

All-*trans* retinoic acid inhibits proliferation of intestinal epithelial cells by inhibiting expression of the gene encoding Krüppel-like factor 5

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Received 28 September 2004; revised 27 October 2004; accepted 27 October 2004

Available online 11 November 2004

Edited by Ned Mantei

Abstract Retinoids are known inhibitors of epithelial cell proliferation. Previous studies indicate that Krüppel-like factor 5 (KLF5) is a pro-proliferative transcription factor. Here, we examined the effect of all-*trans* retinoic acid (ATRA) on proliferation of the intestinal epithelial cell line, IEC6. Treatment of IEC6 cells with ATRA inhibited their proliferation due to G₁ cell cycle arrest. This inhibition was correlated with a decrease in the levels of *KLF5* mRNA and promoter activity. In contrast, constitutive expression of *KLF5* in stably transfected IEC6 cells with a *KLF5*-expressing plasmid driven by a viral promoter abrogated the growth inhibitory effect of ATRA. Moreover, ATRA inhibited proliferation of several human colon cancer cell lines with high levels of *KLF5* expression but not those with low levels of *KLF5* expression. Our results indicate that KLF5 is a potential mediator for the inhibitory effect of ATRA on intestinal epithelial cell proliferation.

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Keywords: Cell cycle; Colon cancer; Krüppel-like factor 5; IKLF; Promoter; Pro-proliferative; Stable transfection

1. Introduction

Krüppel-like factors (KLFs) belong to a class of zinc finger-containing transcription factors that exhibit homology to the general transcription factor Sp1 [1–4]. Numerous studies indicate that KLFs play important roles in regulating cell proliferation and differentiation. Two KLFs are present at high levels in the intestinal epithelium although in different locations – *KLF4* is expressed primarily in the terminally differentiated, post-mitotic epithelial cells [5,6] and *KLF5* in the proliferating crypt compartment [7,8]. The two proteins also exhibit different biological properties – *KLF4* is an inhibitor of cell proliferation [5,9,10] but *KLF5* stimulates cell proliferation when overexpressed [11]. Thus, the two KLFs may function to coordinate proliferation of the intestinal epithelial cells (IEC).

Retinoids, and other derivatives of vitamin A, are known to have important functions in regulating differentiation and proliferation. The effects of retinoids are mediated by nuclear receptors, such as retinoic acid receptors (RARs) and retinoic X receptors (RXRs) [12]. Because of their potent growth-suppressive effects, retinoids are used as a chemotherapeutic or chemopreventive agent in the treatment of a variety of disorders in preclinical and clinical settings [13,14]. All-*trans* retinoic acid (ATRA) is one type of retinoid that binds preferentially to RARs, which form homodimers or heterodimers with RXRs and interact with retinoic acid response elements (RAREs) to mediate cellular response [15–17]. ATRA is an effective chemotherapeutic drug for the treatment of acute promyelocytic leukemia (APL) [18]. In addition, ATRA exhibits an inhibitory effect in a number of colon cancer cell lines [19–21]. Therefore, ATRA may potentially serve as an anti-proliferative agent in the treatment of cancerous cells.

Previous studies have shown that overexpression of *KLF5* stimulates proliferation of intestinal epithelial cells, IEC-18, and NIH 3T3 fibroblasts [11,22]. Moreover, RAR α interacts with *KLF5* and RAR ligands modulate *KLF5* transcriptional activity and consequent cellular proliferation [23]. In the current study, we aimed at determining whether ATRA may modulate proliferation of intestinal epithelial cells, IEC6, and whether such modulation may be dependent on *KLF5*.

2. Materials and methods

2.1. Cell lines

Non-transformed rat intestinal epithelial cells, IEC6, and the human colon cancer cell lines, DLD1, HT29 and RKO, were purchased from the American Type Culture Collection (Manassas, VA). The colon cancer cell line, Caco2-BBE, was previously described [24]. Stable transfection of IEC6 cells was performed by lipofection using pBK-CMV-KLF5-HA [7,11]. Transfected cells were selected with 1 mg/ml G418 and pooled for experimentation. Cells were treated with media containing 10 μ M ATRA or ethanol control for the stated periods.

2.2. Cell proliferation assay

Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum (FBS), 0.1 U/ml insulin, and 1% streptomycin. Cells were seeded at a density of 100 000 cells/6-well dish and treated with ATRA or ethanol for the stated periods of time. Each day, cells were collected, washed with Dulbecco's phosphate-buffered saline (DPBS), trypsinized, and counted using a hemocytometer.

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Abbreviations: APL, acute promyelocytic leukemia; ATRA, all-*trans* retinoic acid; CMV, cytomegalovirus; DPBS, Dulbecco's phosphate buffered saline; FACS, fluorescence-activated cell sorting; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HA, hemagglutinin A; IEC, intestinal epithelial cells; KLF5, Krüppel-like factor 5; MAPK, mitogen-activated protein kinase; PI, propidium iodide; PKC, protein kinase C; RAR, retinoic acid receptor; RARE, retinoic acid response element; RXR, retinoic X receptor

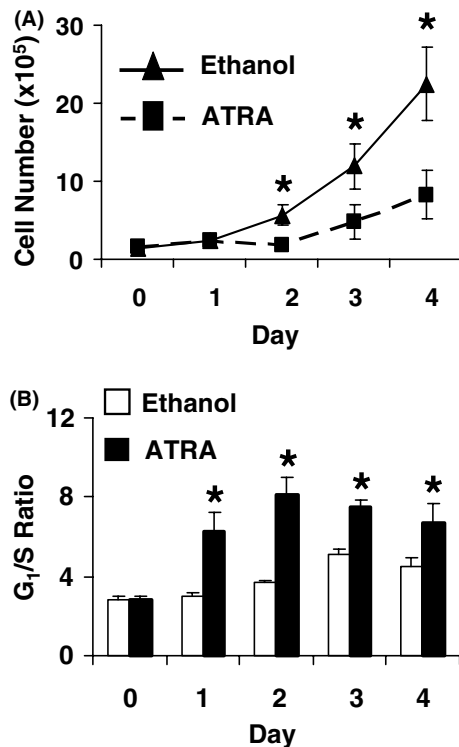


Fig. 1. The effect of ATRA on proliferation and cell cycle profiles of IEC6 cells. (A) IEC6 cells were seeded at a density of 10^5 cells/6-well dish and maintained in media supplemented with 5% FBS and containing either 10 μ M ATRA (closed squares) or ethanol control (closed triangles) for the stated number of days before being counted. $N = 6$. * $P < 0.01$. (B) For cells subjected to fluorescent-activated sorting, IEC6 cells were maintained in media containing 5% FBS and 10 μ M ATRA (closed bars) or ethanol control (open bars) for the stated periods of time. Each day, cells were harvested and analyzed by FACS. Shown are the ratios of cells in the G₁ and S phases of the cell cycle. $N = 5$. * $P < 0.001$.

2.3. Luciferase assay

The mouse -1.5 kb *KLF5* promoter linked to a luciferase reporter in the pGL2-Basic vector was provided by Dr. A. Levine [25] and transfected into IEC6 cells by lipofection along with a Renilla-luciferase reporter as a control. Transfected cells were treated with ethanol or 10 μ M ATRA for 3 days before being harvested for reporter assays using the Dual-Luciferase Reporter Assay protocol (Promega, Madison, WI).

2.4. Fluorescence-activated cell sorting analysis

Cell cycle analysis was performed using fluorescence-activated cell sorting (FACS) as previously described [26,27]. Cells were trypsinized, washed with DPBS, and cooled to -20 °C overnight in 70% ethanol. Cells were then collected by centrifugation and stained with propidium iodide (PI) solution (50 μ g/ml PI, 50 μ g/ml RNase A, 0.1% Triton X-100, and 0.1 mM EDTA) for 25 min. Cells were analyzed using FAC-SCalibur cytometer (Becton–Dickinson).

2.5. Western blot analysis

Cells were washed with DPBS and collected by centrifugation. The cells were re-suspended in 1 \times lysis buffer (Cell Signaling, Beverly, MA). The suspended cells were subjected to sonication and centrifugation. Western blot analysis was then performed using a rabbit polyclonal antibody against *KLF5*, hemagglutinin A (HA) (Santa Cruz Biotech.) or β -actin (Oncogene Research).

2.6. Northern blot analysis

RNA was extracted using the Trizol protocol (Invitrogen, Carlsbad, CA). Twenty micrograms of RNA was loaded onto a denaturing agarose gel containing 10 \times MOPS buffer and 37% formaldehyde, and then transferred to a nylon membrane (Hybond, Amersham, Piscataway, NJ). Radioactive [α -³²P] dATP was used to label cDNA probes for *KLF5* and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). The membrane was hybridized in Quikhyb solution (Stratagene, La Jolla, CA), washed under high-stringency conditions, and scanned using a phosphorimager (Amersham).

3. Results

We first determined whether ATRA affects growth and proliferation of IEC6, a non-transformed rat intestinal epithelial

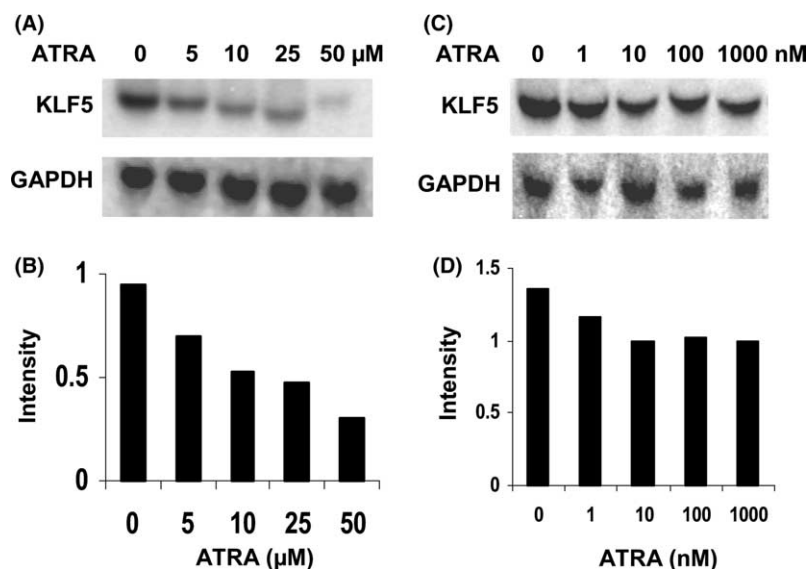


Fig. 2. A dose-response study of ATRA on *KLF5* mRNA levels in IEC6 cells. Northern blot analyses were conducted on RNA isolated from IEC6 cells treated with ATRA. Panel A is the result of a dose-response study in IEC6 cells treated for 3 days with ATRA with a concentration between 0 and 50 μ M. Panel C is a similar experiment except that the concentration of ATRA was between 0 and 1000 nM. Blots were probed with *KLF5* and then *GAPDH*. Panels B and D represent densitometric quantification of band intensities of *KLF5* mRNA after normalizing to that of *GAPDH*.

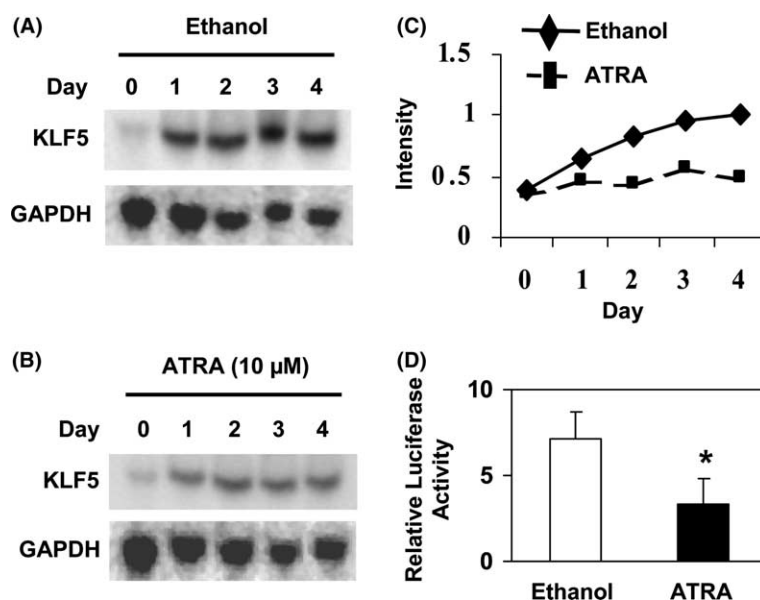


Fig. 3. A time-course and promoter study of ATRA on *KLF5* mRNA levels in IEC6 cells. Northern blot analyses were conducted on RNA isolated from IEC6 cells treated with ethanol control (panel A) or 10 μ M ATRA (panel B) on a daily basis up to 4 days after seeding. Panel C represents the densitometric quantification of band intensities of *KLF5* mRNA after normalizing to that of *GAPDH*. In panel D, a -1.5 kb *KLF5* promoter-luciferase plasmid was transfected into IEC6 cells and treated with 10 μ M ATRA (closed bar) or ethanol control (open bar) for 3 days before being harvested for luciferase assays. Shown are the mean relative luciferase activities after normalizing for the internal Renilla luciferase control. $N = 5$. * $P < 0.01$.

cell line. As shown in Fig. 1A, treatment of IEC6 cells with 10 μ M ATRA resulted in a significant reduction in their rate of proliferation between days 2 and 4 after seeding. This inhibitory effect was the consequence of cell cycle arrest at the G_1/S boundary as demonstrated by the significant increase in the G_1/S ratios of cells treated with ATRA as compared to control (Fig. 1B).

We next determined whether ATRA treatment affects expression of the gene encoding KLF5, previously shown to be a pro-proliferative factor in IEC18 and NIH 3T3 cells [11,22]. As shown in Fig. 2A and B, treatment of IEC6 cells with ATRA from 5 to 50 μ M for 3 days resulted in a dose-dependent reduction in the levels of *KLF5* mRNA. Treatment of cells with lower doses of ATRA, between 1 and 1000 nM, resulted in a less prominent and non-linear reduction in the levels of *KLF5* mRNA (Fig. 2C and D). For this reason, we used a concentration of 10 μ M throughout the present study.

We then performed a time-course study in IEC6 cells treated with ATRA or ethanol control. In the presence of ethanol control, the levels of *KLF5* mRNA increased over time (Fig. 3A). In contrast, the levels of *KLF5* mRNA in cells treated with 10 μ M ATRA remained relatively unchanged for up to 4 days (Fig. 3B). The relative abundance of *KLF5* mRNA in treated and control cells (Fig. 3C) paralleled that of their rates of proliferation as illustrated in Fig. 1A. These results indicate that ATRA inhibits expression of *KLF5* in a dose-dependent and time-dependent fashion, and that inhibition of *KLF5* expression is correlated with a reduction in the rate of proliferation.

We then investigated the mechanism by which ATRA inhibits the expression of *KLF5*. We performed transfection studies in IEC6 cells using a plasmid that contained -1.5 kb of the *KLF5* promoter linked to a luciferase reporter and treated them with ATRA or ethanol control. Fig. 3D shows that treatment of transfected IEC6 cells with 10 μ M ATRA for 3 days resulted in a statistically significant reduction in the activity

of the reporter construct when compared to ethanol control. These findings suggest that the effect of ATRA on *KLF5* expression is mediated at the level of gene transcription.

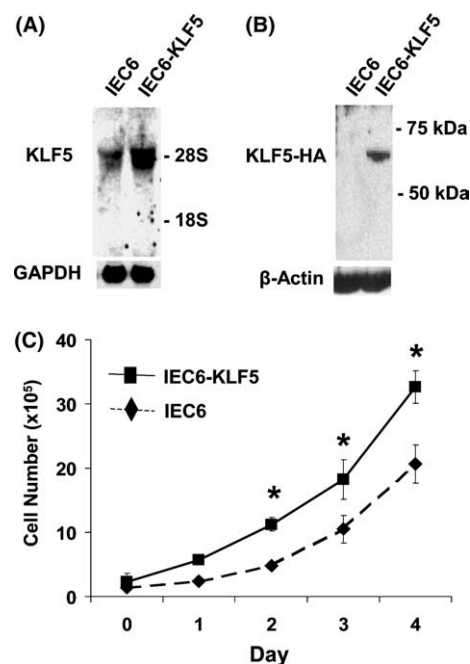


Fig. 4. Establishment of stably transfected IEC6 cells with KLF5. IEC6 cells were transfected with pBK-CMV-KLF5-HA, selected with G418 and pooled for the study. The resultant cells are designated IEC6-KLF5. Shown are the results of Northern blot analysis (panel A) and Western blot analysis (panel B) on the levels of *KLF5* mRNA and HA-tagged KLF5 protein from IEC6 and IEC6-KLF5. In panel C, IEC6 or IEC6-KLF5 was seeded at a density of 10^5 cells/6-well dish and propagated for 4 additional days. Cell numbers were determined on a daily basis. $N = 6$. * $P < 0.01$.

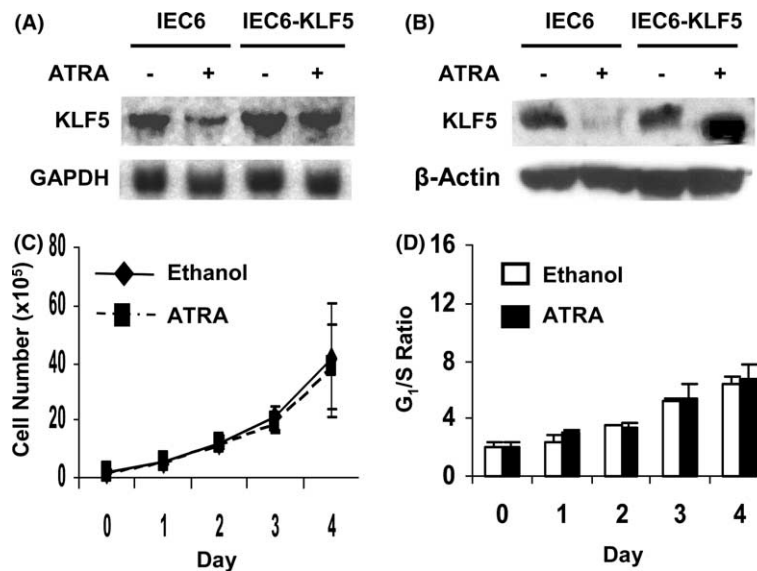


Fig. 5. The effect of ATRA on *KLF5* expression, proliferation, and cell cycle profile in untransfected and *KLF5*-transfected IEC6 cells. IEC6 and IEC6-KLF5 cells were treated with 10 μ M ATRA or ethanol control for 3 days before being harvested for Northern (panel A) or Western (panel B) blot analyses. The antibody used in panel B is directed against *KLF5*. In panel C, IEC6-KLF5 cells were seeded at a density of 10^5 cells/6-well dish and treated with 10 μ M ATRA (closed squares) or ethanol (closed diamonds) for the stated number of days. $N = 6$. In panel D, IEC6-KLF5 cells were treated as in panel C and analyzed by FACS on a daily basis. $N = 5$.

To further demonstrate that *KLF5* mediates the inhibitory effect of ATRA on proliferation of IEC6 cells, we established a stably transfected IEC6 cell line that constitutively expressed

KLF5 under the control of the cytomegalovirus (CMV) viral promoter, which is not subject to regulation by ATRA. This cell line, called IEC6-KLF5, contained a significantly higher le-

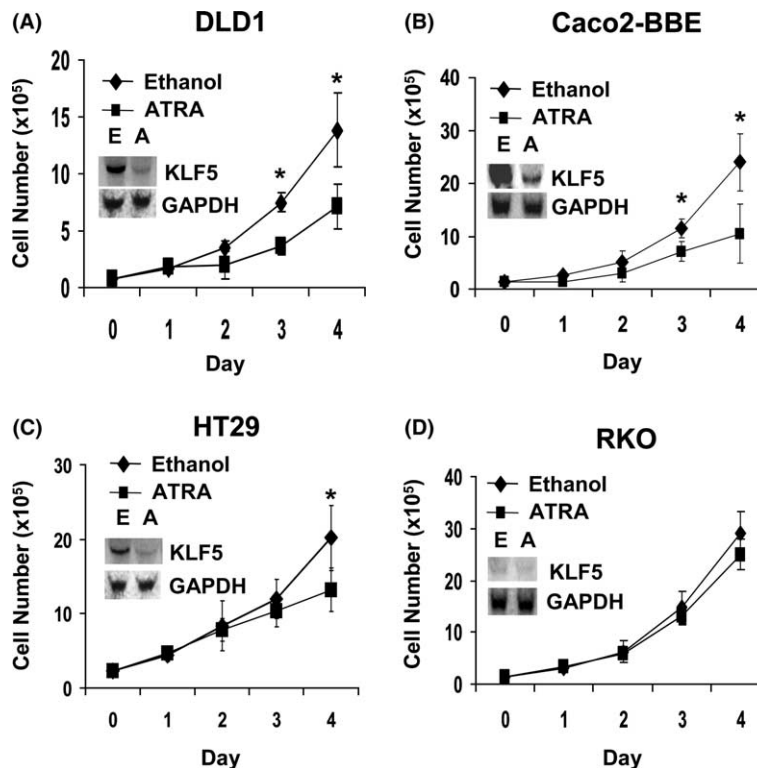


Fig. 6. The effect of ATRA on proliferation of human colon cancer cell lines. Four different colon cancer cell lines were used in the study: DLD1 (panel A), Caco2-BBE (panel B), HT29 (panel C), and RKO (panel D). For each cell line, cells were seeded at a density of 10^5 cells/6-well dish and maintained in the presence of 10 μ M ATRA or ethanol control for up to 4 days. $N = 6$. * $P < 0.05$. The insets show the mRNA levels of *KLF5* and *GAPDH* in cells treated with ATRA (A) or ethanol control (E) for 3 days.

vel of *KLF5* mRNA when compared to untransfected IEC6 cells (Fig. 4A). In addition, when probed with an antibody against HA, IEC6-KLF5 cells contained HA-tagged KLF5 protein, which was absent from untransfected cells (Fig. 4B). As a consequence of stable and constitutive expression of *KLF5*, IEC-KLF5 cells exhibited a higher rate of proliferation when compared to the untransfected IEC6 cells (Fig. 4C). Importantly, treatment of IEC6-KLF5 with 10 μ M ATRA failed to reduce the levels of KLF5 mRNA and protein as shown in Fig. 5A and B, respectively. In comparison, the levels of KLF5 mRNA and protein were reduced in untransfected IEC6 cells that were treated with ATRA (Fig. 5A and B). Moreover, constitutive expression of *KLF5* in IEC6 cells resulted in resistance to ATRA as demonstrated by the lack of effect of ATRA on the rate of proliferation and cell cycle profile, illustrated in Fig. 5C and D, respectively. These results suggest that the inhibitory effect of ATRA on IEC6 cells is primarily mediated by the ability of ATRA to inhibit endogenous *KLF5* expression.

To determine whether ATRA may have an effect on proliferation of human colon cancer cell lines, we cultured several established colon cancer cell lines in the presence or absence of 10 μ M ATRA. The growth rates of three cell lines, DLD1, Caco2-BBE and HT29, were sensitive to ATRA treatment (Fig. 6A, B and C, respectively). However, the rate of proliferation of RKO cells was indifferent to ATRA (Fig. 6D). A difference between the first three cell lines and RKO cells is that the basal level of *KLF5* mRNA is significantly lower in RKO cells as compared to the first three cell lines (Fig. 6, insets). Importantly, the ability of ATRA to reduce cell proliferation is correlated with a reduction in the levels of *KLF5* mRNA in DLD1, Caco2-BBE and HT29 cells (Fig. 6, insets). In RKO cells, the level of *KLF5* mRNA was unchanged with ATRA treatment (Fig. 6D, inset). These results further suggest that KLF5 mediates the inhibitory effect of ATRA on IEC proliferation.

4. Discussion

Retinoids are known to play a vital role in various physiological processes such as embryogenesis, growth, differentiation and reproduction [28]. Studies also showed that retinoic acid can inhibit proliferation of many tumor cells grown in vitro [29]. These findings led to the successful clinical application of retinoids in the treatment or prevention of various human cancers, such as APL [18]. In the case of APL, ATRA is able to induce complete remission in almost all patients by inducing in vivo differentiation of blast cells in APL [18].

Numerous studies have demonstrated that the cellular effects of retinoids are mediated by nuclear receptors, including RARs and RXRs [12]. For example, the inhibitory effect of ATRA on proliferation of the human colon cancer cells, HT29, is mediated through RAR α [19]. Also, induction of RAR in the retinoid-resistant colon cancer cells, LoVo, restored retinoid sensitivity [30]. Despite these findings, the mechanisms by which ATRA inhibits tumor cell proliferation remain poorly established.

In the current study, we characterized the anti-proliferative effect of ATRA on a non-transformed rat IEC line, IEC6. Our results showed that ATRA inhibits cell proliferation in

IEC6 (Fig. 1A). This inhibitory effect is primarily due to an arrest in the G₁ to S transition of the cell cycle (Fig. 1B). A similar effect of ATRA on the cell cycle has previously been demonstrated in myeloid cells [31] and ovarian carcinoma cells [32]. In myeloid cells, ATRA-induced cell cycle arrest is accompanied by downregulation of c-Myc and cyclin E [33], the latter an essential component for G₁ to S transition. ATRA has also been shown to reduce expression of protein kinase C α (PKC α) and activity of the mitogen-activated protein kinase (MAPK) pathway [34].

The Krüppel transcription factor, KLF5, has been shown to positively regulate cell proliferation in NIH 3T3 fibroblasts and IEC [11,22]. Results of our present study indicate that ATRA inhibits expression of *KLF5* in IEC6 cells (Figs. 2 and 3) by inhibiting the promoter activity of *KLF5* (Fig. 3D). The reduction in *KLF5* mRNA levels due to ATRA treatment is correlated with a reduction in the rate of proliferation (Figs. 1 and 3C). The dosage of ATRA that we chose was relatively high at 10 μ M, based on the result of the dose-response experiment in Fig. 2. Several other studies have used lower dosages [35,36]. However, numerous studies have also used higher concentrations of ATRA similar to ours in order to accomplish the inhibitory effects of ATRA in various cell lines [37–40]. Therefore, the different sensitivity of different cell lines to ATRA is probably a cell type-specific event.

IEC6 cells stably transfected with a viral promoter-driven *KLF5*-expression vector contained a higher level of *KLF5* mRNA and protein and a higher rate of proliferation as compared to untransfected IEC6 cells (Fig. 4). When treated with ATRA, the levels of *KLF5* mRNA and protein were not decreased in IEC6-KLF5 cells as opposed to untransfected IEC6 cells (Fig. 5A and B). This is primarily due to the constitutive expression of *KLF5* from a viral promoter that is not affected by ATRA. This constitutive expression resulted in the IEC-KLF5 cells to resist the inhibitory effect of ATRA as demonstrated in Fig. 5C and D. These results suggest that KLF5 is a mediator for the inhibitory effect of ATRA on proliferation of IEC6 cells, although we could not rule out KLF5-independent mechanisms. It is of interest to note that *KLF5* expression is stimulated by the phorbol ester, PMA, a potent activator of PKC α [11,41]. Moreover, studies indicate that *KLF5* expression is stimulated by MAPK activation [41,42]. The fact that ATRA inhibits both PKC α and MAPK [34] may be a mechanism by which it inhibits *KLF5* expression. It should be noted that cyclin D1 has been shown to be a target of regulation by KLF5 [42]. The effect of ATRA on G₁ progression can therefore be a consequence of its ability to inhibit cyclin D1 expression through KLF5.

ATRA has previously been shown to inhibit proliferation of some, but not all, colon cancer cells [19–21,30]. Our study demonstrated that 3 colon cancer cell lines, including DLD1, Caco2-BBE and HT29, are sensitive to the inhibitory effect of ATRA but RKO cells are resistant (Fig. 6). Previous studies suggest that the presence or absence of RARs in cells appears to play a role in determining whether cells are sensitive or resistant to ATRA treatment [21,30]. However, all of the cells that we examined contain RAR α (data not shown). Instead, the ability of ATRA to inhibit cell proliferation is directly correlated with a reduction in *KLF5* mRNA levels in the 3 sensitive cell lines (Fig. 6A, B and C). The lone cell line, RKO, that is resistant to ATRA action, contains little *KLF5* mRNA at baseline, which is not changed with ATRA

treatment (Fig. 6D). These results further support the role of KLF5 in mediating the inhibitory effect of ATRA on cell proliferation. Furthermore, whether a tumor cell is sensitive to ATRA may in part be determined by the degree of *KLF5* expression.

The relationship between KLF5 and retinoids in regulating cell proliferation has also been demonstrated in vivo [23]. This was shown by the ability of RAR ligands to modulate KLF5 transcriptional activity. Moreover, RAR and KLF5 physically interact. A consequence of this relationship is the ability of a RAR agonist, Am80, to inhibit vascular remodeling in vivo, a process that is dependent on KLF5 [23]. Whether RAR and KLF5 physically interact in IEC is unclear at this time.

In conclusion, our study demonstrated an important role for KLF5 in mediating the inhibitory effect of ATRA on proliferation of IEC. We also showed that differential sensitivity of colon cancer cells to ATRA may be due to differential levels of *KLF5* expression. Additional correlation between *KLF5* expression and ATRA sensitivity in other cell lines may further substantiate the role of KLF5 in modulating IEC proliferation.

Acknowledgements: We thank Drs. J. Lingrel and A. Levine for providing the pBK-CMV-KLF5-HA [7] and -1.5 kb KLF5 promoter-luciferase [25] plasmids, respectively. This work was in part supported by National Institutes of Health Grants DK52230, DK64399, and CA84197. V.W.Y. is the recipient of a Georgia Cancer Coalition Distinguished Cancer Clinician Scientist award.

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