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| <b>13. ABSTRACT (Maximum 200 Words)</b><br>Estrogen can be metabolized to hydroxylated catechol estrogen, a genotoxic metabolite of estrogen, which causes DNA damage and tumors in animal models. In situ synthesis of estrogen in the breast through aromatase results in high tissue estrogen concentrations. We hypothesized that overexpression of aromatase in breast tissue increases tissue estradiol concentrations and consequent genotoxic metabolites, and eventually causes breast cancer. To test our hypothesis, we stably expressed aromatase cDNA in MCF-10A cells, a benign breast epithelial cell line. During this funding period, we initially characterized the stable line, MCF-10Aarom using tritiated water release assay and products isolation by thin layer chromatography. We demonstrated that MCF-10Aarom cells expressed functional aromatase. We then conducted in vitro study for transformation using soft agar growth assay and in vivo tumorigenesis in nude mice. Our pilot studies allowed us to set up standards (growth time and colony size) for the soft agar assay. There is no palpable tumor formed in nude mice in which MCF-10Aarom cells were inoculated. We also treated the MCF-10Aarom cells in the culture with androstenedione, estradiol, and aromatase inhibitor letrozole. The samples were sent to Dr. Cavalieri for metabolites measurement. |   |  |  |
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## Introduction

Data from clinical animal studies indicate that estrogen not only promotes breast cancer growth but also plays a role in breast cancer initiation. The carcinogenic effect of estrogen is mediated by hydroxylated catechol estrogen, a genotoxic metabolite of estrogen, which causes DNA damage (1-4). In situ synthesis of estrogen in the breast through aromatase makes the major contribution to the high tissue estrogen concentrations (5). We hypothesized that overexpression of aromatase in breast tissue increases tissue estradiol concentrations and consequent genotoxic metabolites, and eventually causes breast cancer. To test our hypothesis, we have established a stable cell line of benign breast epithelial cell by expressing aromatase gene in MCF-10A cells. During the second annual report period, we fulfilled the functional characterization of aromatase expressed in MCF-10A cells (Specific Aim 1) and started the in vitro and in vivo studies (Specific Aims 2 and 3) as scheduled.

## Body

### I. Aromatase activity in stable line of MCF-10A expressing human aromatase (MCF-10A<sup>arom</sup>)

As an alternative approach, we expressed human aromatase cDNA in MCF-10A cells using pH $\beta$ arom vector (6). MCF-10A cells stably expressing aromatase cDNA were selected by G418. Aromatase activity in positive clones was characterized.

#### 1. Aromatase activity and incubation time

One characteristics of an enzyme is the correlation of enzyme activity with time of exposure to its substrate. Time course of aromatase activity was determined by incubation of MCF-10A<sup>arom</sup> cells with tritium labeled aromatase substrate, [1 $\beta$ -<sup>3</sup>H]androstenedione, and measurement of the product, [<sup>3</sup>H] water, the method known as tritiated water release assay. Production of [<sup>3</sup>H] water increased with incubation time. There is a linear correlation between aromatase activity and incubation time within 3 hours (Fig. 1).

#### 2. Aromatase activity and substrate concentration

Correlation of activity with substrate concentration is another general characteristics of enzymes, based on which the value of  $K_m$  and maximal velocity ( $V_{max}$ ) of an enzyme can be calculated. We incubated MCF-10A<sup>arom</sup> cells at 37 °C for 30 minutes with increasing concentration of aromatase substrate, androstenedione ([1 $\beta$ -<sup>3</sup>H]androstenedione and

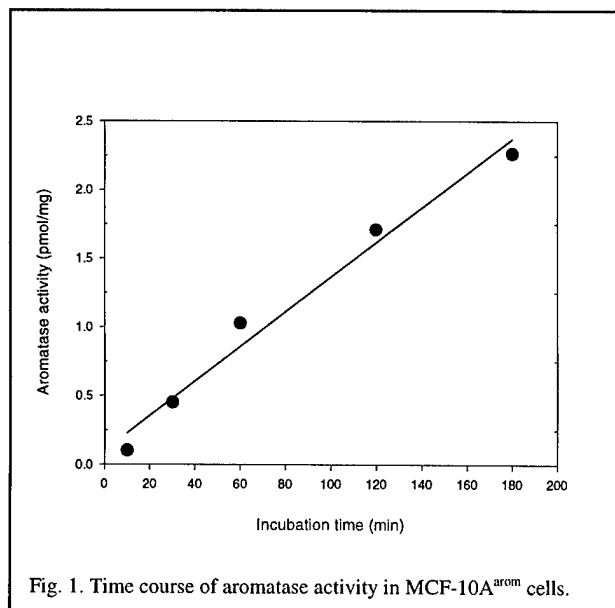


Fig. 1. Time course of aromatase activity in MCF-10A<sup>arom</sup> cells.

unlabeled androstenedione). As shown in Fig. 2, aromatase activity increased linearly with substrate concentration when the substrate concentration is below 10 nM and the rate of increment slows down when the enzyme was saturated with the substrate. The apparent  $K_m$  and  $V_{max}$  are 9.4 nM and 15.2 pmol/mg/h, respectively.

### 3. Inhibition of aromatase activity by letrozole

Aromatase activity was measured by tritiated water release assay in MCF-10A<sup>arom</sup> cells with or without letrozole, a specific aromatase inhibitor. Letrozole induced dose-dependent inhibition of aromatase activity in MCF-10A<sup>arom</sup> cells. Maximal inhibition was about 97% at concentration of  $10^{-7}$  M (Fig. 3).

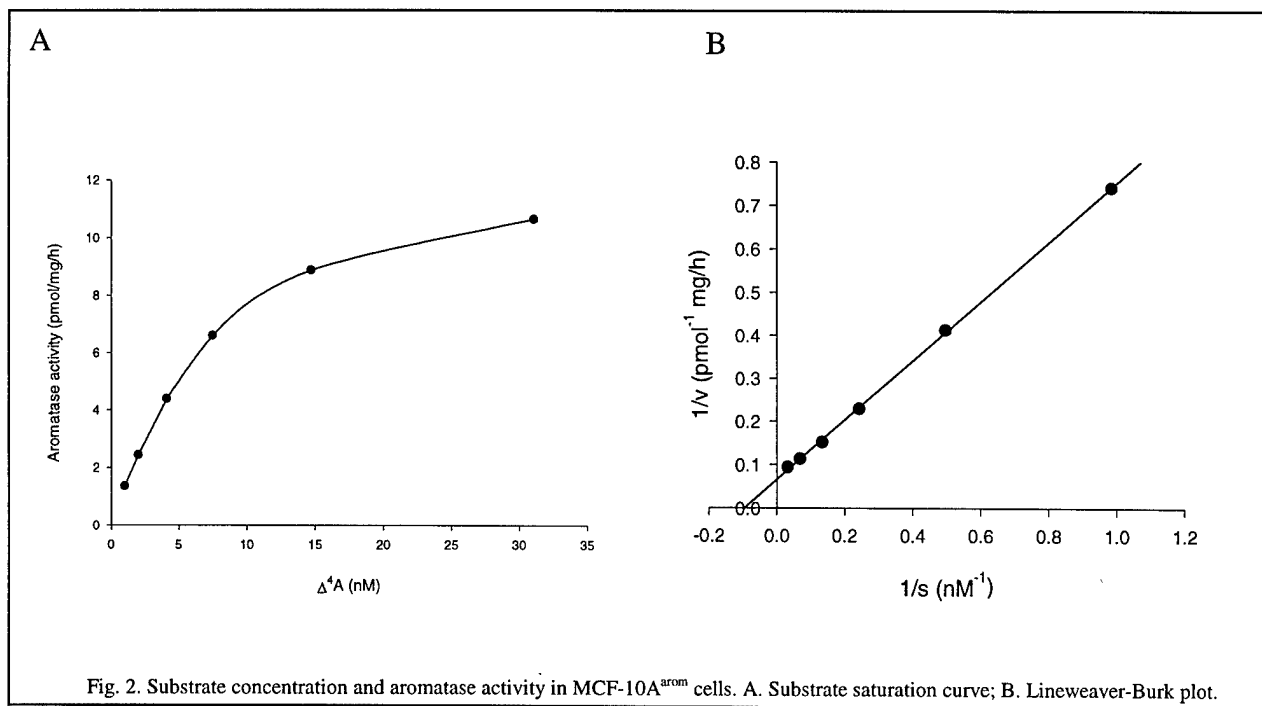


Fig. 2. Substrate concentration and aromatase activity in MCF-10A<sup>arom</sup> cells. A. Substrate saturation curve; B. Lineweaver-Burk plot.

### 4. Confirmation of aromatase activity by product isolation

[<sup>3</sup>H] Water is a byproduct of aromatization reaction using [<sup>3</sup>H] androstenedione as a substrate. Therefore, tritiated water release assay is an indirect way to determine aromatase activity. To further confirm that aromatase stably expressed in MCF-10A<sup>arom</sup> cells can convert androgens to estrogens, [1,2,6,7-<sup>3</sup>H] testosterone was used as a substrate and major estrogens, estrone and estradiol, were isolated by thin layer chromatography.

Our results demonstrate that aromatase

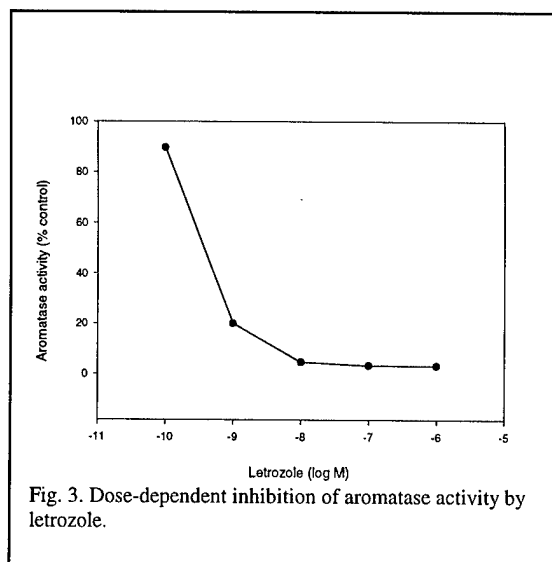


Fig. 3. Dose-dependent inhibition of aromatase activity by letrozole.

expressed from pH $\beta$ rom vector can convert testosterone to estradiol and estrone. This conversion can be effectively inhibited by letrozole. We also found that MCF-10A cells have very high levels of 17 $\beta$ -hydroxysteroid dehydrogenase activity, which catalyzes conversion between testosterone and androstenedione, estradiol and estrone. Fig. 4 shows percentage of tritium radioactivity recovered from the spots of testosterone, androstenedione, estradiol and estrone after 1-hour incubation of MCF-10A<sup>arom</sup> cells with [1,2,6,7 <sup>3</sup>H] testosterone.

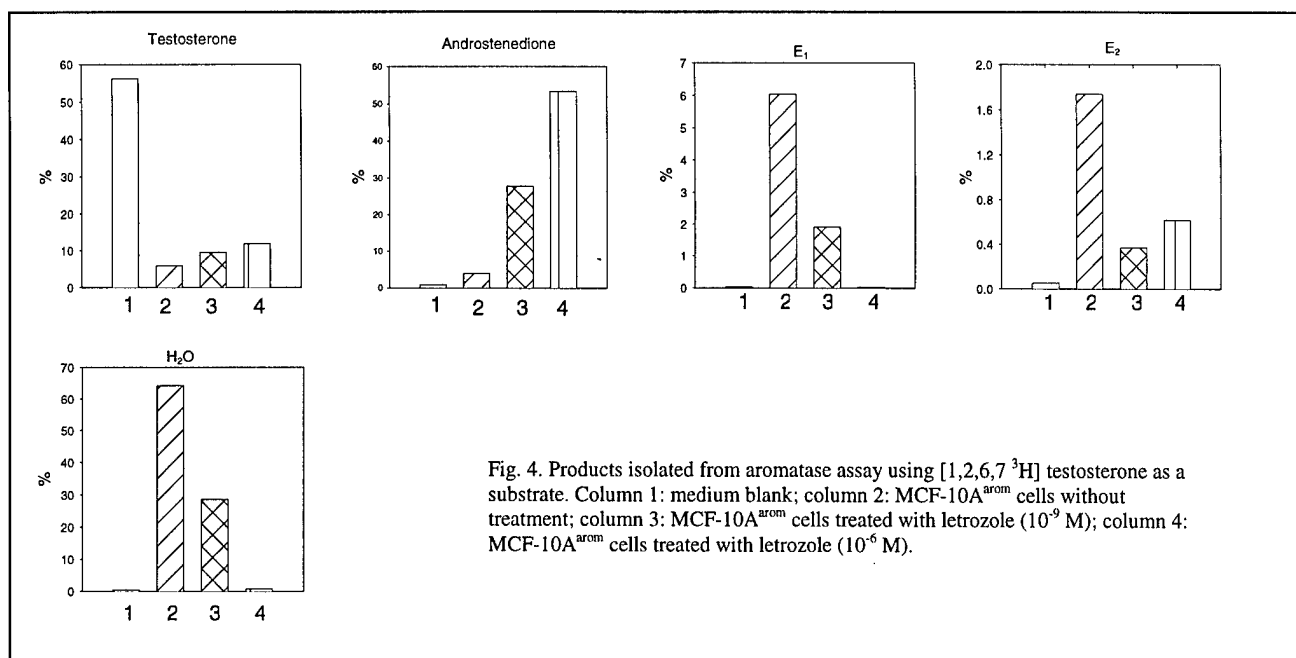


Fig. 4. Products isolated from aromatase assay using [1,2,6,7 <sup>3</sup>H] testosterone as a substrate. Column 1: medium blank; column 2: MCF-10A<sup>arom</sup> cells without treatment; column 3: MCF-10A<sup>arom</sup> cells treated with letrozole (10<sup>-9</sup> M); column 4: MCF-10A<sup>arom</sup> cells treated with letrozole (10<sup>-6</sup> M).

## II. In vitro study

### 1. Anchorage independent growth assay

To determine whether aromatase overexpression results in cellular transformation, the ability of MCF-10A<sup>arom</sup> cells to grow on soft agar (anchorage-independent growth) was assessed.

Russo et. al. demonstrated that pretreatment of MCF-10F cells with estradiol for two weeks increased colony efficiency (7). We are conducting pilot experiments to determine the duration of pretreatment with aromatase substrate, androstenedione; cut-off colony size for evaluation, and the length of observation.

Cell lines used include MCF-10A<sup>arom</sup> cells, MCF-10A (called regular MCF-10A), and MCF-10A<sup>vect</sup> (transfectant with empty pH $\beta$  vector). Regular and control MCF-10A<sup>vect</sup> serve as the controls for MCF-10A<sup>arom</sup> cells.

Treatment: androstenedione ( $\Delta^4$ A, 10<sup>-8</sup> and 10<sup>-6</sup> M), estradiol (10<sup>-8</sup> and 10<sup>-6</sup> M), and vehicle control.

Preliminary results: By the time this report was prepared, we had only data from regular MCF-10A and MCF-10A<sup>arom</sup> cells. The cells were pretreated with vehicle,  $\Delta^4$ A (10<sup>-6</sup> M), or estradiol (10<sup>-6</sup> M) for 84 days. The treated cells were mixed with 0.6% agar in culture medium (1:1) and transplanted to base agar (0.8%) laid in 24 well plates with 8 replicate wells for each treatment.

The density of cells was 2000 per well. Colony formation was monitored under the microscope weekly. Colonies were counted on days 35, 55, and 60 of inoculation.

There was no colony formed in regular MCF-10A cells regardless of treatment. In contrast, MCF-10<sup>arom</sup> cells formed a few colonies. As shown in Table 1, colonies less than 0.25 mm in diameter formed in all three groups as early as 35 days. However, colonies larger than 0.5 mm were only seen in estradiol or androstenedione treated cells. Four colonies in androstenedione treated cells were as big as 1.0 mm in diameter after 55 days of inoculation. Figure 5 shows photos of colonies in different sizes from MCF-10A<sup>arom</sup> cells.

From this pilot experiment we conclude that 1) MCF-10A<sup>arom</sup> cells can form colony in soft agar; 2) The cut-off size of 0.5 mm or larger is a proper for colony counting because smaller colonies represent inconsistently growing cells; 3) the growth rates are low, thus colony counting should not take place earlier than 55 days; 4) a control with empty vector transfection is necessary to exclude the possibility that transfection but not aromatase expression facilitates colony formation.

Table 1. Colony formation of MCF-10A<sup>arom</sup> cells

| Treatment        | Colony size (mm) | Total colony in 8 wells |        |        |
|------------------|------------------|-------------------------|--------|--------|
|                  |                  | Day 35                  | Day 55 | Day 60 |
| Vehicle          | 0.25             | 4                       | 5      | 5      |
|                  | 0.5              | 2                       | 0      | 0      |
|                  | 1.0              | 0                       | 0      | 0      |
| E <sub>2</sub>   | 0.25             | 2                       | 14     | 14     |
|                  | 0.5              | 0                       | 2      | 2      |
|                  | 1.0              | 0                       | 0      | 0      |
| Δ <sup>4</sup> A | 0.25             | 9                       | 11     | 11     |
|                  | 0.5              | 4                       | 3      | 2      |
|                  | 1.0              | 0                       | 4      | 4      |

## 2. Metabolite measurement

MCF-10A<sup>arom</sup> and regular MCF-10A cells in the culture were treated with estradiol, androstenedione plus or minus letrozole for 48 h. Cells and media were collected and sent to Dr. Cavalieri for measurement of estrogen metabolites and depurinated DNA adducts.

## III. In vivo study

To determine whether aromatase overexpression causes genotoxic damage and consequently mammary tumor, tumorigenesis of aromatase expressing MCF-10 cells were assessed in nude mice. In vivo studies are divided into three parts: 1) letrozole dose finding; 2) short experiment (one month) for histological examination of the mammary glands where MCF-10A cells were inoculated and aromatase expression; 3) long term experiment for tumor formation and prevention.

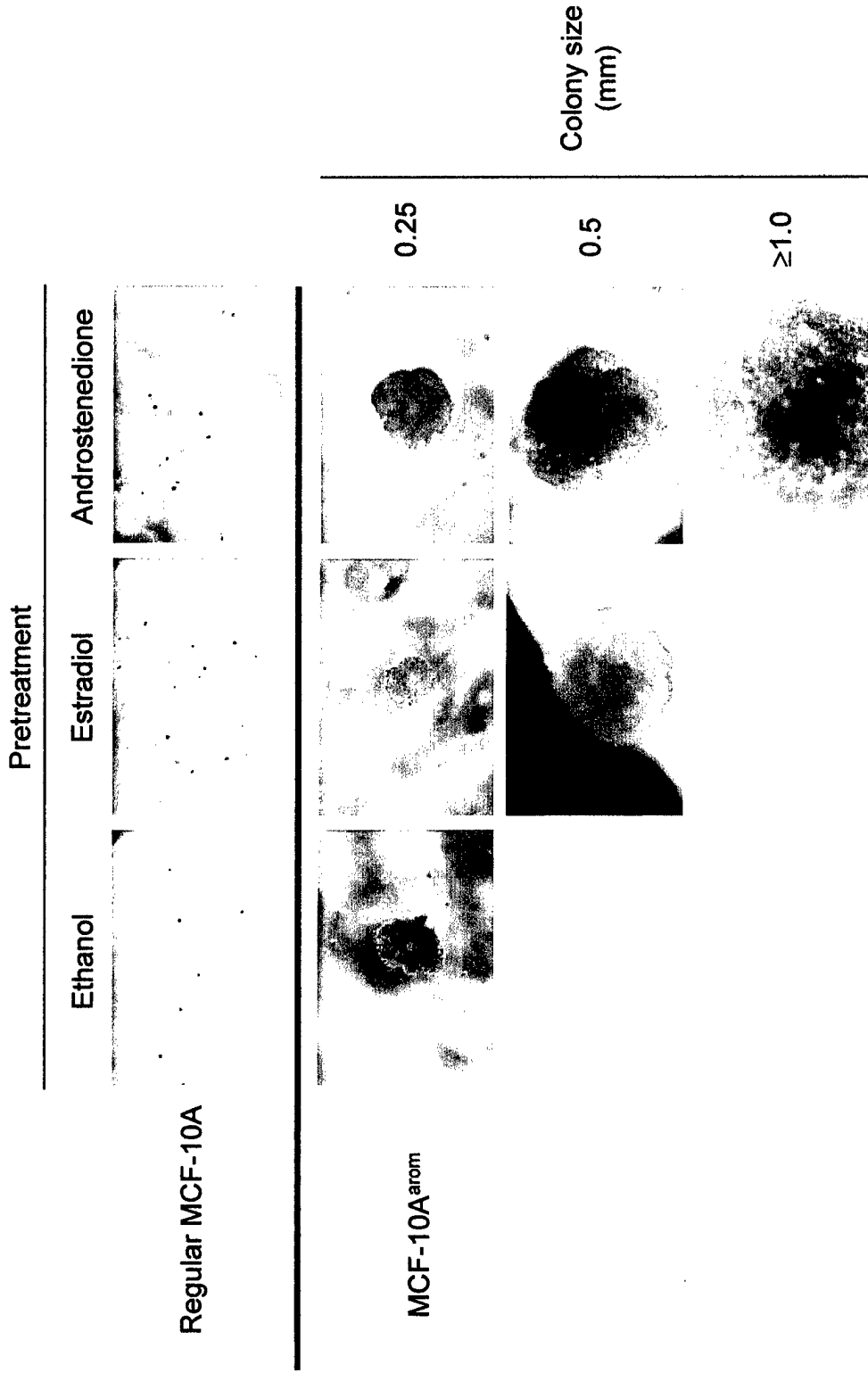


Fig. 5. Colony formation of regular and aromatase expressing MCF-10A cells

## 1. Letrozole dose finding

We reported before that letrozole at the dose of 60  $\mu\text{g}/\text{day}$  completely inhibited growth of aromatase transfected MCF-7 cells in nude mice (8). For the purpose of prevention, duration of letrozole administration will be longer. Therefore, it is necessary to find a minimal effective dose. We inoculated MCF-7Ca cells (MCF-7 cells transfected with aromatase) to six ovariectomized nude mice. The mice received aromatase substrate, androstenedione injection (0.1 mg/mouse/day, s.c.) for tumor to establish. The mice were then divided into the control, letrozole 1  $\mu\text{g}/\text{day}$ , and letrozole 5  $\mu\text{g}/\text{day}$ , three groups. Fourteen days later, the animals were sacrificed and aromatase activity in the tumors was measured. Compared with the control, low dose letrozole (1  $\mu\text{g}/\text{day}$ ) did not inhibit tumor aromatase activity. At the dose of 5  $\mu\text{g}/\text{day}$ , letrozole reduced aromatase activity by 30%. Since letrozole is a competitive inhibitor and there was no letrozole added during the aromatase assay, higher inhibition in vivo was expected. We planned to use 5  $\mu\text{g}/\text{day}$  in future experiment.

## 2. Short term experiment

We inoculated regular MCF-10A and MCF-10A<sup>arom</sup> cells into mammary fat pads of ovariectomized nude mice. Each animal was inoculated with MCF-10A on the right side and MCF-10A<sup>arom</sup> on the left side. Each inoculant consists of 7.5 million cells. The animals were divided into two groups. One group of 10 mice received androstenedione (0.1 mg/mouse/day, s.c.) for one month. The other group of 9 mice served as the control. There was no palpable tumor formed during the one-month period. The animals were sacrificed and mammary tissues collected and fixed in formalin. Uteri were weighed as a biological indicator of circulating estrogen. The average uterine weight of untreated animals was  $11.4 \pm 1.8$  mg. Androstenedione treatment increased uterine weight to  $24.4 \pm 1.3$  mg. This indicated that a certain amount of androstenedione was converted to estrogen that circulated to the uterus. However, we cannot conclude whether this conversion took place in MCF-10A<sup>arom</sup> cells or in peripheral tissues of the animal. Immunohistochemistry staining of mammary tissues for aromatase will be performed to determine survival and function of MCF-10A<sup>arom</sup> cells.

## 3. Long term experiment

This experiment was designed to monitor tumor formation and prevention for up to one year. The procedure for cell inoculation is the same as described above except that three groups of animals are used: the control group, androstenedione group, and androstenedione plus letrozole group. The experiment started in February 2003.

## Key Research Accomplishments

- functional characterization of MCF-10A<sup>arom</sup> cells
- pilot studies for in vitro anchorage independent growth and in vivo tumorigenesis

## Reportable Outcomes

An abstract entitled "Stable Expression of Aromatase in MCF-10A Benign Breast Epithelial Cells for Carcinogenesis Study" was submitted to the 85<sup>th</sup> annual meeting of the Endocrine Society for poster presentation in June 2003, Philadelphia.

## Conclusions

MCF-10A<sup>arom</sup> cells stably express aromatase. Aromatase activity in the cells is linearly correlated with incubation time. Apparent  $K_m$  and  $V_{max}$  of aromatase expressed in MCF-10A cells are 9.4 nM and 15.2 pmol/mg/h, respectively. MCF-10A<sup>arom</sup> cells formed colonies in soft agar. Pretreatment with estradiol or androstenedione facilitate formation of colonies larger than 0.5 mm. No palpable tumor formed from MCF-10A<sup>arom</sup> cells in nude mice.

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## Appendices

Abstract submitted to the Endocrine Society's 85<sup>th</sup> Annual Meeting.

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## The Endocrine Society's 85th Annual Meeting

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**Title:** Stable Expression of Aromatase in MCF-10A Benign Breast Epithelial Cells for Carcinogenesis Study

Wei Yue <sup>1\*</sup>, Jiping Wang <sup>1</sup> and Yuebai Li <sup>1, 1</sup> Internal Medicine, University of Virginia, Charlottesville, VA, 22903 .

The relevance of estrogen to established breast cancer is well documented. Substantial correlative data suggest that estrogens cause breast cancer in women. The commonly held mechanism whereby estrogen causes cancer is that estrogen increases the rate of cell division and spontaneous replication errors while reducing time for DNA repair. However, a body of emerging data suggests another mechanism whereby estrogen is metabolized to genotoxic products that directly initiate mutations. Conversion of androgen substrates to estrogens is catalyzed by aromatase, the rate-limiting enzyme in the estrogen synthesis pathway. Our prior studies demonstrated that in situ aromatization in the breast play a critical role in determination of tissue estradiol concentration and tumor growth. We hypothesized that overexpression of aromatase in the breast increases estrogen concentration and causes breast cancer via metabolite-mediated genotoxicity and estrogen receptor mediated cell proliferation. To test this hypothesis, we plan to establish a benign breast epithelial cell line stably expressing aromatase for evaluation of estrogen metabolism and tumorigenesis. We transfected MCF-10A cells with aromatase gene under the human  $\beta$ -actin promoter and selected positive clones with G418. Thirty G418-resistant clones were collected for evaluation of aromatase activity using [ $1\beta$ - $^3$ H] androstenedione as a substrate. Aromatase activity increased with time of incubation with the substrate. Letrozole, a specific aromatase inhibitor, inhibited aromatase activity in a dose-dependent manner. Km and Vmax of expressed aromatase were determined in three clones with the values of 6-9 nM (Km) and 4-15 pmol/mg/h (Vmax), respectively. Aromatase activity was verified by incubation of MCF-10A<sup>arom</sup> cells with radiolabeled substrate, [1,2,6,7  $^3$ H]testosterone and

quantitation of estradiol ( $E_2$ ) and estrone ( $E_1$ ) after thin-layer chromatography. About 8% radioactivity was recovered from  $E_2$  and  $E_1$  spots, which was dose-dependently reduced by letrozole. The majority of the radioactivity was found in the water phase after ether extraction, which was reduced by 90% with letrozole. In the presence of letrozole, more than 90% substrate was converted to androstenedione through 17 $\beta$ -hydroxysteroid dehydrogenase compared to 5% without letrozole. Aromatase activity in MCF-10A<sup>arom</sup> cells is comparable to those in other aromatase transfected cell lines reported previously.

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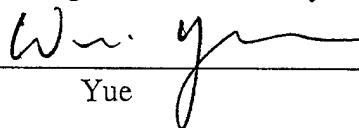
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