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13. ABSTRACT *(Maximum 200 words)*

The object of this proposal is to understand the tumor suppressor function of maspin, a novel serine protease inhibitor, and to test directly maspin as a therapeutic agent for breast cancer. Transgenic and knockout mouse models will be employed to study the effects of gain and loss of maspin function on mouse mammary tumorigenesis and development. We hypothesize that overexpression of maspin should be protective against mammary tumorigenesis and metastasis, while loss of maspin will render mice more susceptible to tumor formation and metastasis.

The proposal is based upon our previous experiments, primarily performed in conventional cell culture models, demonstrating that maspin has tumor suppressor activity. Recently, we have established transgenic mice overexpressing maspin in the mammary gland, and generated maspin knockout mice. We have crossed maspin transgenic mice with a breast tumor WAP-Tag strain. Our data suggest that maspin functions directly as a metastasis inhibitor. We have also shown in this report the mechanism by which maspin inhibits normal mammary development during pregnancy in WAP-maspin transgenic mice, and we are testing maspin against tumor progression in mice. Continuation of these tasks in the next few years will help us understand the role of maspin in tumor metastasis and angiogenesis, and hopefully leading to the development of new therapies for the treatment of breast cancer.

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FOREWORD

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Introduction

Breast cancer is the most common malignancy in western countries. Among women in the United States between the ages of 40 and 55 years, breast cancer is the leading cause of death (1). In the past, study of breast cancer has lagged behind other forms of cancer. Although the balance is being shifted, principally owing to women's awareness and increased funding, there are great gaps in our understanding of almost every aspect of this disease. Recently, The National Cancer Institute established a Breast Cancer Progress Review Group (BCPRG) to identify and prioritize scientific needs and opportunities that are critical to hasten progress against the disease. The proposal by this group has provided a guideline for individual breast cancer researcher to follow (Web site for this proposal: http://wwwosp.nci.nih.gov/planning/prg).

The object of this proposal is to understand the tumor suppressor function of maspin, a novel serine protease inhibitor, and to test directly maspin as a therapeutic agent for breast cancer. Transgenic and knockout mouse models will be employed to study the effects of gain and loss of maspin function on mouse mammary tumorigenesis and development. We hypothesize that overexpression of maspin should be protective against mammary tumorigenesis and metastasis, while loss of maspin will render mice more susceptible to tumor formation and metastasis. We will take advantage of the powerful tool of mouse genetics by crossing these mice with other well characterized mouse breast cancer models to test the tumor suppressor activity of maspin. Mammary tumorigenesis and normal mammary development will be studied using a variety of established techniques, including histopathology and whole mount analyses. Finally, we plan to deliver maspin locally as a drug to determine its therapeutic efficacy as an anti-tumor agent.

The following tasks were proposed for the first 12 month period of study.

Task 1. Analysis of the tumor suppressor activity of maspin by crossing WAP-maspin mice with WAP-Tag mice. Months 1-24. Total 100 mice will be used.

a. Generate four groups of mice by crossing WAP-maspin heterozygotes with WAP-Tag heterozygotes.
b. Continuous mating to activate the transgenes.
c. Collect mammary gland samples for histopathology and other studies.
d. Record tumor progression, and take tumor samples for histopathology.

Task 3. Tumor inhibition by local Evac implants of maspin. Months 6-20. Numbers of mice: 40

a. Prepare large quantity of recombinant GST-maspin protein and make maspin Evac pellets.
b. Implant pellets in the mammary fat pad near the site of mammary tumor development in WAP-Tag mice.
c. Biopsy of tumor samples, examine proliferation and apoptosis.

Task 4. Characterization of physiological functions of maspin by mean of targeted overexpression. Months 6-24. Number of mice: 40

a. Do proliferation and apoptosis experiments using mammary gland biopsies from WAP-maspin mice to study effects on lobuloalveolar development.
b. Deliver maspin pellets to the mammary gland of normal 4-6 week old virgin mice and post-lactation mice to study effects on ductal morphogenesis and involution.

Body

Materials and methods

Animals

WAP-Tag mice were provided by Dr. Priscilla Furth as a collaboration. WAP-maspin transgenic mice were established in this laboratory. Mouse tumors were provided by Dr. Dan Medina. All animals were maintained within the PI's animal facility at Baylor.

Antibodies

Polyclonal anti-maspin antibody was made by Zymed, Inc. as a custom service. Anti-Tag antibody was purchased from Pharmingen. All secondary antibodies were purchased from Zymed, Inc.

Northern and Western analysis

RNAs and proteins were isolated from mammary glands from virgin, pregnant, lactating stages. Total RNAs were isolated using Gibco/BRL Trizol regent. For northern blot, roughly 20 ug RNA will be loaded each lane. For Western blot analysis, protein extracts were prepared by lysing the cells in RIPA buffer. Total 100 ug protein extract will be loaded for electrophoresis.

Immunohistochemical analysis

Mammary glands were removed under anesthesia from normal and transgenic females at different stages of development. Mammary tissues were fixed in 10% neutral formalin buffer and embedded in paraffin and sectioned at 5 μm. For maspin immunostaining, tissues were boiled in citrate buffer (Zymed, Inc.) for ten minutes for antigen retrival. The antibody was produced in rabbit against a fifteen amino acid peptide located in the reactive site loop of maspin (AbS4A) (2). The antibody was purified using an AbS4A sulfo-linked affinity column (Sulfolink kit, Pierce, IL). The sections were stained with the affinity purified maspin antibody at a dilution of 1:400, followed by a secondary goat anti-rabbit antibody staining, and the color was developed by Zymed's AEC chromogen kit. For specific peptide blocking, a concentration of 10 nM of AbS4A peptide was preincubated with antibody for thirty minutes at room temperature. For PCNA staining, a PCNA staining kit was purchased from Zymed (Zymed, Inc., CA) and slides were stained following the instruction of the kit. The anti-Tag antibody (pharminogen) will be diluted 1:400 times and used as instructed by Pharmingen.

Results and Discussion

Task 1. Analysis of the tumor suppressor activity of maspin by crossing WAP-maspin mice with WAP-Tag mice.

WAP-Tag transgenic mice (3) that are highly susceptible to mammary tumorigenesis were mated with WAP-maspin to generate four groups of mice: Tag +/maspin+ (25), Tag+/maspin-(15), Tag-/maspin+(16), Tag-/maspin-(15). They were mated continously to activate transgene expression. Tumor progression data were recorded. Tumors first appeared in Tag+ mice about three months after first pregnancy, with 100% of them developed tumors by 6 months. Tumor growth was monitored by measurement with caliper biweekly and was followed until the tumor size reached 2.0 cm in diameter or tumor volume reached 10 % of body weight before the mice were sacrificed. At the moment, 80% of total mice were sacrificed and their samples harvested. Tumor free curves between biogenic and Tag+ were compared. No significant difference was observed for tumor incidence between these two groups (Fig. 1). We conclude maspin overexpression does not prevent tumor initiation. Lung tissues were collected from tumor mice. Tumor metastasis to lung was evaluated by examining lung sections by H&E staining. Our data showed tumor metastasis were significantly reduced in transgenic mice (26%) comparing to the Tag+ mice (67%). The incidence of metastasis will be further examined by immunohistochemical analysis using an anti-Tag antibody. Serial sections of lung were made to collect such data as incidence of metastasis and numbers of tumor foci per lung. The final number may vary after we finish collecting the remaining 20% of samples and analyzing immunohistochemical data.

These results indicated that overexpression of maspin in vivo can block tumor metastasis. At the moment, it is still too early to conclude that maspin does not inhibit tumor growth. We are currently analyzing the tumor growth curves of Tag+ mice and comparing them to biogenic mice.

The mechanism of metastasis inhibition in biogenic mice will be evaluated. Tumor sections will be analyzed for apoptosis rate and microvessel density. The TUNEL assay has been established in this laboratory for apoptosis study and CD31 immunostaining has also been established to examine the MVD.

One lesson we have learned from this study is that although WAP-Tag mice is a very good tumor model but it is not very amenable for metastasis study. It lacks marker for detecting lung metastasis and requires labor intensive multiple sectioning of lung samples and histology. Also, the rate of metastasis is not particular high comparing to other tumor model such as polyoma middle T antigen mice whose mets rate to lung can reach as high as 97% and metastasis can be quantitated by more accurate RNAase protection assay for milk genes (4). To study the effect of maspin gene dosage on mets rate and maspin mediated gene therapy, we plan to use polyoma middle T mice in the future.

Task 4. Characterization of physiological functions of maspin by mean of targeted overexpression. Months 6-24.

We have found that overexpression of maspin at midpregnancy inhibits mouse mammary gland development (Fig. 2). One hypothesis is that maspin may be involved not only in extracellular matrix

remodeling but also as a regulator for cell proliferation. On the other hand, over-expression of maspin may inhibit mammary gland development by inducing extensive apoptosis. To understand whether the induction of transgene expression caused any changes of alveolar cells in proliferation and apoptosis, PCNA staining and TUNEL assay were carried out with midpregnant mammary samples. Our data indicate that the maspin transgene does not change the proliferation rate, but increases significantly the apoptosis of alveolar cells. The increase was sustained even to the late stage of pregnancy when normal gland had little apoptosis (Table 1). Since milk protein genes can function as differentiation markers for the mammary gland, we compared their expression patterns in transgenic and wildtype control mice. Western blot analysis showed that WAP and β-casein were highly expressed in wild-type mammary gland at day 19 pregnancy and throughout the three stages of lactation. However, neither WAP nor β-casein was detected in our assay at day 19 pregnancy in transgenic glands as compared to control. The levels of both milk proteins were present in lactating day 1 transgenic glands, but at a reduced level. Following three days of lactation, WAP and β-casein levels in the transgenic mice increased to that of control (Fig. 3). The decreased expression of milk genes could arise from the reduced number of alveoli, as well as lower expression by each alveolar epithelial cell.

In summary, maspin transgene expression resulted in a decrease in both the number of lobular-alveolar structures and the size of each alveolar unit during pregnancy and early lactation, and this effect is due to the increased rate of apoptosis.

Task 3. Tumor inhibition by local Evac implants of maspin. Months 6-20.

We have prepared maspin Evac to delivery maspin to mammary tumors. Instead of using WAP-Tag tumor mice, we started by using Balb/c mice and tumor tissues isolated from syngenic Balb/c mice since this model was well characterized by Dr. Dan Medina (5). All tumors and mice were provided by Dr. Daniel Medina as a collaboration. The study was carried out as followings. Tumor tissues were microscopically dissected into pieces for implanting to the fat pad of 8 week old Balb/c female mice. They were allowed to grow for two weeks inside mammary gland. Maspin slow release Evacs were surgically implanted beside the tumor sites and the incisions were closed. Two weeks later, tumors were excised and their sizes were measured. Initial experiment showed maspin treated tumors have reduced size but due to the large variation of tumor size and small scale of first experiment, the difference was not proved to be statistically significant. In view of recent data from biogenic experiment, it is also possible that maspin may not inhibit tumor growth. Another possibility is that the delivery efficiency needs to be optimized. We are currently developing an adeno-maspin mediated gene delivery. Hopefully, we can determine the efficacy by which maspin inhibits tumor progression in the next 12-months.

Conclusion

All three tasks proposed in the grant were initiated in the first year of proposal. We have obtained very informative data suggesting maspin functions directly as a metstasis inhibitor. We have uncovered the mechanism by which maspin inhibits normal mammary development during pregnancy in WAP-maspin transgenic mice, and we are testing maspin against tumor progression in mice. Continuation of these tasks in the next few years will help us understand the role of maspin in tumor

metastasis and angiogenesis, and hopefully leading to the development of new therapies for the treatment of breast cancer.

Reference

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Figure legends

Fig. 1 Tumor initiation curves for Biogenic (Group 1) and Tag+ (Group2) mice. Most mice developed t in less than 30 weeks. No significant difference was observed in tumor initiation rate between two groups.

Fig. 2 Histological analysis of mammary tissues from the following mice: (A) wildtype at day 15 pregnancy; (B) transgenics at day 15 pregnancy; (C) wildtype at day 19 pregnancy; (D) transgenics at day 19 pregnancy. (E) and (F) (same magnification) were high power pictures from (C) and (D) respectively. Note the reduced numbers of alveolar structures and the smaller lumen size in the transgenics (D, F). Photographs were taken with a 10X objective for (A-D) and with a 40X objective for (E-F).

Fig.3 Western blot analysis of milk proteins in the mammary glands of transgenic and C57BL/6 mice. Lanes 1-4 were from C57BL6 mammary tissues and lanes 5-8 were from transgenic mice. Age-matched samples used were from day 19 of pregnancy (lane 1, 5); day 1 of lactation (lane 2, 6); day 3 of lactation (lane 3, 7); and day 10 of lactation (lane 4, 8). Aliquots of whole cell extracts (20 μg) were loaded on each lane and separated by 10% SDS-PAGE electrophoresis and transferred to PVDF membrane. The blot was probed first with WAP antibody and striped for a second probing with β-casein antibody. Staining of a duplicate protein gel by Coomassie blue confirmed equal loading of all samples.

Fig.1

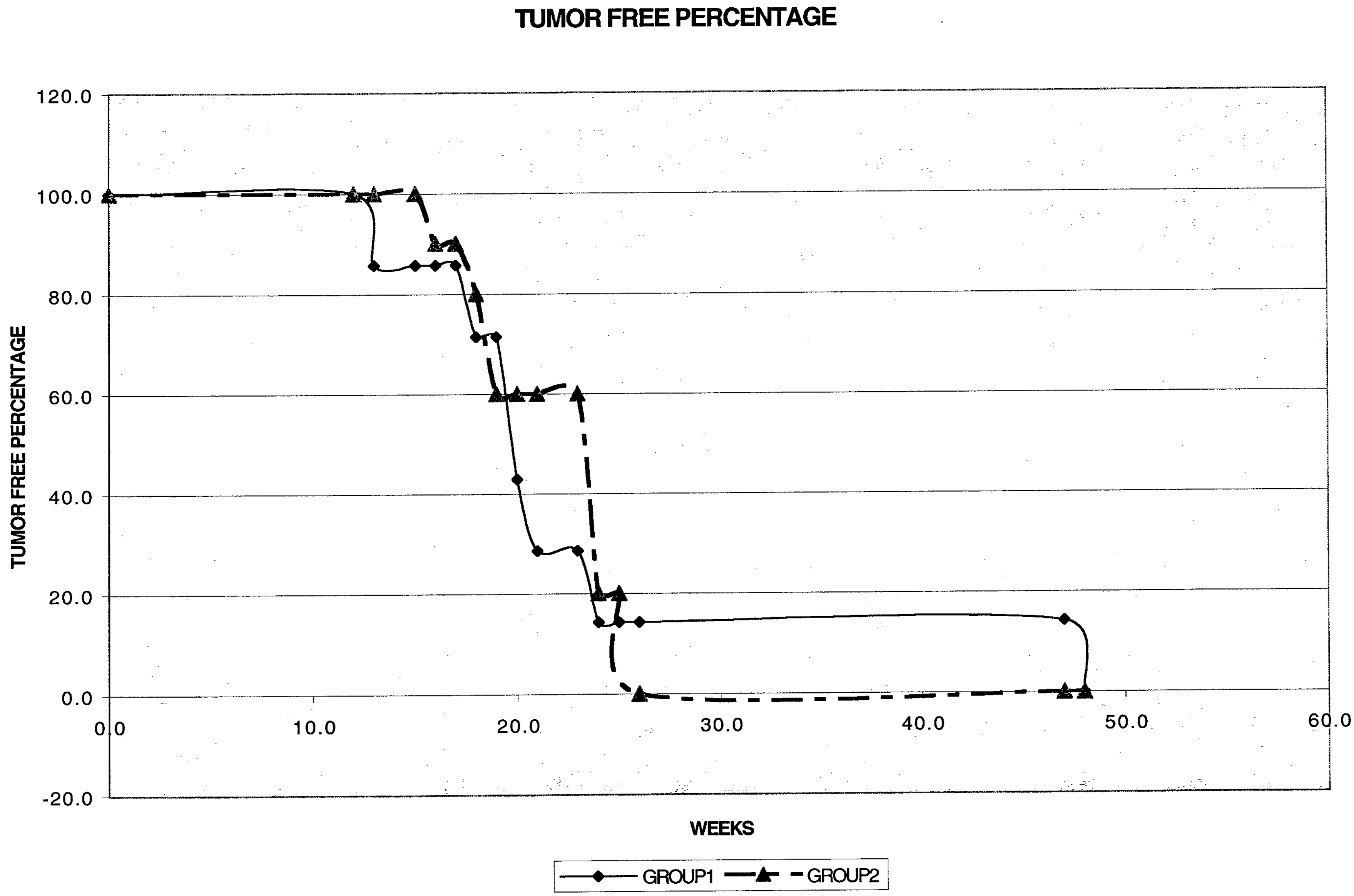

Fig.2

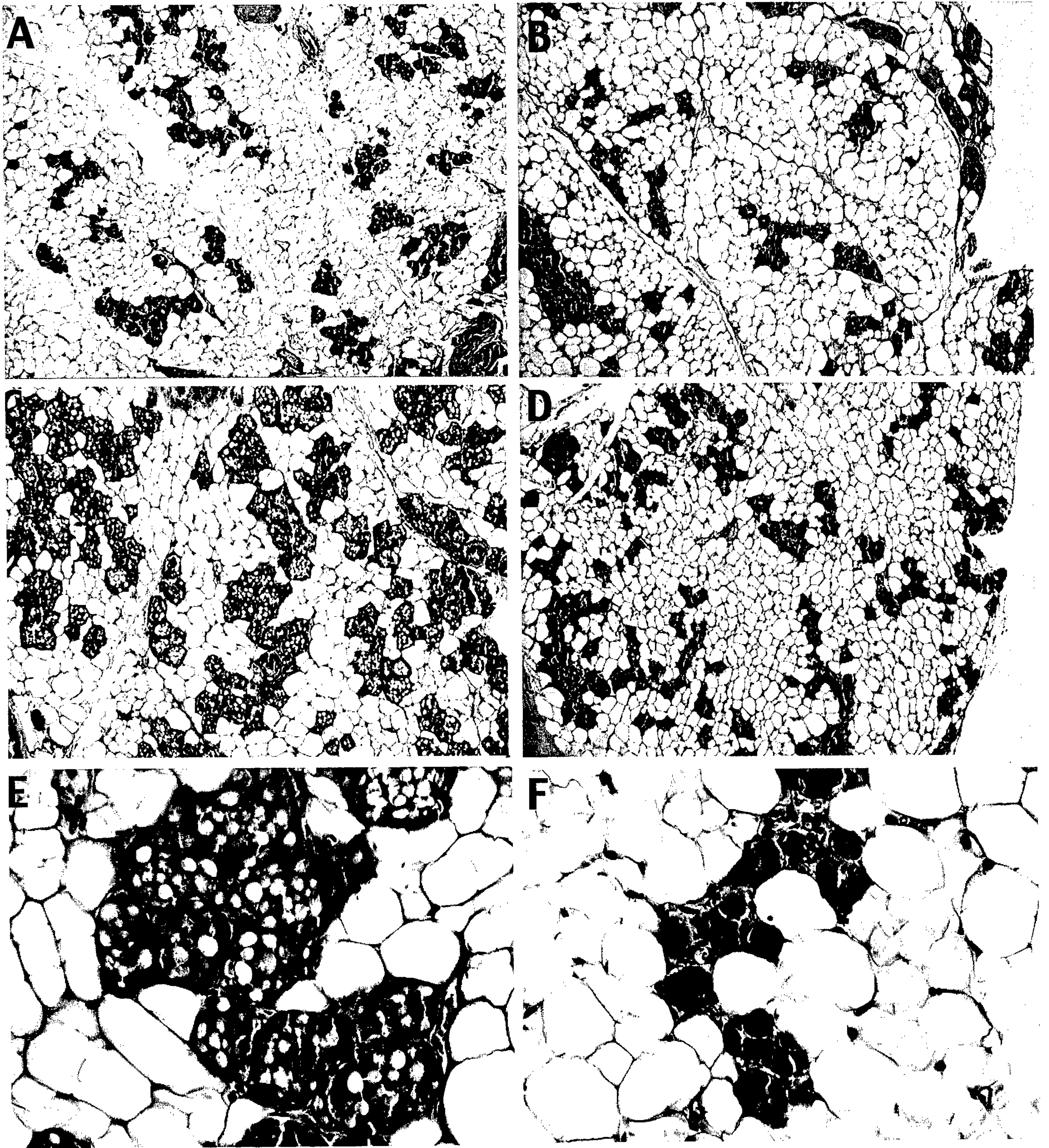

Fig.3

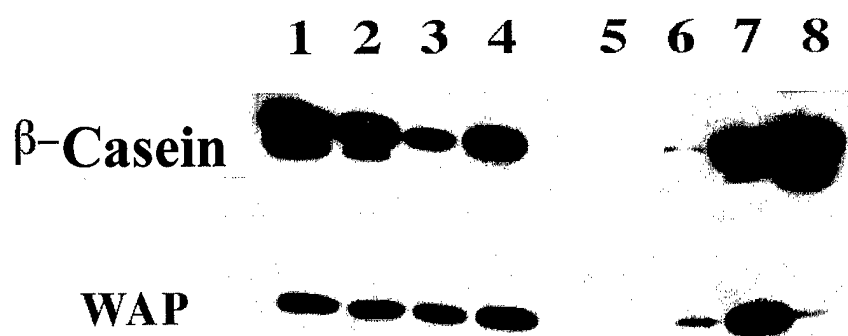

Table 1. Proliferation and apoptosis rate in normal and transgenic mice

		WT	Transgenic	P value
15 day Pregnant	Proliferation	11.55±1.85	10.50±2.58	$P < 1.0$
	Apoptosis	0.99±0.05	2.29±0.26	$P < 0.004$
19 day pregnant	Apoptosis	0.54±0.05	2.35±0.64	$P < 0.05$

Values are presented as mean ± SD. Each value was obtained from at least three samples. About 1000 cells per samples were counted .