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# All-trans Retinoic Acid Induced Differentiation of Rat Mammary Epithelial Cells Cultured in Serum-free Medium

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Retinoids are applied to not only cancer prevention but also cancer chemotherapy by stimulating differentiation of cells. We studied differentiation inducing effect of all-trans retinoic acid (ATRA) by studying proportion of high dense fractions of stem-like cells and the size of S phase fraction in cell cycle. From mammary organoids obtained from 7- to 8-week old F344 female rat mammary gland, we cultured rat mammary epithelial cells (RMEC) and treated physiological doses of $10^{-6}$, $10^{-7}$, and $10^{-8}$ M ATRA from the first day and then cultured for 4, 7, and 14 days. After that, immunostaining was performed using peanut agglutinin (PNA) and anti-Thy-1.1 monoclonal antibody (Thy-1.1) that can be used as markers of differentiation. We separated four different cell subpopulations by flow cytometry: cells negative to both reagents (B-), PNA-positive cells (PNA+), Thy-1.1-positive cells (Thy-1.1+), and cells positive to both reagents (B+). We observed continuous decreases of high dense fractions of stem-like cells (PNA+ subpopulations) for 14 days and as much decreases as high doses of ATRA, which were thought to be proportional to doses of ATRA. We labeled RMEC with bromodeoxyuridine and investigated cell cycle fractions that went through S phase. We observed a tendency of decrease of S phase fraction with time in culture, which is thought to be related to continuous decreases of PNA+ subpopulations and inhibitory role of ATRA on cell cycle. These results suggest that physiological doses of ATRA could stimulate differentiation of RMEC and convert stem-like RMEC to differentiated cells in SFM for a relatively long period of 14 days.

**Key words :** All-trans retinoic acid, Mammary epithelial cell, Differentiation, Flow cytometry

## INTRODUCTION

Retinoic acid (RA) and related compounds play important regulatory roles in growth and differentiation of a wide variety of cell types. Moreover, retinoids are required to maintain normal differentiation and proliferation of epithelial tissues in general (Dembinski and Shiu, 1987). RA showed inhibitory effects on the growth of squamous metaplasia formation from rat mammary epithelial cells cultured in reconstituted basement membrane, Matrigel (Kim *et al.*, 1996).

The glands of young adult (7~8 week-old) female rats contain more than one functional type of differentiated epithelial cells including ductal epithelia, alveolar epithelia and myoepithelia; they have also been postulated to contain pluripotent self-renewing stem cells (Clifton, 1990; Clifton and Gould, 1985; Dulbecco *et al.*, 1986; Kim and Clifton, 1993; Kim and Paik, 1995; Rudland and Barraclough, 1988). In recent years, stem-like cells from rat mammary glands were isolated with flow cytometry using peanut lectin and anti-Thy-1.1 antibody and demonstrated the clonal growth potential in the transplanted sites (Kim and Clifton, 1993, 1996). Peanut lectin (PNA) has been used to identify luminal mammary epithelial cells that line the mammary ducts *in vivo* (Newman *et al.*, 1979a, b). It also binds to the small cuboidal cells in primary cultures only after treatment with neuraminidase (Warburton *et al.*, 1985).

Kim and Clifton (1993) sorted four different RMEC with PNA and anti-Thy-1.1 antibody, such as PNA-positive cells (PNA+), Thy-1.1-positive cells, cells negative to both reagent, and cells positive to both reagents, from intact rat mammary glands with flow cytometry and transplanted in hyperprolactinemic syngenic recipient rats to study the clonogenicity of these cells. We found that PNA+ cells contained the highest numbers of clonogenic cells among subpopulations of rat mammary epithelial cells (RMEC). Moreover, single

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sorted PNA+ cells gave rise to multicellular colonies in a reconstituted basement membrane, Matrigel (Kim and Clifton, 1996; Kim *et al.*, 1993). Culture of the heterogeneous mammary epithelial cells in serum-free medium also gives rise to several morphologically different cell types *in vitro* (Kim *et al.*, 1997).

To study the effects of all-trans retinoic acid (ATRA) on the proliferation and differentiation of mammary stem-like cells cultured in serum-free medium, mammary organoids were collected, cultured in the presence of various concentration of ATRA, and examined the patterns of *in situ* immunocytochemical staining patterns, and analyzed S phase fractions with bromodeoxyuridine labeling technique.

## MATERIALS AND METHODS

### Cell culture

Serum-free medium (SFM) was MEGM (Mammary Epithelial Growth Medium, Clonetics, San Diego, CA, USA). MEGM was supplements-added MEBM (Mammary Epithelial Basal Medium) with epidermal growth factor (EGF) (10 ng/ml), human transferrin (10 μg/ml), gentamicin sulfate (50 μg/ml), insulin (5 μg/ml), and hydrocortisone (0.5 μg/ml). ATRA was prepared as stock solution in ethanol, and aliquots were stored at -20°C. Each ATRA ($10^{-6}$, $10^{-7}$, and $10^{-8}$ M) was added to the medium immediately before each feeding and was present continuously thereafter.

### Mammary epithelial organoids isolation and culture

Rat mammary epithelial organoids were prepared as described previously (Kim and Clifton, 1993). In brief, virgin female F344 rats, 50-55 days old, were killed and their inguinal mammary fat pads were removed, and digested with collagenase solution (Type III, 2 mg/ml, Worthington Biochemical, Freehold, NJ, USA) in MEBM supplemented with gentamicin sulfate (50 μg/ml) with shaking at 37°C for approximately 3 hr. After digestion, the suspension was washed in MEBM with 10% fetal bovine serum (FBS, HyClone, Logan, UT, USA) and centrifuged, and the pellet which contained cells, cell clumps, and mammary organoids was collected. The mammary digest was distributed to 10 cm polystyrene culture dishes (Lux, Naperville, IL, USA) in MEBM with 10% FBS and incubated at 37°C for 2 hr to allow the rapidly adhering cells which are predominantly fibroblasts and other cells of mesenchymal origin to attach to the dish. The supernate, which contained the free epithelial cells, cell clumps, and organoids (ductal and endbud fragments) were collected, pooled and washed by centrifugation. The organoids and cells were resuspended in MEBM with 10% FBS and the organoids were collected on a 40 μm pore filter (Tetko, Briarcliff Manor, NY, USA) which allowed the dispersed cells and small cell clumps to pass. The organoids were resuspended by backwashing the filter, were distributed in culture dishes in an appropriate MEGM with 5% FBS in the presence or absence of ATRA at 37°C in a humidified 5% $CO_2$/air atmosphere for 1 day. Next day, MEGM with 5% FBS was removed and MEGM with or without ATRA was added. Each 10 cm petri dish contained one inguinal fat pad-equivalent of mammary organoids. The medium was changed 3 times weekly.

To make single cell suspension and count cell numbers, the cultured cells were collected with 0.2% trypsin-EDTA with chick serum for 6 min, washed with MEBM and resuspended in 0.05% trypsin-EDTA and incubated at 37°C for 9 min with shaking. The resultant cells were washed and resuspended in MEBM. Three ml 0.05% DNase (Worthington Biochemical) was added per 10 ml suspension, and the mixture was broken up by pipetting and filtered in sequence through 40, 20 and 10 μm pore size Nytex filters. The concentration of morphologically intact cells was determined by mixing 1 vol of cell suspension with 1 vol of 0.5% trypan blue in 0.85% saline and counting by phase microscopy in a hemacytometer.

### Labeling cells with BrdU

Bromodeoxyuridine (BrdU, 10 μg/ml) was added to organoid cultures which were then incubated at 37°C for 1 hr. The cells were then harvested, washed in PBS and fixed in 3 ml cold 70% ethanol with 0.5% Tween-20. The fixed cells ($2\times10^6$) were treated with 0.04% pepsin (Sigma) in 0.1 N HCl at 37°C for 30 min. They were then washed once in PBS and resuspended in 1.5 ml of 2 N HCl with 0.5% Triton X-100 (Sigma) for 30 min to partially denature the DNA. After incubation, 3 ml borax (sodium tetraborate, 0.1 M $Na_2B_4O_7$, pH 8.5, Sigma) was added to neutralize any excess acid. The cells were then washed once with 3 ml PBS-TB (PBS with 1% BSA and 0.5% Tween-20, Sigma). The pellets consisting of permeabilized cells and nuclei were resuspended in 1 ml RNase (50 μg/ml in PBS) and incubated at 37°C for 20 min. Forty μl anti-BrdU antibody (Becton Dickinson, Mountain View, CA, USA) was added to these cells and nuclei and they were incubated at room temperature for 30 min. Afterward, the cells and nuclei were washed once with 3 ml PBS-TB and resuspended in 200 μl PBS-TG (PBS with 0.5% goat serum and 0.5% Tween-20) containing 2.8 μg FITC conjugated F(ab')2 goat-anti-mouse IgG (Tago, Burlingame, CA, USA) and incubated at room temperature for 30 min in the dark. After incubation, the cells and nuclei were washed with 3 ml PBS-TB. The labeled cells were analyzed by Becton Dickinson FACScan flow cytometer. Laser excitation at 488 nm and standard FITC em-

ission filters were used. Ten thousand cells were analyzed for each sample. Analysis of the DNA distribution was done with Lysis II version 1.0 software (Becton Dickinson).

### Immunostaining of monodispersed cells

The concentration of monodispersed cells in suspension was adjusted in PBS with 1.0% bovine serum albumin (BSA, Sigma) to $2\times10^7$ cells/ml. Fifty μl aliquots of the cell suspension ($1\times10^6$ cells) were distributed in plastic tubes. Staining with 50 μl FITC-peanut lectin (PNA-FITC, 1.25 μg/ml, Vector Laboratories, Burlingame, CA, USA) and/or 8 μl phycoerythrin-conjugated anti-Thy-1.1 (Thy-1.1-PE) monoclonal antibody (Bioproducts For Science, Indianapolis, IN, USA) was carried out at 4°C for 30 min. Some cell samples were single-labeled with either PNA-FITC or Thy-1.1-PE and others with both. A negative control for the lectin staining was prepared by pre-incubation with 0.2 M galactose (Sigma) for PNA-FITC before staining the cells as above. A negative control for anti-Thy-1.1 antibody was aliquots of cell suspension incubated with PE-conjugated $IgG_1$ isotype (Becton Dickinson). The stained cells were then washed and the final concentrations were adjusted to $1\times10^6$ cells/ml in PBS with 1% BSA.

### Flow cytometric analyses

Fluorescence activated flow cytometric analyses of the monodispersed stained cells from 4, 7, and 14 days as primary and subcultures were performed with a Becton Dickinson FACScan. Cells were excited at 488 nm with 15 mW for analysis. Green FITC fluorescence was measured with a 530/30 band pass filter, and orange PE fluorescence with either a 585/42 or 575/26 band pass filter. Forward light scatter, side scatter, and fluorescence signals were collected in list mode files. Data were analyzed with Lysis II version 1.0 software (Becton Dickinson). Paint-A-Gate software (Becton Dickinson) was used for the analysis of double-positive cells.

### Immunostaining of cultured cells *in situ*

About ~100 organoids were distributed into each well of 24-multiwell Primaria culture plates (Becton Dickinson), and were cultured in serum-free medium from 1 to 14 days as described as above. On days 1, 2, 4, 7, and 14 of culture, the selected culture plates were washed with PBS with 1% BSA and stained with PNA-FITC and Thy-1.1-PE as above. The cells were viewed under a Zeiss Axiovert 100 fluorescence phase microscope.

## RESULTS

### Cell growth and immunostaining of cells *in situ*

The organoid cultures which had been plated with one inguinal fat pad-equivalent of mammary tissue each had received ~$2\times10^4$ (±20%) organoids per 10 cm dish. In the presence or absence of ATRA, monolayer cells spreaded out from the organoids with time in culture. *In situ* immunostaining of cultured organoids and cells with PNA-FITC and Thy-1.1-PE showed very distinctive cell growth patterns. There were three morphologically different organoids: elongated, spherical, and mixed types (data not shown). The majority

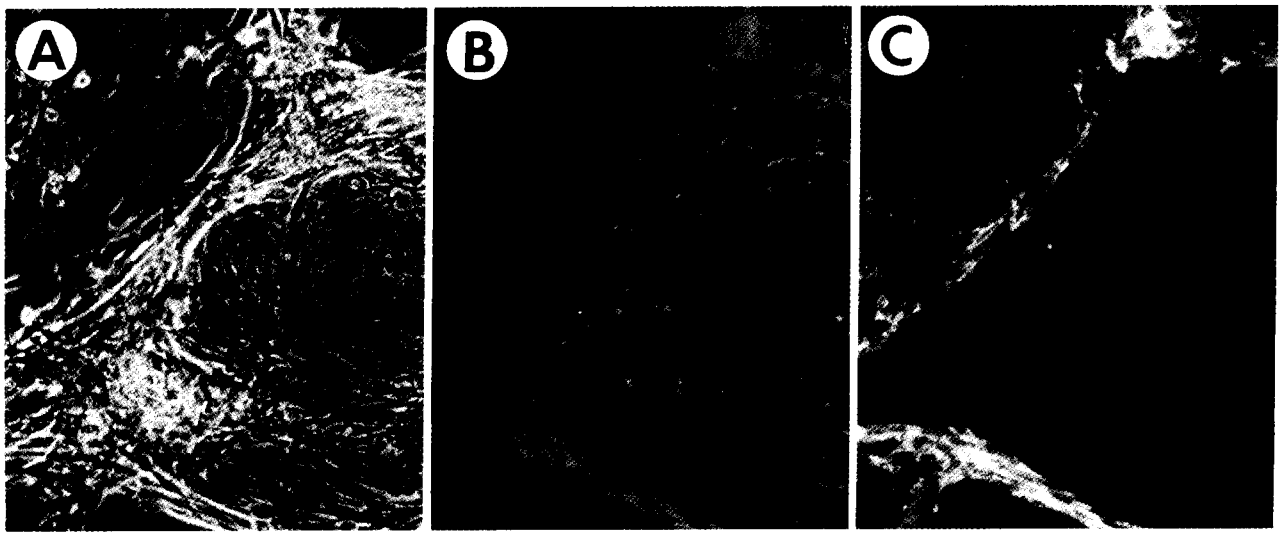


**Fig. 1.** Immunostaining of RMEC in primary cultures in the presence or absence of ATRA for 4, 7, and 14 days with PNA-FITC and Thy-1.1-PE. Unstained cells (A) and immunostained cells were viewed under a Zeiss Axiovert 100 fluorescence phase microscope with a 485/17 band pass filter for green FITC fluorescence (B) and a 546/12 band pass filter for orange PE fluorescence (C). Phase contrast × 100.

of these organoids proliferated into the different types of colonies. Most cells in elongated organoids were positive to Thy-1.1-PE antibodies. However, most of cells in spherical shape organoids were positive to PNA-FITC, and cells in mixed type were positive to both markers. Representative staining patterns are shown in Fig. 1.

### Flow cytometric analysis of immunostained cultured cells

Multiparameter flow cytometric analysis of cells stained with both PNA-FITC and Thy-1.1-PE showed four different populations of cells: Thy-1.1-positive cells (Thy-1.1+), cells positive to both reagents (B+), cells negative to both reagents (B-), and PNA-positive cells (PNA+). Cells cultured in the presence or absence of ATRA showed very different distribution patterns of RMEC subpopulations. Generally, fractions of Thy-1.1+ cells were slightly increased with time and their relative sizes were small (Fig. 2A). Especially, fractions of Thy-1.1+ cells treated with ATRA were smaller than those of untreated control group. There were small amount of B+ cells throughout in culture (Fig. 2B). The fractions of B- cells were increased with time. Especially, ATRA treated groups showed greater changes in the sizes of each fraction than untreated group (Fig. 2C). The fraction of PNA+ cells were decreased almost constantly with time in culture (Fig. 2D). Moreover, the fractions of PNA+ cells in ATRA treated groups were lesser than those of untreated group.

### Growth kinetics of epithelial cells

We compared the BrdU distributions of organoid cultures in the presence or absence of ATRA after different culture times. The fractions of cells in the S phase of cell cycle were well separated based on differences in DNA content in which BrdU was incorporated in place of thymidine. Cells in gate 1 area were BrdU incorporated and emitted higher fluorescence than the cells in left site (Fig. 3). Therefore, we compared the size of cells in gate 1 area among ATRA treated or untreated groups. The sizes of each S phase fraction were changed with time in culture (Fig. 4). Generally, cells cultured in the presence of ATRA showed larger S phase fractions in early culture time than those of control. However, the cells in late stage of culture showed lesser amounts of S phase than those of control group.

## DISCUSSION

The present study has shown that a) serum-free de-

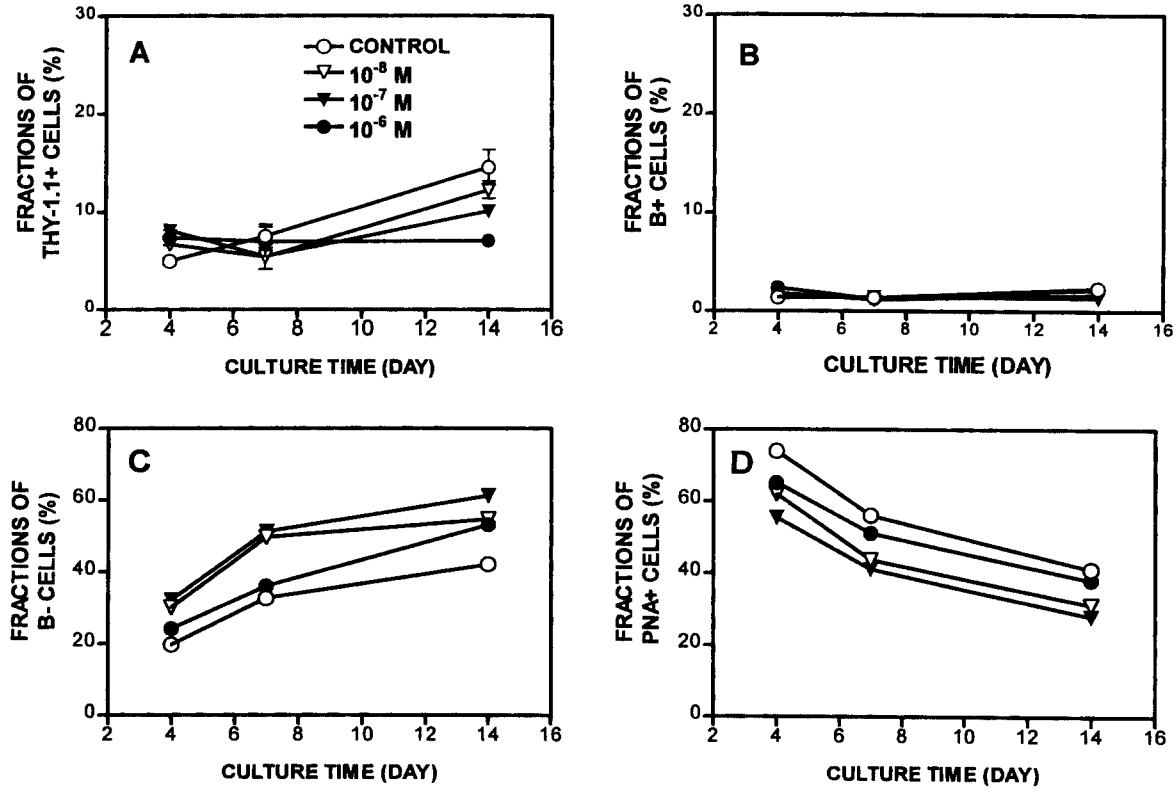


**Fig. 2.** FACScan analyses of Thy-1.1+ (A), B+ (B), B- (C), and PNA+ (D) fractions of RMEC subpopulations in primary culture with or without treatment of ATRA (0, $10^{-8}$, $10^{-7}$, and $10^{-6}$ M) at different culture times (4, 7, and 14 days).

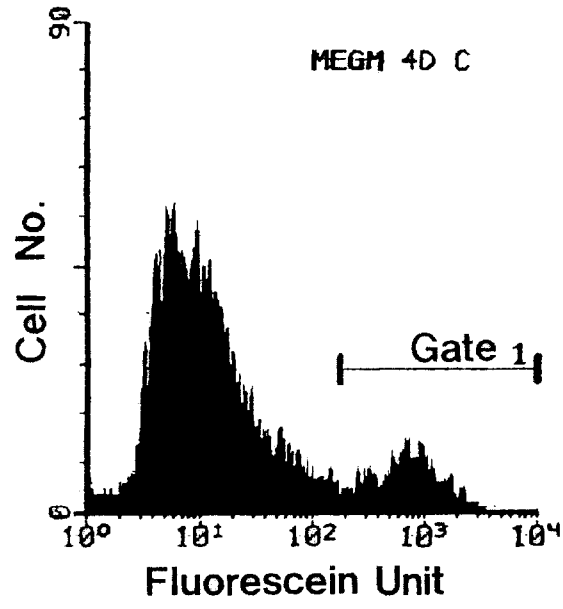


**Fig. 3.** FACScan analysis of S-phase cell fractions of RMEC in primary culture in the presence or absence of ARTA with DNA-labeling of BrdU. Gate 1 showed S-phase cell fraction.

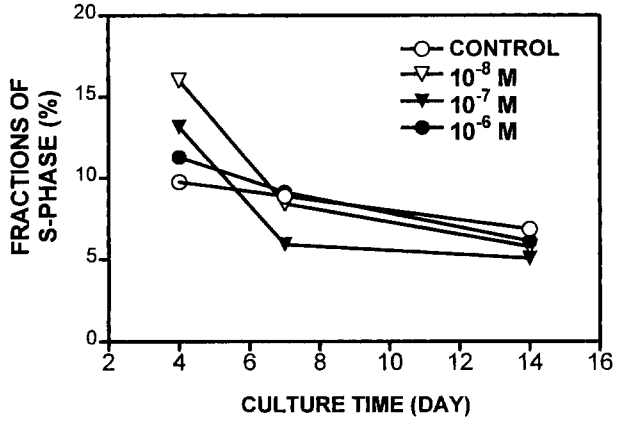


**Fig. 4.** FACScan analysis of S-phase cell fractions of RMEC in primary culture with treatment of different dose of ATRA (0, $10^{-8}$, $10^{-7}$, and $10^{-6}$ M) at different culture times (4, 7, and 14 days).

fined medium (*e.g.*, MEGM) was able to support the growth of primary normal rat mammary epithelial cells; b) four different subpopulations of RMEC were discerned with PNA-FITC and Thy-1.1-PE; c) the presence or absence of ATRA in culture medium showed the changes of morphological and clonal growth potential of cultured cells; d) ATRA enhanced proliferation of RMEC in early phase of cell culture and decreased proliferation in late phase; and e) especially, the fractions of PNA+ cells, which are candidate cells of mammary stem cells, were decreased more rapidly in culture with ATRA.

As we early reported (Kim and Clifton, 1993), there were three morphologically major cell types were observed by phase-contrast microscopy: a) small cuboidal, cobble stone appearance epithelial cells growing in tightly packed colonies, b) larger epithelioid cells that grew in colonies and often surrounding small cuboidal epithelial cells and colonies, and c) elongated cells often found in areas between the epithelial colonies. Several other minor cell types were sometime observed. Cells with spindle shape were coming from tubular-shape organoids which contained red blood cells. At day 1, most of out-growth cells from spherical organoids were tightly packed cobble stone appearance. Most of out-growth cells were completely negative to either Thy-1.1-PE or PNA-FITC. The cells in outer layer of out-growth were also completely negative to either markers and the cells in inner layer of out-growth were positive to PNA-FITC and negative to Thy-1.1-PE (data not shown). Most of out-growth cells from elongated organoids were positive to Thy-1.1-PE in both media. At day 2, the cells which were negative to PNA-FITC became positive cells. The Thy-1.1+ cells remained as it were. Most of mono-layer cell colonies were established by day 7. PNA+ cells had a cuboidal, cobble stone appearance and Thy-1.1+ cells were spindle shaped (Fig. 1B, 1C). Most of cells located near the center of the colonies were positive to PNA-FITC and cells located at the colony boundaries were positive to Thy-1.1-PE. By 14 days in culture, most of remained organoids were degenerated and finally disappeared. Most of Thy-1.1+ cells were elongated or flat shapes and had come to predominate; only a small area of PNA+ cells remained in organoids. Some of colonies positive to PNA-FITC remaining at 14 days had very weak strength of FITC fluorescence. Although the numbers of B+ cells are small, the presence of these cells supports the conversion of epithelial cells into a mesenchyme-like phenotype (Barraclough *et al.*, 1984).

The biological effects of vitamin A on the mammary gland are uncertain. Sankaran and Topper (1982) reported that mammary glands of rats fed a vitamin A-free diet showed similar response to lactogenic hormones from control rats. They concluded that vitamin A does not exert a physiological role in either maintenance of the mammary epithelium or its potential for hormone-dependent phenotypic expression. However, mammary epithelial cells cultured in reconstituted basement membrane, such as Matrigel, showed very distinctive response in the presence of vitamin A derivatives (Kim *et al.*, 1996). RA derivatives induced differentiation of squamous cells in squamous metaplasia colonies into ductal epithelial cells.

The possible interaction of vitamin A and EGF in the regulation of mammary gland development and function has been studied (McGrath *et al.*, 1985; Komura *et al.*, 1986). RA alone in these studies appeared to have no significant effect on the mammary

epithelial cell growth but increased the mitogenic effect of EGF in mammary explants. The synergistic effect of retinoic acid was apparently due to its effect in increasing binding of EGF to mammary cells. Many of the actions of RA are generally mediated through two families of nuclear receptors, the retinoic acid receptors and the retinoid X receptors.

Recently, RA derivatives are clinically effective in preventing the development of cancer (Guchelarr *et al.*, 1993; Hong and Sporn, 1997; Lee *et al.*, 1993; Tallman and Wiernik, 1992). Moreover, ATRA inhibited cell cycle progression in MCF-7 human breast cancer cells (Zhu *et al.*, 1997). They demonstrated that ATRA affected on some of the key cell cycle regulatory proteins in MCF-7 cells. ATRA decreased cyclin D3 and CDK4 as the early phase, reduced pRB expression and phosphorylation, as well as inhibited the fuction of E2F1 transcription factor which controls progression from G1 to S phase. Recent approaches to therapy for various types of cancer have focused on drugs that induce the differentiation of cancer cells. Clinical trials of differentiation-inducing agents, such as ATRA, 13-cis RA, and 9-cis RA, are in progress in patients with leukemia, myelodysplastic syndrome, and solid tumors.

In summary, the serum-free defined medium was able to support the growth of primary normal rat mammary epithelial cells. The cells cultured in the medium contained four different subpopulations of RMEC discerned with PNA-FITC and Thy-1.1-PE. Stem-like PNA+ cells were disappeared and lost proliferative potentials in the presence of ATRA. The manipulation of ATRA in culture medium showed the change of proliferation and differentiative clonal growth potential of cultured cells. These results suggest that physiological doses of ATRA could stimulate differentiation of RMEC and convert stem-like RMEC to differentiated cells in SFM.

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