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Metabolism and Regulation of Gene Expression by 4-Oxoretinol versus All-*trans* Retinoic Acid in Normal Human Mammary Epithelial Cells

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Abstract

We previously demonstrated that 4-oxoretinol (4-oxo-ROL) activated retinoic acid receptors (RARs) in F9 stem cells. We showed that 4-oxo-ROL inhibited the proliferation of normal human mammary epithelial cells (HMECs). To understand the mechanisms by which 4-oxo-ROL regulates HMEC growth we examined gene expression profiles following 4-oxo-ROL or all-*trans* retinoic acid (tRA). We also compared growth inhibition by tRA, 4-oxo-ROL, or 4-oxo-RA. All three retinoids inhibited HMEC proliferation. Gene expression analyses indicated that 4-oxo-ROL and tRA modulated gene expression in closely related pathways. The expression of many genes, e.g. ATP-binding cassette G1 (ABCG1); adrenergic receptorβ2 (ADRB2); ras-related C3 botulinum toxin substrate (RAC2); and short-chain dehydrogenase/reductase 1 gene (SDR1) was changed after 4-oxo-ROL or tRA. Metabolism of these retinoids was analyzed by high-performance liquid chromatography(HPLC). In 1 μM tRA treated HMECs all of the tRA was found intracellularly, and tRA was the predominant intracellular retinoid. In 1 μM 4-oxo-ROL treated HMECs most 4-oxo-ROL was esterified to 4-oxoretinyl esters, no tRA was detected, and 4-oxo-ROL and 4-oxo-RA were observed intracellularly. In 1 μM 4-oxoretinoic acid (4-oxo-RA) treated HMECs little intracellular 4-oxo-RA was detected; most 4-oxo-RA was in the medium. Our results indicate that: (a) 4-oxo-ROL regulates gene expression and inhibits proliferation of HMECs; (b) 4-oxo-ROL and tRA regulate some of the same genes; (c) more tRA is found in cells, as compared to 4-oxoretinoic acid, when each drug is added at the same concentration in the medium; and (d) the mechanism by which 4-oxo-ROL exerts its biological activity does not involve intracellular tRA production.

Keywords

ABCG1; biomarkers; cell growth arrest; gene expression profiling; gene microarray; HPLC; normal human breast epithelial cell; 4-oxo-RA metabolism; 4-oxoretinol; retinoic acid; RA metabolism; retinoic acid receptors; retinoid metabolism; SDR1; transcription; transcriptomics

INTRODUCTION

Retinoic acid (tRA) and some of its synthetic derivatives influence cell growth and differentiation. At a molecular level, RA and various other synthetic retinoids act via binding and activating nuclear receptors, the retinoic acid receptors and retinoid X receptors,

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which are transcription factors that directly regulate the transcription of certain “target” genes (Altucci and Gronemeyer, 2001; Mongan and Gudas, 2007). Retinoids inhibit the proliferation of many types of cells by regulating the expression of several cell cycle proteins (Faria et al., 1998a; Li et al., 2004; Love and Gudas, 1994; Niles, 2000a; Teixeira and Pratt, 1997; Zhou et al., 1997). Retinoids also play important roles during embryonic development. Vertebrate embryos that were either vitamin A-deficient or exposed to excess retinoids exhibit extensive abnormalities (Gudas, 1994; Lohnes et al., 1995; Means and Gudas, 1995; Ross et al., 2000; Smith et al., 1998; Zile, 1998; Zile et al., 2000).

Aberrant signaling in the retinoid signaling pathway is involved during the process of carcinogenesis. For example, abnormal metabolism of retinoids and the reduced expression of RARβ have been observed in many malignant cells, as well as in the transition from pre-malignant lesions to aggressive carcinomas (Arapshian et al., 2000; Love and Gudas, 1994; Niles, 2000b; Qiu et al., 1999; Sun and Lotan, 2002; Swift et al., 2006; Swisshelm et al., 1994; Widschwendter et al., 2001; Xu et al., 1994; Yang et al., 2001). The expression of the gene encoding LRAT (lecithin:retinol acyltransferase) is lower in human breast carcinoma patient specimens and cell lines as compared to normal breast tissues (Chen et al., 1997; Sheren-Manoff et al., 2006). LRAT esterifies retinol (vitamin A) in various types of epithelial cells, including breast epithelial cells (Randolph et al., 1991). Moreover, postmenopausal breast cancer patients with low plasma retinol showed a poorer prognosis than those with higher plasma retinol levels (Formelli et al., 2009). Bexarotene (LG1069, Targretin) and other synthetic retinoid X receptor selective retinoids have shown efficacy in preventing breast cancer in several animal models (Abba et al., 2009; Abba et al., 2008; Bischoff et al., 1999; Brown et al., 2008; Gottardis et al., 1996; Li et al., 2008; Liby et al., 2008; Wu et al., 2002), in inhibiting human breast cancer cell proliferation (Kim et al., 2006; Wang et al., 2006; Wu et al., 1997), and in clinical trials (Esteva et al., 2003). For these reasons, retinoids have been regarded as useful therapeutic and chemopreventive agents for many types of cancers, including human breast cancers (Alberts et al., 1999; Hong and Sporn, 1997; Li and Brown, 2009; Liby et al., 2007; Lotan, 1996; Miller, 1998; Recchia et al., 2009; Zanardi et al., 2006).

Among the natural retinoids, all-*trans* RA (tRA) is thought to be the most biologically active. Research suggests that other retinoids are also functionally important (Chiu et al., 2008; Ross et al., 2000). Major natural metabolites of vitamin A (retinol) include 4-hydroxyretinol, 4-oxoretinol, anhydroretinol, 14-hydroxy-4,14-retroretinol, all-*trans* retinoic acid, 4-hydroxyretinoic acid, 4-oxoretinoic acid, and 3,4-didehydroretinoic acid. We previously showed that 4-oxoretinol (4-oxo-ROL) is not metabolized to tRA and is capable of activating RARs in F9 cells (Achkar et al., 1996). Studies from our laboratory and others in *Xenopus* embryos showed that exogenous 4-oxo-ROL could induce dose-dependent progressive axial truncation during the embryonic development (Achkar et al., 1996; Blumberg et al., 1996). Recent research in mouse skin has also shown that 4-oxoretinol was not converted to tRA and that 4-oxoretinol exerted direct retinoid activity in vivo (Sorg et al., 2008), confirming these prior studies in amphibians and cultured skin cells (Achkar et al., 1996; Blumberg et al., 1996) in which we and others showed that the 4-oxometabolites of retinol were bioactive compounds rather than inactive catabolites. We have also demonstrated that 4-oxoretinol can induce cell growth arrest and granulocytic differentiation of cultured human promyelocytic leukemia cells (Faria et al., 1998b), and that 4-oxoretinol is more effective than tRA in inducing CRABP1 and CRABP2 transcripts in murine embryonic stem cells cultured without LIF (Lane et al., 1999; Lane et al., 2008).

4-oxo-ROL also inhibited the proliferation of cultured mammary epithelial cells and breast carcinoma cells, including the estrogen receptor (ER) negative breast carcinoma MDA-MB-231 line (Chen et al., 1997). Thus, 4-oxo-ROL has the potential to be an alternative

chemotherapeutic agent for the types of breast cancer that respond poorly to tRA treatment (Chen et al., 1997). In this research we compared the gene expression profiles of primary cultures of normal human mammary epithelial cells (HMECs) after tRA or 4-oxo-ROL treatment. We also examined the uptake and metabolism of tRA, 4-oxo-ROL, and 4-oxo-RA in the HMECs.

MATERIALS AND METHODS

Materials

All-*trans* retinoic acid was from Sigma (St Louis, MO). All-*trans* 4-Oxoretinol (4-oxo-ROL) was synthesized as described (Achkar et al., 1996) and stored under nitrogen at −70°C prior to use. Trimethylsilyl diazomethane was purchased from Aldrich (Milwaukee, WI). HG-U133A oligonucleotide microarray chips were from Affymetrix (Santa Clara, CA).

Cell and Culture Conditions

Normal human mammary epithelial cells (HMECs) were purchased from Clonetics Corp. (now Lonza) (Walkersville, MD). For maintenance of the cell strain, HMECs were cultured in 5% CO_2 in mammary epithelial growth medium (MEGM) with appropriate supplements (Clonetics). All experiments were performed using cells between passages 4 and 6.

Cell Proliferation Assays

Proliferation assays were performed as described previously (Hoffman et al., 1996). The HMECs were plated in 24-well plates at 1×10^4/well and cultured in MEGM with or without drug treatment. The medium was replaced every other day. The cells were trypsinized and counted every day using a Coulter Counter through day 6. Triplicate wells were counted for each time point. The data were analyzed with a Prism program. Data are presented as the mean ± SD.

cRNA Preparation and Microarray Analysis

HMECs were given fresh medium a day before the addition of tRA or 4-oxo-ROL. The HMECs were treated with tRA (1μM) or 4-oxo-ROL (1μM) for 24 hrs. Total cellular RNA was extracted from approximately 0.5×10^7 HMECs using a RNeasy kit (Qiagen, Valencia, CA). The oligonucleotide HG-U133A gene chips (Affymetrix, Santa Clara, CA) were used for gene expression analysis. The cRNA synthesis, hybridization, staining, and scanning were performed using the standard protocol from the manufacturer. The experiments were repeated. The microarray data were processed using Microarray Suite 5.0 (Affymetrix) and GeneSpring software 5.1 from Silicon Genetics (Redwood City, CA). The microarrays were run by the Weill Cornell Microarray Core Facility.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from the HMECs and the cDNA was synthesized using a RT-PCR kit from Invitrogen (Carlsbad, CA). The PCR was performed using the following conditions: twenty-six cycles at 94°C for 20 sec, 57°C for 30 sec, and 72°C for 2 min, with a final extension at 72°C for 10 min. The gene specific primers for PCR are listed below: for ABCG1, the upstream primer is 5-CCTGCTGTACTTGGGGATCGGGAACG-3, and the downstream primer is 5-CCAGCGCGGCAAACAGCACAAAG-3; for ADRβ2, the upstream primer is 5-ATAGAAGCCATGCGCCGGACCACGAC-3, and the downstream primer is 5-TAAGGCCTGACACAATCCACACCATC-3; for RAC2, the upstream primer is 5-TGCCTTC TCATCAGCTACACCACCAA-3, and the downstream primer is 5-AGCCAGCTTCTTCTCC TTCAGTTTCTC-3; for SDR1, the upstream primer is 5-TTCAAAGGGCGGACATAGAGAC AGGAT-3, and the downstream primer is 5-

TTTTGGAACGGGAGGCAGAGCAT-3. The GAPDH (upstream primer 5-GGGCTCTCCAGAACATCATCC-3; downstream primer 5-CAGCGTCAAAGGTGGAGGAGTG-3) was used as a control gene. An aliquot of 10 μl from each PCR product (total 50μl) was loaded on a 1.5% agarose gel. The gel images were recorded with a FluorChem 8800 system (Alpha Innotech, San Leandro, CA).

Analysis of Retinoids by High-Performance Liquid Chromatography

The HMECs were cultured and treated with retinoids in 10 ml of MEGM in 100 mm tissue culture plates. At harvest, the cell density was 2.9×10^6 to 4.3×10^6 per plate. The cells were washed with PBS twice and scrapped in 0.5 ml cold PBS. An aliquot of 0.5 ml (out of 10 ml) cell culture medium was also saved for extraction of retinoids. The retinoids were extracted with 350 μl of organic phase from HMECs and the medium in the dark environment as previously described (Chen et al., 1997; Guo and Gudas, 1998). The high-performance liquid chromatography (HPLC) was performed using a Waters Millenium system (WatersCorp., Milford, MA). Each sample (100 μl) was loaded on an analytical 5-μm reverse-phase C_{18} column(Vydac, Hesperia, CA) and eluted at a flow rate of 1.5 ml/min. Two mobilephase gradient systems were used as previously described (Chen et al., 1997; Guo and Gudas, 1998). Retinoids were detected at the wavelength of 340 nm. Retinoids were identified by an exact match of the retention time of an unknown peak with the retinoid standard. The concentrations of the nonradiolabeled retinoids were determined by first calculating their maximum absorption values. Solution concentrations in mol/liter of the retinoids were obtained by dividing the measured maximum absorbance by the molar extinction coefficient ε.

RESULTS

The Growth of HMECs in the Presence of tRA and 4-oxo-ROL

All-*trans* RA is a known growth inhibitor for many cell types, including HMECs. To determine whether the HMEC strain was growth inhibited by 4-oxo-ROL treatment, we added either 4-oxo-ROL (1μM) or tRA (1μM) to the culture medium, and the cell number was counted each day through day 6. The results indicated that the proliferation of HMECs was inhibited by addition of either tRA or 4-oxo-ROL to the cell culture medium. On day 6 the HMECs treated with tRA and with 4-oxo-ROL were effectively growth inhibited as compared to control HMECs (Fig. 1).

Comparison of the Gene Expression Profiles after RA and 4-oxo-ROL Treatment

All-trans RA exerts its biological activities via activation of the nuclear receptors, the RARs. Once they are bound to retinoid agonists, the RARs and the participating co-regulatory proteins rapidly alter the expression of their downstream target genes. To identify the downstream genes regulated by 4-oxo-ROL or tRA we analyzed the gene expression profiles of HMECs that were harvested at 8 hr or 24 hr following tRA or 4-oxo-ROL addition. The HG-U133A oligonucleotide gene chips from Affymetrix were used in our study. Altered mRNA expression (a change in expression over two-fold) was found for a large number of genes as compared to the control, untreated cells (Fig. 2A and B). We also compared the gene expression profiles of 4-oxo-ROL versus tRA treated HMECs. For most genes whose transcripts were present (red dots), the difference in mRNA expression was similar and less than two-fold (Fig. 2C). The microarray assay was repeated twice. Fifty eight genes are shown in Fig. 2D and Table 1. The genes were selected from 24 hr treated HMECs based on the criteria that the expression was changed over two-fold by either tRA or 4-oxo-ROL as compared to the control, and that the changes were consistent in the independent microarray analyses. The order of genes is according to the fold-change by tRA. The genes showing reduced expression are presented as negative numbers. Some genes

on the list belong to the family of known RA target genes [e.g., short-chain dehydrogenase/reductase 1 gene (Cerignoli et al., 2002)]. Many genes (e.g., ABCG1 gene) not previously studied as retinoid regulated also showed a response to RA or 4-oxo-ROL in the microarray assays.

Gene Expression Analysis by RT-PCR

To confirm and validate the gene expression patterns observed in the microarray analyses we synthesized gene specific primers and used RT-PCR approaches to examine gene expression in tRA versus 4-oxo-ROL treated HMECs. The RT-PCR results for four genes, short-chain dehydrogenase/reductase 1 (SDR1) (Cerignoli et al., 2002); adrenergic receptor β-2 (ADRB2); ATP-binding cassette G1 (ABCG1); and ras-related C3 botulinum toxin substrate 2 (RAC2); are shown (Fig. 3A). Similar patterns of expression, as compared to the microarray analyses, were detected for all four genes. The increased SDR1 mRNA levels and the decreased ADRβ2 mRNA levels were seen in both RA and 4-oxo-ROL treated HMECs (Fig. 3B). 4-Oxoretinol treatment resulted in a larger increase in ABCG1 gene expression (2.1-fold by tRA and 2.5-fold by 4-oxo-ROL at 24 hr; 1.6-fold by tRA and 2.3-fold by 4-oxo-ROL at 48 hr; 1.6-fold by tRA and 2.1-fold by 4-oxo-ROL at 72 hr), which agreed with the microarray results.

Analysis of Retinoid Extracts by HPLC

To delineate the mechanisms by which 4-oxo-ROL and tRA regulate gene expression in HMECs and the intracellular concentrations of these retinoids, we extracted the retinoids from the control, tRA treated, 4-oxo-ROL treated, and 4-oxo-RA treated HMECs. We also extracted the retinoids from each corresponding cell culture medium. All of the samples were subjected to the HPLC analysis.

Control HMECs

In untreated, control HMECs we could not detect any retinoids in the cellular extracts (Fig. 4A) or in the cell culture medium (Fig. 4B). The limit of detection in these assays was 0.7 pmol of retinol, which is equivalent to 70 nM in 1×10^7 cells, assuming a cell volume of 1 pl (Chen and Gudas, 1996; Chen et al., 1997).

tRA treated HMECs

In HMECs cultured in the presence of 1 μM tRA for 24 hr, tRA (retention time: 22.24 min) was the only intracellular retinoid (Fig. 4C, indicated by arrow) and only 20% of the tRA remained in the medium (Fig. 4D, indicated by arrow). Thus, 80% of the 1 μM tRA was associated with or taken up by the HMECs, resulting a ~1.4 mM intracellular concentration of tRA (assuming a cell volume of 1 pl). In contrast, the medium concentration of tRA was 103 nM at 24 hr.

4-oxo-ROL treated HMECs

In HMECs cultured for 24 hr in the presence of 1 μM 4-oxo-ROL, multiple peaks were observed (Fig. 4E). The major intracellular retinoids were 4-oxoretinyl esters (multiple peaks between 48 min to 60 min; intracellular concentration = 1.3 mM), 4-oxo-ROL (retention time = 18.84 min, indicated by arrow with double stars; intracellular concentration = 145 μM), and 4-oxo-RA (retention time = 10.43 min, indicated by single star; intracellular concentration = 34 μM) (Table 2). All-*trans* RA was not detected in 4-oxo-ROL treated HMECs (for tRA, the limit of detection in these assays was 0.4 pmol, which is equivalent to 40 nM in 1×10^7 cells, assuming a cell volume of 1 pl) (Fig. 4E). When the HPLC tracing was examined at high resolution, two minor peaks (Fig 4J, peaks *a′* and *b′*) were identified

in the 4-oxo-ROL treated HMECs. These peaks are all-*trans*-4-hydroxy-ROL (peak *a′*) and 13-*cis*-4-hydroxy-ROL (peak *b′*) according to our previous study (Achkar et al., 1996).

In the cell culture medium of 4-oxo-ROL treated cells, 4-oxo-RA was the major retinoid at 24 hr (Fig. 4F, star). The medium concentration of 4-oxo-RA was 226 nM. Under our experimental conditions (2.9×10^6 cells treated in 10 ml of cell culture medium), approximately 96% of the 4-oxo-RA produced by HMECs from 4-oxo-ROL was in the medium (Fig. 4F, star). A small amount of 4-oxo-ROL (medium concentration = 61 nM) (Fig. 4F, double star) was also detected in the medium. 4-Oxoretinyl esters were not detected in the cell culture medium (Fig. 4F).

4-oxo-RA treated HMECs

4-Oxo-RA is an agonist for the retinoic acid receptors (Idres et al., 2002). In HMECs cultured for 24 hr in the presence of 1 μM 4-oxo-RA the intracellular concentration of 4-oxo-RA was 60 μM, and the medium concentration was 493 nM (Fig. 4G and H). The intracellular 4-oxo-RA was less than 5% of the total 4-oxo-RA (Table 2); most of the 4-oxo-RA was in the medium.

DISCUSSION

Target Genes of tRA and 4-oxo-ROL

We examined the effects of 4-oxo-ROL and tRA on gene expression and the proliferation of the normal human mammary epithelial cells (HMECs). Studies on changes (Table 1) in transcript levels indicate that a large number of genes are targets of tRA or 4-oxo-ROL in HMECs. The target genes are involved in various biological functions. Some are associated with the regulation of cell proliferation and differentiation. For example, the leukemia inhibitory factor (LIF) gene is up-regulated by both tRA and 4-oxo-ROL in HMECs. LIF inhibits the proliferation of normal breast epithelial cells by arresting cell growth in the G0/G1 phase (Grant et al., 2001). The prostate differentiation factor, which is a member of the transforming growth factor-β (TGF-β) superfamily, is up-regulated by tRA and 4-oxo-ROL. It is expressed in various tissues and functions as a regulatory factor for cell growth and differentiation (Paralkar et al., 1998). Many other target genes, such as Id-1H (inhibitor of DNA binding 1, dominant negative helix-loop-helix protein)(Hara et al., 1994), cyclin A1 (Yang et al., 1999), RGC-32 protein (Badea et al., 2002), cullins (Kipreos et al., 1996; Pause et al., 1997), and prohibitin (Wang et al., 2002) are also associated with cell proliferation and differentiation. These target genes may mediate the cell growth arrest in response to retinoids. The SDR1 gene is also up-regulated by 4-oxo-ROL and tRA (Table 1, Fig. 3A). Since SDR-1 protein is capable of reducing retinal to retinol (Haeseleer et al., 1998; Napoli, 2001), the induction of SDR-1 mRNA by 4-oxo-ROL and RA may be involved in the homeostasis of retinoids in HMECs as well as in neuroblastoma cells (Cerignoli et al., 2002).

Other target genes of 4-oxo-ROL or tRA include nuclear proteins (e.g., pirin), signal transduction proteins (e.g., inositol 1,4,5-triphosphate receptor type I and MAP kinase-interacting serine/threonine kinase 2), proteases and protease inhibitors (e.g., cathepsin H, kallikrein 7, serine protein inhibitor), and genes associated with the carcinogenesis (e.g., early growth response 1 gene, tumor necrosis factor superfamily member 10, and transforming growth factor, beta receptor II). Many of these genes have previously been shown to be important in different stages during the development of carcinomas.

A significant overlap is found among most of the genes regulated by tRA and 4-oxo-ROL. Many genes are similarly regulated by tRA versus 4-oxo-ROL (Table 1, Fig. 3), indicating that tRA and 4-oxo-ROL modulate gene expression through closely related, but not identical

pathways. We and others reported previously that all-*trans* retinol and tRA increase CYP26a1 transcript levels but that 4-oxo-ROL does not increase CYP26a1 mRNA levels as much as tRA in embryonic stem cells (Lane et al., 1999; Lane et al., 2008). 4-Oxo-ROL also does not induce CYP26a1 mRNA in epidermal cells in mice (Sorg et al., 2008). However, we showed that 4-oxo-ROL is more potent than tRA in terms of increasing CRABP1 and CRABP2 mRNA levels in embryonic stem cells cultured in the absence of LIF (Lane et al., 2008). Neither tRA nor 4-oxo-ROL induced CYP26a1 mRNA to a level > 2-fold in the HMECs (Table 1).

The metabolism of tRA vs. 4-oxo-ROL in HMECs

The addition of 1 μM tRA to the cell culture medium results in a high concentration of tRA within the HMECs (Fig. 4 and Table 2). Since tRA is known to be the most active ligand of RARs and since it is also the only retinoid found at detectable levels in the tRA treated HMECs, there is little question that exogenously added tRA is responsible for the modulation of gene expression and the growth arrest of HMECs. Additionally, tRA is not metabolized to a significant degree by HMECs; at 24 hr we can account for almost 100% of the tRA added to the cell culture at time 0 – 80% is in the cells, and ~20% is in the medium (Fig. 4C and D)

4-Oxo-ROL was identified as a major intracellular polar retinoid after 4-oxo-ROL treatment (Fig. 4). 4-Oxo-ROL selectively binds and activates certain subtypes of RARs and serves as a natural ligand of RARs in some cells (Achkar et al., 1996; Blumberg et al., 1996). It was previously demonstrated by our laboratory that 4-oxo-ROL, but not tRA, is a major product of retinol metabolism in murine F9 cells, and that the 4-oxo-ROL functions as an active ligand in F9 cells (Achkar et al., 1996). Two minor polar retinoids, all-*trans*-4-hydroxy-ROL and 13-*cis*-4-hydroxy-ROL (peak *a′* and *b′* shown in Fig 4J) are also detected in 4-oxo-ROL treated HMECs. Although they are active metabolites (Achkar et al., 1996), their intracellular concentration is relatively low and not comparable to other active ligands, such as 4-oxo-ROL. At 24 hr after 4-oxo-ROL (1μM) addition, the majority of the 4-oxo-ROL is metabolized to 4-oxo-retinyl esters in HMECs (Fig 4E, multiple peaks between 48 min to 60 min). The functions of these 4-oxo-retinyl esters have not been characterized in detail. Our results show that some of the 4-oxo-ROL is oxidized to 4-oxo-RA in HMECs (Fig. 4E). Although 4-oxo-RA can activate the RARs (Gaemers et al., 1996; Idres et al., 2001; Idres et al., 2002; Pijnappel et al., 1998; Pijnappel et al., 1993; Sani et al., 1996; Sonneveld et al., 1999), our data show that most of the 4-oxo-RA produced from 4-oxo-ROL is not present within HMECs. Instead, the majority of 4-oxo-RA is in the cell culture medium (Fig. 4, Table 2).

The oxidative pathway for retinol in normal human mammary epithelial cells is different from that in the mouse F9 cells. In the HMECs, all-*trans* retinol can be metabolized to tRA, and this metabolic pathway is important for the growth arrest of HMECs during all-*trans* retinol treatment (Hayden et al., 2001; Taibi et al., 2008). In contrast, in human breast cancer this conversion of retinol to retinoic acid does not occur, and retinol is metabolized to 4-oxo-ROL in MCF-7 breast cancer cells (Chen et al., 1997). Although the oxidation pathway that converts retinol to RA is lost in human breast carcinoma MCF-7 and MDA-MB-231 cells (Mira et al., 2000), inhibition of cell proliferation in these two cell lines by 4-oxo-ROL was demonstrated (Chen et al., 1997).

Our results show that when HMECs are treated with 4-oxo-RA, the intracellular concentration of 4-oxo-RA at 24 hr after drug addition is 60 μM, which is only approximately 4% of the intracellular concentration of tRA (1.4 mM) detected in tRA treated HMECs (Table 2). The mechanism for the differential uptake of 4-oxo-RA and tRA in HMECs is not clear. It is known that two cellular retinoic acid binding proteins, CRABP1

and CRABP2, are important for transporting and binding tRA in the cytoplasm (Chen et al., 2003; Fiorella et al., 1993; Noy, 2000). Like tRA, 4-oxo-RA binds to the CRABPs with a high affinity (Fiorella et al., 1993; Fiorella and Napoli, 1991). We have recently generated Cyp26a1 knockout embryonic stem cells. These Cyp26a1$^{-/-}$ cells can't metabolize tRA to 4-oxo-RA (only a small amount after RA addition) and they don't fully differentiate. These data suggest a role for both tRA and 4-oxo-RA in the stem differentiation process in terms of transcriptionally activating different subsets of genes {Langton, 2008 #127}.

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Abbreviations

ABCG1	human ATP-binding cassette, sub-family G, member 1
ADRβ2	human adrenergic receptor, beta-2
HMEC	human mammary epithelial cell
HPLC	High-performance liquid chromatography
4-oxo-RA	all-*trans* 4-oxoretinoic acid
4-oxo-ROL	all-*trans* 4-oxoretinol
RA	tRA, retinoic acid
RAC2	human ras-related C3 botulinum toxin substrate 2
RARs	retinoic acid receptors
RT-PCR	reverse transcription polymerase chain reaction
RXR	retinoid X receptors
SDR1	short-chain dehydrogenase/reductase 1
tRA	all-*trans* retinoic acid

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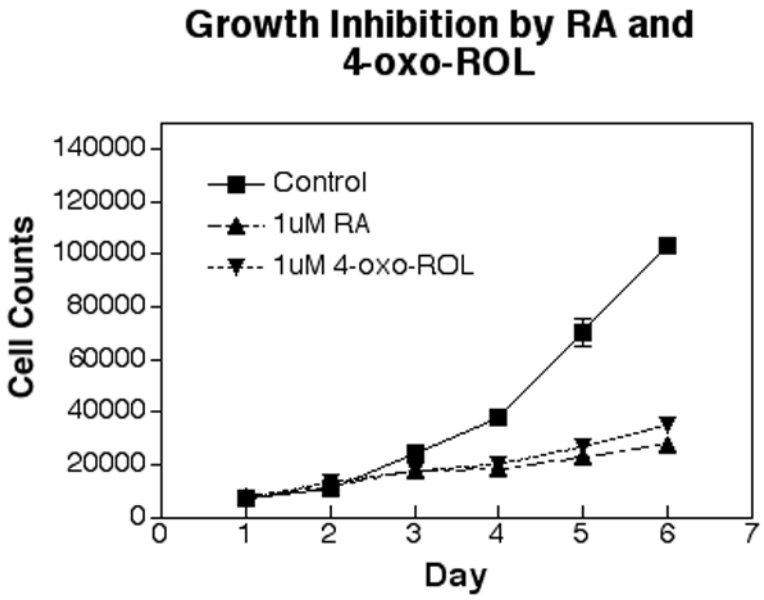

Figure 1. Inhibition of HMEC growth by tRA and 4-oxo-ROL

HMECs were grown in MEGM or MEGM supplemented with tRA (1μM) or 4-oxo-ROL (1μM). The medium was changed every other day during the experiment. Cell numbers were counted each day and represented as the mean ± SD (n=3).

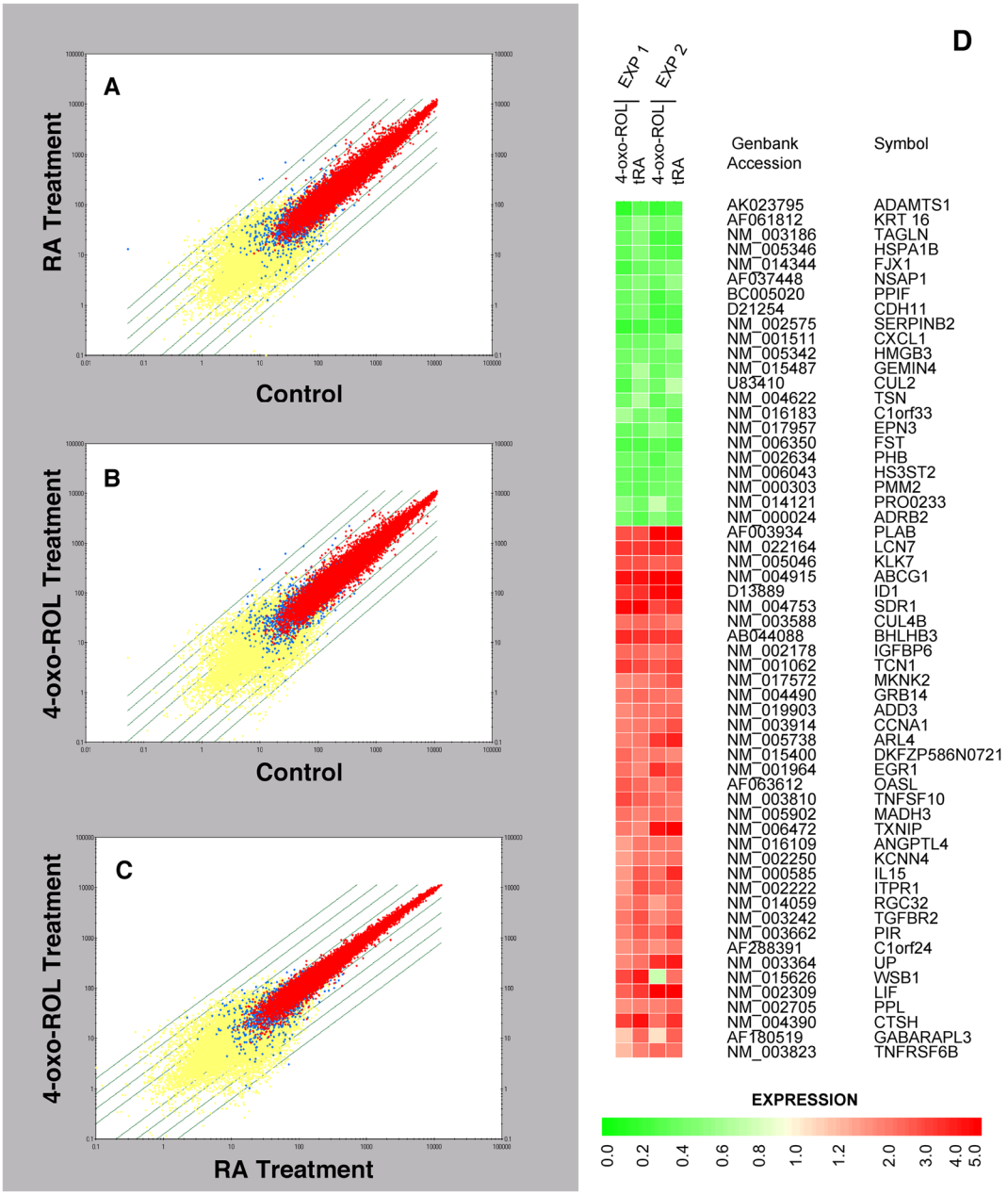

Figure 2. Comparison of gene expression profiles after tRA or 4-oxo-ROL treatment

Total RNA was extracted from control, tRA treated (1 μM, 24 hrs), and 4-oxo-ROL treated (1 μM, 24 hrs) HMECs. A standard microarray analysis was performed as described in Materials and Methods. This microarray experiment was performed twice, with different RNA preparations, at both 8 hr (not shown) and 24 hr after drug addition. **A**: Comparison of gene expression in the tRA treated vs control HMECs. The green lines indicate the changes of 2, 4, 8, and 16-fold (from inside to outside) in expression. Red dots represent the transcripts present; blue dots represent marginal transcripts; yellow dots represent absent transcripts. **B**: Comparison of gene expression in 4-oxo-ROL treated vs control HMECs; **C**: Comparison of gene expression in 4-oxo-ROL treated vs tRA treated HMECs. **D:** The hierarchical clustering of the fifty-eight genes whose expression was changed two-fold or more by either RA or 4-oxo-ROL. (The expression level in the control HMECs was

arbitrarily set as 1.0 for each gene. Green, decreased expression; Red, increased expression.).

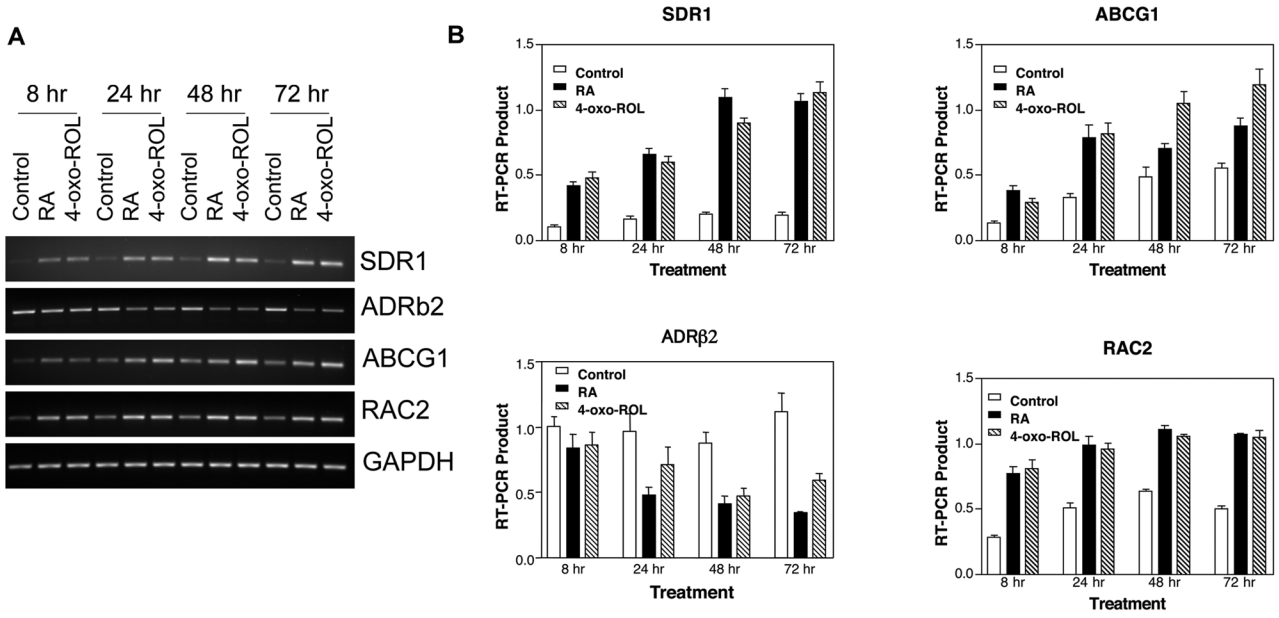

Figure 3. Time course of gene expression examined by RT-PCR in tRA or 4-oxo-ROL treated HMECs

The mRNA levels of SDR1, ADRβ2, ABCG1, and RAC2 genes in 1 μM tRA treated and 1 μM 4-oxo-ROL treated HMECs were examined by RT-PCR (28 cycles) (panel A). All RT-PCR experiments were performed three times and results within 15% were obtained. The results from all three experiments were quantitated relative to GAPDH. These genes are starred (*) in Table 1 (panel B). Quantitative data are shown as mean ± standard deviation (panel B). The y-axes are arbitrary units.

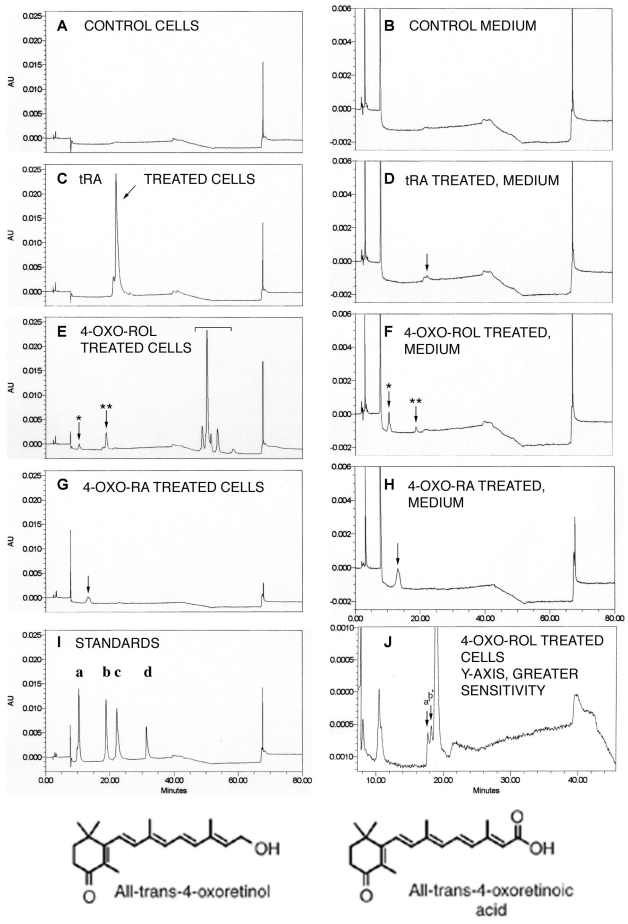

Figure 4. Analysis of retinoids in HMECs treated with tRA, 4-oxo_ROL, or 4-oxo-RA for 24

HMECs were given fresh medium 24 hr before addition of 1 μM tRA, 1 μM 4-oxo-ROL, or 1μM 4-oxo-RA. Intracellular retinoids were extracted with 350 μl of organic solvent from the control and HMECs treated with retinoids at 24 hr after drug addition. (The cell number for the 4-oxo-RA treatment was 4.3×10^6 cells; the cell number for the other treatments were 2.9×10^6 cells). Retinoids were also extracted from each corresponding cell culture medium (0.5 ml out of 10 ml), so only 5% of the total amount is shown in the tracing. An aliquot of 100 μl of each sample was subjected to HPLC analysis. This experiment was performed three times with similar results (within 10%); one experiment is shown. **A**: HMECs, control. **B**: medium, control. **C**: HMECs, tRA treated (24 hr). Peak of tRA is indicated by arrow. **D**: medium, tRA treated (24 hr). Peak of tRA is indicated by arrow. **E**: HMECs, 4-oxo-ROL treated (24 hr). Peak of 4-oxo-RA is indicated by arrow with *. Peak of 4-oxo-ROL is indicated by arrow with **. Multiple peaks from 48 min to 60 min (bracketed) are 4-oxo-ROL esters. **F**: medium, 4-oxo-ROL treated (24 hr). Peak of 4-oxo-RA is indicated by arrow with *. Peak of 4-oxo-ROL is indicated by arrow with **. **G**: HMECs, 4-oxo-RA treated (24 hr). Peak of 4-oxo-RA is indicated by arrow. **H**: medium, 4-oxo-RA treated (24 hr). Peak of 4-oxo-RA is indicated by arrow. (There was a slight shift in the retention times for 4-oxo-RA in panel G versus H; the identities of the 4-oxo-RA peaks were also confirmed by treatment with trimethyl diazomethane, which shifts these peaks to the retention time of the ester (data not shown)). **I**: Retinoid Standards: peak ***a***: 4-oxo-RA, retention time = 10.43 min; peak ***b***: 4-oxo-ROL, retention time = 18.84 min; peak ***c***: tRA, retention time = 22.24 min; peak ***d***: all-*trans* retinol, retention time = 31.38 min. **J**: Lower

concentrations of two polar retinoids were observed in the 4-oxo-ROL treated HMECs. peak *a′*: all-*trans*-4-hydroxy-ROL; peak *b′*: 13-*cis*-4-hydroxy-ROL. *Bottom of figure:* chemical structures of 4-oxoretinol and 4-oxoretinoic acid.

Table 1

Effects of tRA and 4-oxo-ROL on Gene Expression of HMECs

Gene	Genbank Accession	Fold-change by tRA[a]	Fold-change by 4-oxo-ROL[b]
* ATP-binding cassette, sub-family G (WHITE), member 1	NM_004915	7.02	8.13
prostate differentiation factor	AF003934	4.46	4.64
inhibitor of DNA binding 1, dominant negative helix-loop-helix protein	D13889	4.39	4.08
* short-chain dehydrogenase/reductase 1	NM_004753	4.32	3.88
leukemia inhibitory factor (cholinergic differentiation factor)	NM_002309	4.15	4.42
cathepsin H	NM_004390	4.06	2.70
lipocalin 7	NM_022164	3.50	3.58
thioredoxin interacting protein	NM_006472	3.48	3.26
basic helix-loop-helix domain containing, class B, 3	AB044088	3.47	3.61
interleukin 15	NM_000585	3.31	1.96
SOCS box-containing WD protein SWiP-1	NM_015626	3.26	1.90
uridine phosphorylase	NM_003364	3.24	2.73
transcobalamin I (vitamin B12 binding protein, R binder family)	NM_001062	3.16	2.99
ADP-ribosylation factor-like 4	NM_005738	2.99	2.70
kallikrein 7 (chymotryptic, stratum corneum)	NM_005046	2.83	2.70
inositol 1,4,5-triphosphate receptor, type 1	NM_002222	2.64	2.07
transforming growth factor, beta receptor II (70/80kDa)	NM_003242	2.59	1.96
Pirin	NM_003662	2.55	2.19
2′-5′-oligoadenylate synthetase-like	AF063612	2.50	2.39
cyclin A1	NM_003914	2.50	2.07
GABA(A) receptors associated protein like 3	AF180519	2.45	1.13
MAP kinase-interacting serine/threonine kinase 2	NM_017572	2.43	2.04
insulin-like growth factor binding protein 6	NM_002178	2.33	2.24
early growth response 1	NM_001964	2.32	2.87
tumor necrosis factor (ligand) superfamily, member 10	NM_003810	2.32	2.67
RGC32 protein	NM_014059	2.29	1.67
growth factor receptor-bound protein 14	NM_004490	2.25	2.12
adducin 3 (gamma)	NM_019903	2.21	2.03
periplakin	NM_002705	2.18	1.81
cullin 4B	NM_003588	2.15	2.30
tumor necrosis factor receptor superfamily, member 6b, decoy	NM_003823	2.13	1.83
potassium intermediate/small conductance calcium-activated channel, subfamily N, member 4	NM_002250	2.12	1.77
chromosome 1 open reading frame 24	AF288391	2.11	1.72
angiopoietin-like 4	NM_016109	2.10	1.84
MAD, mothers against decapentaplegic homolog 3 (Drosophila)	NM_005902	2.06	2.47

Gene	Genbank Accession	Fold-change by tRA[a]	Fold-change by 4-oxo-ROL[b]
DKFZP586N0721 protein	NM_015400	1.87	2.25
cullin 2	U83410	−1.50	−2.71
translin	NM_004622	−1.60	−2.42
gem (nuclear organelle) associated protein 4	NM_015487	−1.61	−2.31
NS1-associated protein 1	AF037448	−1.71	−2.34
keratin 16 (focal non-epidermolytic palmoplantar keratoderma)	AF061812	−1.82	−2.30
chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	NM_001511	−1.86	−2.40
prohibitin	NM_002634	−2.09	−2.44
high-mobility group box 3	NM_005342	−2.10	−2.22
PRO0233 protein	NM_014121	−2.16	−1.48
four jointed box 1 (Drosophila)	NM_014344	−2.16	−2.74
peptidylprolyl isomerase F (cyclophilin F)	BC005020	−2.18	−2.90
epsin 3	NM_017957	−2.20	−1.95
phosphomannomutase 2	NM_000303	−2.33	−2.28
heat shock 70kDa protein 1B	NM_005346	−2.36	−2.67
transgelin	NM_003186	−2.39	−2.86
60S acidic ribosomal protein PO	NM_016183	−2.43	−1.66
heparan sulfate (glucosamine) 3-O-sulfotransferase 2	NM_006043	−2.45	−2.38
cadherin 11, type 2, OB-cadherin (osteoblast)	D21254	−2.45	−2.81
* adrenergic, beta-2-, receptor, surface	NM_000024	−2.53	−2.00
a disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 1	AK023795	−2.98	−4.67
follistatin	NM_006350	−3.03	−2.85
serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 2	NM_002575	−3.56	−3.40

The cRNAs were synthesized from control HMECs and the HMECs treated with tRA (1μM) or 4-oxo-ROL (1μM) for 24 hrs. The oligonucleotide HG-U133A gene chips (Affymetrix, Santa Clara, CA) were used for gene expression analysis. The microarray data were processed using GeneSpring software 5.1 from Silicon Genetics (Redwood City, CA).

a,*b*The average fold change in mRNA expression of two experiments in presence of tRA(*a*) or 4-oxo-ROL(*b*) as compared to control.

*RT-PCR was performed for these genes.

Table 2

Retinoids in HMECs and in the Cell Culture Medium

Treatment[a]	Intracellular Retinoids (μM)[b]				Retinoids in Medium (nM)[c]			
	4-oxo-RA	4-oxo-ROL	tRA	esters	4-oxo-RA	4-oxo-ROL	tRA	esters
Control	-[d]	-	-	-	-	-	-	-
tRA	-	-	1435	-	-	-	103	-
4-oxo-ROL	34	145	-	1348	226	61	-	-
4-oxo-RA	60	-	-	-	493	-	-	-

[a] The stock concentration of each retinoid was adjusted to 1 mM with ethanol. For treatment, the cell culture medium was supplemented with each retinoid act 1 μM. For the control, the same volume of ethanol (< 0.2%)was added to the cell culture medium. The cells were cultured in 5% CO_2, 37°C, for 24 hrs before harvest. The experiment was repeated twice with very similar results; average values are shown.

[b] Each retinoid was quantitated using the area under the peak, as compared to the corresponding standard of known concentration. The 4-oxo-ROL esters were quantitated using a 4-oxo-ROL standard. The cell volume was estimated to be 1 pl (Chen and Gudas, 1996; Chen et al., 1997).

[c] The volume of cell culture medium was 10 ml.

[d] "-" indicates that no corresponding retinoid was detected.