ2−/− cells exhibited lower levels of cyclins D1, D2, D3, and E in the absence of RA, but did not exhibit further changes in the levels of these cell cycle regulators after RA addition. Since RA significantly increased the level of p27 protein (approximately 24-fold) in F9 Wt as compared to the F9 RARβ2−/− cells, we chose to study p27 in greater detail. The p27 mRNA level and the rate of p27 protein synthesis were increased in RA-treated F9 Wt cells, but not in F9 RARβ2−/− cells. Moreover, RA increased the half-life of p27 protein in F9 Wt cells. Reduced expression of RARβ2 is associated with the process of carcinogenesis and RARβ2 can mediate the growth arrest induced by RA in a variety of cancer cells. Using both genetic and molecular approaches, we have identified some of the molecular mechanisms, such as the large elevation of p27, through which RARβ2 mediates these growth inhibitory effects of RA in F9 cells.

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Introduction

Vitamin A (retinol) and its metabolites and derivatives are called retinoids. Retinoid acid (RA), a metabolite of retinol, is one of the most biologically active forms of retinoids. RA plays important roles in embryonic development and in the regulation of cell proliferation and differentiation [1,2]. It exerts its biological activities by binding to nuclear retinoic acid receptors (RARs) and retinoid X receptors (RXRs), which are members of the nuclear hormone receptor superfamily. There are three RARs and three RXRs encoded by different genes (α, β, γ). Each RAR and RXR gene encodes several protein isoforms, generated by different promoter usage or alternate splicing. The RARβ2 isoform, the most abundant RARβ isoform, is transcriptionally induced by RA in many cell types [3]. RARs and RXRs form heterodimers and act as ligand-inducible transcription factors by binding to retinoic acid responsive elements (RAREs) and retinoid X responsive elements (RXREs) in the enhancer regions of RA responsive genes. RAR coactivators and corepressors are also involved in the efficient transcription of RA-responsive genes [4–7].

F9 murine teratocarcinoma stem cells, which possess many of the properties of embryonic stem (ES) cells, express all three RARs and RXRs, and each RAR and RXR has unique actions in mediating the effects of RA on F9 cell growth inhibition and differentiation. Both F9
RARα and RARγ null cell lines exhibit specific alterations in gene expression [8,9]. F9 RXRα and RARβ2 knockout cell lines fail to grow arrest upon RA treatment [10,11]. That RARs and RXRs mediate the effects of RA in animals has been demonstrated by the phenotypes of single and compound knockout mutants of RAR and RXR isotype genes [12,13].

Retinoids such as RA have been successfully used for the chemoprevention and chemotherapy of various types of cancer because they inhibit proliferation and induce the differentiation of many types of malignant cells, such as F9 murine teratocarcinoma, promyelocytic leukemia, breast, lung, head and neck, renal, and prostate cells [1,14,15]. There are data indicating that RARβ plays an important role in mediating the growth inhibitory actions of RA. Conversely, the loss of RARβ expression occurs during the process of carcinogenesis. Reduced expression of RARβ is a common feature of premalignant lesions and carcinogenesis. For example, RARβ mRNA levels are reduced in premalignant oral lesions, head and neck squamous cell carcinomas [16–22], esophageal carcinoma [23,24], lung cancer [25–29], breast carcinoma [30–33], and cervical cancer [34]. Malignant cells with decreased expression of RARβ become resistant to RA treatment [28,35,36], whereas the up-regulation of RARβ parallels RA-induced growth suppression in immortalized bronchial epithelial cells, esophageal cancer cells, and non-M3 acute myeloid leukemia blast cells [37–39].

In addition to the correlation of RARβ levels with cell growth arrest in tumor cells, direct genetic evidence that RARβ is required for RA-induced growth arrest has been reported by this laboratory [11]. Faria et al. [11] reported that an RARβ2 double knockout F9 teratocarcinoma cell line completely lost responsiveness to the growth inhibitory effects of RA. The absence of growth arrest was accompanied by a lower level of induction of several RA-responsive genes in F9 RARβ2−/− cells as compared to F9 Wt cells. As experimental data indicate that the loss of RARβ expression plays a role in the process of carcinogenesis, it is important to elucidate the molecular mechanisms by which RARβ mediates the growth inhibitory effects of RA and to determine which cell cycle regulatory proteins are involved in this signaling pathway.

Some of the regulation of the mammalian cell cycle occurs at a restriction point in G1 phase, a point at which cyclins and cyclin-dependent kinases (CDKs) interact with each other to allow the cells either to progress through the restriction point or to growth arrest in G1. In mid-G1, the D-type cyclins (D1, D2, and D3) bind and activate their catalytic subunits CDK4 and CDK6. In many cell types, the phosphorylation of the retinoblastoma (Rb) tumor suppressor protein by the cyclin D–CDK complex releases the transcription factor E2F, which activates the transcription of cyclin E. In late G1, cyclin E associates with CDK2 to initiate the synthesis of DNA. The activities of CDKs are also regulated by cyclin inhibitors (CKIs). In mammalian cells, two families of CKIs, the Cip/Kip and Ink4 families, have been identified. The Cip/Kip family includes p21, p27, and p57, and the Ink4 family includes p16, p15, p18, and p19 [40,41]. The p27 protein plays a pivotal role in the regulation of the proliferation and differentiation of many cell types. Down-regulation of p27 has been observed in carcinogenesis and metastasis and the level of p27 has been used to evaluate cancer progression [42]. The Rb and CKI pathways both control G1/S progression in differentiated mammalian cells. However, the Rb pathway has been reported not to play a significant role in the regulation of ES cells, and ES cells exhibit a cell cycle with a shortened G1 phase [43]. In this study, we examined the levels of cell cycle regulatory proteins in F9 Wt vs. F9 RARβ2 null cells both before and after RA treatment.

Materials and methods

Cell culture

Murine F9 wild type (Wt) teratocarcinoma cells and the F9 RARβ2−/− line [11] were cultured as described previously [44]. Cells were cultured in the presence or absence of 1 μM all-trans-retinoic acid (RA; Sigma, St. Louis, MO) as indicated in the figure legends. The measurement of cell number was performed with an electronic particle counter (Coulter Electronics, Hialeah, FL).

Cell cycle distribution

Murine F9 Wt cells and F9 RARβ2−/− cells were treated with or without 1 μM RA for 96 h. Cells (1 × 10⁶) were fixed in 75% ethanol, washed with phosphate-buffered saline, and resuspended in 150 μl RNase A (500 U/ml) for 20 min at 37°C. The cells were then stained with 150 μl of propidium iodide (50 μg/ml) for at least 20 min at 4°C in the dark. The DNA content of the cells was then analyzed by flow microfluorimetry. Three independent measurements were made and averaged. Statistical significance was determined using a Student’s t test.

Western blot analysis

Cells were lysed in final sample buffer (60 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, and 100 μM DTT), sonicated for 30 s and boiled for 10 min. For Western analysis, equal amounts of protein extracts (up to 100 μg) were fractionated by SDS-polyacrylamide gel electrophoresis and blotted onto nitrocellulose. The antibodies used in this study were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) (anti-cyclin D1, sc-718; anti-cyclin D2, sc-593; anti-cyclin D3, sc-182; anti-cyclin E, sc-481; anti-CDK2, sc-163; anti-CDK4, sc-260; anti-CDK6, sc-177; anti-p27, sc-528; anti-actin, sc-1616; anti-rabbit IgG-HRP, sc-2030; anti-goat IgG-HRP, sc-2056) and Oncogene Research Products (La Jolla, CA) (anti-p21, PC55). All of the other primary and the secondary
antibodies were diluted into Tris-buffered saline containing 0.05% Tween-20 and 5% milk and used at a dilution of 1 μg/ml and 1:10,000, respectively. SuperSignal West Pico Chemiluminescent Substrate detection system (Pierce, Rockford, IL) was used to detect the secondary antibody according to the manufacturer’s instructions.

**RNA isolation and Northern analysis**

Total cellular RNA was extracted using RNA-STAT 60 (Tel-Test, Friendswood, TX) following the manufacturer’s protocol. Northern blot analysis was performed as described [44], and 20 μg mRNA was loaded for each lane. The Northern experiments were performed three times using independent RNA preparations. The probes used were: 400-bp NdeI–BamHI fragment of mouse p27 cDNA, and a 650-bp PstI fragment of mouse actin cDNA.

**Biosynthetic radiolabeling of proteins**

Cells (5 × 10^5/60 mm dish) were incubated for 20 min with 1 ml labeling medium (methionine-free DME with 2 mM glutamine, and 10% dialyzed calf serum) to deplete intracellular pools of methionine, and then cultured in the

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**Fig. 1.** (A) Analysis of the growth of F9 Wt and RARβ_{2-/-} cells after treatment with 1 μM RA. The cells were plated in duplicate wells at a density of 3000 cells/well. The cell numbers were counted on the indicated days. The experiment was performed three times with very similar results. The values represent the mean ± SD of three independent experiments. (B) F9 Wt and RARβ_{2-/-} cells were treated with 1 μM RA for 96 h. The DNA content of the cells was measured by flow microfluorimetry. The experiment was repeated three times with very similar results. (C) Statistical analysis of the cell cycle distribution of F9 Wt and RARβ_{2-/-} cells after treatment with 1 μM RA for 96 h. The values represent the mean ± SD of three independent experiments. *P < 0.05.
presence of 50 μCi/ml \[^{35}\text{S}\]-methionine in the above labeling media for 30 min. Cells were incubated in 1 ml lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.5% NP-40, 0.1 mM sodium fluoride, and 10 μM PMSF) for 30 min at 4°C. Lysates were cleared by centrifugation and the total amount of protein was estimated. Equal amounts of protein extract (1 × 10^7 cpm/lane) were incubated with 2 μg of anti-p27 antibody (sc-528, Santa Cruz Biotechnology) overnight at 4°C. The immune complexes were bound to protein A-agarose and washed with lysis buffer. The immunoprecipitated protein was electrophoresed on a 10% SDS-polyacrylamide gel and subjected to autoradiography.

**Pulse-chase experiments**

Cells (5 × 10^5/60 mm dish) were labeled with 100 μCi/ml \[^{35}\text{S}\]-methionine in the above labeling media for 1 h, then incubated in \[^{35}\text{S}\]-methionine-free media for the times indicated in the figure legend. Cells were lysed as described above. Equal amounts of protein extract (3 × 10^7 cpm/lane) were incubated with 2 μg of anti-p27 antibody (sc-528, Santa Cruz Biotechnology) overnight at 4°C. The immunoprecipitated protein was electrophoresed and subjected to autoradiography as described above.

**Inhibition of proteasome degradation activity**

Cells were treated in the presence or absence of 1 μM RA for 48 h and then exposed to 50μM calpain inhibitor I [N-
acetyl-Leu-Leu-Norleu-Al (LLnL) (Sigma), a proteasome activity inhibitor [45], for 6 h. Western analysis of p27 was performed as described previously.

Results

The effects of RA on the cell cycle distribution in F9 Wt and F9 RARb2−/− knockout cell lines

The importance of RARb2 in mediating the RA-induced growth arrest in F9 Wt cells is shown in the comparison of the growth of F9 Wt vs. RARb2−/− cells treated with RA (Fig. 1A). To ascertain whether the RA-induced cell proliferation arrest was cell cycle phase-specific, the cell cycle distributions of the F9 Wt and the F9 RARb2−/− cells before and after treatments with RA for 96 h were determined (Figs. 1B and C). After RA treatment, the F9 Wt cells exhibited shifts in their cell cycle distribution profiles, with an increase from 28% to 60% in the percentage of cells in G1/G0 and a decreased percentage of cells in S and G2. In contrast, no statistically significant change was observed in the F9 RARb2−/− cells after RA treatment; the percentages of cells in G1/G0 were 41% and 35%, respectively, before and after RA treatment (Figs. 1B and C). Thus, RA increases the number of F9 Wt cells in the G1/G0 phase of the cell cycle, but this is not true for the F9 RARb2−/− cells. In F9 Wt cells, RA has been reported to induce apoptosis [10,46]; this may explain the signal in the sub-G1 area of Fig. 1B (F9 Wt cells treated with RA).

Altered cell cycle regulatory proteins in F9 Wt and F9 RARb2−/− cell lines upon RA treatment

We next examined the levels of G1 cell cycle regulatory proteins in F9 Wt cells and RARb2−/− cells (Fig. 2A). In F9 Wt cells, RA treatment decreased the levels of cyclins D1, D3, E, and cdk6, and increased the levels of cyclin D2 and p27 by several fold. In fact, F9 Wt cells exhibited a very large increase in p27 protein levels (approximately 24-fold at 48 h, Fig. 2B).

The F9 RARb2−/− cells showed a very different pattern of expression of these cell cycle regulatory proteins. In the untreated, control RARb2+ −/− cells the levels of cyclins D1, D2, D3, E, and the p27 protein were lower when compared to F9 Wt cells. In contrast to the F9 Wt cells, RA treatment did not significantly alter (<2-fold) the levels of these cyclins and p27 in the F9 RARb2−/− cells. The basal levels of other cell cycle regulators, such as cdk2, cdk4, cdk6, and p21, and the changes in the levels of these proteins in response to RA treatment were similar in F9 Wt and RARb2−/− cells (Fig. 2A). These results showed that RA regulates several cell cycle regulatory proteins, including p27, in the F9 Wt cells, but not in the F9 RARb2−/− cells.

RARb2 is required for the increase in p27 in F9 Wt cells upon RA treatment

We explored the potential role of the RARb2 protein in mediating the increase in p27 levels in F9 Wt cells by culturing F9 Wt, RARb2−/−, RARα−/−, and RARγ−/− cell lines in the presence of RA for 48 h. An increase in p27 protein after RA was observed in all of the cell lines except the RARb2−/− line, though the increase in p27 was much greater in F9 Wt than in RARα−/− or RARγ−/− cells (Fig. 3). Since only the F9 RARb2−/− cells are not growth-inhibited by RA (Refs. [8,9,11] and Fig. 1A), these data suggest that p27 is an effector involved in the regulation of cell proliferation downstream of RARb2. Our data also suggest, however, that RARα and RARγ are required, in addition to RARb2, for the maximal increase in p27 following RA addition. That the F9 RARα−/− and RARγ−/− cells exhibit growth arrest upon RA treatment while accumulating less p27 than the F9 Wt cells suggests that a threshold level of p27 is required for growth inhibition.

p27 is regulated at the mRNA and translational levels by RA in F9 Wt cells

The effects of RA on the synthesis of p27 protein were tested by biosynthetic protein labeling. RA treatment resulted in 2.1- and 2.5-fold increases in p27 protein synthesis in F9 Wt cells at 24 and 48 h, respectively, concomitant with 2.2- and 3.6-fold increases in p27 mRNA level at 24 and 48 h, respectively (Figs. 4 and 5). These increases in p27 protein and mRNA were not observed in...
RA-treated F9 RARβ2−/− cells (Figs. 4 and 5). Thus, RA increases the p27 mRNA level, which contributes to the increase in p27 protein in the F9 Wt cells, but this does not occur in the F9 RARβ2−/− cells.

p27 protein can be stabilized by RA in F9 Wt cells

It is known that the level of p27 protein can be regulated at both the translational and post-translational levels in other cell types [47–51]. To determine whether RA can affect the rate of degradation of p27 in F9 Wt cells, pulse-chase experiments were performed in untreated vs. RA-treated F9 Wt cells. These data indicated that the p27 protein exhibited a longer half-life in RA treated as compared to control F9 Wt cells (Fig. 6).

p27 is degraded through the ubiquitin-mediated proteasome pathway in both F9 Wt and F9 RARβ2−/− cells

We next investigated whether any differences existed in p27 degradation pathways between F9 Wt and F9 RARβ2−/− cells. We confirmed that the degradation of the p27 protein occurs in part through the ubiquitin-mediated proteasome pathway in both F9 Wt and RARβ2−/− cells by culturing them in the presence or absence of RA and/or LLnL, a proteasome activity inhibitor, for 48 h (Fig. 7). In F9 Wt cells, there was a 19-fold increase in p27 protein level after LLnL treatment under control conditions (compare lanes 1 and 4, Fig. 7A), while there was a 49- vs. 22-fold increase in p27 protein level in F9 Wt cells treated with RA and LLnL.
vs. RA alone (compare lanes 2 and 3, Fig. 7A). These data indicate that the p27 protein in F9 Wt cells is degraded through the proteasome pathway, as the addition of LLnL to the F9 Wt cells resulted in a large increase in the p27 level. The addition of LLnL also increased the p27 protein level in F9 RARβ2−/− cells (compare lanes 5 and 8, Fig. 7). These data showed that the ubiquitin-mediated proteasome pathway is involved in the degradation of p27 protein in both F9 Wt cells and the F9 RARβ2−/− cells.

The level of Skp2 protein in F9 Wt and F9 RARβ2−/− cells

The ubiquitin-proteasome degradation pathway plays an important role in regulating the abundance of many critical regulatory proteins, including the cell cycle regulators. The recognition of p27 for proteasome degradation in some cell types is mediated by the SCF complex, which is composed of Skp1, Cul1, and any one F-box protein [52]. Skp2, an F-box protein and a component in the SCF complex involved in the ubiquitin-mediated proteasome degradation pathway, is a ligase that can target the phosphorylated p27 at Thr187 for its degradation. The basal levels of Skp2 protein in both F9 Wt and F9 RARβ2−/− cells, and the changes in the levels of Skp2 protein after RA treatment were similar in both F9 Wt and RARβ2−/− cells (Fig. 8). Thus, we conclude that a change in Skp2 level does not play a crucial role in the differential regulation of p27 in F9 Wt vs. F9 RARβ2−/− Cells.

Discussion

RA-induced growth arrest in F9 cells is associated with the up-regulation of p27

Various pieces of data support a specific role for RARβ2 in mediating the growth inhibitory effects of RA in F9 cells and other cell types (Fig. 1 and references [8–11,53–60]). Despite the critical role of RARβ in mediating RA-induced growth inhibition in a variety of cell types, the downstream steps involved in this signaling pathway are unclear. We have shown here that the p27 protein level was greatly up-regulated by RA in F9 Wt cells but not in F9 RARβ2−/− cells. Our findings are consistent with reports that p27 mRNA and/or protein is increased by RA in a variety of cells undergoing differentiation and growth inhibition [45,61–67]. Overexpression of p27 in oligodendrocyte
progenitor cells, vascular smooth muscle, mammary carcinoma, and neuroblastoma cells inhibits their proliferation [68–71], while p27−/− mice display increased proliferative capacity and pituitary tumors [72–76].

While F9 Wt, RARα−/−, and RARγ−/− cells growth arrest upon RA treatment, the increase in p27 protein levels in the RARα−/−, and RARγ−/− cell lines is lower than that in the F9 Wt cells (Fig. 3). These data demonstrate that the RAR isoforms exert partially redundant functions on the regulation of p27 in F9 cells. The fact that there was no increase in the level of p27 protein in F9 RARβ2−/− cells after RA treatment (Figs. 2 and 3), despite the presence of the RARα and RARγ proteins in the RARβ2−/− cells, suggests that the RARβ2 isoform plays the major role in the regulation of p27 by RA.

In contrast to its effect on p27, RA did not affect the level of p21 protein in F9 Wt or in F9 RARβ2−/− cells (Fig. 2A), although the p21 gene is RA-responsive in certain cell types [77]. We also tested the effect of RA on the Ink4 family member, p16, in both F9 Wt and F9 RARβ2−/− cells. The p16 protein plays an important role in some cell types in regulating cell growth and exhibits tumor suppressor activities [78]. However, the p16 protein signal was not detectable in either F9 Wt or RARβ2−/− cells (data not shown).

Another group recently reported a decrease in p27 levels in F9 Wt extracts following RA addition [79]. Since the p27 protein is present in both the nucleus and cytoplasm, the methods used to lyse the cells and prepare extracts can affect the ability to assess quantitatively total p27 levels. For example, using the extract preparation method reported by this group [79], which involves a high-speed centrifugation of cell extracts in a nonionic detergent and subsequent electrophoresis of the supernatant proteins, we were also unable to detect the increase in p27 protein in the supernatant, after centrifugation, from F9 Wt cells treated with RA (data not shown). However, in the same experiment, F9 Wt cells lysed directly into sodium dodecyl sulfate final sample buffer (see Materials and methods) exhibited a large (approximately 20-fold) increase in p27 level in response to RA (e.g., Fig. 2).

RA up-regulates p27 in F9 Wt cells at the mRNA, translational and post-translational levels

The regulation of p27 in many cell types has been reported to occur mainly at the post-transcriptional level through the regulation of either the efficiency of translation or the ubiquitin-mediated proteasome degradation of p27 [47–51]. Our results showed an increase in the rate of p27 protein synthesis in F9 Wt cells at 24 and 48 h after RA treatment, which is attributable, at least in part, to a corresponding increase in p27 mRNA level at the same time points after RA (Figs. 4 and 5). Although RA may increase the stability of the p27 mRNA, the transcriptional activation of the p27 gene by RA, using luciferase reporter assays, has also been reported; however, whether RA response elements (RAREs) are present in the p27 promoter region was not addressed in these studies [61,80]. We have searched for RAREs in the mouse p27 promoter region using the TESS software program (University of Pennsylvania, Philadelphia, PA). We detected a half-site RARE in the p27 promoter region at −679 S′ of the transcription start site. This putative RARE has not been tested in functional assays, however. Moreover, as a half-site RARE is not sufficient to direct RA responses, other more complex elements in the p27 gene may be involved in the transcriptional activation of this gene by RA.

The translation of eukaryotic proteins is initiated by the recognition of the mRNA S′ cap by eukaryotic initiation factor 4E (eIF4E), which is down-regulated under conditions facilitating cell cycle arrest. p27 mRNA can be translated predominantly through cap-independent mechanisms under conditions in which eIF4E activity is low [81]. Likely, the increased p27 mRNA level, its cap-independent translation, and the increased protein stability that is discussed later, together are responsible for the elevated p27 protein level in F9 Wt cells after RA treatment. Whether p27 can directly interact with the RARβ receptor is being tested.

It has been reported that RA increases the level of p27 in neuroblastoma cells by down-regulating the ubiquitin-proteasome pathway [45]. A decreased level of Skp2 protein is correlated with the accumulation of p27 following RA treatment in breast cancer cell lines [82]. The fact that both the basal level of Skp2 protein and the decrease in expression of Skp2 protein are similar in the absence or presence of RA in both F9 Wt and RARβ2−/− cell lines suggests that (i) the Skp2-dependent ubiquitination pathway does not play a crucial role in the degradation of p27 in F9 cells, (ii) post-translational modification affects the activity of Skp2, or (iii) other factors, in addition to Skp2, are required for efficient p27 ubiquitination, for example, the recruitment of Skp1 and Cul-1 upon the binding of Skp2 to phosphorylated p27 [83].

Cyclins involved in the RA-induced growth arrest in F9 Wt cells

Our data demonstrated that the levels of cyclin D1, D3, and E proteins, as well as cyclin D1 mRNA (data not shown) were reduced in F9 Wt cells after RA, while F9 RARβ2−/− cells showed no such changes. The decreases in the levels of these cyclin D proteins would be expected to release p27 from sequestration by the cyclin D–CDK complex to which it is bound. The released p27 can bind to and inhibit the activity of cyclin E/CDK2 kinase. The increased binding of p27 and the reduced level of cyclin E (Fig. 2A) have been shown to prevent the cyclin E/CDK2 kinase from phosphorylating target genes, such as nuclear protein mapped to the AT locus (NPAT). The phosphorylation of NPAT may promote S phase entry [84]. The down-regulation of cyclin E by RA treatment in F9 Wt cells would also be expected to decrease the phosphorylation of p27 at
Thr^{187} and the subsequent proteasome degradation of p27 [85], contributing to the up-regulation of p27. Other investigations have shown an RA-associated down-regulation of cyclin D1, D3, and cyclin E expression in F9 Wt cells [86,87]. In contrast to the cyclins D1, D3, and E, cyclin D2 was up-regulated by RA in F9 Wt cells, but not in F9 RARβ2−/− cells (Fig. 2A). In addition to promoting cell cycle progression, cyclin D2 may function as a negative regulator of cell growth [88]. The mechanisms by which the levels of the cyclin D and E proteins are reduced in the untreated F9 RARα cells was up-regulated by RA in F9 Wt cells, but not in F9 RARβ2−/− cells (Fig. 2A). These decreased levels of Thr^{187} and the subsequent proteasome degradation of p27 RARα was up-regulated by RA in F9 Wt cells, but not in F9 RARβ2−/− cells (Fig. 2A). In addition to promoting cell cycle progression, cyclin D2 may function as a negative regulator of cell growth [88]. The mechanisms by which the levels of the cyclin D and E proteins are reduced in the F9 RARα cells as compared to F9 Wt cells in the absence of RA require further investigation. These decreased levels of cyclin D and E proteins in the untreated F9 RARβ2−/− cells may be related to the higher number of these cells in G1/G0 phase of the cell cycle (Fig. 1C).

The observation that the levels of CDKs were similar between F9 Wt and F9 RARβ2−/− cells after RA addition suggests that the growth inhibitory actions of RA do not result from the changes in the levels of CDKs. Although CDKs are not RA-regulated in F9 cells, they have been reported to be involved in the growth inhibitory effects of RA in many other cell types [89–92].

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