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PRINCIPAL INVESTIGATOR: Limin Liu, Ph.D.

CONTRACTING ORGANIZATION: Weill Medical College of Cornell University
New York, New York 10021

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Limin Liu, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Weill Medical College of Cornell University
New York, New York 10021

E-Mail: lil2002@med.cornell.edu

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13. ABSTRACT (Maximum 200 Words)

This study was designed to identify the target genes of retinoids in the normal human mammary epithelial cells (HMEC) and breast cancer cells. In our early studies, we found that the IL-1β gene was a responsive gene to retinoic acid (RA) and its expression was up-regulated as early as two hours after RA treatment. In the recent studies, we examined the gene expression profiles in the human mammary epithelial cells following RA and 4-oxo-retinol (4-oxo-ROL) treatment. Our results indicated that both 4-oxo-ROL (1μM) and RA (1μM) strongly inhibited the proliferation of HMEC. The microarray analyses show that a large number of genes, including transcription factors, nuclear receptor co-regulators, cell cycle regulatory proteins and protein kinases, are regulated by RA and 4-oxo-ROL.Although many genes were found to be regulated by both RA and 4-oxo-ROL, 4-oxo-ROL was not converted to RA in HMEC, which suggests that 4-oxo-ROL regulates gene expression by a mechanism different from RA.

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breast cancer, microarray analysis, retinoic acid, human mammary epithelial cell, gene expression

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INTRODUCTION

Retinoic acid (RA) and some of its synthetic derivatives can act as chemopreventive agents for certain malignant diseases, including breast cancers (Hu et al. 1991; Jing et al. 1996; Lotan 1996; Guo et al. 2000; Xu et al. 2001). At a molecular level, RA acts via binding and activating its nuclear receptors, which are transcription factors that directly regulate the transcription of certain "target" genes (Langston and Gudas 1992; Allenby et al. 1993; Boylan et al. 1995; Faria et al. 1999). To understand the chemopreventive mechanisms of RA in the process of breast carcinogenesis, the downstream target genes of RA nuclear receptors must be identified. The identification and characterization of RA target genes in cultured *normal* human breast epithelial cells are the major goals in the current proposal. The comprehensive characterization of RA target genes will increase our understanding of the molecular mechanisms which underly the RA associated growth arrest and differentiation of human mammary epithelial cells (HMEC). These studies will also provide an important foundation for designing more specific and more effective agents for the prevention and therapy of breast carcinoma, and potentially for the other types of carcinomas as well. The major goals of this project include: 1. To identify the genes regulated by RA in normal breast epithelial cells; 2. To determine how RA affects the expression of its target genes; 3. To characterize the functions of the isolated RA target genes on the growth and differentiation of HMEC; 4. To compare the effects of RA on the expression of RA target genes and on the growth of human mammary epithelial cells and breast cancer cells; 5. To monitor the gene expression profile in HMEC after RA treatment by using oligonucleotide microarray analysis. In the previous report, we included the result of the characterization on one of the RA target genes, the interleukin-1β (IL-1β) gene. In the current report, we will present the latest data obtained from microarray analysis.

BODY

The present study was designed to identify genes that were regulated by RA in cultured normal human mammary epithelial cells. In our previous studies, we found that the IL-1β gene is transcriptionally regulated by RA in HMEC. Either RA or IL-1β could significantly inhibit the proliferation of HMEC. However, the addition of soluble IL-1 receptor antagonist (sIL-1ra) to the cell culture medium did not block RA-induced HMEC growth inhibition, whereas sIL-1ra did block the growth inhibition of HMEC by IL-1β, indicating that the IL-1β was not a dominant factor in RA-induced growth arrest of HMEC. In the current report, we include the results of the microarray analysis data, which revealed a large group of genes that are responsive to RA and 4-oxo-retinol (4-oxo-ROL, a natural oxidative derivative of all trans retinol) in HMEC.

RESULTS

Comparison of Gene Expression Profiles in HMECs after Treatment with RA and 4-oxo-ROL. To identify the genes regulated by RA, we performed the gene expression study using Affymetrix HG-U133A oligonucleotide gene chips. As we expected, the expression of a large number of genes was changed in response to RA treatment. We used the following criteria to choose the genes for further study: 1. Genes must be presented in all chip assays; 2. The genes were up- or down-regulated at least 2-fold by RA; 3. The changes of gene expression were reproducible in two independent microarray assays. We selected thirty-two genes as up-regulated genes by RA, and nine genes as down-regulated by RA. We also examined the gene expression profile in 4-oxo-ROL treated HMEC and found a noticeable overlapping between RA-regulated genes and 4-oxo-ROL regulated genes. Seventeen genes were up-regulated and five genes were down-regulated by both RA and 4-oxo-ROL Table 1 and 2).

Gene Expression Analysis with RT-PCR. To confirm gene expression patterns, we synthesized the gene specific primers for several genes, and used the RT-PCR approach to examine their expression in response to RA and 4-oxo-ROL treatment. The RT-PCR results of 4 genes, which are short-chain dehydrogenase/reductase 1 (SDR1), adrenergic receptor β-2 (ADRB2), ATP-binding cassette, sub-family G, member 1 (ABCG1), and ras-related C3 botulinum toxin substrate 2 (RAC2), were shown in Fig 1 and 2. SDR1 and RAC2 genes were up-regulated, and ADRB2 gene was down-regulated by both RA and 4-oxo-ROL. The up-regulation of ABCG1 gene expression was up-regulated by both RA and 4-oxo-ROL, while the regulation by 4-oxo-ROL was more marked.

The Growth of HMEC after Culture in the Presence of RA and 4-oxo-ROL. To determine whether the normal human mammary epithelial cell (HMEC) line responds to 4-oxo-ROL treatment, we added either 1μM 4-oxo-ROL or 1μM RA in the HMEC culture medium, and the cell growth in the presence of RA or 4-oxo-ROL was observed up to day 6 after drug addition. The growth curves were presented in Fig 3. The results show that the proliferation of HMECs was significantly inhibited by either RA or 4-oxo-ROL treatment. By day 6, the number of RA treated HMECs was 27.00%, and the 4-oxo-ROL treated HMECs was 33.99%, respectively, of the number of the control HMECs.

Analysis of Retinoid Extractions with HPLC. In order to understand the metabolic processes of RA and 4-oxo-ROL after being uptaken by HMEC, we extracted the retinoids from the RA and 4-oxo-ROL treated HMECs and analyzed the retinoids with HPLC. The results indicated that the RA was the only major retinoid in the RA-treated HMECs (Fig 4B). In contrast, multiple peaks of retinoids were identified from the 4-oxo-ROL treated cells, and the major retinoids were retinol esters and 4-oxo-ROL (Fig 4C). We could not detect RA in 4-oxo-ROL treated HMEC. A small peak that showed a same retention time with 4-oxo-RA standard (peak A, 10.43min) was detected in the 4-oxo-ROL treated HMEC. In order to identify whether this peak was 4-oxo-RA, we treated the extracts with trimethylsilyl

diazomethane before running the HPLC (Chen et al. 1997; Guo and Gudas 1998). Trimethylsilyl diazomethane is able to shift the retention time of 4-oxo-RA peak from 10.43 min to 26.94 min (Fig 5C). As shown in Fig 5B, a tiny peak shifted by trimethylsilyl diazomethane was detected in the 4-oxo-ROL treated sample, and it indicated that a tiny amount of 4-oxo-RA was produced in the 4-oxo-ROL treated HMEC.

KEY RESEARCH ACCOMPLISHMENTS

1. Forty-one genes were identified as RA target genes in HMEC. Many of them are transcription factors, nuclear receptor co-regulators, cell cycle regulatory proteins and protein kinases;
2. 4-oxo-ROL is an active retinoid in HMEC. It inhibits the proliferation of HMEC and regulates the expression of many downstream genes of RARs. Many genes regulated by 4-oxo-ROL are also regulated by RA.
3. 4-oxo-ROL is not converted to RA in HMEC. It regulates the gene expression of HMEC through a pathway independent to RA formation.

REPORTABLE OUTCOMES

1. Limin Liu and Lorraine J. Gudas (2002) Retinoic acid induces expression of the interleukin-1β gene in cultured normal human mammary epithelial cells and in human breast carcinoma lines. J Cell Physiol. 193(2):244-52
2. Limin Liu, Fadilla Derguini, Lorraine J. Gudas. All trans 4-oxo-Retinol and All trans Retinoic Acid regulate Gene Expression and Inhibit the Proliferation of Normal Human Mammary Epithelial Cells. Manuscript in preparation.

CONCLUSIONS

1. RA inhibits the proliferation of HMEC through via activating its nuclear receptors and modulating the expression of the downstream genes. Using HG-U133A microarray, we identified thirty-two genes as up-regulated, and nine as down-regulated genes by RA. These genes includes transcription factors, cytokines, growth factor-related protein, cell cycle regulatory proteins, and protein kinases. Many genes are proven regulators of the cellular proliferation and differentiation.
2. 4-oxo-retinol (4-oxo-ROL) is the oxidative polar product of all *trans* retinol. In the present study, we examined the biological activity of 4-oxo-ROL in HMEC. Our results indicated that the 4-oxo-ROL (1μM) could efficiently inhibit the proliferation of HMEC and actively modulate the expression of its downstream genes. Since many genes regulated by 4-oxo-

ROL are also regulated by RA, it is suggested that 4-oxo-ROL exerts its biological activity through activating the RARs.

3. The retinol esters and the intact 4-oxo-ROL are major retinoids in the 4-oxo-ROL treated HMEC. Although a tiny amount 4-oxo-RA was detected, no RA was found in the 4-oxo-ROL treated HMEC. These results suggest that the activity of 4-oxo-ROL is independent to RA. The previous study suggests that 4-oxo-ROL could directly bind and activate the RARs in F9 cells (Achkar et al. 1996). Our results suggest that a direct activation of RARs by 4-oxo-ROL may also occur in HMEC. We did not address whether the retinol esters directly contribute to the HMEC growth inhibition and gene expression in the present study.

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Table 1. Genes Up-regulated by RA/4-oxo-ROL in HMEC

Genes with Increased Expression	
RA	4-oxo-ROL
Prostate differentiation factor	Prostate differentiation factor
Basic helix-loop-helix domain containing, class B, 3	Basic helix-loop-helix domain containing, class B, 3
P3ECSL	P3ECSL
2'-5'-oligoadenylate synthetase-like	2'-5'-oligoadenylate synthetase-like
Inhibitor of DNA binding 1, dominant negative helix-loop-helix protein	Inhibitor of DNA binding 1, dominant negative helix-loop-helix protein
Pirin	Pirin
Growth factor receptor-bound protein 14	Growth factor receptor-bound protein 14
Cyclin A1	Cyclin A1
Kallikrein 7 (chymotryptic, stratum corneum)	Kallikrein 7 (chymotryptic, stratum corneum)
Transcobalamin I (vitamin B12 binding protein, R binder family)	Transcobalamin I (vitamin B12 binding protein, R binder family)
MAD (mothers against decapentaplegic, Drosophila) homolog 3	MAD (mothers against decapentaplegic, Drosophila) homolog 3
Leukemia inhibitory factor (cholinergic differentiation factor)	Leukemia inhibitory factor (cholinergic differentiation factor)
ATP-binding cassette, sub-family G (WHITE), member 1	ATP-binding cassette, sub-family G (WHITE), member 1
Insulin-like growth factor binding protein 6	Insulin-like growth factor binding protein 6
Tumor necrosis factor (ligand) superfamily, member 10	Tumor necrosis factor (ligand) superfamily, member 10
Short-chain dehydrogenase/reductase 1	Short-chain dehydrogenase/reductase 1
Cathepsin H	Cathepsin H
Angiopoietin-like 4	DKFZP586N0721 protein
RGC32 protein	Cullin 4B
G protein-coupled receptor kinase 7	Early growth response 1
Chromosome 1 open reading frame 24	Thioredoxin interacting protein
GABA(A) receptors associated protein like 3	
Transforming growth factor, beta receptor II (70-80kD)	
Tumor necrosis factor receptor superfamily, member 6b, decoy	
Interleukin 15	
ADP-ribosylation factor-like 4	
Potassium intermediate/small conductance calcium-activated channel, subfamily N, # 4	
Inositol 1,4,5-triphosphate receptor, type 1	
Periplakin	
Uridine phosphorylase	
Adducin 3 (gamma)	
DKFZP564A122 protein	

Table 2. Gene Down-regulated by RA/4-oxo-ROL

Genes with Decreased Expression	
RA Treatment	4-oxo-ROL Treatment
A disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 1	A disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 1
Heparan sulfate (glucosamine) 3-O-sulfotransferase 2	Heparan sulfate (glucosamine) 3-O-sulfotransferase 2
Follistatin (207345_at)	Follistatin (207345_at)
Serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 2	Serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 2
Phosphomannomutase 2	Phosphomannomutase 2
60S acidic ribosomal protein PO	Four jointed box 1 (Drosophila)
Epsin 3	Keratin 16 (focal non-epidermolytic palmoplantar keratoderma)
PRO0233 protein	NS1-associated protein 1
Adrenergic, beta-2-, receptor, surface	Cadherin 11, type 2, OB-cadherin (osteoblast)
	Transgelin
	Gem (nuclear organelle) associated protein 4
	Follistatin (204948_s_at)
	GRO1 oncogene (melanoma growth stimulating activity, alpha)
	High-mobility group (nonhistone chromosomal) protein 4
	Cullin 2
	Heat shock 70kD protein 1B
	Translin
	Peptidylprolyl isomerase F (cyclophilin F)
	Prohibitin

Fig 1

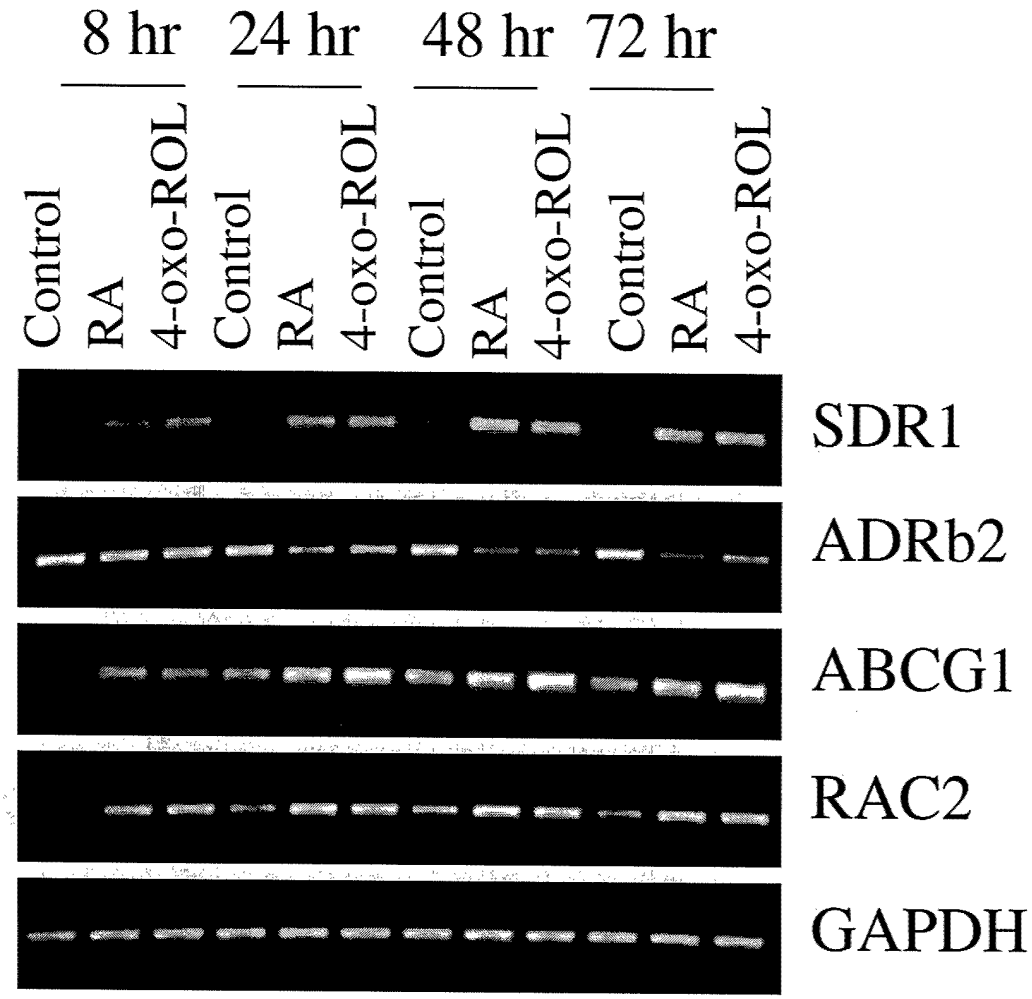
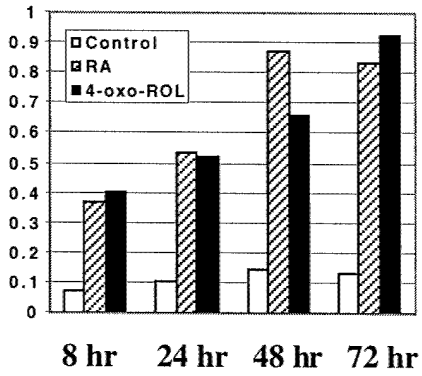

8 hr
24 hr
48 hr
72 hr
Control
RA
4-oxo-ROL
SDR1
ADRb2
ABCG1
RAC2
GAPDH

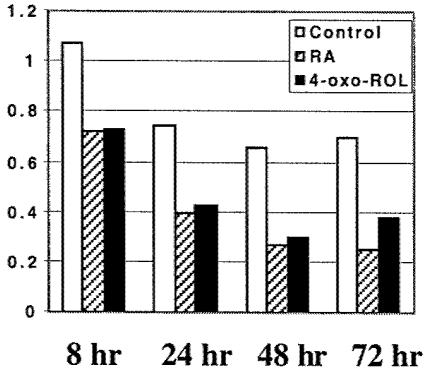
Fig 2

Quantitative Analysis of SDR1, ADRb2, ABCG1, and RAC2 Gene Expression

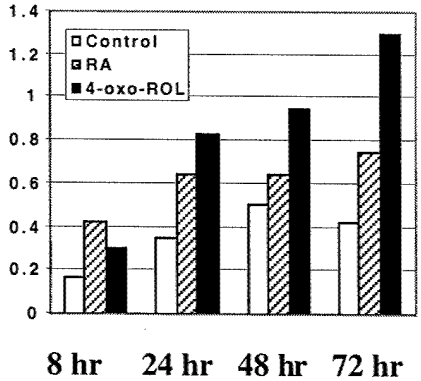
A SDR1



B ADRb2



C ABCG1



D RAC2

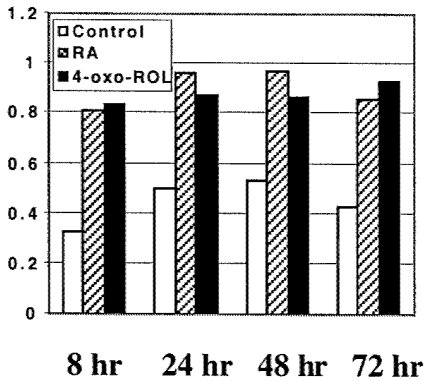


Fig 3

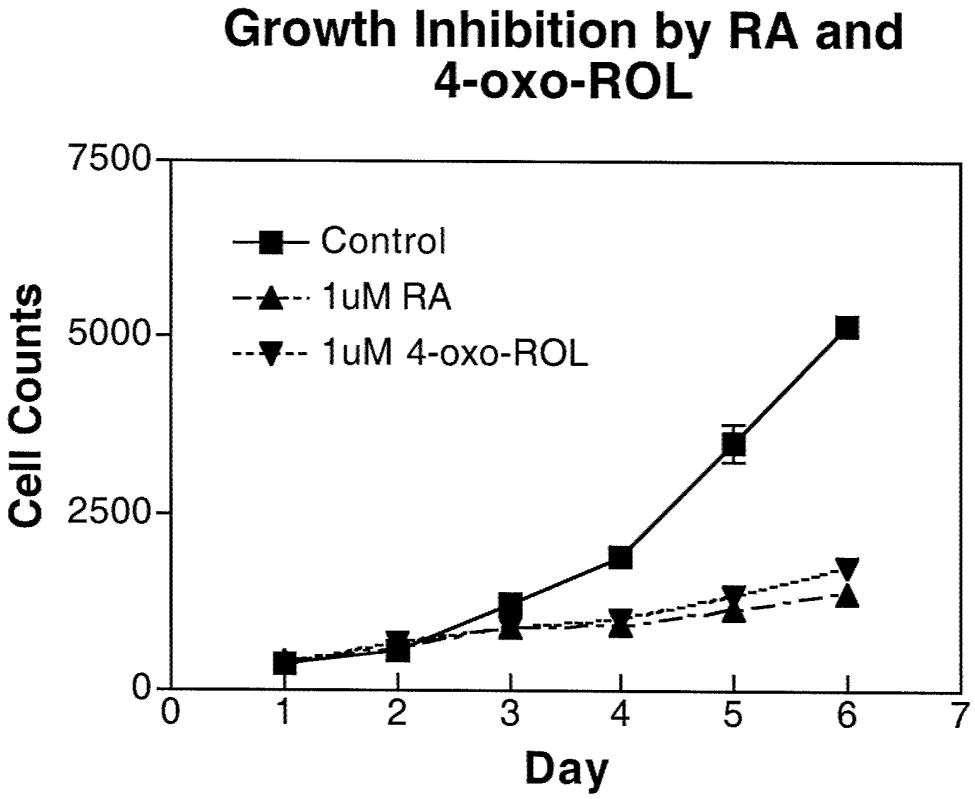

Growth Inhibition by RA and 4-oxo-ROL
7500
5000
2500
0
Cell Counts
Control
1uM RA
1uM 4-oxo-ROL
0
1
2
3
4
5
6
7
Day

Fig 4

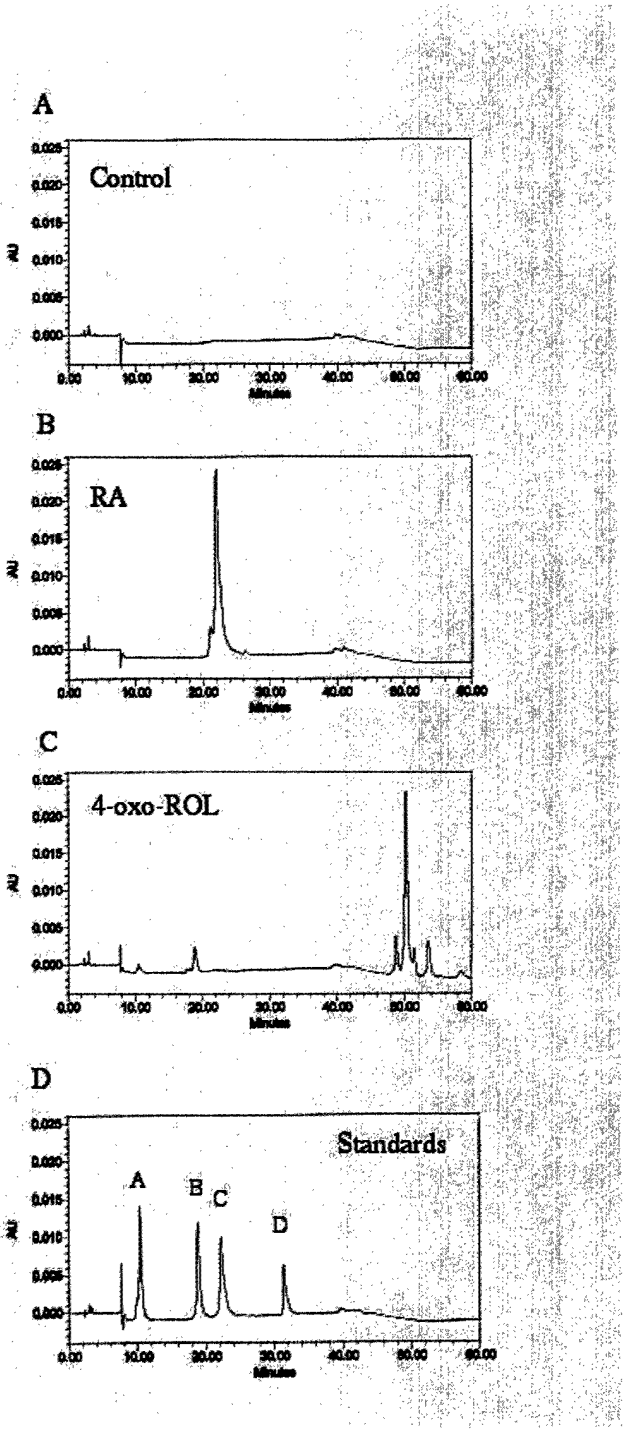


Fig 5

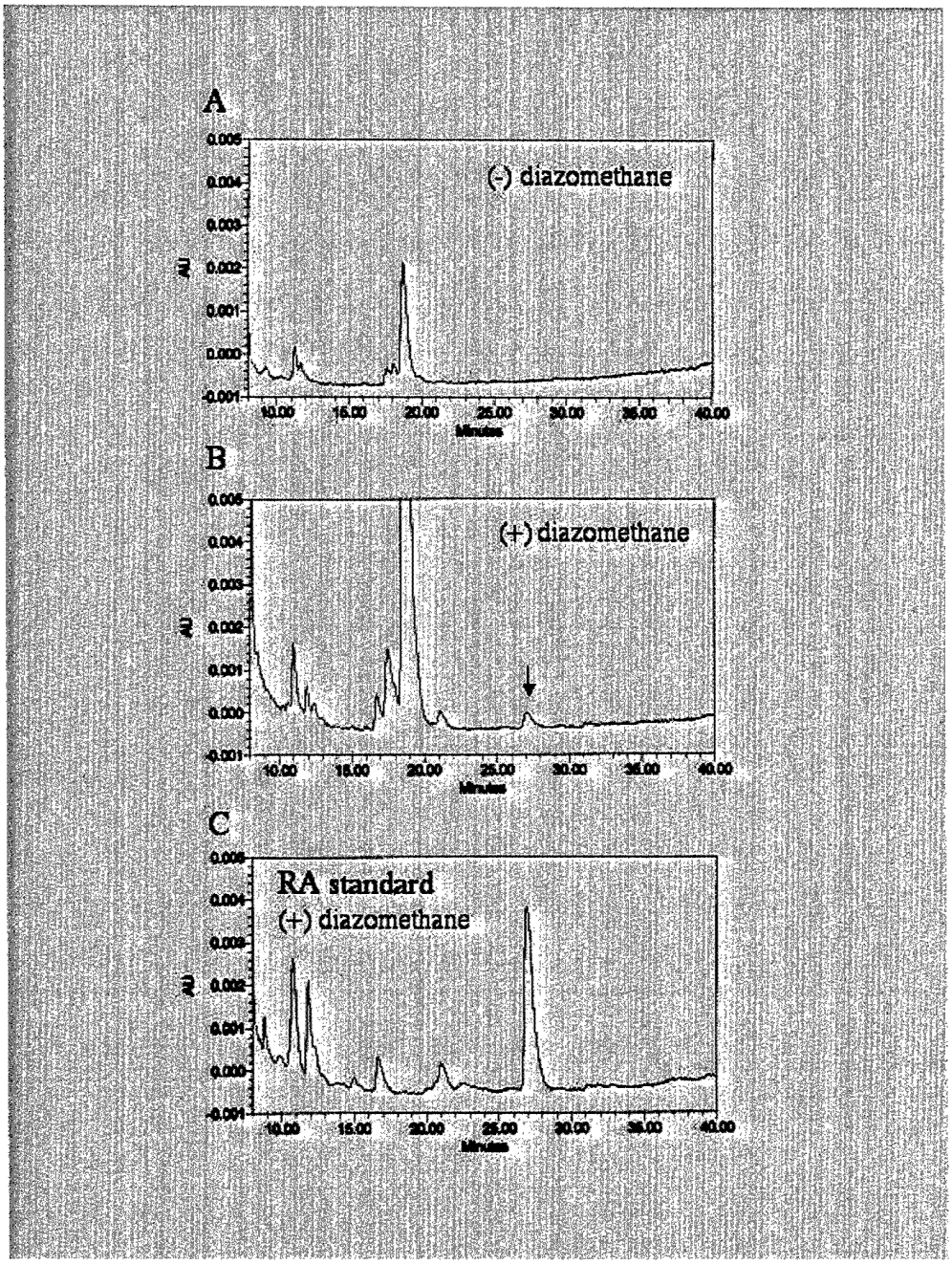

A
(-) diazomethane
B
(+) diazomethane
C
RA standard
(+) diazomethane
AU
Minutes

FIGURE LEGENDS

Figure 1. Examination of the gene expression by RT-PCR. The HMECs were treated with 1 μM RA or 1 μM 4-oxo-ROL. The expression of SDR1, ADRB2, ABCG1, and RAC2 mRNAs were then examined with RT-PCR. The PCR products were visualized on 1.6% agarose gel.

Figure 2. Quantitative analysis of RT-PCR results. The equally loaded RT-PCR products of SDR1, ADRB2, ABCG1, and RAC2 mRNAs were quantitatively measured with FluorChem 8800 system (Alpha Innotech, San Leandro, CA) and calibrated by the intensity of GAPDH.

Figure 3. Inhibition of HMEC growth by RA and 4-oxo-ROL. The HMECs were seeded in 24 well plates at $1x10^4$/well. Cells were grown in MEGM, or MEGM supplemented with RA (1 μM) 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 4. Analysis of retinoids extractions by HPLC. HMECs were fed with fresh medium at 24-hour before the addition of RA or 4-oxo-ROL. Retinoids was extracted from control, RA treated, and 4-oxo-ROL treated HMECs and analyzed with HPLC. The results for 24-hr treatment were shown. A. Control; B. RA treatment; C. 4-oxo-ROL treatment; D. Standards.

Figure 5. Shift of the retention time of the methylated 4-oxo-RA derivative. The methylated 4-oxo-RA derivative was synthesized by reaction with trimethylsilyl diazomethane. The 4-oxo-RA peak is shifted from 10.43 min before the treatment to 26.94 min after the treatment.