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p21 Is Required for atRA-Mediated Growth Inhibition of MEPM Cells, Which Involves RAR

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Abstract All-trans retinoic acid (atRA), a metabolite of vitamin A, is essential for embryonic development. Thus the spatial and temporal dispersal of RA must be tightly controlled. Previous studies show that excessive atRA led to growth inhibition and p21 accumulation in mouse embryonic palatal mesenchymal (MEPM) cells. We reported here the identification of p21 as a required mediator during atRA-induced growth inhibition. atRA caused a G1 arrest in the cell cycle with an increase in the proportion of cells in G0/G1 and a decrease in the proportion of cells in S phase. In addition to a marked effect on cell cycling, atRA also triggered DNA fragmentation, reflected by an increase of the fraction of cells in the sub-G_1 population. Western blot analysis revealed that atRA treatment led to an increase in p21 level and a decrease in cyclin D1 protein and Rb phosphorylation. Using luciferase assay with reporter gene regulated by p21 promoter, we showed that atRA increased the reporter activity in a dose-dependent manner; and p21 siRNA blocked the growth inhibition by atRA, suggesting that p21 is required for atRA-mediated growth inhibition. Moreover, the induction of p21 by atRA was partially attenuated when RAR was silenced with specific siRNA. atRA stimulated RARE-driven reporter gene activity dose-dependently. Using chromatin immunoprecipitation, we demonstrated that RAR protein could bind to the p21 promoter. Taken together, our results indicate p21 is responsible for atRA-induced growth inhibition of MEPM cells and RAR plays a role during this process. J. Cell. Biochem. 104: 2185–2192, 2008. © 2008 Wiley-Liss, Inc.

Key words: all-trans retinoic acid; mouse embryonic palatal mesenchymal (MEPM) cells; RAR; p21; cell cycle

Cleft palate (CP), with or without cleft lip, represents one of the most common birth defects observed in humans, occurring in approximately 1 in 700 live births [Gorlin et al., 2001; Murray, 2002]. Formation of mammalian secondary palate is a multi-step process that includes mesenchymal cell proliferation, palatal shelf outgrowth, elevation, fusion and eventually disappearance of the midline epithelial seam. Disturbance of any events may lead to clefts in the palate [Ferguson, 1998; Wilkie and Morriss-Kay, 2001; Hilliard et al., 2005]. Embryonic palatal mesenchymal (EPM) cells are derived from embryonic palate. Primary culture of murine and human EPM cells has been used extensively to investigate the mechanism of the interaction between genetic and environmental factors in the process of palatogenesis [Dhulipala et al., 2004; Yu and Xing, 2006].

Retinoic acid (RA), the active metabolite of vitamin A, plays a major role as physiological regulator of important biological processes, such as early embryonic development, and the development of certain organs and systems, etc. [Ross et al., 2000; Zile, 2001; Vermot and Pourquié, 2005; Bowles and Koopman, 2007]. While normal embryo development requires retinoids, too much or too little, at the wrong stage or at the wrong time, can adversely affect the developing embryo [Morriss-Kay et al., 1991]. Embryos exposed to low concentrations of RA [0.0001–0.1 μM] develop normally; whereas those exposed to higher concentrations [0.5–100 μM] develop characteristic dose-dependent defects [Vandersea et al., 1998]. Embryonic exposure to RA causes a wide spectrum of severe malformation in the offspring of animal and humans [Lammer et al., 1985; Coberly et al., 1996; Collins and Mao, 1999].

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RA mediates its signals via two types of nuclear hormone receptors: retinoic acid receptor (RAR) and retinoic acid receptor X (RXR). Activated and dimerized receptors control gene expression via binding to retinoic acid response elements [Ziouzenkova and Plutzky, 2008]. All isoforms of the RAR (α, β, and γ) and RXR (α, β, and γ) are expressed in the mesenchyme of the embryonic mouse palate (E11–E15). A high concentration of RA has been shown to inhibit cell growth and induce apoptosis in embryonic palatal mesenchyme and induce CP [Okano et al., 2007]. Other target tissues exposed to high levels of RA also show reduced bromodeoxyuridine (BrdU) incorporation [Dalvin et al., 2004; Li and Kim, 2004]. These studies highlight the inhibition of embryonic cell proliferation by RA, both in vivo and in vitro and suggest closer examination of mechanisms regulating cell growth may be warranted.

Cell cycle progression in eukaryotic cells is finely regulated by the sequential activation of cyclin-dependent kinases (CDKs), which are activated after interaction with their corresponding cyclins [Sherr and Roberts, 1999]. The activity of these CDKs is restrained by two groups of CDK inhibitors. Of these, p21 plays an essential role in growth arrest [Sherr and Roberts, 1995] and also directly inhibits DNA polymerase activity by binding to the PCNA subunit [Waga et al., 1994]. p21 retards S phase progression by inhibition of cyclin-dependent kinases [Ogryzko et al., 1997] and functions as a G2 checkpoint by binding to cyclin B1-CDC2 complexes, which are integral in the G2/M transition [Dulic et al., 1998; Barboule et al., 1999]. Deregulation in the expression of p21 would be expected to result in abnormal cell proliferation and to predispose the cells to transformation [Chen et al., 1995; Cardinali et al., 1998].

Palatal morphogenesis involves delicately controlled mechanisms between adjoining palatal epithelial and mesenchymal tissues that share a specific spatial and temporal relationship. Environmental influences that induce effects on cell cycle and apoptosis can result in reduced proliferation of cells actively cycling at the time of such exposure. During embryonic palatal development, such changes can lead to CP. Notably, inhibition of embryonic cell growth by atRA seems to be a multifaceted process involving the induction of both apoptosis and cell cycle arrest [Yu et al., 2005]. The present study was undertaken to obtain insights into the initial cell cycle response, identify direct RAR target genes that are involved in this response, and examine the contribution of RAR to the process.

MATERIALS AND METHODS

Materials

atRA and 5′-bromo-2′-deoxyuridine (BrdU) were bought from Sigma (St. Louis). Anti-BrdU FITC antibody was from Becton Dickinson. atRA was dissolved in DMSO as 100 mM stocks and stored in aliquots in dark brown tubes at −80°C. Dilutions to the working concentration had DMSO concentrations <0.01%.

Cell Culture

Cells were derived from palatal mesenchyme of gestational day 13 ICR mouse embryos (date of vaginal plug detection was considered day 0 of gestation). The palate shelves were dissected in sterile, cold phosphate-buffered saline (PBS) and were pooled, minced, and converted into single cell suspensions by incubating with 0.25% trypsin/0.05% EDTA in PBS for 10 min at 37°C with constant shaking. Digested samples were briefly triturated, filtrated through 70-μm mesh, and cells were seeded on cell-culture dishes and grown to confluence in DMEM medium (Gibco BRL) containing 5% fetal calf serum (FBS, Hyclon Co.) at 37°C in a 95% air/ 5% CO_2 atmosphere, with media replaced every other day.

Fluorescence-Activated Cell Sorting

Cells were seeded in six-well plates (1×10^5/per well) in DMEM supplemented with 10% FBS and grown overnight. Cells were treated with atRA of 3.0 μM for 48 h and then incubated with 30 Ag/mL of BrdU for 20 min at 37°C. The cells were collected, suspended in 1 mL PBS, rapidly injected into 10 mL 70% ethanol, and incubated overnight at 4°C. Cells were centrifuged and resuspended in 1 mL of 2 N HCl/0.5% Triton X-100. Following a 30-min incubation, cells were centrifuged, resuspended in 1 mL of 0.1 mol/L $Na_2B_4O_7 \cdot 10H_2O$ (pH 8.5) and collected, and 1 mL of dilution buffer (0.5% Tween 20 and 1% bovine serum albumin in PBS) was added. Anti-BrdU FITC antibody was added and incubated for 30 min. Stained cells were washed with dilution buffer and

resuspended in 1 mL PBS containing propidium iodide (PI) of 5 Ag/mL and analyzed by flow cytometry.

Western Blot

Total cellular protein extracts were prepared using 2× SDS-lysis buffer supplemented with protease and phosphatase inhibitors (Cell Signaling Technology, Cambridge, MA). Protein concentration was determined using the DC Protein Assay from Bio-Rad (Hercules, CA). Equal amounts of cellular protein extracts were run on denatured polyacrylamide gels and transferred onto nitrocellulose membranes (Bio-Rad) by standard blotting procedures. p21, cyclin D_1 and pRb antibodies were all purchased from Santa Cruz Biotechnology. Proteins reactive with primary antibody were visualized with a horseradish peroxidase-conjugated secondary antibody and enhanced chemiluminescence reagents (Amersham Bioscience, Arlington Heights, IL).

Luciferase Reporter Gene Assay

Cells were seeded at a density of 1×10^5 cells/well in 6-well plates so that they were 50% confluent the day of transfection. The cells were then transfected with 1 μg cDNA of p21 promoter-driven luciferase reporter (or a RAR response element-driven luciferase reporter gene, pRARE) and 0.5 μg of β-galactosidase as an internal control vector DNA using Lipofectamine 2000 (Invitrogen Co.). After 24 h transfection, the cells were exposed for an additional 48 h to various concentrations of atRA as indicated. The cells were washed and lysed, and the luciferase activities were measured using the luciferase assay system (Promega, Madison, WI) and corrected for transfection efficiency by β-galactosidase activity. Each experiment was repeated at least three times.

Gene Silencing Using siRNA

Pan-RAR siRNA, which is a generous gift from Dr. Zhang (UNC at Chapel Hill), was used to knockdown RAR expression and mouse p21 siRNA (Santa Cruz, sc-29428) was used to knockdown p21 expression. MEPM cells at 50% confluence were transfected with a final concentration of 100 nM siRNA by using the siIMPORTER siRNA transfection reagent (Upstate Biotechnology) according to the manufacturer's instructions. Twenty-four hours after transfection, the cells were treated with alcohol (control) or 3.0 μM atRA for 48 h. The cells were then collected and processed for analysis of cell cycle distribution and cyclin D1 expression. The efficiency of target gene knockdown was verified by Western blotting.

Chromatin Immunoprecipitation Assay

Cells were seeded in 100-mm plates in the presence or absence of 3 μM atRA for 72 h. Cells were then directly treated with the crosslinking reagent formaldehyde (1% final concentration) for 10 min at 37°C, rinsed twice with cold PBS, and swollen on ice in SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris–HCl, pH 8.1) for 10 min. Nuclei were collected and sonicated on ice. Supernatants were obtained by centrifugation for 10 min and diluted 10-fold in ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris–HCl, pH 8.1, 167 mM NaCl). Shared chromatin was immunoprecipitated with RAR antibody (a kind gift from Dr. Hai, INSERM, France) or with IgG as negative control. Then 20 μl of protein A/G PLUS-agarose were added and incubated for 1 h at 4°C with rotation to collect the antibody/chromatin complex. Crosslinked, precipitated chromatin complexes were recovered and reversed according to Upstate's protocol (Upstate, Chicago, IL). Final DNA pellets were recovered and analyzed by PCR to amplify the p21 promoter sequences using the primers 5′-agactctgagcagcctgag-3′ (forward) and 5′- aaccctcatttgcagatggt -3′ (reverse).

Statistical Analysis

Data in this report are presented as mean ± SEM from three separated experiments, as described in the figure legends. Statistical differences were evaluated using the one-way ANOVA. Statistical significance was defined as a P value <0.05.

RESULTS

atRA Induces a G_1 Arrest With Increased Levels of Cell Cycle Regulators

MEMP cells were treated with atRA of 3 μM for 48 h and then the cell cycle distribution was examined using a BrdU incorporation assay. Cells were also stained with PI, and the fraction of cell populations in different cell cycle phases was determined by flow cytometry. The data in Figure 1A showed that RA treatment resulted in a marked increase in the cell population in

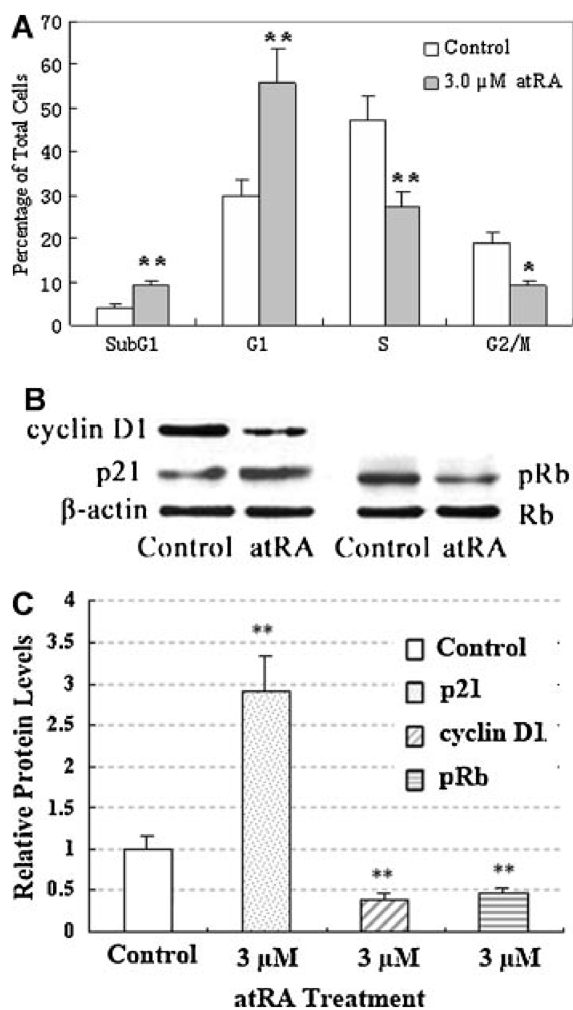

Fig. 1. atRA induced growth inhibition of MEPM cells. **A**: MEMP cells were treated with vehicle DMSO or atRA (3.0 μM) for 48 h before incubation with BrdU (20 min). Cells were then fixed in ethanol, stained with FITC-conjugated anti-BrdU antibodies or with PI, and analyzed by fluorescence-activated cell sorting using flow cytometry. **B**: MEPM cells were treated with vehicle DMSO or 3.0 μM atRA for 48 h, total cellular protein was extracted. The protein levels of p21, cyclin D1 and phosphorylated Rb were analyzed by Western blot with specific antibodies. Each lane contains 35 μg total protein. **C**: Western bolting signals were transferred to Adobe Photoshop software through high resolution scanning (Microtek ScanMaker 630) of the XB-1 films (Kodak) and digitized with Image Quant software. From four independent experiments, for all different samples, the ratio between the tested target protein and internal standard β-actin band was calculated (for p-Rb, the internal standard is total Rb band). Within each separate experiment, ratios of the samples were compared to the ratio obtained from control cells, which were set at 1. Data here are mean ± SEM, n = 3. Each experiment was performed in triplicate. Columns, mean (n = 3); bars, SE. $^*P < 0.05$, significant difference from control values.

the G_1 phase, leading to an overall 1.87-fold increase at 48 h. The increase was accompanied by a corresponding decrease in S phase, showing that RA inhibits the G_1-S transition. In addition to a marked effect on cell cycling, RA also triggered DNA fragmentation, reflected by an increase of the fraction of cells in the sub-G_1 population. In accordance with a G_1 arrest, the expression levels of cyclin D1 protein and phosphorylated Rb were decreased, whereas p21 were significantly increased upon 48 h of atRA treatment as demonstrated in Figure 1B.

atRA Treatment Up-Regulated p21 and RARE Activity

To investigate the responsiveness of 21 promoter element to different concentrations of atRA, we performed a transcriptional activation assay using a p21 promoter-driven luciferase reporter. As shown in Figure 2A, atRA induced

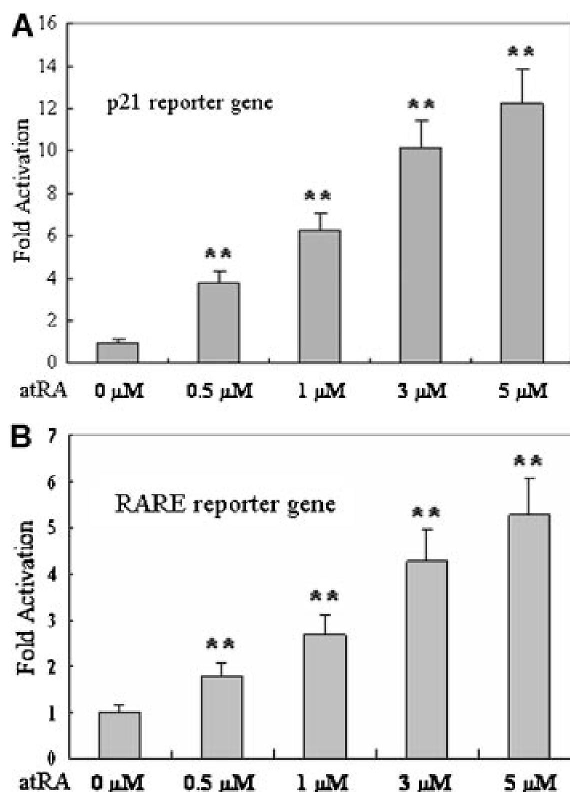

Fig. 2. Luciferase reporter gene assay. MEPM Cells were transfected with p21 promoter-driven luciferase reporter vector (**A**) or a RAR response element-driven luciferase reporter gene (**B**) and treated with the indicated various concentrations of atRA for 48 h. Luciferase activities were measured in light units of optical density (OD) of β-galactosidase. Data here are mean ± SEM, n = 3. Each experiment was performed in triplicate. Columns, mean (n = 3); bars, SE. $^*P < 0.05$, significant difference from control values.

transcription of the reporter gene in a dose-dependent manner. The result attested to the expression and functionality of p21 in atRA-treated cells and demonstrated the specificity of reporter response. Additionally, atRA treatment upregulated the expression of a reporter gene construct driven by the RAR response element (RARE) and did so also in a dose-responsive manner (Fig. 2B). These data support such a hypothesis that atRA-mediated induction of p21 in cells might be involved in a ligand-dependent manner.

Knockdown of p21 siRNA Blocked Growth Inhibition of MEPM Cells by atRA

To further determine the functional significance of p21 protein induction in atRA-mediated cell cycle arrest, we used siRNA technology to knock down p21 expression. Similar to untransfected cells, atRA treatment caused the induction of p21 and the reduction of cyclin D1 and pRb protein expression in control nonspecific siRNA-transfected MEPM cells (Fig. 3A). Transient transfection of cells with p21-targeted siRNA resulted in the near-complete abrogation of atRA-mediated induction of p21 and reduction of cyclin D1 and pRb, respectively (Fig. 3A). Next, we determined the effect of p21 knockdown on atRA-mediated cell cycle distribution. As shown in Figure 3B, the atRA-mediated G0–G1 phase cell cycle arrest was observed in control nonspecific siRNA-transfected cells but cells transfected with a p21-targeted siRNA had no cell cycle arrest in atRA-treated cells. Collectively, these results indicated that p21 is likely responsible for atRAl-induced cell cycle arrest.

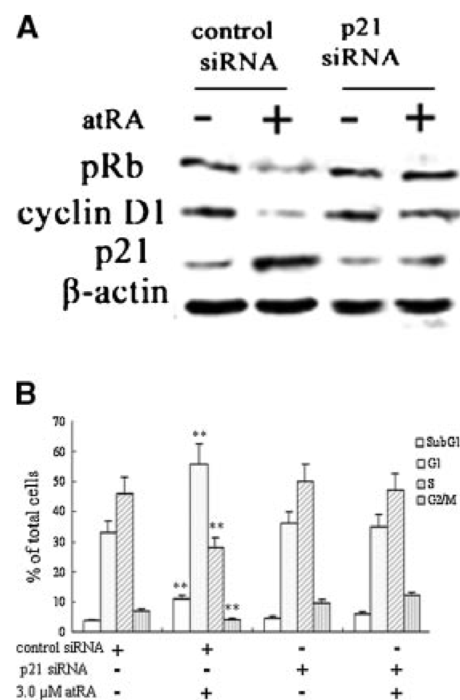

Fig. 3. p21 siRNA blocked growth inhibition of MEPM cells by atRA. MEPM cells were transfected with specific siRNA against p21 or with control siRNA. Following transfection, MEPM cells were treated with 3.0 μM RA or with alcohol vehicle as a control, and incubated for 48 h. **A**: Western blot was performed to examine the protein levels of cell cycle regulators using lysates from control nonspecific siRNA-transfected MEPM cells and MEPM cells transfected with p21-specific siRNA and treated with 3.0 μM atRA for 48 h. **B**: Fluorescence-activated cell sorting by flow cytometry was performed to detect cell cycle distribution in control nonspecific siRNA-transfected MEPM cells and p21-specific siRNA-transfected MEPM cells following a 48 h treatment with vehicle DMSO or atRA of 3.0 μM. Columns, mean (n = 3); bars, SE. *$P < 0.05$, significantly different compared with DMSO-treated control.

Knockdown of RAR Partially Attenuated Growth Inhibition of MEPM Cells by atRA

To examine the effect of RAR on atRA-mediated growth inhibition and p21 induction in cells, we next used the siRNA against RAR, as described in Materials and Methods Section, to knockdown RAR expression. As shown in Figure 4A, the siRNA against pan-RAR effectively knocked down RARα, β, and γ expression in cells. Because RAR in cells was not significantly knocked down by control siRNA and expression level of β-actin as a housekeeping protein was not affected by either siRNA (Fig. 4A), this inhibitory effect of RAR by siRNA in cells was specific. We next examined atRA-mediated cell cycle distribution of cells in the presence or absence of 3 μM through fluorescence-activated cell sorting by flow cytometry. As demonstrated, atRA-induced growth inhibition (Fig. 4C) and p21 induction (Fig. 4B) were attenuated when compared with control siRNA plus atRA treatment; RAR siRNA could not completely block G1 arrest by atRA when compared with control siRNA. These data suggest that atRA-mediated growth inhibition and induction of p21 is partially through RARs by a ligand-dependent manner.

p21 Is Associated With RAR

Since we observed that the induction of p21 by atRA treatment in cells might be in a ligand-dependent manner. We then want to know if there is a physical interaction between RAR and

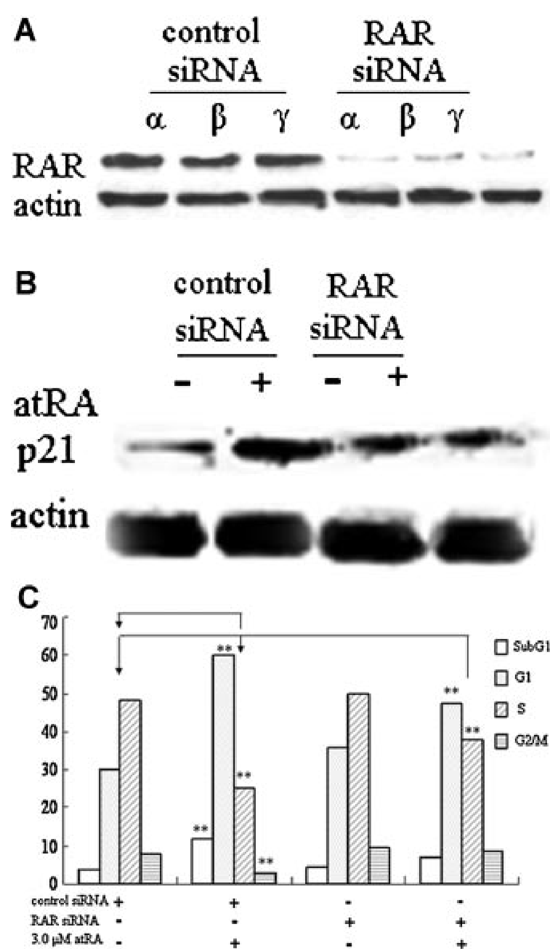

Fig. 4. Knockdown of RAR by siRNA partially attenuated growth inhibition and p21 induction in MEPM cells by atRA. MEPM cells were transfected with siRNA against RAR or with control siRNA using the siIMPORTER siRNA transfection reagent. Following transfection, MEPM cells were treated with 3.0 μM atRA or with DMSO vehicle as a control, and incubated for 48 h. **A**: Western blot was performed to examine the efficiency of RAR knockdown. **B**: Western blot was performed to detect the induction of p21 using lysates from control siRNA-transfected MEPM cells and MEPM cells transfected with pan-RAR siRNA and treated with 3.0 μM atRA for 48 h. **C**: Fluorescence-activated cell sorting by flow cytometry was performed to detect cell cycle distribution in control siRNA-transfected MEPM cells and pan-RAR siRNA-transfected MEPM cells following a 48 h treatment with 3.0 μM atRA. Columns, mean (n = 3); bars, SE. *$P < 0.05$, significantly different compared with vehicle alcohol-treated control.

the p21 promoter. For this purpose, we performed chromatin immunoprecipitation (ChIP) assay to determine whether RAR is recruited onto chromosomally integrated genomic sequences, which contain the p21 promoter in the context of cells. As shown in Figure 5, the anti-RAR antibody, but not the control IgG antibody, precipitated the p21 promoter fragment spanning p21 RAREs region in cells

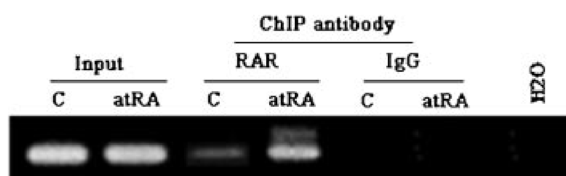

Fig. 5. Recruitment of RAR to the p21 promoter in vivo. MEPM Cells were treated for 48 h with 3.0 μM of atRA and processed for ChIP with a polyclonal RAR antibody. A dummy affinity-purified IgG was used as control in extracts obtained from atRA-treated cells. Input material and immunoprecipitates were analyzed by PCR with oligonucleotide primers specific for the p21 promoter (see Materials and Methods Section). The cross-linked protein-DNA complexes were immunoprecipitated with the anti-RAR antibody (**lanes 3** and **4**) or with an isotype control IgG as negative control (**lanes 5** and **6**). PCR of the input DNA is shown in **lanes 1** and **2**. H_2O is the PCR control (**lane 7**).

treated with 3.0 μM and not in the untreated control cells.

DISCUSSION

Retinoic acid (RA), the active metabolite of vitamin A, regulates cellular growth and differentiation during embryonic development. The physiologic doses (usually ≤0.01 μM) of RA play an important role in embryogenesis. In excess, this reagent is highly teratogenic to both animals and humans [Lammer et al., 1985; Coberly et al., 1996, Collins and Mao, 1999]. Studies indicated that exposure to teratogenic levels of RA decreases cell proliferation and increases cell death in the developing embryo [Webster et al., 1986; Sulik et al., 1988; Watanabe et al., 1988]. An understanding of the mechanisms by which RA causes cell growth inhibition may facilitate our understanding of teratogenic actions of this kind of agent because this knowledge could lead to the identification of mechanism-based biomarkers potentially useful in future teratogenic screening. Recent studies have established a link between RA and cell growth regulators [Chen and Gardner, 1998; Hayes and Morriss-Kay, 2001; Dalvin et al., 2004]. Of these, the Cdk inhibitor p21 is the most prominent candidate. p21 plays an essential role in the regulation of G1-S transition by binding to and inhibiting kinase activity of Cdk/cyclin complexes [Schafer, 1998; Molinari, 2000; Taylor and Stark, 2001]. The cyclin D1/Cdk4 and cyclin D1/Cdk6 kinase complexes hyperphosphorylate Rb protein, leading to its dissociation from the transcription factor E2F1, which regulates expression of genes necessary for cell cycle progression

[Schafer, 1998; Molinari, 2000; Taylor and Stark, 2001]. Deregulation in the expression of p21 would be expected to result in abnormal cell proliferation and to predispose the cells to transformation [Chen et al., 1995; Cardinali et al., 1998].

Our present results indicated that atRA treatment resulted in a marked increase in the cell population in the G_1 phase, leading to an overall 2.0-fold increase at 48 h. The increase was accompanied by a corresponding decrease in S phase, showing that RA inhibits the G_1-S transition. In addition to a marked effect on cell cycling, RA also triggered DNA fragmentation, reflected by an increase of the fraction of cells in the sub-G_1 population. In accordance with a G_1 arrest, the expression level of cyclin D1 protein and phosphorylation of Rb were suppressed, whereas p21 was significantly increased upon 48 h of atRA treatment as demonstrated in Figure 1B. As described above, p21 is a critical regulator in cell growth. We next want to know its status in growth inhibition of MEPM cells by atRA. To this end, we used specific siRNA to knockdown p21 expression. Quantifying the western blots revealed the degree of p21 protein repression to be $\sim$80%. As expected, p21 siRNA succeed in blocking the growth inhibition and restoring the level of pRb and Cyclin D1 of MEPM cells by atRA. Moreover, atRA treatment increased p21 promoter activity in a dose-dependent manner by using MEPM cells transiently transfected with p21 promoter-driven luciferase reporter vector. These findings propose that p21 activation is required in growth inhibition of EMPM cells by atRA.

RA induces gene expression through binding to the nuclear receptors RAR (RA receptor) and RXR (retinoid X receptor) [Chambon, 1996; Wei, 2003]. atRA acts as a ligand for RAR, while the isomer 9-*cis*-RA can be bound by both RAR and RXR. For each receptor, there are three subtypes (α, β, and γ) and several isoforms, which differ in their tissue distribution [Dolle et al., 1994; Mollard et al., 2000]. RAR and RXR bind to each other forming functional heterodimers. Activated and dimerized receptors control gene expression via binding to retinoic acid response elements [Ziouzenkova and Plutzky, 2008]. We herein show that pan-RAR siRNA partially attenuated growth inhibition of MEPM cells and blocked the induction of p21 by atRA. Moreover, atRA treatment increased RAREs activity in a dose-dependent manner by using MEPM cells transiently transfected with RARE-driven luciferase reporter vector. Further chromatin immunoprecipitation assays demonstrated that RAR associated with the p21 promoter (Fig. 5). Conclusively, p21 induction by atRA might be partially, not completely, through a ligand-specific manner; and p21 might be a target of RAR in atRA-mediated growth inhibition of MEPM cells.

Numerous studies have shown that environmental influences that induce effects on cell cycle can result in reduced proliferation of cells actively cycling at the time of such exposure. During embryonic development, such changes can lead to defective functioning and/or smaller size of the organ. The induction of CP by atRA varies depending on the stage of development exposed. In vivo studies have indicated that, after exposure of embryonic mice to RA on GD 10, abnormally small palatal shelves form. After exposure on GD 12, shelves of normal size form, but fail to fuse, as the normal growth and differentiation process of palatal mesenchymal cells is disturbed. Recent studies demonstrated that high concentration of RA led to cell growth arrest in embryonic palatal mesenchyme [Ho et al., 2004; Okano et al., 2007], which is consistent with our earlier and present in vitro study. In summary, we have provided further evidence that RAR has an important role in MEPM cells responding to atRA treatment, and most importantly, our data for the first time indicate p21 as an essential target gene mediating the growth inhibitory effects in MEPM cells. As RAR siRNA could not completely block the inhibitory effects of atRA there should be other underlying pathways through which p21 mediates growth inhibition of MEPM cells by atRA. Further study should focus on this issue.

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