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TITLE: Exploiting for Breast Cancer Control a Proposed Unified Mechanism for Reduction of Human Breast Cancer Risk by the Hormones of Pregnancy

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

In Year 1 we showed that administration of pregnancy-associated hormones to carcinogen-exposed rats not only reduced the appearance of mammary cancers (as does pregnancy), but also led to generation of AFP in serum at near pregnancy levels, in support of our hypothesis. The Year 2 plan was designed to confirm that AFP (not the hormones of pregnancy) is the proximal inhibitor of breast cancer. We planned to perform similar studies in rodents that would be passively immunized against AFP, in which inhibition should fail. However, due to the cost of those experiments, we first employed an in vitro, pseudo-human model for passive immunization. We challenged cultures of HepG2 human liver cancer cells with hormones of pregnancy and demonstrated elevated secretion of AFP into the culture media. When these media were added to cultures of T47D human breast cancer cells, cell proliferation was inhibited. However, preventing inhibition by adding anti-AFP antibodies was not achieved, failing with three different antibodies. Investigation disclosed that the antibodies employed failed to deplete the AFP content of the media. We are now investigating a large panel of antibodies, as well as their use in higher concentrations, before beginning studies using the in vivo model. Achievement of a strategy that effectively neutralizes AFP is critical to completing the work that was proposed to evaluate whether AFP is the proximal breast cancer inhibitor elicited by the hormones of pregnancy.

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Prevention; Breast Cancer Prevention; Mechanism; Chemoprevention

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INTRODUCTION

Seeking explanations for the breast cancer lifetime risk reduction associated with pregnancy, several investigators have administered different pregnancy-associated hormones to carcinogen-treated rats and have observed significant reduction in breast cancer appearance. We think it is highly likely that such treatments elicit secretions of AFP, which in turn is the agent that inhibits breast cancer growth. We have isolated AFP from rodent and human sources and have shown that it inhibits the growth of breast cancer cells seeded in culture or as xenografts in immune deficient mice. The purpose of the work described herein was to determine whether the hormones of pregnancy (estrogen, progestin, hCG) would elicit AFP in a quantity sufficient to inhibit the growth of breast cancer cells thus providing evidence that AFP is indeed the proximal agent that explains the risk reducing effect of pregnancy on breast cancer. Not only is this of teleological relevance, but it provides a rational bases for developing active analogs of AFP that can be used as non-toxic preventive and therapeutic agents in the management of the breast cancer epidemic in the human population.Our experiments reported have shown that the cancer inhibiting hormone treatments do in fact elicit AFP which appears in the serum, and further studies will show that it is the proximal breast cancer inhibiting agent.

BODY
Materials and Methods

Animals

Female Sprague Dawley rats were obtained from Taconic Farms and were placed immediately on a controlled diet (Agway Pro-Lab 2000; Agway Corporation, Syracuse, NY). They were allowed free access to food and water, and maintained on a 12-hour light-dark cycle at a constant temperature (22°C) for the duration of the study

Animals for Year 1 Hormone Study – Animals obtained at 34 days of age and subjected to carcinogen treatment as described below.

Animals for amniotic fluid (AF) standard curve- Pregnant females obtained at 15 days gestation
.
Animals for progesterone treatment- were 50 days old and were injected with progesterone, as described below, without prior administration of carcinogen

Carcinogen Treatment
Animals for Year 1 Hormone Study- There were 30 rats in each experimental group (unless otherwise specified) to assure a 95% probability of detecting a difference between groups (ratios) of 40%, which was the difference seen for pregnancy (Grubbs et al A. 1983). NMU was obtained from the National Cancer Institute carcinogen repository (MRI, Inc. Kansas City, MO) and was dissolved in sterile physiological saline, (1% w/v), buffered to pH 5.0 with 3% acetic acid. At 50 days of age, rats were randomized, and while under anesthesia were numbered using an ear punch, and a single intracarotid injection of NMU, 50mg/kg body weight was administered.

Hormone Treatments
Each hormone treatment administered followed the doses and schedules specified in the publications being replicated.

Estriol (E_3)
Ten days following carcinogen administration, 30 female rats received a subcutaneous silastic capsule (0.078 inch IDx 0.125 inch OD, 2cm long; Dow Corning, Midland, MI, USA) packed with 30mg E_3 (Sigma Aldrich, St. Louis, MO). All silastic capsules were implanted subcutaneously dorsally, while animals were under isoflurane anesthesia. Implants were left in place for 21 days to mimic pregnancy, and then were removed (3).

Human Chorionic Gonadotropin (hCG)
Twenty-one days after carcinogen administration, 30 female rats received an intra-peritoneal injection of 100IU lyophilized hCG, (Sigma Aldrich, St. Louis, MO) reconstituted with de-ionized water, pH 7.2 , daily, for 60 days (4).

Estradiol (E_2) $_+$ Progesterone (P_4)
Ten days following carcinogen administration, 30 female rats received 20µg estradiol (E_2, obtained from Sigma Aldrich, St. Louis, MO) plus 4mg progesterone (P_4, obtained from Sigma Aldrich, St. Louis, MO) dissolved in sesame oil, daily, by 0.2ml subcutaneous injection, for 40 days (2).

E_3 $_+$ P_4
Ten days following carcinogen administration, 30 female rats received two individual subcutaneous silastic capsules (0.078 inch IDx 0.125 inch OD, 2cm long; Dow Corning, Midland, MI, USA) packed with 30mg E_3 (Sigma Aldrich, St. Louis, MO) and 30mg P_4 (Sigma Aldrich, St. Louis, MO),

respectively. Each silastic capsule was implanted subcutaneously dorsally, on either side of the upper spine, while animals were under isoflurane anesthesia. Implants were left in place for 21 days to mimic pregnancy, and then removed (3).

The reason that some groups received silastic implants of test agents while other groups received injections of test agents was due to our adherence to the protocols described in references 1-3.

Treatment with Pregnancy
Ten days following carcinogen administration, 30 female rats were introduced to males (three females per male). Females stayed with males for 7 days, after which they were removed and separated. Twenty-one days later, 19 females bore litters, which were allowed to breast feed for 15 days. Females that did not become pregnant were excluded from the study (1).

P_4
Six female rats that were not given NMU received 4mg P_4 (Sigma Aldrich, St. Louis, MO) dissolved in sesame oil, daily, by 0.2ml subcutaneous injection, for 30 days.

Mammary Carcinogenesis
For tumor detection and monitoring of growth, carcinogen-treated animals were palpated twice weekly, starting at day 30 after NMU exposure, continuing for 123 days.

Statistics
The amount of inhibition provided by each hormone treatment was analyzed by comparing the percent incidence (number of rats bearing tumors) of the treatment group with that of the NMU-only controls (Fischer's Exact , $P<0.05$ was considered significant).

Blood Samples
Blood was drawn from each of the 6 groups of rats at 4 separate times; at the beginning, middle and end of the treatment, and once 14 days after the conclusion of the treatment. Blood samples were collected from the tail vein. While rats were anesthetized under 3% isoflurane, 1ml of blood was drawn with a 25G. Blood was transferred to glass centrifuge tubes and allowed to clot, uncovered, on the bench at room temperature for 4 hours. Serum was drawn off of each sample and spun 3 times (1500rpm, for 10 min) after which supernatant was collected. Samples were stored at -80 C until used in AFP assay.

AFP Assays
The grant application outlined the planned procedure to analyze rat serum and quantify rat AFP by Enzyme Linked Immunosorbance Assay using a commercially available anti rat AFP. As it turns out, the anti rat AFP antibodies supplied by Santa Cruz Biotechnologies are evidently non-precipitating, thus they are only suitable for use in a Western Blot, and can not be configured into an ELISA.

Detection of Rat AFP
AFP concentration in blood samples was estimated by constructing a standard curve of amniotic fluid and comparing band densities. To construct the standard curve, amniotic fluid was drawn from the amniotic sac of 15 day pregnant Sprague Dawley female rats then centrifuged three times at 2,500 rpm for five minutes to clarify. Amniotic fluid samples diluted with 1 x phosphate buffered saline (PBS) were run on a 10% Tris HCl agarose gel (15ul sample loaded). Protein from the gel was transferred onto a PVDF membrane by a semidry transfer apparatus. After transfer the PVDF membrane was blocked with 5% milk in Tween Tris Buffered Saline (TTBS) for 2 hours at room temperature, then incubated overnight at 4C in goat anti rat AFP IgG HRP conjugated antibody (1:200 in 5% milk TTBS) (Santa Cruz Biological, Santa Cruz, CA). The following day the PVDF was agitated on a shaker at room temperature and washed 6 times over the course of 1.5 hours with TTBS. The membrane was rinsed 6 times with MQ H20 before being incubated for 1

minute in Western Lightening Western Blot Chemiluminescence Reagent (Perkin Elmer, Boston, MA), a 1:1 mixture of luminal and oxidizing reagent. Film was exposed to PVDF membranes and developed, and bands were visualized.

Serum samples were prepared using an immunoprecipitation technique that removed excess nonspecific IgG from the serum sample. The samples were incubated for 1 hour with Protein AG Beads to remove non specific IgG, by adhering it to the beads. Samples were spun for 5 minutes @ 2,500 rpm, supernatants were collected and incubated for 1.5 hours at 4C with anti rat AFP antibody (goat anti rat AFP IgG, Santa Cruz Biological). The supernatant/antibody mixture was again incubated with Protein AG beads for 1.5 hours at 4C and samples were spun 5 minutes @ 2,500 rpm. The supernatants were discarded. The beads were cleaned 3 times with 1 x PBS, centrifuged for 5 minutes 2,500 rpm. The pellet was re suspended in 1x sample buffer (details), boiled for 6 minutes to uncouple protein from the bead and 15ul of the supernatant was applied to the gel. After electrophoretic resolution, proteins were transferred to PVDF membranes and blotted as previously described above.

Evaluation of AFP production after treatment of human hepatoma (HepG2) cells in culture
Our goal in this experiment was to determine whether treating human hepatoma (HepG2) cells with E_2+P, hCG, E_3, or E_3+P that prevent mammary cancer in rats would elicit increased secretion of human AFP. Treatment with P alone which does not prevent mammary cancer in rats was also evaluated. AFP protein secreted into the culture medium was quantifed by ELISA. Production of AFP in the treated cells was compared to that of control cells which were not hormone treated.

HepG2 cells were maintained and grown as a monolayer in αMEM (Life Technologies, Inc., Gaithersburg, MD) supplemented with 5% serum (40% calf serum, 60% FCS), penicillin G (100 units/ml), and streptomycin (100 μg/ml). Cells were released from monolayer with 0.25% trypsin and 0.25% EDTA. 24-well cell culture dishes were plated with $0.1*10^6$ cells/well. Wells received 2ml of medium every 3 days.

Hormone Treatments- E3, E3 +P, E2+P and P (Sigma) were dissolved in 95% ethanol and brought to 10-5M, 10-6M, 10-7M, 10-8M. Final ethanol concentration in each well was 0.095%. hCG (Sigma) (10,000 IU/vial) was dissolved in sterile deionized water at three hormone concentrations (4.1*10-8M, 1*10-8M, 1*10-9M). Control cells were grown in maintainance medium alone.

After the cells reached confluence they were treated with hormones daily for 21 days. Each group had triplicates. The medium containing AFP was removed every 3 days and replaced with fresh medium and hormones. The collected media containing the secreted AFP was stored at -20°C to prevent AFP degradation.

Quantification of human AFP protein- The levels of human AFP protein was quantified by routine assay using the Beckman-Coulter Access at the Albany Medical Center Clinical Chemistry Laboratories.

HepG2 / AFP/ T47D Proliferation Assay
To complete the "in vitro human model" we examined the inhibition of cultured human breast cancer cells with human AFP that was elicited from human liver cells by treatments with the hormones of pregnancy. 1 x 10^5 T47D human breast cancer cells were seeded in 16mm collagen-coated wells contained in a 24-well plate. Cells were grown in estrogen depleted medium. Estrogen stimulation was accomplished by addition of 10^{-10}M E_2 . Supernatants from HepG2 cells or control medium were added daily. After 7 days of growth, cells were trypsinized into suspension, diluted, and enumerated by counting in a hemocytometer.

Published studies have shown that carcinogen-exposed rats subsequently treated with pregnancy-associated hormones, estradiol and progesterone (E2+P), estriol (E3) or human chorionic gonadotropin (hCG), are at a reduced risk of developing carcinogen induced breast cancer (1-4). On the other hand, treating carcinogen exposed rats with progesterone (P) alone does not reduce the risk of breast cancer (5). As shown in Fig 1e we have confirmed these findings. The Unified Mechanism we hypothesize asserts that the hormones are not the proximal agents by which breast cancer risk is decreased, but rather that they induce the release of AFP from the adult rat liver, and that AFP is the proximal inhibitor of breast cancer development. AFP is a glycoprotein produced during pregnancy by the fetal liver (of rodents and other mammals) and has been shown to inhibit the growth of human breast cancer grown as xenografts in immune deficient mice. Elevated levels of AFP have been shown to be associated with reduced life time risk of breast cancer in humans. It was thus logical to suspect that AFP might work either in conjunction with or downstream from the hormones of pregnancy to reduce breast cancer incidence. In the investigation reported herein, virgin female rats exposed to carcinogen were subsequently treated with pregnancy hormones and exhibited elevated levels of AFP in the sera compared to control rats (Fig. 3). hCG and estrogens combined with progesterone show a more sustained level of AFP elevation than progesterone alone. This is consistent with the cancer incidence data which showed that hCG and estrogens combined with progesterone resulted in greater cancer incidence reduction than did progesterone alone.

To assess whether these hormones could also stimulate the human liver to produce AFP, cultured human hepatoma (HepG2) cells were exposed to the pregnancy hormones (10^{-8}M for steroids and 10^{-9}M for hCG). Human AFP secreted into the medium was quantified by ELISA. The results show that hormone treatments that reduce the breast cancer incidence in rats also stimulate AFP production by HepG2 cells above the levels produced in untreated controls (Fig. 4). On the other hand, treatment with P alone which does not reduce the risk of breast cancer also does not stimulate AFP production by HepG2 cells. These results support the hypothesis that hormones of pregnancy induce production of AFP by the liver and are consistent with the idea that AFP is the proximal protective agent that yields a reduced incidence of breast cancer in women who have experienced pregnancy.

In our second specific aim we propose the treatment of SCID mice bearing human breast cancer xenografts with hormones of pregnancy to elicit murine AFP production, which in turn would block tumor growth. In SCID mice that have been passively immunized against AFP, and then treated as above, the cancer in the animals would experience exposure to the hormones of pregnancy and NOT to AFP elicited by these hormones. We expect that in the latter group, inhibition of tumor growth will not occur.

Since these are costly, time- consuming experiments, and any unplanned repetition that might be required could not be done within the grant time and budget parameters, we employed an in vitro test of our ability to achieve passive immunization against AFP with prevention of breast cancer growth.

In the studies shown in Table 1, AFP enriched supernatent from HepG2 cells treated with the hormones of pregnancy inhibited the growth of T47D human breast cancer cells in culture. However, in data not shown, preventing inhibition by adding anti-AFP antibodies was not achieved failing with three different antibodies. Investigation disclosed that the antibodies employed failed to deplete the AFP in the supernatants (Table 2). We are now investigating a large panel of antibodies, as well as their use in higher concentrations, to validate that the anti AFP antibody approach will indeed deplete AFP from the HepG2 supernatants. We feel that a validated AFP depletion step is needed before beginning in vivo studies which will emply the AFP-neutralization step through passive immunization with anti-AFP.

We expect that the treatment of HepG2 cells with hormones that are known to be protective against breast cancer will upregulate transcription and elicit higher levels of AFP mRNA and AFP in comparison to the controls. On the other hand, we expect that progesterone alone, which does <u>not</u> protect against breast cancer

will <u>not</u> upregulate transcription in comparison to the controls. This will be evaluated in year 3 of this study. If the results support the hypothesis, i.e. if each of the hormone treatments which are known to be protective individually lead to increased levels of AFP mRNA production, it would support the hypothesis that the hormones work through a common mechanism of eliciting AFP production in the liver. We have already shown that the hormones E_3, E_2+P and hCG stimulate AFP production from hepatoma cells; an increase in AFP mRNA levels by these hormones would further support the proposed mechanism.

Results
Inhibition of Mammary Tumors in rats
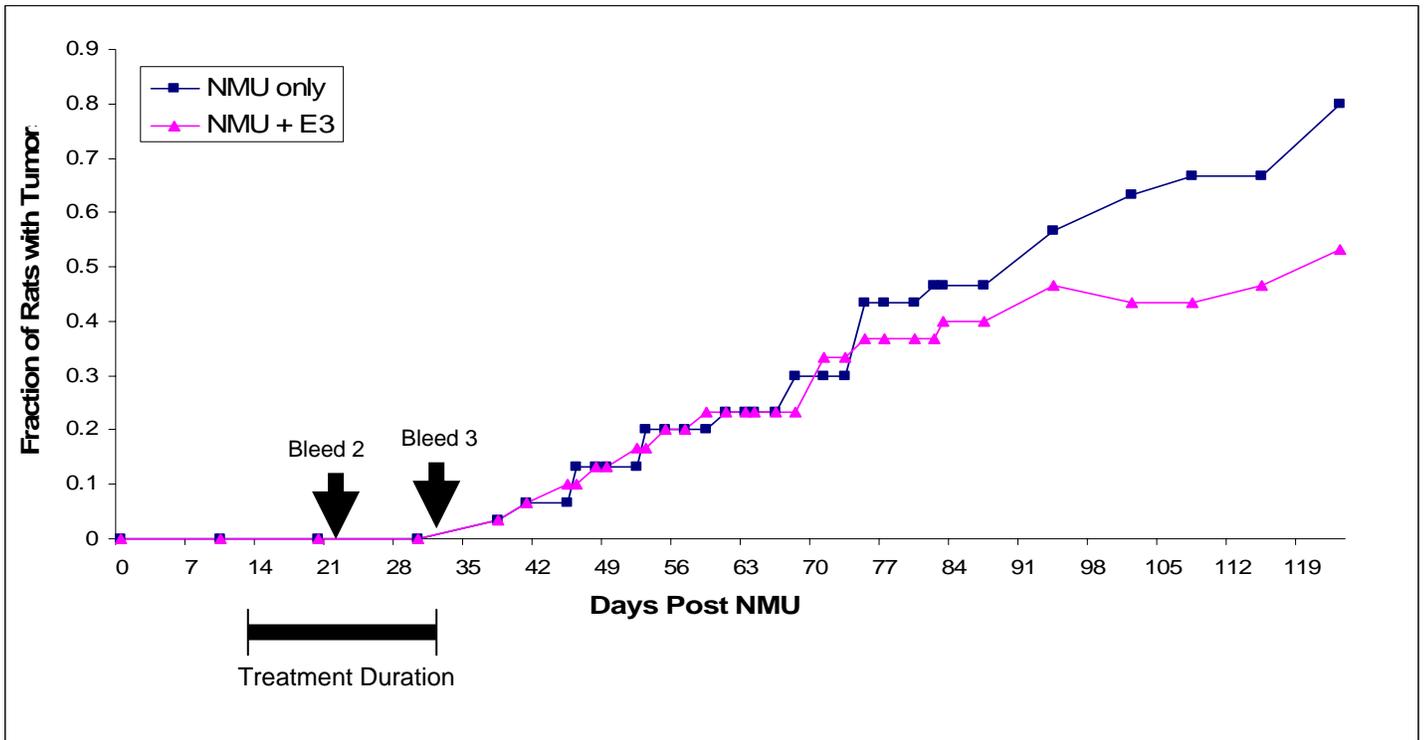
Figure 1a. Breast Cancer Incidence in Estriol (E3) Treated Sprague Dawley rats. Thirty Sprague-Dawley female virgin rats received NMU at the age of 50 days. After thirteen days, rats received a single subcutaneous silastic implant (0.078 inch IDx 0.125 inch OD, 2cm long) containing 30mg estriol and left under the skin for 21 days (2). At Day 102, tumor incidence was decreased by 27% (p< 0.026).
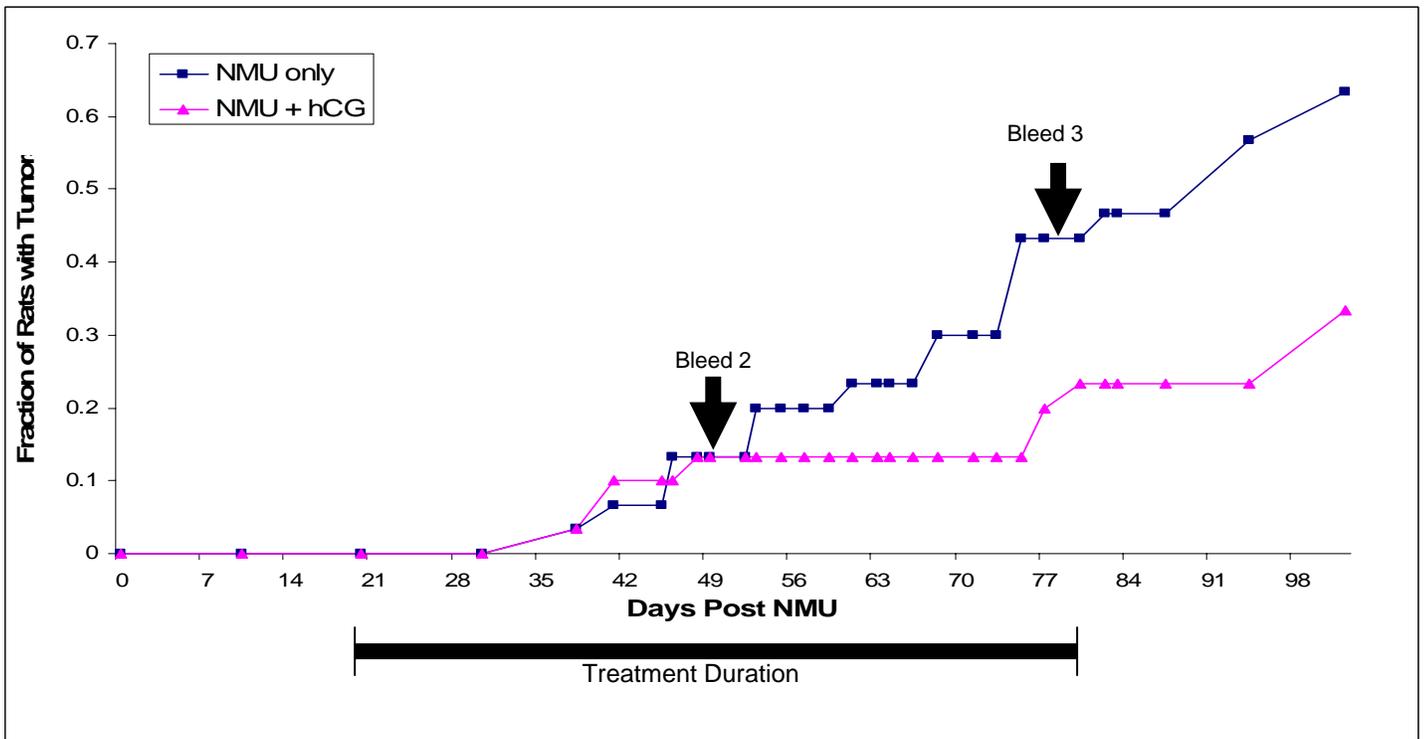

Figure 1b. Breast Cancer Incidence in Human Chorionic Gonadotropin (hCG) Treated Rats. Thirty Sprague-Dawley female virgin rats received NMU at the age of 50 days. After twenty-one days, rats were administered an i.p. injection of 100 IU hCG daily, for 60 days (4). At Day 102, tumor incidence was decreased by 30% (p< 0.02).
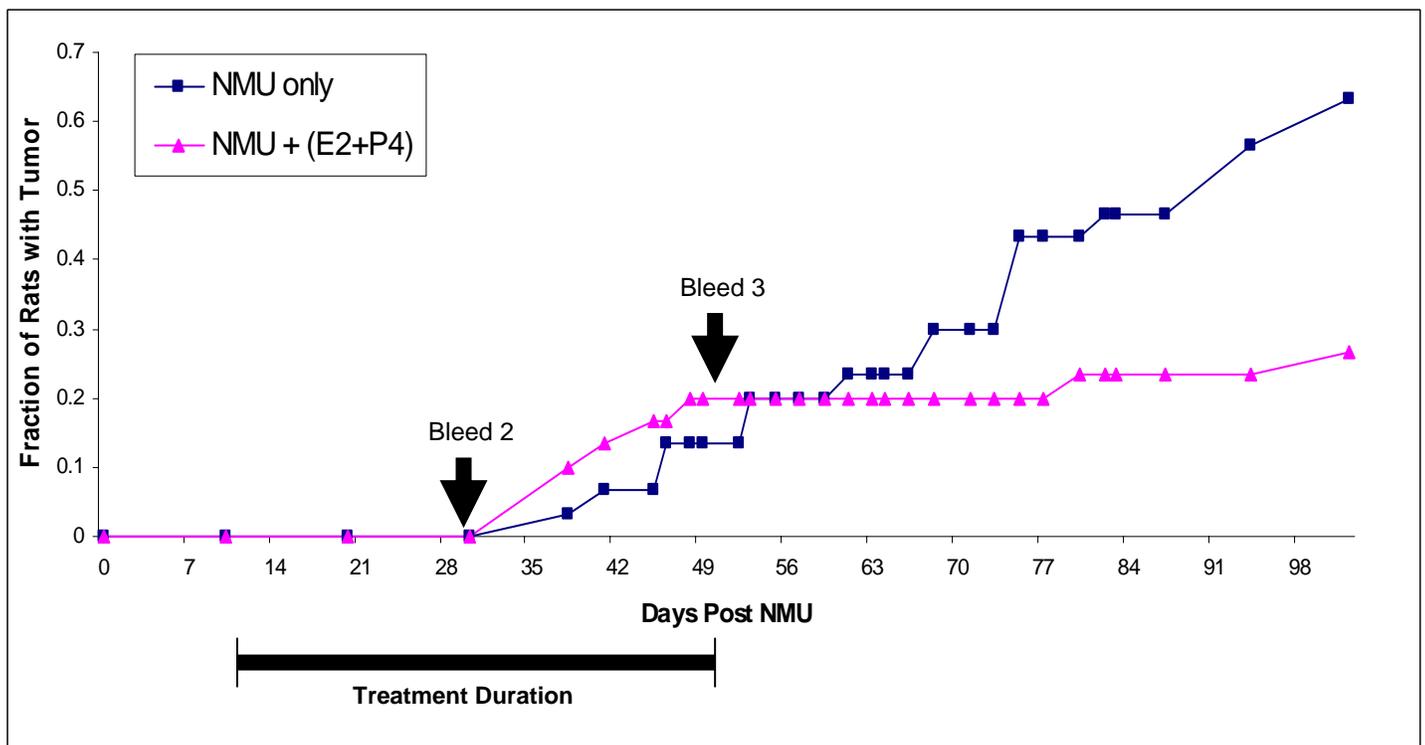
Figure 1c. Breast Cancer Incidence in Estrogen (E2) + Progesterone (P4) Treated Rats. Thirty Sprague-Dawley female virgin rats received NMU at age 50 days. After ten days, rats were administered a daily s.c. injection of 20μg E2 + 4mg P4, for 40 days (1). At Day 102, tumor incidence was decreased by 37% (p< 0.0036).
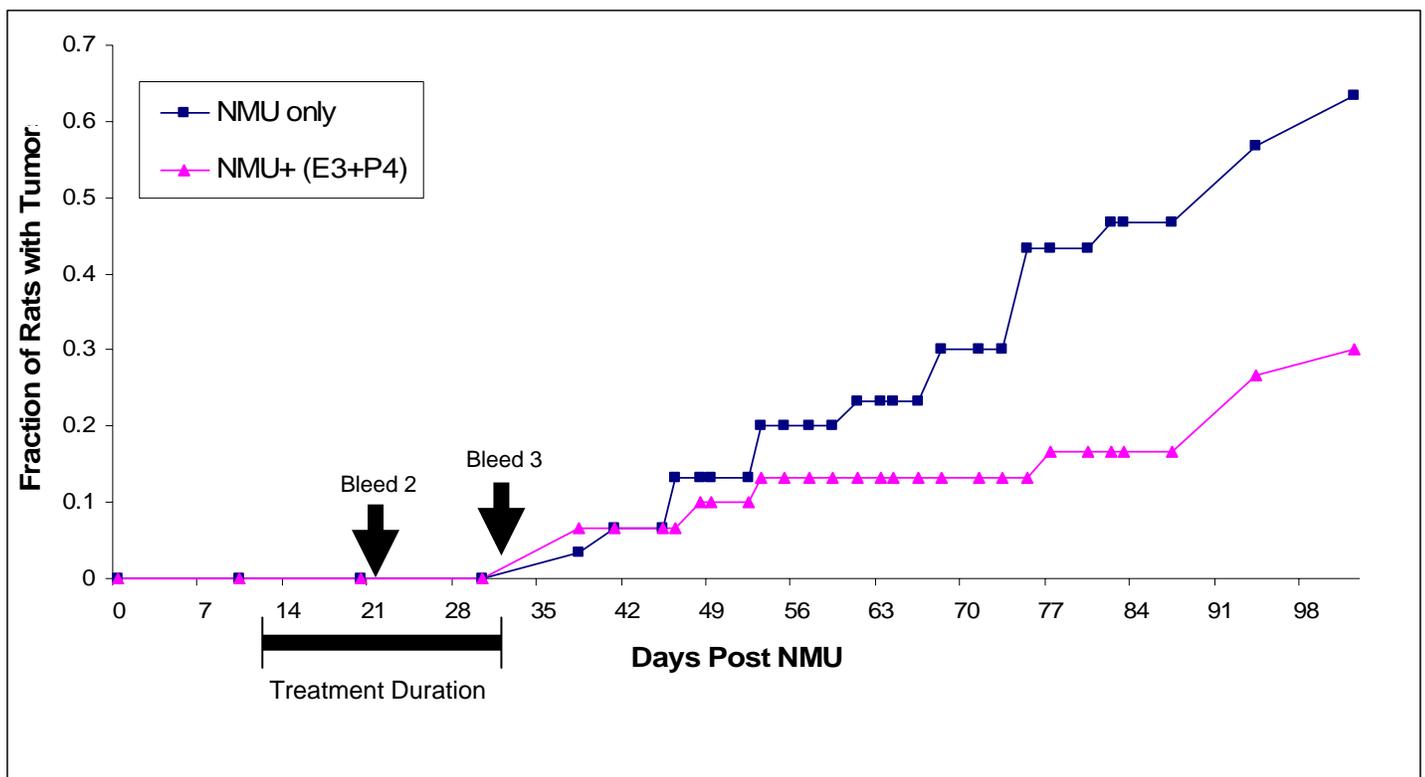

Figure 1d. Breast Cancer Incidence in Estriol (E3) + Progesterone (P4) Treated Rats. Thirty Sprague-Dawley female virgin rats received NMU at the age of 50 days. After thirteen days, rats received two subcutaneous silastic implants (0.078 inch IDx 0.125 inch OD, 2cm long) , containing 30mg estriol and 30mg progesterone respectively, which were left in place for 21 days (2). At Day 102, tumor incidence was decreased by 33% (p< 0.008).

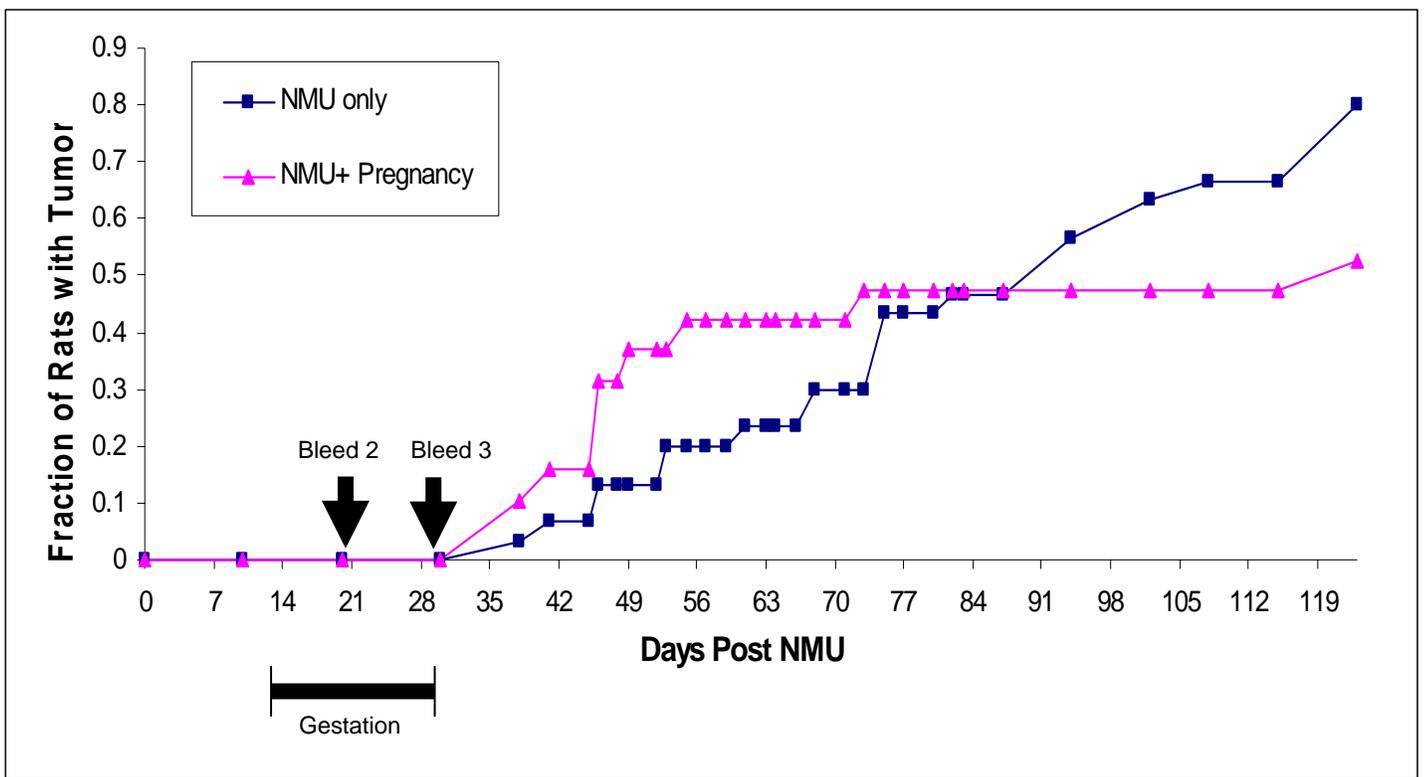**Figure 1e. Breast Cancer Incidence in Mated Female Rats.** Thirty Sprague-Dawley female virgin rats received NMU at the age of 50 days (50mg/kg, intracarotid) . After 10 days, 30 female rats were introduced to males (three females per male). Females stayed with males for 7 days, after which they were removed and separated. Twenty-one days later, 19 females bore litters, which were allowed to breast feed for 15 days (1). Females that did not become pregnant were excluded from the study. At Day 123, tumor incidence was decreased by 28% (p< 0.035). Note Crossover at Day 84.

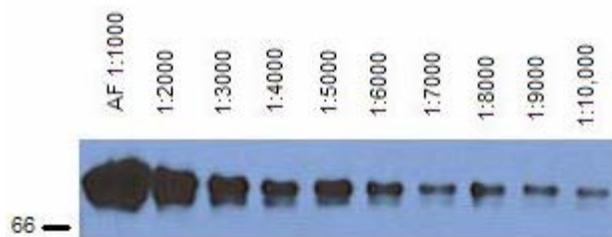## Detection of Rat ARP by Western Blot
AFP Standard Curve

Figure 2. Western Blot of AFP Standard Curve. AFP levels were quantified in blood samples using a standard curve of amniotic fluid diluted in 1 x phosphate buffered saline (PBS). Amniotic fluid was drawn from the amniotic sac of 15 day pregnant Sprague Dawley female rats. Amniotic fluid was centrifuged three times at 2,500 rpm for five minutes to clarify. A western blot was run by diluting samples with 1 x PBS, loading 15ul of sample onto a 10% Tris HCl gel. Following electrophoresis protein was transferred to a PVDF membrane, blocked with a 5% dry milk solution, then blotted using a goat anti rat AFP HRP (1:200 in 5% milk TTBS) conjugated antibody (Santa Cruz Biological).
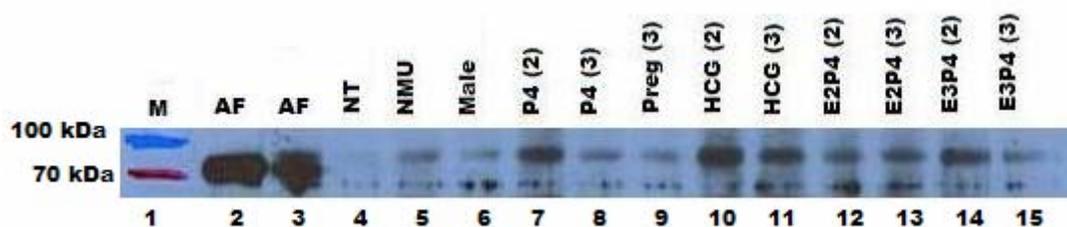

Figure 3. Western blot detection of AFP in serum of hormone treated rats.

NMU treated Sprague Dawley female rats subsequently treated with pregnancy-associated hormones were bled at the midpoint of hormone treatment (B2) and at the conclusion of hormone treatment (B3). Rat sera was separated from whole blood, AFP was isolated by immunoprecipitation (see Methods Section) and subsequent samples were run on a gel (Lanes 9-15) alongside an amniotic fluid standard (Lane 2, diluted 1:1000 in 1 x Phosphate Buffered Saline). Samples that underwent immunoprecipitation were not diluted (Lanes 3-15). Also included were a series of immunoprecipitated controls; amniotic fluid (Lane 3), untreated female rat sera (Lane 4), NMU treated female rat sera (Lane 5), and untreated male rat sera (Lane 6). Following electrophoresis protein was transferred to a PVDF membrane, blocked with a 5% dry milk solution, blotted overnight using a goat anti rat AFP (1:1000 in 5% milk TTBS), then with a rabbit anti goat IgG HRP (1:5000, Santa Cruz Biological). For the hormone treatments that lead to a decrease in carcinogen-induced cancer (hCG, E2+P4, P is more intense in Bleed 2 and Bleed 3 in all four of the hormone treatments that successfully inhibit breast cancer appearance. Progesterone treatment, which does not effectively block cancer appearance, shows AFP in Bleed 2 and less in Bleed 3, indicating that it is not persistent.
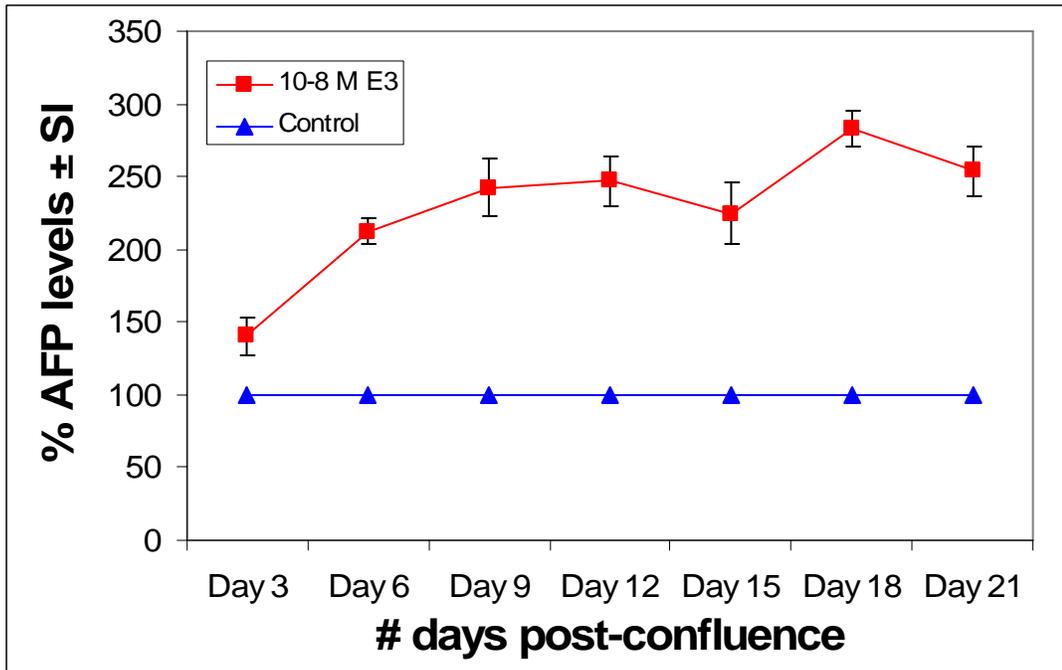
Figure 4a- AFP levels from HepG2 cells treated with 10-8M estriol (E3). 0.1*106 HepG2 cells were plated per well in a 24-well cell culture dish. After confluence was achieved, hormone treatment was started. The culture medium was extracted every 3 days and the concentration of AFP was determined by ELISA.
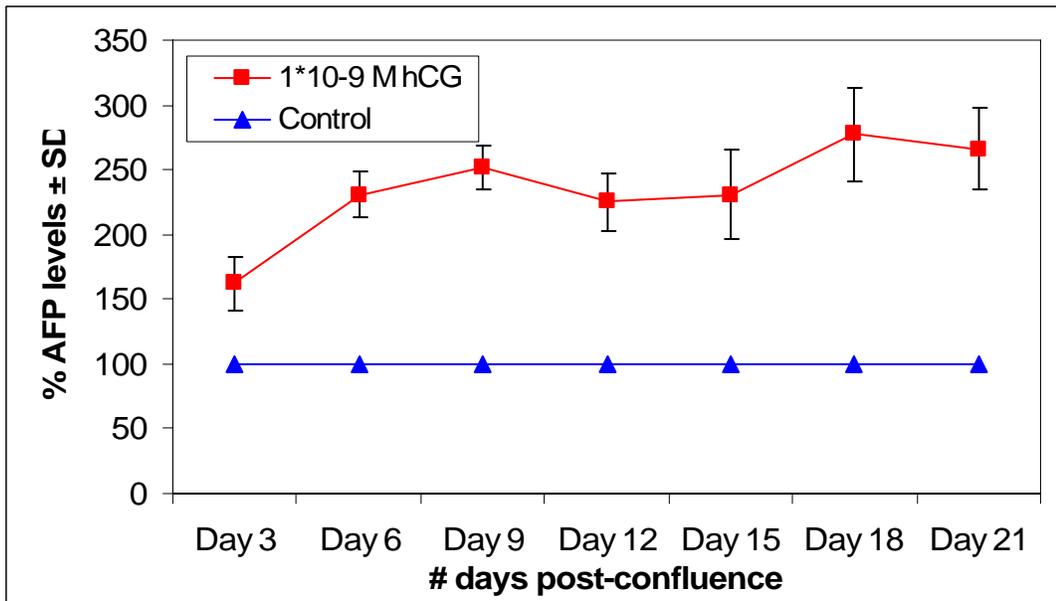

Figure 4b- AFP levels from HepG2 cells treated with 10-9M human chorionic gonadotropin (hCG). 0.1*106 HepG2 cells were plated per well in a 24-well cell culture dish. Confluence was achieved on Day 0 when hormone treatment was started. The culture medium was extracted every 3 days and the concentration of AFP was determined by ELISA.
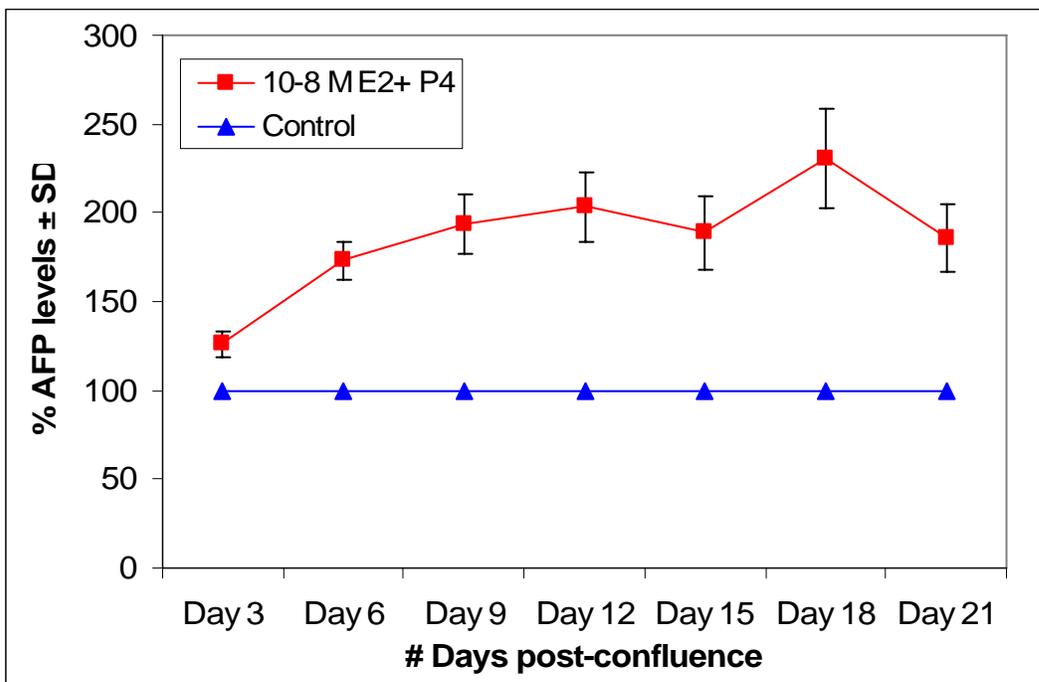
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Figure 4c- AFP levels from HepG2 cells treated with 10-8M estradiol (E2) + 10-8M progesterone (P). 0.1*106 HepG2 cells were plated per well in a 24-well cell culture dish. Confluence was achieved on Day 0 when hormone treatment was started. The culture medium was extracted every 3 days and the amount of AFP quantified by ELISA.
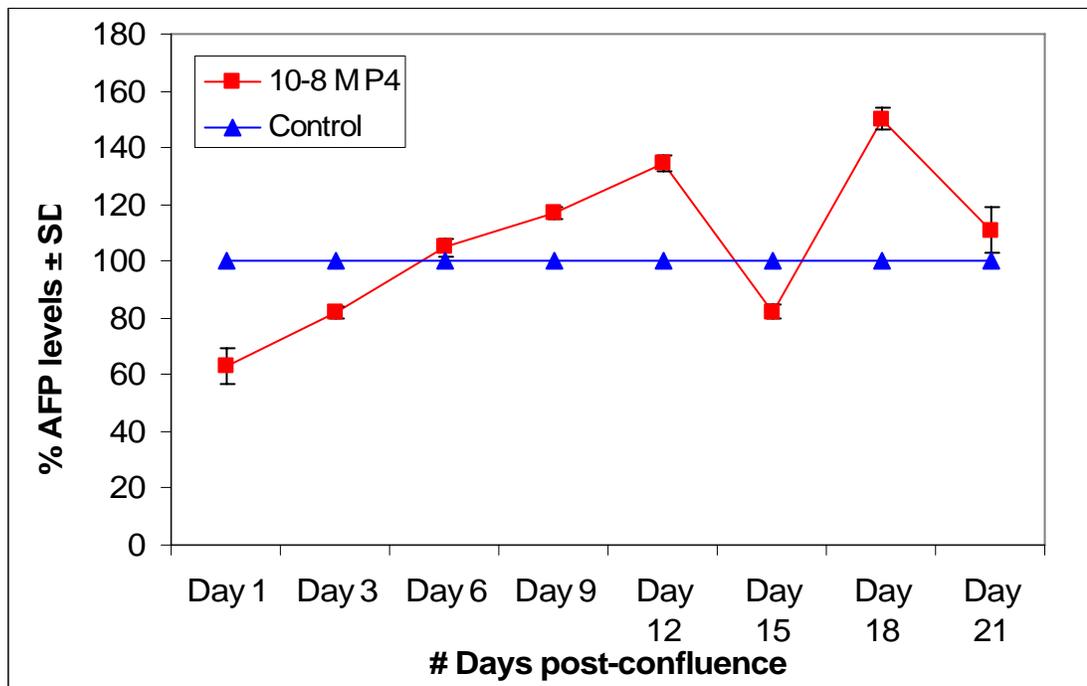

Figure 4d- AFP levels from HepG2 cells treated with 10-8M progesterone (P). 0.1*106 HepG2 cells were plated per well in a 24-well cell culture dish. Confluence was achieved on Day 0 when hormone treatment was started. The culture medium was extracted every 3 days and the concentration of AFP was determined by ELISA.
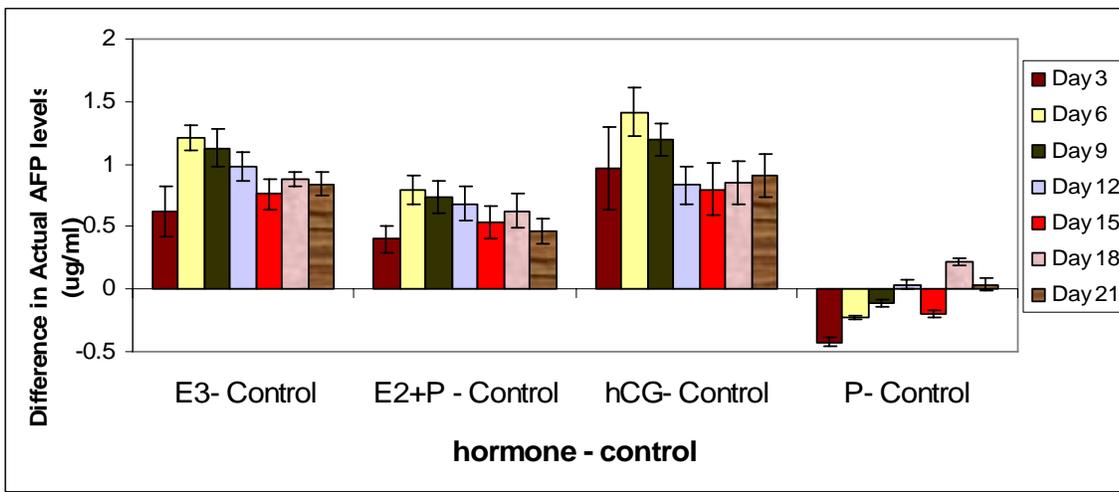
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Figure 4e. Difference in AFP levels between hormone treated (lowest concentrations) and control groups. 0.1*106 HepG2 cells were plated per well in a 24-well cell culture dish. The medium was extracted every 3 days and the concentration of AFP was determined by ELISA. All three hormone treatments (especially at the lowest concentration) stimulate AFP production in HepG2 cells above levels in the controls.

Table 1: Effect of HepG2 Supernatants on Estrogen-Stimulated Growth of T47D Human Breast Cancer Cells in Culture.

Treatment	Mean Cell Number x $10^5 \pm$ SE
No E_2	4.5 ± 0.3
E_2 at 10^{-10} M	11.9 ± 0.9
E_2 + spnt from untreated HepG2 cells	8.4 ± 0.3
E_2 + spnt from hCG-treated HepG2 cells	8.2 ± 0.5
E_2 + hCG at 10^{-9} M	10.4 ± 0.9

1.2 x 10^5 T47D human breast cancer cells were seeded in estrogen free medium in 16mm wells of a 24 well plate. Test agents were added daily during medium replenishment beginning 24 hours after seeding. Supernatants (spnt) were obtained from HepG2 cells that were either untreated or hCG-treated. AFP in untreated spnt was 24ug/ml final concentration, and in hCG (10^{-9}M)–treated, AFP was 27ug/ml final concentration. Cells were enumerated in a hemocytometer 8 days after seeding. Results represent the mean $\pm$ the standard error of four replicate wells.

Table 2: Strategies to Neutralize AFP in HepG2 Supernatants

Expt	AFP generated in HepG2 Supe (µg/ml)	AFP remaining in Neutralized Supe (µg/ml)	Amount of AFP depleted (µg/ml)	Condition
1	$16.8 + 1.7$	$15.7 + 1.7$ *	6.5 %	*single antibody with Protein A/G beads
2	$18.8 + 1.8$	$11.6 + 1.5$ **	38.3 %	** double antibody with Protein A/G beads followed by Blue Sepharose

The average amount of AFP in the HepG2 supernatant used to treat T47D cells over the 7 day period and the AFP levels remaining in the neutralized supernatants were measured as indicated above using the Beckman-Coulter Access Assay System. The supernatants were neutralized either with a single anti-human AFP antibody with Protein A/G beads or with a combination of two anti-human AFP antibodies with Protein A/G beads followed by Blue Sepharose.

KEY RESEARCH ACCOMPLISHMENTS

1. Determined that $E_2 + P_4$ given to NMU-treated rats reduces mammary cancers by 37%.

2. Determined that $E_3 + P_4$ given to NMU-treated rats reduces mammary cancers by 33%.

3. Determined that E_3 given to NMU-treated rats reduces mammary cancers by 27%.

4. Determined that parity in NMU-treated rats reduces mammary cancers by 28%.

5. Determined that hCG given to NMU-treated rats reduces mammary cancers by 30%.

6. Established sensitivity of Western Blot assay for the detection of rat AFP in serum at 1.5 nM.

7. Administering $E_2 + P_4$ has elicited rat AFP in rat sera.

8. Administering $E_3 + P_4$ has elicited rat AFP in rat sera.

9. Administering E_3 has elicited rat AFP in rat sera.

10. Administering hCG has elicited rat AFP in rat sera.

11. Incubating HepG2 human liver cancer cells with $E_2 + P_4$ causes secretion of human AFP.

12. Incubating HepG2 human liver cancer cells with $E_3 + P_4$ causes secretion of human AFP.

13. Incubating HepG2 human liver cancer cells with E_3 causes secretion of human AFP.

14. Incubating HepG2 human liver cancer cells with hCG causes secretion of human AFP.

15. Incubating with Progesterone (which does not block mammary cancer in vivo) does not cause secretion of human AFP.

16. Human AFP produced by hCG stimulation of HepG2 cells when given to cultures of T47D breast cancer cells inhibits growth of the breast cancer cells.

17. Commercial anti-AFP antibody, which detects AFP on Western Blots, does not neutralize AFP in solution or render it incapable of interfering with breast cancer cell growth.

REPORTABLE OUTCOMES

Publications

1. Parikh RR, Gildener-Leapman N, Narendran A, Lin HY, Lemanski N, Bennett JA, Jacobson HI, Andersen TT. Prevention of N-methyl-N-nitrosourea-induced breast cancer by alpha-fetoprotein (AFP)-derived peptide, a peptide derived from the active site of AFP. Clin Cancer Res. 2005 Dec 1;11(23):8512-20.

2. Bennett JA, Defreest L, Anaka I, Saadati H, Balulad S, Jacobson HI, Andersen TT.Pep. An anti-breast cancer peptide that is orally active. Breast Cancer Res Treat. 2006 Mar 15; [Epub ahead of print]

Posters

1. Jacobson HI, Andersen TT, Bennett JA, Lemanski N. "Exploiting for Breast Cancer Control a Proposed Unified Mechanism for Reduction of Human Breast Cancer Risk by the Hormones of Pregnancy". U.S. Department of Defense Era of Hope Meeting, 2005.

2. Andersen TT, Bennett JA, Jacobson HI, Defreest L, Joseph L, Lemanski N, Bacon S, Gildener-Leapman N, and Georgekutty J. "A Safe and Effective Novel Drug for the Prevention and Treatment of Breast Cancer." U.S. Department of Defense Era of Hope Meeting, 2005.

3. Defreest L, Andersen TT, and Bennett JA. "Inhibition of Breast Cancer Growth by AFP Derived Peptide: Study of Mechanism". U.S. Department of Defense Era of Hope Meeting, 2005.

4. Jacobson HI, Andersen TT, Bennett JA, Lemanski N. "Exploiting for Breast Cancer Control a Proposed Unified Mechanism for Reduction of Human Breast Cancer Risk by the Hormones of Pregnancy". American Association for Cancer Research, Frontiers in Cancer Prevention Meeting, 2005.

5. Andersen TT, Georgekutty J, Anaka I, Parikh R, Gildener-Leapman N, Jacobson HI, and Bennett JA. "A Peptide that Prevents Breast Cancer is Fully Active after Oral Administration: AFPep". American Association for Cancer Research, Frontiers in Cancer Prevention Meeting, 2005.

6. Bennett JA, Defreest L, Narendran A, Georgekutty J, Lemanski N, Jacobson HI, and Andersen TT. "Therapeutically cooperative and mechanistically distinct effects of an alpha-fetoprotein-derived peptide (AFPep) in combination with tamoxifen in models of breast cancer". American Association for Cancer Research Annual Meeting, 2006.

7. Mesfin FB, Andersen TT, Friedlich D, Popp AJ, Jacobson HI, and Bennett JA. "The effect of novel alpha-fetoprotein-derived peptide and tamoxifen on proliferation of cultured human glioblastoma cells". American Association of for Cancer Research Annual Meeting, 2006.

Mentoring

1. Mentoring medical students who have applied for and have been admitted to candidacy for the degree of Doctor of Medicine with Distinction in Research (MDDR).
   a. Anu Agarwal
   b. Rahul Parikh
2. Mentor for graduate students
   a. Lori DeFreest
   b. Leroy Joseph

CONCLUSIONS

We have replicated the five published procedures for inhibiting appearance of carcinogen-induced breast cancer in rats. In each of the five, statistically significant inhibition was noted. Clearly the truncated curves in Figure 1-5 indicate that maintaining the animals for longer observation would show even greater degrees of inhibition. We have used larger groups of rats than did most of the previous studies in order to produce firm results.

We have collected a total of about 120 serum samples taken at times straddling the intervals of treatment for each group. We have developed a western blot assay for rat AFP in serum, and have examined many of the samples that were collected.

The analysis provides qualitative AFP data (present or absent) and quantitative data showing the time course of AFP induction that each inhibition protocol produces. Quantitative data can show differences in the maximum serum AFP concentration obtained by each treatment group.

The carcinogen-treated rats in which cancer appearance was inhibited by administration of pregnancy-associated hormones all developed elevated pregnancy-like levels of serum alphafetoprotein, which persisted through the treatment period. This is in accord with our primary hypothesis.

This phenomenon is not unique to rat tissues. We have shown that in cell cultures, human HepG2 liver cancer cells secrete human AFP when challenged with these same hormones. Further, these AFP-containing culture media when added to cultures of human T47D breast cancer cells effectively retard the growth of the cells.

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