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Retinoic acid stability in stem cell cultures

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ABSTRACT It has been reported that retinoids, such as retinoic acid (RA) and retinol (ROL), dissolved in aqueous solutions are susceptible to oxidative damage when exposed to light, air, and relatively high temperatures, conditions that are normal for culturing stem cells. Thus, questions arise regarding the interpretation of results obtained from studies of mouse embryonic stem cells exposed to retinoids because their isomerization state, their stability in culture conditions, and their interactions with other potential differentiation factors in growth media could influence developmental processes under study. Media samples were supplemented with retinoids and exposed to cell culture conditions with and without mouse embryonic stem cells (mESC), and retinoids were extracted and analyzed using HPLC. To determine whether retinoids are stable in media supplemented with fetal bovine serum (FBS) or in chemically-defined, serum-free media, mESC adapted to each type of growth media were investigated. Studies reported here indicate there was little loss or isomerization of *at*-RA, *9-cis*-RA, *13-cis*-RA, or ROL in cell cultures grown in serum-supplemented media when cell cultures were maintained in the dark and manipulated and observed under yellow light. In contrast, the stability of both *at*-RA and ROL were determined to be greatly reduced in serum-free media as compared with serum-supplemented media. Addition of 6 mg/ml bovine serum albumin was found to stabilize retinoids in serum-free media. It was also determined that ROL is less stable than RA in cell culture conditions.

KEY WORDS: *retinoic acid, cell culture condition, retinoid integrity*

Introduction

Retinoic acid (RA) has been shown to influence cell proliferation, differentiation, maturation, and apoptosis (Duong and Rochette-Egly 2011; Gudas and Wagner, 2011; Caricasole *et al.,* 2000; Baharvand *et al.,* 2007; Crnek *et al.,* 1991). To decipher mechanisms underlying these effects, *in vitro* studies have been carried out using cells cultured in growth media supplemented with RA (Gubler and Sherman 1985; Williams and Napoli 1985; Gudas and Wagner 2011; Strickland and Mahdavi 1978; Zouboulis *et al.,* 1999; Redfern and Todd 1988). In these and many other published studies (too numerous to be cited here), a variety of protocols have been followed and a broad range of RA concentrations (nanomolar to micromolar) have been used to investigate RA effects on cell processes. In some protocols, RA has been provided in growth media supplemented with serum and in others, in serum-free media.

Retinoids dissolved in aqueous solutions are susceptible to oxidative damage when exposed to light, air, and high temperature in the absence of protein or antioxidants such as vitamin C and E (Szuts and Harosi 1991; Klaassen *et al.,* 1999). Additionally, retinoids are adsorbed by glassware and plasticware (Klaassen *et al.,* 1999; Noy 1992). These cautionary dictums raise important questions regarding the stability and integrity of retinoids in cell cultures. Early studies analyzing the metabolism and turnover of RA and ROL in cell cultures provided qualitative evidence that retinoids survive cell culture conditions intact in medium supplemented with fetal bovine serum (FBS) (Williams and Napoli 1985; Gubler and Sherman 1985), but a specific quantitative study analyzing the status of retinoids in serum-deprived and serum-supplemented media under cell culture conditions has not been previously reported.

This study was designed to determine whether retinoids are stable in growth media maintained under cell culture conditions. The results indicate that with careful handling, RA and ROL retain their integrity in cultures when serum or bovine serum albumin (BSA) is present in growth media. In contrast, even with careful handling, *at*-RA isomerizes and ROL is lost when added to serum-free media and maintained under cell culture conditions (without cells) for 24h.

Abbreviations used in this paper: BSA, bovine serum albumin; DMEM, Dulbecco´s minimal essential media; FBS, fetal bovine serum; mESC, mouse embryonic stem cell; RA, retinoic acid; ROL, retinol.

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Experimental Protocols

Chemicals

Retinoids were purchased from Sigma-Aldrich, St. Louis MO. HPLC-grade reagents were obtained from Fisher Scientific Inc. (Pittsburgh, PA).

Media and cells

R1 mouse embryonic stem cells (R1 mESCs, from William Stanford, University of Toronto, Canada) were adapted to growth in serum-supplemented growth media. Cells were maintained in high glucose Dulbecco's Minimal Essential Media (DMEM) with GlutaMax ™ (Invitrogen, Carslbad, CA) supplemented with 15% FBS (Atlanta Biological, Lawrenceville, GA) and 50 U/ml Penicillin/50 μg/ml Streptomycin. C57/BL6 mESCs (BL6 mESCs,) were adapted to growth in chemically defined, serum-free media and were maintained in ESGRO Complete Plus Medium (Millipore Corporation, Billerica, MA).

Retinoid supplementation studies

All operations were carried out under yellow light because retinoids do not absorb light in the yellow range of the electromagnetic spectrum (570-585nm). Concentrated stock solutions of *at*-RA, *9-cis*-RA, *13-cis*-RA, and ROL were prepared in ethyl acetate and stored in amber glass vials at -20°C in the dark. Ethanol stock solutions (approximately 2mM) were prepared from the concentrated stocks, sterile filtered through a 0.2 μm syringe filter, and stored in glass vials at -20°C in the dark for use in the retinoid-supplementation studies (described below). The absorbance and spectrum of each sterile retinoid stock was determined with a UV-VIS spectrophotometer, and concentrations calculated using the following absorption coefficients: 44,300L/mol × cm at 350nm for *at*-RA in ethanol; 39,800 L/mol × cm at 354 nm for *13-cis*-RA in ethanol; 36,500 L/mol × cm at 343 nm for *9-cis*-RA in methanol; 46,000 L/mol × cm at 325 nm for ROL in ethanol.

Retinoid supplementation studies without cells

5mL media was supplemented with 0.5μM *at*-RA, *9-cis* RA, *13-cis* RA, ROL, or vehicle (≤ 0.04% ethanol, control), and the media was either analyzed immediately or was snap frozen in liquid nitrogen and stored at -80°C until time of analysis. For overnight incubations (18 to 24 h, hereafter referred to as 24 h), media was added to cell culture dishes and incubated in the dark at 37°C in a humidified atmosphere containing 7.5% CO_2.

Retinoid supplementation studies with cells

mESCs were seeded in media without retinoids and allowed to settle overnight. The media was replaced with fresh, retinoid-supplemented media. After 24 h incubation, the media was removed and processed as described above. When cultures were exposed to retinoids for longer than 24 h, the media was replaced with fresh, retinoid-supplemented media every 24h.

BSA-supplemented media

In some experiments, BL6 mESC serum-free media was supplemented with 0.5 mg/ml (Futterman and Heller 1972) or 6mg/ml BSA (Quinlan *et al.,* 2005). Dissolution of BSA and mixing with *at*-RA and ROL were as described (N'soukpoe-Kossi *et al.,* 2007). Retinoid solutions were added drop by drop to BSA-supplemented media with constant stirring to produce desired final concentrations. Media was gently rocked for 15min at room temperature to allow time for the retinoids to bind to the BSA and to ensure media homogeneity. Three different media preps each were prepared for *at*-RA and for ROL. 5mL from each of the preparations was snap frozen (0h samples) and stored at -80°C until the day of analysis. Another 5mL from each sample was incubated in cell culture dishes for 24h.

TABLE 1

RECOVERY OF SUPPLEMENTED RETINOIDS FROM R1ES MEDIA AND CELL CULTURES

Retinoid added	Retinoid analyzed	R1ES Media No Incubation Without Cells (Percent Recovery)	R1ES Media 24 HR Incubation Without Cells (Percent Recovery)	R1ES Media 24 HR Incubation With Cells (Percent Recovery)
MEDIA + Vehicle	*at*-RA	nd	nd	nd
	ROL	0.03 ± 0.01 μM	0.03 ± 0.01 μM	0.02 ± 0.00 μM
MEDIA + *at*-RA	*at*-RA	0.31 ± 0.03 μM (61%)	0.34 ± 0.06 μM (69%)	0.06 ± 0.01 μM (11%)
	Pooled RA	0.33 ± 0.01 μM (66%)	0.38 ± 0.06 μM (76%)	0.06 ± 0.01 μM (12%)
MEDIA + ROL	ROL	0.30 ± 0.00 μM (59%)	0.28 ± 0.05 μM (55%)	0.18 ± 0.03 μM (34%)
MEDIA + 9-*cis*-RA	9-*cis*-RA	0.37 ± 0.07 μM (74%)	0.34 ± 0.04 μM (68%)	0.15 ± 0.01 μM (30%)
	Pooled RA	0.37 ± 0.07 μM (74%)	0.35 ± 0.05 μM (70%)	0.15 ± 0.01 μM (30%)
MEDIA + 13-*cis*-RA	*13-cis*-RA	0.35 ± 0.04 μM (69%)	0.32 ± 0.03 μM (61%)	0.22 ± 0.02 μM (45%)
	Pooled RA	0.37 ± 0.04 μM (74%)	0.34 ± 0.03 μM (68%)	0.25 ± 0.02 μM (50%)

Mean ± s.d. concentration and mean percent recovery for 3 independent samples are given. nd=not detectable.

HPLC method

HPLC analyses were carried out using an Agilent Technologies 1100 HPLC system as described in (Asson-Batres *et al.,* 2009). Separation was with an Agilent Technologies Zorbax Eclipse XDB, 3mm mesh, 150 mm C18 column. Solvent A was 40 mM ammonium acetate, 70 mM acetic acid in water; solvent B was acetonitrile/methanol/n-butanol (80:4:3) containing 40 mM ammonium acetate, 70 mM acetic acid. A gradient was run from 80% solvent B to 100% B for 15 min, held at 100% B for 5 min, and returned to 80% solvent B for 5 min, with a 10 min post run equilibration. Flow rate was 0.25 mL/min. Column temperature was 30°C. Detection was with a photodiode array detector (DAD) set to collect at 345 (maximal absorbance of RA), 325 (maximal absorbance of ROL), and 250 (reference) nm. Bandpass widths for DAD spectra were set at 16 nm. Samples were set up in a thermostatted compartment maintained at 20ºC, and 10 μL injections were made with an automated sample injector. After each day's analyses, the column was washed overnight with 10% acetonitrile/90% water, followed by a wash with 90% acetonitrile/10% water.

Extraction of retinoids from cell culture media

0.5 mL cell culture media was brought to a final concentration of 0.2 M with acetic acid and mixed by vortexing with 1.0 mL ice cold 100% ethanol. Two to three volumes of hexane were added, and

the sample was vortexed for 2 min and centrifuged at 4000g × 20 min × 4°C. The organic layer was transferred to a glass tube and dried under a gentle stream of argon gas. 100 μL HPLC solvent B was added to the dried residue. The samples were vortexed briefly, allowed to stand at room temperature for 5 min, and the resuspended sample was transferred to an autosampler vial.

Standard curves and sample concentrations

Standard curves were generated by separating 5–6 concentrations of each retinoid. Peak areas were plotted against concentration, and the best fit line was determined by least squares regression analysis. These standard curves were used to calculate daily standard and sample recoveries: *at*-RA, $Y = 92x - 7$ ($r^2 = 0.999$); ROL $Y = 79x + 16$ ($r^2 = 0.999$); *9-cis*-RA, $Y = 90x - 16$ ($r^2=0.999$); *13-cis*- RA, $Y = 83x - 9$ ($r^2=0.999$).

Sample retinoid peaks were identified by comparing their retention times and spectra with those of authentic standards. An appropriate internal standard to correct for normal day-to-day variability in chromatographic separations of RA isomers was not available for these analyses. Thus, unextracted retinoid standards were included in all sample runs, and the sample recoveries of each run were corrected using the percent recovery of the appropriate standard for that day. Retinoid standards for each analysis were freshly prepared on the day of the run by diluting ethanol stocks to 2.5μM with solvent buffer. This concentration was the expected theoretical concentration of the extracted samples at the point of injection onto the HPLC column.

TABLE 2A

RECOVERY OF SUPPLEMENTED RETINOIDS FROM BL6 SERUM-FREE MEDIA AND CELL CULTURES*

Retinoid added	Retinoid analyzed	BL6 Media No Incubation Without Cells (Percent Recovery)	BL6 Media 24 HR Incubation Without Cells (Percent Recovery)	BL6 Media 24 HR Incubation With Cells (Percent Recovery)
MEDIA + Vehicle	ROL	nd	nd	nd
MEDIA + *at*-RA	*at*-RA	0.28 ± 0.01 (55%)	0.13† (27%)	0.04 ± 0.03 μM (8%)
	Pooled RA	0.31 ± 0.01 (62%)	0.29† (58%)	0.12 + 0.03 μM (24%)
MEDIA + ROL	ROL	0.15 ± 0.02 (30%)	0.02 ± 0.01 (4%)	nd†

Mean ± s.d. concentration and mean percent recovery for 3 independent samples are given. *A proprietary amount of ROL is present in the chemically defined serum-free media that was used to grow BL6 mESCs; however, this amount was not detectable by our method of HPLC analysis. † N=2. nd=not detectable

TABLE 2B

RECOVERY OF SUPPLEMENTED RETINOIDS FROM BL6 SERUM-FREE MEDIA SUPPLEMENTED WITH BSA

Retinoid added	Retinoid analyzed	BL6 Media + 6 mg/ml BSA No Incubation Without Cells (Percent Recovery)	BL6 Media + 6 mg/ml BSA 24 HR Incubation Without Cells (Percent Recovery)	BL6 Media + 0.5 mg/ml BSA 24 HR Incubation Without Cells (Percent Recovery)
MEDIA + *at*-RA	*at*-RA	0.28 ± 0.08 (56%)	0.31 ± 0.03 (62%)	0.16 ± 0.01 (32%)
	Pooled RA	0.28 ± 0.08 (56%)	0.44 ± 0.03 (88%)	0.27 ± 0.04 (54%)
MEDIA + ROL	ROL	0.34 ± 0.01 (68%)	0.18 ± 0.03 (36%)	0.09 ± 0.03 (18%)

Mean ± s.d. concentration and mean percent recovery for 3 independent samples are given.

Image analysis

Brightfield images of living cells were acquired with an OptixCam digital camera mounted in the eyepiece of a Hund Wetzler inverted microscope. Images were edited for presentation in Adobe Photoshop CS2. HPLC traces were obtained with ChemStations ver 10.1 and exported to Adobe Illustrator CS2. Layers were merged into a single image and saved as TIFF files at 300dpi resolution.

Statistical analysis

Preliminary studies were carried out to design the formal test of retinoid stability that ultimately yielded results reported in Tables 1 and 2. Three independent analyses were carried out for each control and experimental treatment group. All similar samples were extracted and analyzed on the same day. Values in Tables 1 and 2 are the mean ± one standard deviation of the three measurements for the indicated groups. In two cases, one of the three samples was judged to be artifactual, reported in Table 2 (where sample size, N=2). Additional samples prepared and analyzed on another day confirmed the results (data not shown).

Results

Effects of at*-retinoic acid (RA) and retinol (ROL) on mouse embryonic stem cells (mESCs)*

The retinoid stocks used in this study were stored in ethanol, and ethanol was added as a vehicle control to mESC cultures. Embryonic stem cells grown in media supplemented with ≤0.04% ethanol for 24h (Fig. 1, group I) or 48h (Fig. 1, group II) retained the morphologic features of undifferentiated, pluripotent stem cell colonies (arrowheads, Fig. 1 A,E,I,M). BL6 mESCs exposed to 0.1 to 10μM *at*-RA in chemically defined, serum-free media displayed a differentiated morphology at 24h and 48h (arrows, Fig. 1 B-D and 1J-L). Other studies carried out in the lab (to be published elsewhere) have confirmed that undifferentiated BL6 mESCs express the pluripotent markers, OCT3/4 and SSEA I, and when grown in the concentrations of *at*-RA shown in Fig. 1, do express differentiation markers, including the neuron lineage markers, neuron specific β-tubulin III and GAP-43. The results shown in Fig. 1 portray effects of *at*-RA on BL6 mESCs maintained in serum free media. Similar results were observed with R1 mESCs maintained in DMEM supplemented with FBS (data not shown).

BL6 mESCs grown in serum free media supplemented with 0.1 and 1μM ROL also exhibited a differentiated morphology after 24 and 48h exposure (Fig. 1 F-G and 1 N-O). BL6 mESCs exposed to 10 μM ROL died within 2h exposure to the retinoid (Fig. 1H and 1P).

Analysis of retinoids in cell culture media

To investigate whether *at*-RA is stable in media maintained under cell culture conditions and to determine whether *at*-RA is depleted or broken down after 24h, *at*-RA status was monitored in media using an HPLC method that was designed for optimal recovery of acidic retinoids (Asson-Batres *et al.,* 2009). This method also recovers ROL. The data indicated that retinoid stability was affected by the media formulation.

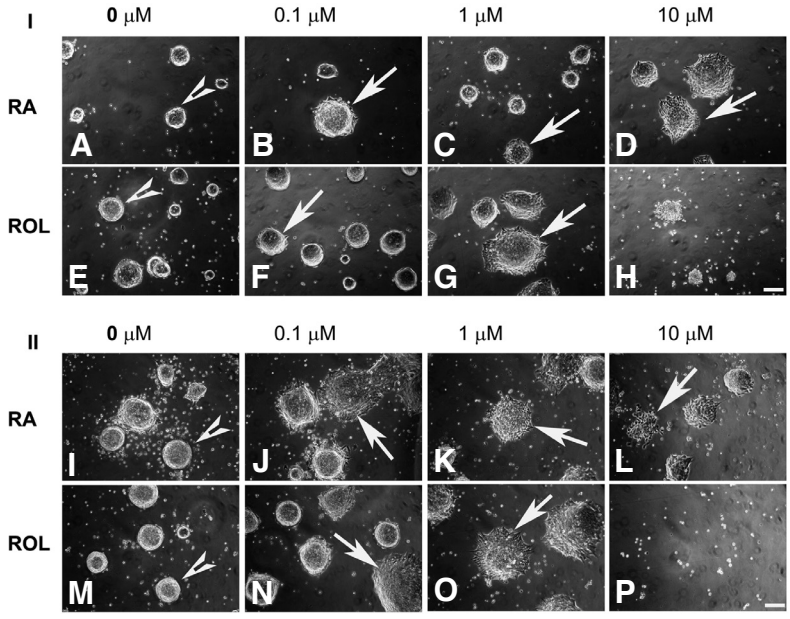

Fig. 1. BL6 mouse embryonic stem cells (mESCs) exposed to increasing concentrations of *at*-retinoic acid (*at*-RA) or retinol (ROL) in chemically defined, serum free media for 24h (I) and 48h (II) exhibit a differentiated morphology. *ESGRO Complete Plus media was supplemented with vehicle* **(A,E, I,M)**, *0.1, 1, or 10 μM* at-*RA* **(B,J; C,K; D, L)** *(respectively), or with 0.1, 1, or 10 μM ROL* **(F,N; G,O; H,P)** *(respectively). Bright-field images were acquired with an OptixCam digital camera attached to a Hund Wetzlar inverted microscope. Arrowheads indicate undifferentiated cell colonies; arrows indicate colonies that are undergoing differentiation. Note that 10μM ROL kills cells within 2h of exposure. Scale bar represents 100 μm.*

Retinoids in serum-supplemented media

R1 mESC media supplemented with vehicle had no detectable RA, but did have 0.01 to 0.03μM ROL (Table 1; Fig. 2 A,C,D). Since ROL is present in FBS, this latter result was expected. The extraction efficiency for retinoids added to control (unincubated, 0h) mESC serum-supplemented media was approximately 60-70% for all three RA isomers and ROL.

Maintenance of retinoid-supplemented media (without cells) under the increased temperature and humidified air conditions associated with cell culture had no effect on the recovery of any of the RA species or ROL, and, there was little or no isomerization of added *at*-RA, *9-cis*-RA, *13-cis*-RA or ROL (Table 1 and Fig. 2). When cells were incubated in media with added retinoids, there was a notable reduction in the levels of the retinoids in the spent media after 24h. The greatest reduction was in *at*-RA levels, which were reduced to approximately 20% of those recovered from unincubated (0h) and cell-free incubated media (24h), followed by *9-cis*-RA and ROL levels, which were reduced to approximately 50% of those in cell-free media (Table 1 and Figs. 2 B,C), and *13-cis*-RA levels, which were least affected, with measured levels in spent media only reduced to about 70% of levels in cell-free, unincubated and incubated media (Table 1 and Fig. 2D).

Retinoids in serum-free media

Fifty five percent of *at*-RA added to unincubated BL6 media was recovered as a single peak eluting at the same time as authentic *at*-RA standard (Table 2A, Fig. 3A). After 24h incubation without cells, 27% *at*-RA was recovered (Table 2A). Growth of cells in the *at*-RA supplemented media further reduced recovery levels to 8% of the original amount added (Table 2A, Fig. 3B). Pooled RA recovery data indicate there was little isomerization of added *at*-RA in the unincubated media, but 24h incubation with or without cells led to 50% isomerization of *at*-RA (Table 2A).

When serum-free media was supplemented with ROL, recovered ROL levels declined after overnight incubation even in the absence of cells (Table 2A). This loss of ROL was accompanied by the recovery of unidentified species in the HPLC trace (compare Figs. 2D and E). Growth of cells in the ROL-supplemented serum-free media further reduced recoverable ROL to non-detectable levels (Table 2A). Albumin, a component of FBS, that has the capacity to bind lipophilic molecules, including retinoids (Futterman and Heller 1972; Quinlan *et al.,* 2005), was added to BL6 mESC media in some experiments to determine whether it would improve the stability of *at*-RA and ROL in this serum free medium. BL6 media supplemented with 6mg/ml BSA, a concentration equivalent to that found in the R1

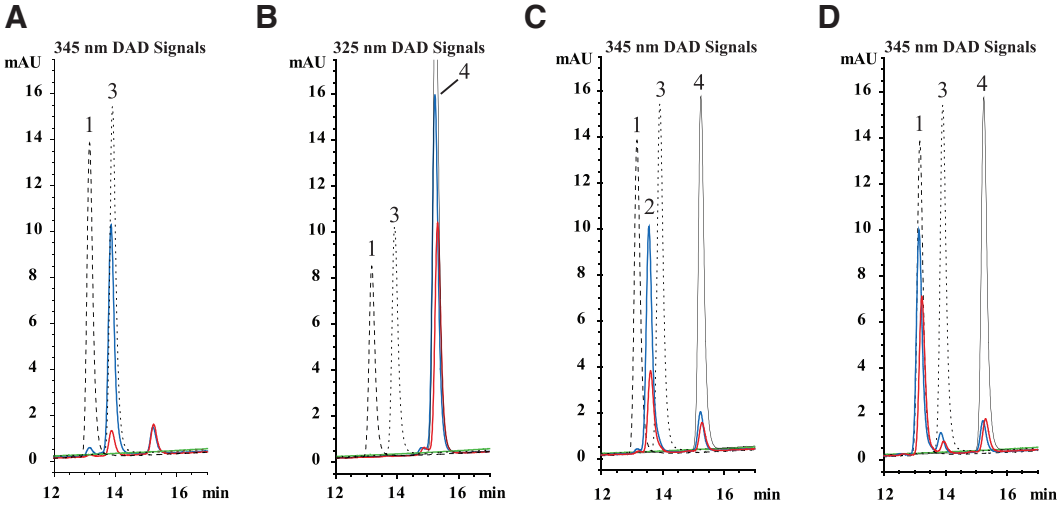

Fig. 2. R1 mESCs metabolize retinoids. *Retinoids were added to serum-supplemented media and incubated at 37ºC for 24 h with (red traces) and without (blue traces) R1 mESCs.* **(A)** at-*RA-supplemented media.* **(B)** *ROL-supplemented media.* **(C)** 9-cis-*RA-supplemented media.* **(D)** 13-cis-*RA-supplemented media. Standard peaks are* 13-cis-*RA (1, dashed trace),* at-*RA (3, dotted trace), and ROL (4, solid black trace), respectively.* 9-cis-*RA eluted at 13.6 min (sample peak labeled 2 in C). Blank traces are shown in green. Signals were detected at 345 and 325nm using a diode array detector (DAD) as indicated.*

mESC serum-supplemented media had a protective effect on the stability and recovery of *at*-RA after incubation for 24h without cells (Table 2B; Fig. 3C). This concentration of BSA also increased the recovery of ROL from unincubated media. However, after 24h incubation under cell culture conditions, 6mg/mL BSA only partially protected ROL (Table 2B; Fig. 3F). Supplementation of serum-free media with 0.5mg/mL BSA had no stabilizing effect on any of the retinoids analyzed (Table 2B).

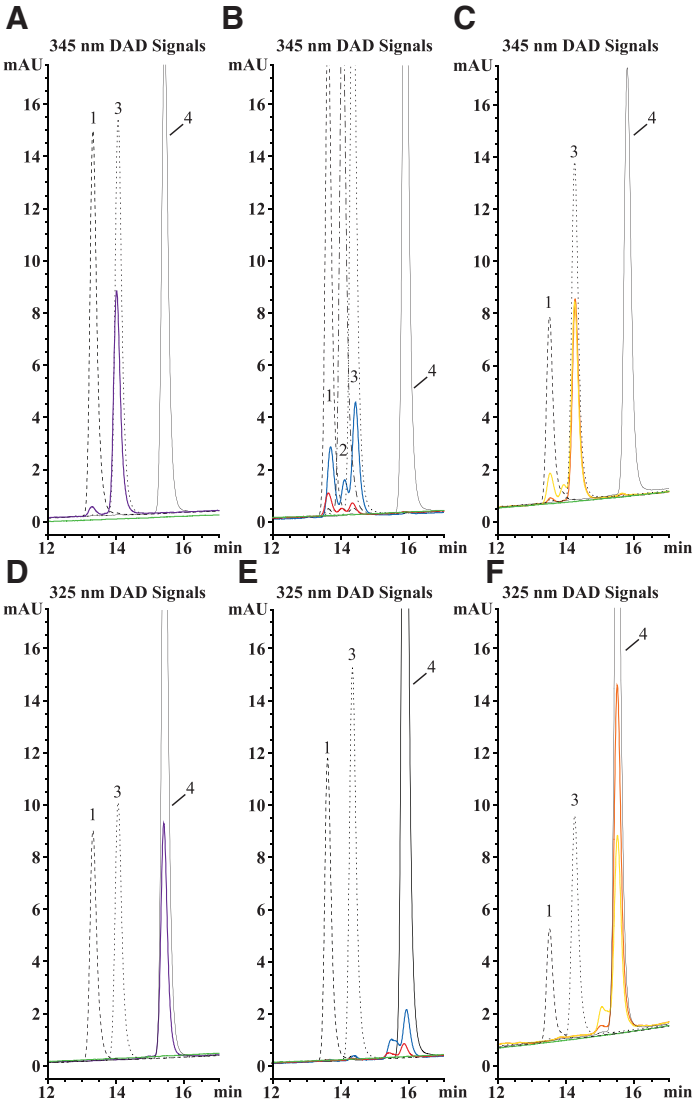

Fig. 3. Retinoids are unstable in serum free media. *Retinoids were added to chemically defined, serum,-free media and analyzed immediately (0h) or incubated overnight with and without BL6 mESC.* **(A)** at-*RA-supplemented media without cells, 0h, purple trace.* **(B)** at-*RA-supplemented media incubated for 24h with (red trace) and without cells (blue trace).* **(C)** *Media supplemented with* at-*RA and 6mg/ml BSA incubated without cells, 0h (orange trace) or 24h (yellow trace).* **(D)** *ROL-supplemented media without cells, 0h (purple trace).* **(E)** *ROL-supplemented media incubated for 24h with (red trace) and without cells (blue trace).* **(F)** *Media supplemented with ROL and 6mg/ml BSA incubated without cells, 0h (orange trace) or 24h (yellow trace). Standard peaks shown in each panel are* 13-cis-*RA (1, dashed trace),* at-*RA (3, dotted trace), and ROL (4, solid black trace), respectively.* 9-cis-*RA (2, dot-dash trace) standard peak is shown in panel B. Blank traces are shown in green. Signals were detected at 345 and 325nm using a diode array detector (DAD) as indicated.*

Discussion

The purpose of this study was to determine whether retinoids are stable under cell culture conditions. Media with and without serum was supplemented with retinoids and incubated with and without cells, and retinoids were extracted and analyzed using HPLC. Special handling of retinoids was required to ensure the results reported here. All operations from the preparation of retinoid stocks to addition of retinoids to cultures in the cell culture hood were conducted under yellow light. When viewing cells using brightfield microscopy, a yellow filter was inserted in the light path to block out light that could photodamage the retinoids. Culture dishes were wrapped in aluminum foil and kept in the dark during incubation. Retinoid stocks were stored at -20˚C in the dark and their concentrations and spectra were checked routinely using a spectrophotometer and their integrity was verified using HPLC.

Surprisingly, there was little loss or isomerization of *at*-RA, *9-cis*-RA, *13-cis*-RA, or ROL in serum-supplemented media (without cells) exposed to cell culture conditions for 24h. Recoveries of *at*-RA and *13-cis*-RA obtained in this study were generally similar or better than those of Tsukada *et al.,* (Tsukada *et al.,* 2002), who reported recoveries of approximately 65% and 78% of *at*-RA and *13-cis*-RA, respectively, from unincubated serum-supplemented media, and recoveries of 55% for *at*-RA, but only 30% for *13-cis*-RA, from serum-supplemented media maintained under cell culture conditions without cells for 24h.

The stability of both *at*-RA and ROL were determined to be greatly reduced in serum-free media. Recovery of *at*-RA from unincubated, serum-free media control samples (55%) were comparable to recoveries from serum-supplemented media (61%). However, in contrast with recoveries of 69% *at*-RA from cell-free, serum-supplemented media after 24h incubation, only 27% of *at*-RA was recovered from serum-free media incubated without cells. Over half of the total RA detected in these 24h serum-free media samples showed up as isomerized forms of RA. Only 30% of ROL was recovered from unincubated, serum-free media, and this amount dropped to 4% after 24h incubation without cells, with apparent loss of ROL and appearance of unidentified products on the HPLC trace.

Protein has been shown to stabilize retinoids (Klaassen *et al.,* 1999) (N'soukpoe-Kossi *et al.,* 2007). To test whether the greater stability of *at*-RA and ROL in serum-supplemented media versus serum-free media is due to the presence of FBS, BSA was added to retinoid-supplemented, serum-free media samples. Adding 6 mg/mL BSA to serum-free media resulted in recoveries of *at*-RA from 24h incubated samples (no cells present) that were comparable to those obtained with extractions from serum-supplemented media (62% versus 69% recovery, respectively). Unlike *at*-RA, the recovery of ROL from BSA-supplemented, serum-free media incubated for 24h (no cells present) was only partial, increasing only from 4% to 36%. The results confirm a stabilizing effect of protein on retinoids in growth media and, further, show that stabilization is dependent on protein concentration (0.5 mg/ml BSA was ineffective) and retinoid type (e.g., ROL was less stable than *at*-RA).

Addition of *at*-RA to serum-supplemented growth media induced differentiation of serum-adapted R1 mESC, and the levels of *at*-

RA, *9-cis*-RA, *13-cis*-RA, and ROL in serum-supplemented spent media decreased to 11%, 30%, 45%, and 34% of the original amount added, respectively after 24h. These results suggest that R1 mESC took up each of the retinoid species, with greatest uptake evident in cells exposed to *at*-RA, followed by *9*-cis-RA and ROL. Internalization of retinoids by R1 mESC is consistent with reports that AB1 ESC take up large amounts of [^{3}H]RA from the medium within minutes to hours of exposure (Chen and Gudas 1996) and work showing that the half life of RA taken up from the medium by cultured cells ranges from 3.5 to 6h (Williams and Napoli 1985; Redfern and Todd 1988). As shown here, cells deplete retinoids after 24h incubation and thus, need to be refed daily with freshly prepared retinoid-supplemented growth media during the course of a multiple day exposure experiment.

Regardless of the decreased stability of retinoids in serum-free, chemically defined media, BL6 mESC still differentiated in response to 0.1uM to 10uM *at*-RA. Several explanations of this result are possible. (1), *at*-RA may induce differentiation within hours of exposure before significant amounts of degradation or isomerization of *at*-RA occurs; (2) the small amount of non-degraded *at*-RA still present in serum-free media after 24h may be sufficient to promote or maintain differentiation processes since RARs are activated by nM amounts of *at*-RA; or (3) other RA isomers may promote or contribute to cell differentiation processes.

BL6 mESC grown in chemically defined serum free media supplemented with 0.1 to 1uM ROL were also induced to differentiate. These results are in contrast with those of Chen & Khillan (Chen and Khillan 2010), who have reported that ROL suppresses differentiation and maintains pluripotency in mESC by a mechanism that does not involve RA signaling. Unlike RA-supplemented cultures, mESC exposed to 10uM ROL died within hours for unexplained reasons. Zouboulis *et al.,* (Zouboulis *et al.,* 1999) also observed that 10uM ROL was toxic to cultured cells, reporting that cell proliferation was essentially inhibited at this concentration in human keratinocytes.

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