J Mol Med (2006) 84:933–941
DOI 10.1007/s00109-006-0085-x

ORIGINAL ARTICLE

Apoptosis and tumor inhibition induced by human chorionic gonadotropin beta in mouse breast carcinoma

Shu-Qun Shi · Li Xu · Gang Zhao · Ying Yang · Jing-Pian Peng

Received: 14 March 2006 / Accepted: 1 June 2006 / Published online: 5 August 2006

Abstract For many breast cancer patients, human chorionic gonadotropin beta (hCGβ), which is a subunit of a hormone produced by the trophoblast and is essential for maintaining pregnancy, is expressed in the breast cancer cells. However, the mechanism as to how the CGβ in cell affects the cancer development is not very clear. Mouse breast carcinoma 4T1 with stably hCGβ expression (4T1-hCGβ) was established and transplanted into the Balb/c mouse abdominal mammary gland. hCGβ suppressed breast cancer cell viability in vitro, and dramatically inhibited tumor growth and attenuated tumor vessel formation in vivo. An 86–88% reduction in tumor volume in animals injected with breast cancer expressing hCGβ, as opposed to those injected with breast cancer without hCGβ expression, was observed. The production of p21 was promoted by hCGβ, whereas the Cdk2 was decreasing. These indicate that p21 signal pathway is involved in this process. Significant apoptosis was also detected in hCGβ-expressing breast cancer cells as well as the enhancement of Bax protein expression. Moreover, hCGβ blocked the blood vessels formation by inhibiting the expression of MMP9 and VEGF. Further hormone secretion analyses show that the anti-tumor activity induced by hCGβ is not related to the endocrine function.

S.-Q. Shi · L. Xu · G. Zhao · Y. Yang · J.-P. Peng (✉)
Molecular Immunology Group,
State Key Laboratory of Reproductive Biology,
Institute of Zoology, Chinese Academy of Sciences,
Beijing 100080, People's Republic of China
e-mail: pengjp@ioz.ac.cn

S.-Q. Shi · L. Xu · G. Zhao
Graduate School of the Chinese Academy of Sciences,
Chinese Academy of Sciences,
Beijing 100080, People's Republic of China

SHU-QUN SHI
is currently a Ph.D. student at the Institute of Zoology, Chinese Academy of Sciences. His research interests are genetic immunization and signal pathway in tumorigenesis.

JING-PIAN PENG
received his Ph.D. in physiology from the China Agriculture University. He is presently a professor of physiology at the Institute of Zoology, Chinese Academy of Sciences. His research is focused on reproductive biology and immune regulation in pregnancy.

Keywords Breast cancer · Tumor model · hCGβ · Tumor repression

Abbreviations

hCGβ	human chorionic gonadotropin beta
GFP	green fluorescent protein

Introduction

Breast cancer is the most frequent spontaneous malignancy diagnosed among women in the western world. With the

development of economy and society, this disease is continuously increasing in incidence in many large Chinese cities [1, 2]. All tested breast cancer cell lines and 76% primary breast tumors express hCGβ [3]. Further study shows that the expression of hCGβ from specific encoding genes has independent, prognostic value for relapse-free survival after surgery in breast cancer, and the hCGβ-3/β-, hCGβ-5/β-, and hCGβ-8/β-actin ratios were borderline, significantly associated with ER status [4]. It is reported that treatment with hCG inhibits rat mammary carcinogenesis induced by the chemical carcinogen 7,12-dimethylbenz anthracene (DMBA) through activation of programmed cell death [5]. Treatment of MCF-7 human breast cancer cells with highly purified hCG in vitro results in a modest dose-dependent and hormone-specific decrease in cell proliferation [6]. However, very little is known about hCGβ's role in breast cancer and by which mechanisms hCGβ affects breast cancer tumorigenesis in vivo.

4T1 is a cell line derived from the poorly immunogenic Balb/c spontaneous mammary carcinoma. This tumor shares many characteristics with human mammary cancers, making it an excellent animal model [7]. Moreover, 4T1 is a 17ß-estradiol-nonresponsive mammary cancer cell [8]. hCGβ was transfected into 4T1 cells and the stable cell line was established for inoculation into the immunocompetent mouse to evaluate the impact of hCGβ on breast tumorigenesis.

Breast cancer incidence rates are influenced by many factors. It has been firmly established in epidemiological studies that early full-term pregnancy affords lifetime protection against the development of breast cancer [9]. Despite a wealth of literature supporting the role of endocrinological processes in mediating parity-related refractoriness, the cellular and molecular mechanisms that underlie hormone-induced refractoriness are largely unresolved [10–12].

Recently published data demonstrate that p53 plays a pivotal role in hormone-induced protection, and that some steroid hormones functionally activate p53 in mammary epithelial cells [13, 14]. The absence of p53 function increases risk for spontaneous tumorigenesis in the mammary gland, and hormonal stimulation enhances tumor risk in p53-null mammary epithelial cells as well as the incidence of aneuploidy [15].

To test whether the p53 pathway also contributes to the inhibition of the breast cancer growth induced by hCGβ, the expression of p53 and p53-regulated molecule p21 is also investigated in this study. As a potent Cdk inhibitor, p21 is commonly perceived to be the major effector of p53-mediated cell cycle arrest [16]. Mammalian cell growth is governed by a series of Cdks. p21 can inhibit these kinases in vitro so as to invoke G1 and G2-M growth arrest [17, 18].

Bax, which is a proapoptotic Bcl-2 family member, acts as a critical downstream effector of the p53-mediated apoptotic pathway. Bax mediates apoptosis triggered by Myc in lymphoma, and this function is related to the mdm2-p53 pathway [18]. Breast cancer apoptosis induced by curcumin was also accompanied by an increase in p53 level followed by Bax expression level [20].

In the present study, we report the generation of an animal model of breast carcinoma in the Balb/c mouse overexpressing hCGβ. This engineered murine model offers a valuable tool to investigate the effects of hCGβ in the biology of breast carcinoma and tumor–host interactions in vivo.

Materials and methods

Construction of engineered 4T1 cell lines

Plasmid containing hCGβ cDNA was digested with *Hin*dIII and *Bam*HI, and hCGβ cDNA fragment was inserted into the *Hin*dIII and *Bam*HI sites of pGEFP-N1 (Clontech). The desired recombinant was confirmed by restriction analysis and sequencing. Vector constructs for transfection were purified with an endotoxin-free plasmid mega kit (Qiagen).

4T1 mammary tumor cells was purchased from American Type Culture Collection (ATCC) and maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL) supplemented with 10% fetal bovine serum (FBS, Hyclone). Cells were individually transfected with 2 μg of plasmid containing hCGβ and mock vector alone using the lipofectamine 2000 (Invitrogen) reagent by its technical illustration. After 1 day, the transfected cells were split 1:20. Selection was initiated with 600 μg/ml Geneticin G418 (determined by the kill-curve assay) in the culture medium to select the cells that express resistance to this marker 2 days after transfection.

Flow cytometry and cell sorting

Four weeks later, green fluorescent protein (GFP) positive cells were isolated from transfected cells using FACScan (Becton Dickinson). After positive selection, the cells were cultured for expansion. 4T1 stably expressing GFP and hCGβ/GFP was named 4T1-GFP and 4T1-hCGβ, respectively. Before inoculation, the cells were analyzed by cell sorting using CellQuest flow cytometry analysis software (Becton Dickinson) [21]. The positive cells were examined under a fluorescent microscope (Nikon) or confocal microscope (Zeiss).

In vitro cell proliferation assay

The effects of hCGβ on cell proliferation were estimated with an MTS assay using the CellTiter 96 aqueous one-solution cell proliferation assay (Promega), which is a colorimetric method for determining the number of viable cells. Cells were plated in 96-well plates at 5×10^3 cells per well in DMEM supplemented with 10% FBS. Subsequently, the MTS assay reagent was added to each well on 1 day, 2 days, and 3 days after cell culture, and absorbance at 490 was measured using a microplate reader (Bio-Rad, 3550). Each measurement was made in triplicate, and the results were described as a ratio of transfectants vs untreated control cells.

To assess the cell viability with crystal violet assay, 5×10^4 cells were seeded on six-well plate per well. Two days later, the cells were washed with cold phosphate-buffered saline) PBS and stained with 0.5% crystal violet (Sigma).

In vivo tumor growth

The female Balb/c mice were purchased from the Institute of Genetics, Chinese Academy of Sciences. Mice were kept under controlled conditions of light and temperature, with free access to a standard mouse chow and water. All experiments were conducted according to the guidelines of the Beijing Animal Care for Laboratory Animals. Mice were injected intramuscularly in the abdominal mammary gland with parental or transfected 4T1 tumor cells, as reported [22]. Primary tumors were measured every 3 or 5 days after tumor inoculation, using vernier calipers. Animals were killed 3 weeks after inoculation, and the primary tumors were surgically removed and weighed.

Annexin V–propidium iodide (PI) staining

Detection of early apoptotic cells was performed by means of the annexin V–PI detection kit (Baosai, Beijing, China). Briefly, single tumor cells were prepared and approximately 10^6 cells were washed with cold PBS, incubated in the dark at 4°C with annexin V-FITC and PI for 15 min, and then analyzed by dual-color flow cytometry. Cells that were annexin V-FITC-positive and PI-negative were considered as early apoptotic cells.

Immunohistochemistry analysis of tumor cell proliferation and vessel formation

To perform histological analysis, primary tumors were rinsed and fixed in 4% paraformaldehyde, infused with 20% sucrose overnight, and frozen in liquid nitrogen. Frozen tumor tissues were sectioned in 80μm slices. Sections were stained with hematoxylin and eosin. To assess proliferation and angiogenesis in tumor, the sections were stained with the proliferating cell nuclear antigen (PCNA) antibody (Zymed) and CD31 antibody (Zymed). Briefly, sections were treated with hydrogen peroxide to inhibit endogenous peroxidase. After washing with PBS, the slides were blocked with 5% normal goat serum. Then the samples were stained with PCNA monoclonal antibody (mAb, 1:75) or CD31 monoclonal antibody (1:75) diluted in PBS supplemented with 0.5% normal goat serum. Goat anti-mouse IgG conjugated with horseradish peroxidase (HRP) (Jackson) was used as a signal generator and diaminobenzidine as substract chromogen. All sections for immunohistochemistry were counterstained with hematoxylin. As a negative control for specificity, 5% goat serum was used in place of primary antibody.

Western blotting

Breast tumor protein extracts were prepared in RIPA buffer [1×PBS, 1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitor cocktail (Roche)]. After brief sonication and centrifugation, the supernatant was collected for Western blotting. The protein concentration of the supernatant was measured (dye reagent concentrate, Bio-Rad), and approximately 50 μg of denatured protein was separated on 12.5 or 10% SDS-PAGE and electroblotted onto nitrocellulose membranes (Amersham Pharmacia). After blocked with 3% nonfat dry milk, the membrane was incubated with goat anti-MMP9 ploYclonal antibody (C-20, SantCruz), rabbit anti-p53 ploYclonal antibody (FL-393, SantCruz), rabbit anti-Cdk2 ploYclonal antibody (M2, SantCruz), mouse anti-p21 mAb (F-5, SantCruz), mouse anti-Bax mAb (B-9, SantCruz), mouse anti-Mdm mAb (SMP14, SantCruz), mouse anti-VEGF mAb (C-1, SantCruz). The membrane then was washed and incubated with horseradish peroxidase (HRP) conjugated to rabbit anti-goat, goat anti-rabbit, or goat anti-mouse IgG (Jackson). Detection by the chemiluminescence reaction was carried using the ECL kit (Pierce).

Transfected cell lysates were prepared by sonicating followed by centrifugation. Similar Western blots were carried out to detect hCGβ expression using the anti-hCGβ mAb (Calbiochem).

Hormonal analyses

As hCGβ is a functional subunit of an important hormone, to test the possibility of whether the repression of tumor growth is caused by the autocrine effect, the hCGβ level in culture medium and the sera following 4T1-hCGβ tumor injection were detected with immunoradiometric assay. Progesterone and estradiol in mouse serum were measured using the Diagnostic Products Corporation (DPC, Los

Angeles, CA, USA) immunoradiometric Coat-a-Count assay for progesterone and estradiol as described [23]. Standard and samples (50 μl in duplicate) were analyzed and counted using a Packard COBRA γ-counter to 2% efficiency. The data were calculated using the COBRA software.

Data analysis

The values except special explanation are reported as mean± SEM. Statistical analysis was performed using one-way analysis of variance (ANOVA). Differences were considered statistically significant with $p<0.05$.

Results

Stable hCGβ expression in 4T1 cells

The hCGβ cDNA was successfully inserted in the pEGFP-N1 backbone upstream of enhanced GFP. The transfected cells were selected with Geneticin G418 and isolated by cell sorting. After approximate 20 passages, 97.8% GFP-positive cells were detected in the cultures transfected with pEGFP-hCGβ by flow cytometry analysis (Fig. 1a).

The results of microscopy showed that the 4T1 breast carcinoma cells stably transfected with recombinant con-

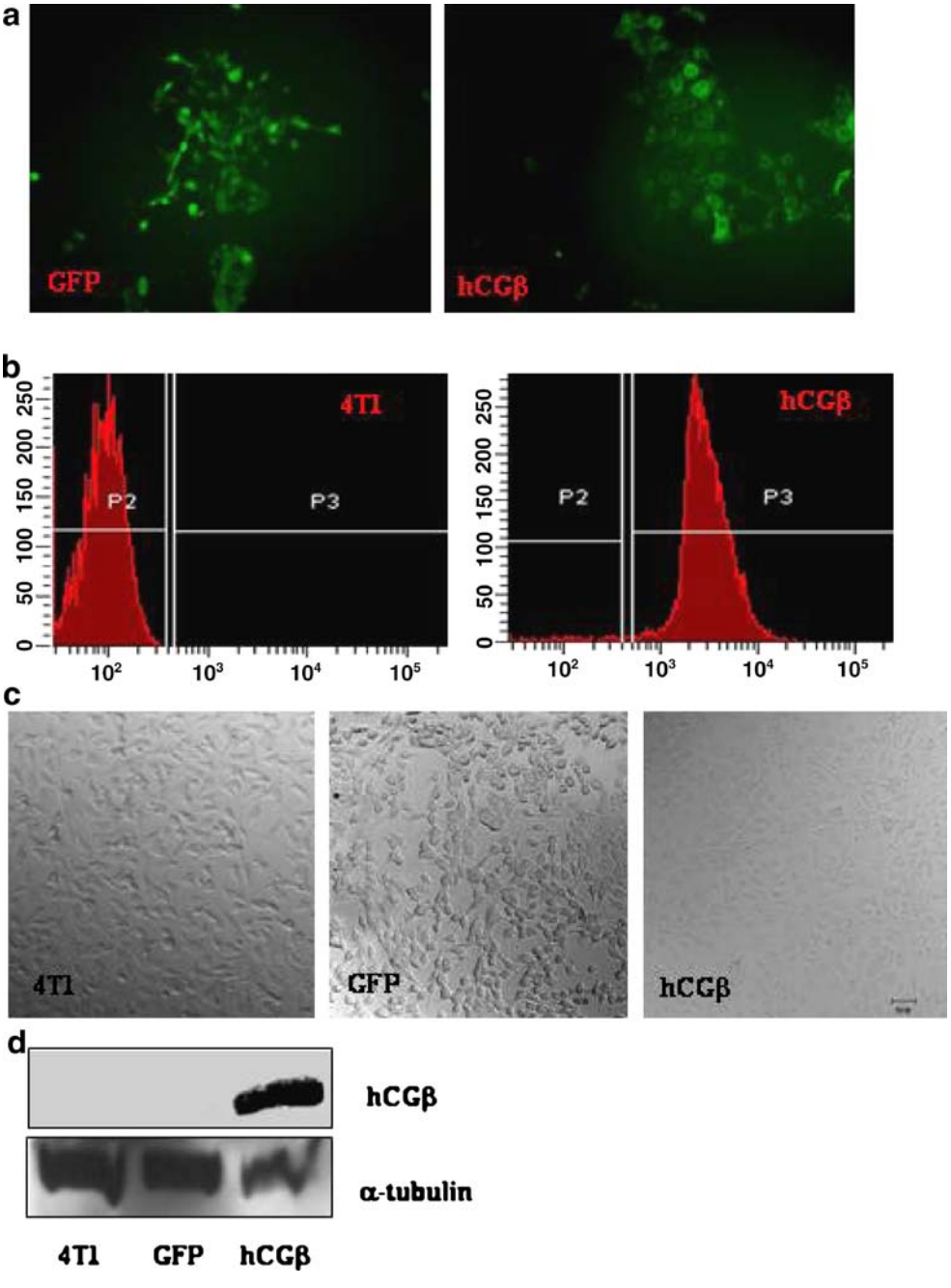

Fig. 1 Stable expression of hCGβ in 4T1 cells after transfection and sorting. 4T1 cells were transfected with pEGFP or pEGFP-hCGβ. G418 geneticin selection and flow cytometry isolation were performed to obtain the stably transfected cells. **a** GFP-positive engineered 4T1 cells after isolation are observed under a fluorescent microscope. Magnification: ×100. **b** Flow cytometry assessment of the purity of cells sorted based on hCGβ/GFP expression. The purity of the population is 97.8% (gate P3). **c** The morphology of 4T1 cells, mock-transfected cells, and cells that are transfected with cDNA encoding hCGβ/GFP are observed with confocal microscope. *Bar*=50 μm. **d** Total hCGβ protein level was analyzed by Western blotting. The expression of α-tubulin served as an internal control

struct could express hCGβ protein (Fig. 1b). These cells expressing hCGβ were somewhat dispatched and flat compared with the mother 4T1 cells and the cells only expressing GFP (Fig. 1c). As shown in Fig. 1d, the correct expression of hCGβ-GFP fusion protein was identified with the specific antibody by Western blotting assay.

The effects of hCGβ on 4T1 cell proliferation

4T1 parent cells and the engineered cells were incubated for 24, 48, and 72 h, and the cell proliferation and viability was determined using the MTS and crystal violet assay. MTS assay and crystal violet staining revealed that hCGβ significantly inhibited 4T1 breast cancer cell proliferation, whereas no statistical difference of cell viability was observed between the 4T1 and 4T1-GFP groups (1 day: p=0.253, 2 days: p=0.073, 3 days: p=0.172) (Fig. 2).

hCGβ in cell dramatically inhibits primary tumor growth

From Fig. 3, 3 weeks after 10,000 tumor cells were injected into the mammary gland of Balb/c mouse, the result of tumor weight showed that the expression of hCGβ significantly inhibited the growth of primary breast tumor

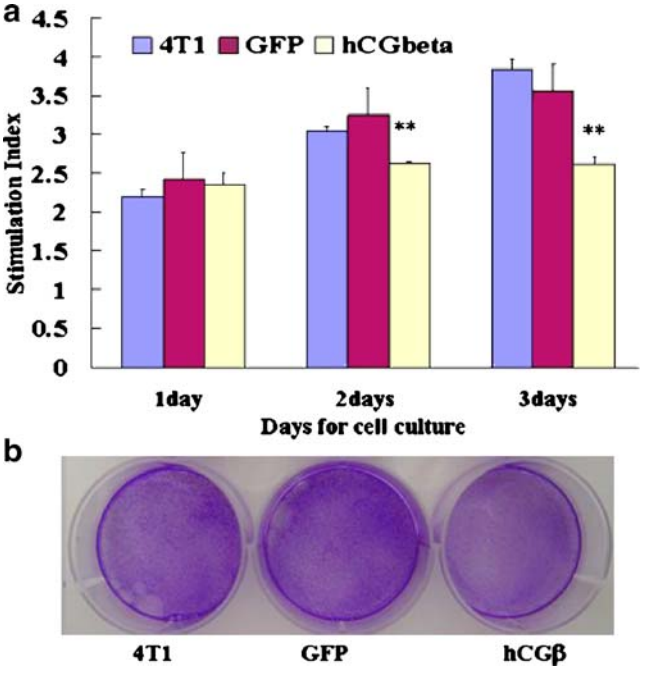

Fig. 2 Cell proliferation and viability assays. **a** 4T1 and engineered 4T1 cells stably transfected with pEGFP or pEGFP-hCGβ were cultured in incubator for 1, 2, and 3 days, and cell proliferation were determined using an MTS assay. Results are expressed as a ratio of transfected viable cells vs untreated control cells (1) and shown as mean±SEM. **b** 0.5% Crystal violet staining of 4T1, and 4T1 cell expressing GFP or hCGβ/GFP after 2 days culture. One representative photo is shown

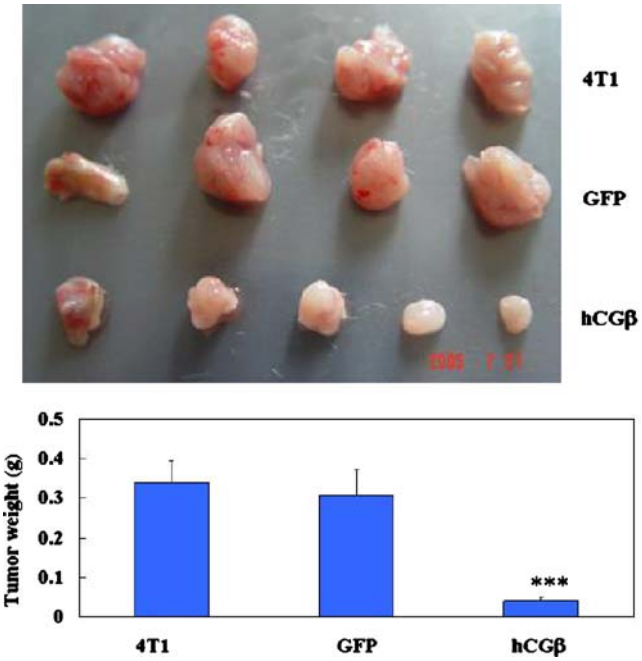

Fig. 3 hCGβ expression in tumor dramatically inhibits primary tumor growth in vivo. Three weeks after the mice were inoculated with 10^4 parent or genetically modified 4T1 cells, primary tumors were excised, photos were taken (**a**), and the tumors were weighted (**b**). ***p<0.001

(p<0.001). No significant difference of tumor growth was observed between the parent 4T1 and 4T1 expressing GFP alone (p=0.657).

Effect of hCGβ on tumor vessel density and tumor cell proliferation

From the sections stained with hematoxylin and eosin (H&E), we found that the nucleoli of hCGβ-expressing tumor cells were irregular and somewhat deeply stained. Tumor necrosis was also observed in some areas of 4T1-hCGβ slices (Fig. 4a–c). Tumor sections were stained for CD31 to allow vessels to be counted. Microvessel density was reduced in hCGβ-expressing tumors as compared with control tumors (Fig. 4d–f). To investigate whether hCGβ expression has indirect effects on tumor cell proliferation, we evaluated the proliferating tumor cells by immunohistochemical staining for PCNA. Figure 4g–i showed that hCGβ-expressing breast tumors demonstrated less proliferating (PCNA-positive) tumor cells than did tumors in the 4T1 and 4T1-GFP group. However, there were no differences in morphology and no obvious differences detected by immunostaining for CD31 or PCNA between 4T1 and 4T1-GFP. Moreover, further immune blotting assay also demonstrates that the expression of MMP9, which accelerates breast tumor growth and angiogenesis, and VEGF, which performs the function to promote blood vessel formation, are decreasing in 4T1-hCGβ tumor (Fig. 6b).

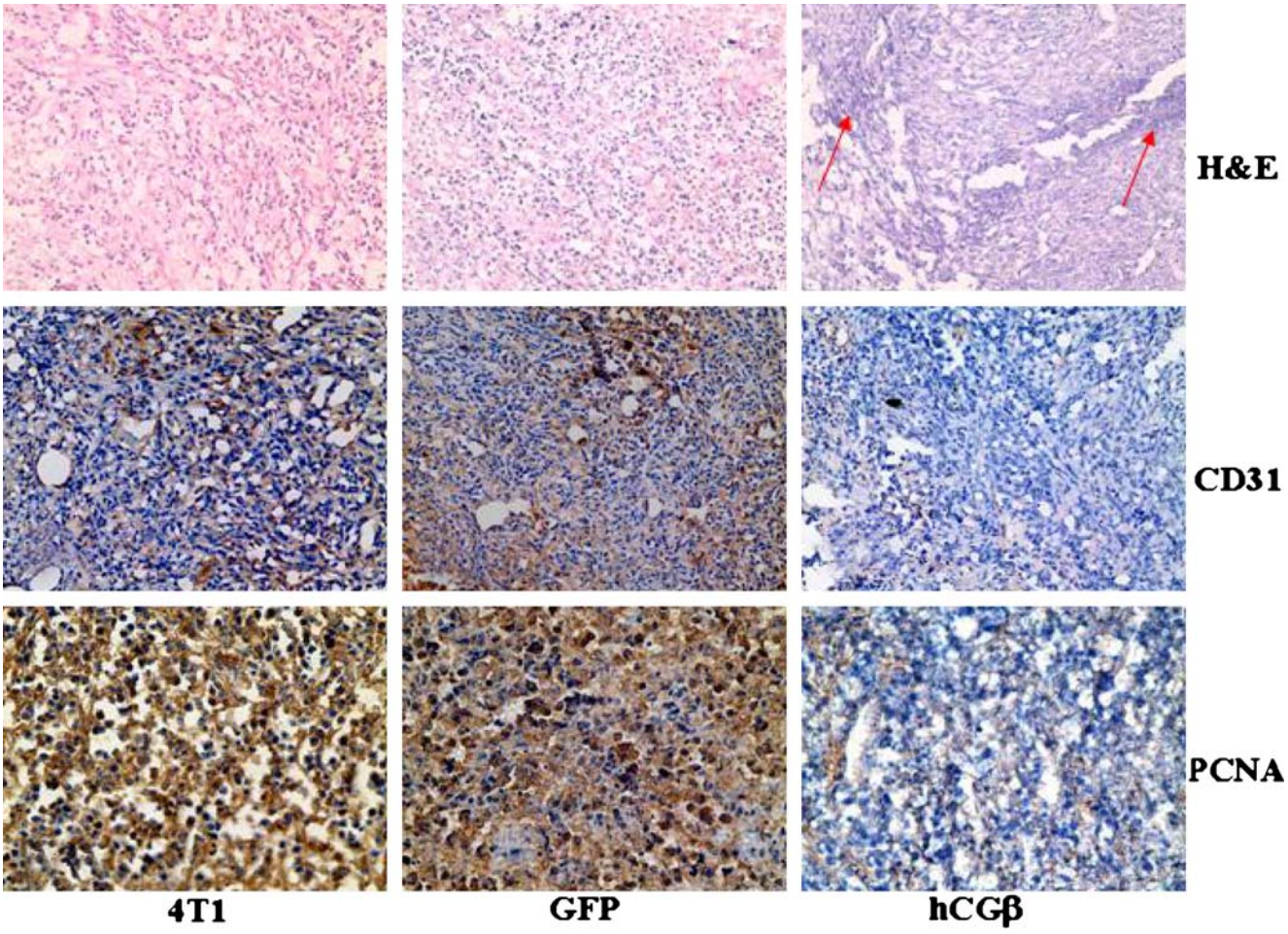

Fig. 4 Immunohistochemical analysis of vessel density (CD31) and tumor cell proliferation (PCNA) in breast tumors. Frozen tumor sections were stained with H&E (**a**–**c**), anti-CD31 antibody (**d**–**f**), and anti-PCNA antibody (**g**–**i**), and counterstained with hematoxylin to visualize nuclei. Images were obtained at ×200 (H&E, CD31) or at ×400 (PCNA) magnification. *Red arrow* denotes the irregular nucleoli and necrosis area

Apoptosis induced by HCGβ through an increase of Bax

Apoptosis in tumor cells after stable transfection with hCGβ was determined by annexin V/PI staining. Little or no apoptosis was seen in parental 4T1 or 4T1 expressing only GFP tumor cells, whereas considerable cell death was detected in the 4T1-hCGβ engineered tumor (Fig. 5). Further investigation showed that the Bax protein level was enhanced in the 4T1-hCGβ tumor cells (Fig. 6a).

Expression of hCGβ down-regulates the proteins involved in p53 pathway

To determine whether p53 pathway is involved in the process against breast cancer induced by hCGβ, immunoblotting was performed to detect the expression of p53, p21, Mdm2, and Cdk2 (Fig. 6a). Compared with parental tumor cells and cells with mock vector alone, the p53, p21, and Mdm2 signals of tumor cells expressing hCGβ were considerably higher. However, the expression of Cdk2 was down-regulated by hCGβ. This indicates that p53 pathway play important roles in anti-tumor mechanism associated with hCGβ stable transfection.

Effect of hCGβ in tumor on hormone secretion

The culture medium and the sera after 4T1-hCGβ tumor injection were detected by immunoradiometric assay; no significant hCGβ was measured ($p>0.05$). In addition, no estradiol in sera was detected either in mice with 4T1-GFP tumor cells or in mice with 4T1-hCGβ. Moreover, no statistic differences of progesterone in sera were observed between the mice that had been inoculated with breast cancer with and those without hCGβ ($p=0.636$). This implies that hCGβ reducing breast cancer in mice is not relative to the endocrine function.

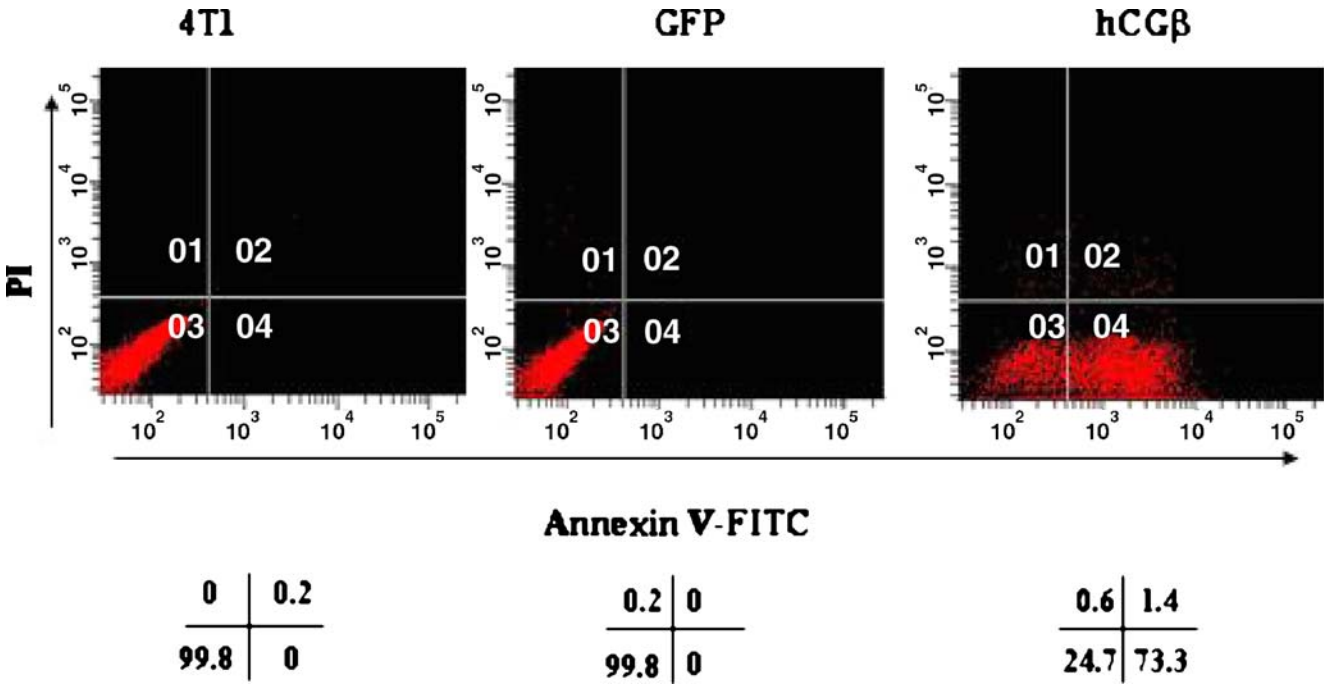

Fig. 5 Measurement of phosphatidylserine (PS) externalization and propidium iodide (PI) exclusion on tumor cells. Single tumor cell suspension was prepared and stained with FITC-Annexin V and PI. After incubation in the dark at 4°C for 15 min, the cells were analyzed by flow cytometry. The data shown are representative of two or three experiments

Discussion

In previous reports, human chorionic gonadotropin and its beta subunit may exert multifaceted functions on breast cancer cells through induction of apoptosis, inhibition of cell proliferation by inhibiting NF-κB and AP-1 activation [6, 24]. Although such interactions may be partly studied in cultured cell lines in vitro or in nude mouse model transplanted with human cancer in vivo. An animal model using immunocompetent mouse is best suited to investigate the mechanism of CGβ on breast tumorigenesis and growth. In this study, we develop a model of mouse breast carcinoma with stable expression of hCG beta subunit in the Balb/c mouse. 4T1 breast cancer is derived from Balb/c mouse, and its growth characteristics parallel highly invasive human metastatic mammary carcinoma, and at the time of surgery, the extent of disease is comparable with human stage IV breast cancer [7].

Inoculation of these genetically modified cells into mouse may be used as an ideal model to explore the effects of CGβ in the biology of breast carcinoma. In this study, we demonstrate that the expression of hCGβ in breast cancer cell could dramatically inhibit the growth of primary tumor. Immunostaining assay for CD31 showed that the vessel formation was suppressed by hCGβ, while the angiogenesis is essential for tumor growth and metastases formation [25]. As it is reported that solid tumor growth depends on the development of new blood supply, which is supported by VEGF [26], and increasing evidence suggests that MMP9 contributes to the formation of a microenvironment that promotes tumor growth during the early stages of tumorigenesis [27], the decreasing expression of MMP9 and VEGF further suggests that hCGβ inhibits breast tumor growth, and this correlates to reducing the angiogenesis.

First described in 1979, and initially believed to be an oncogene, p53 was the first tumor suppressor gene to be identified. p53 is functionally active to promote cell cycle arrest and provide temporal assistance for DNA repair to eliminate and inhibit the proliferation of abnormal cells, thereby preventing neoplastic development. Abrogation of the negative growth regulatory functions of p53 occurs in many, perhaps all, human tumors [28]. p53 plays pivotal roles in hormone-induced protection in mammary tumorigenesis [14, 29]. Absence of p53 is sufficient to cause spontaneous development of mammary tumors [15]. The expression of p53 in 4T1 was ambiguous; however, in a recently published paper, p53 has been proved to express in this mammary cancer cell line [30]. On the basis of our current findings, the expression of p53 was indeed detected in 4T1 cells, and hCGβ stimulates the accumulation of p53, Mdm2, and p21 proteins. This result is indicative of p53 activation. Meanwhile, the Cdk2 was decreasing in hCGβ-expressing tumor cells. Apoptosis triggered by hCGβ transgene is associated with the enhancement of Bax. Therefore, we conclude that p53 signal pathway is critical

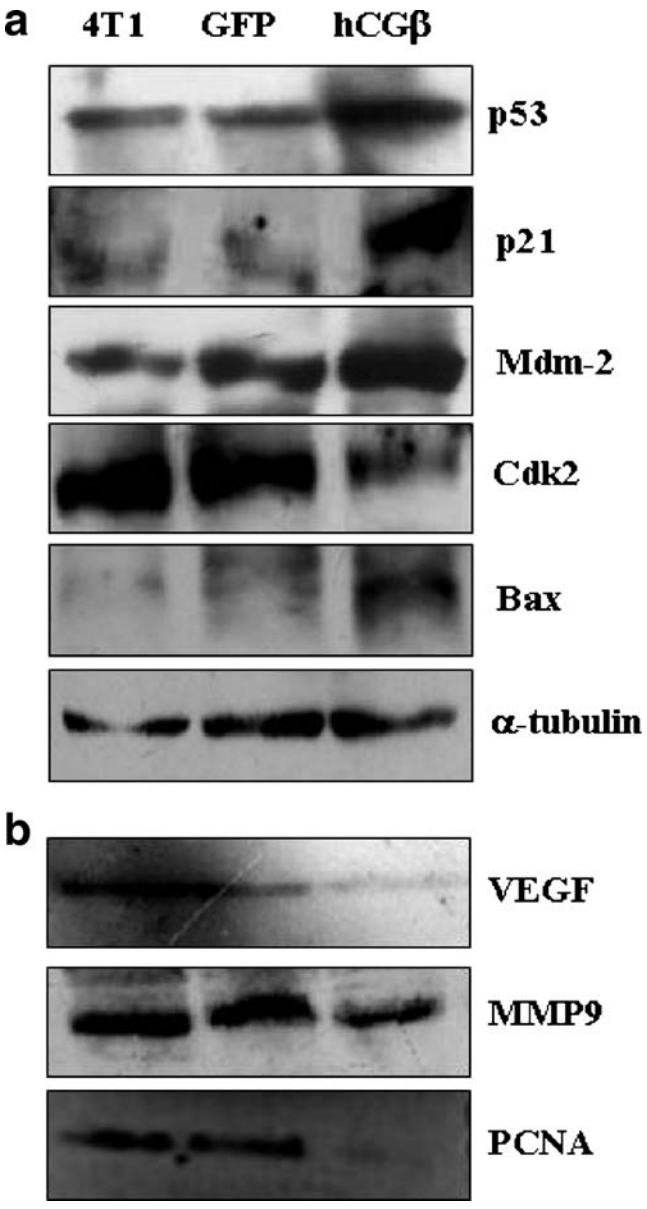

Fig. 6 Western blotting analysis of protein expression regulated by hCGβ in vivo. Fifty micrograms total cellular proteins isolated from the primary tumors were separated by 10 or 12.5% SDS-PAGE and then transferred onto a nitrocellulose membrane. Immune blotting of the membrane was performed using the p53, p21, Mdm2, CDK2, and Bax (**a**) and VEGF, MMP9, and PCNA antibody (**b**). The expression of α-tubulin served as an internal control

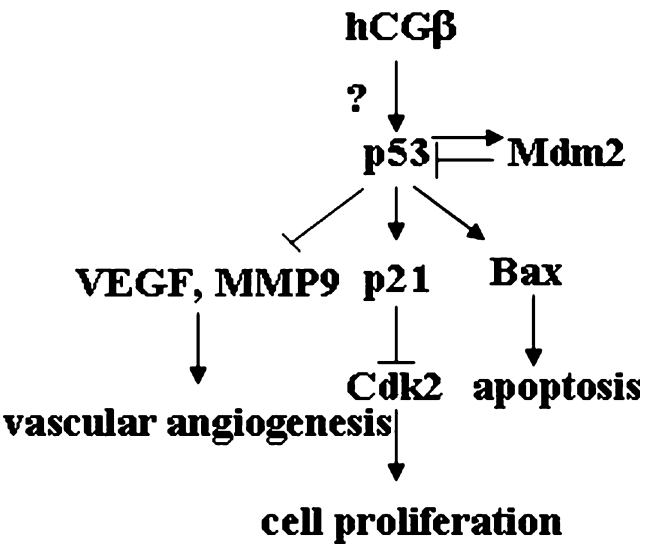

Fig. 7 Model depicting the possible signal pathway of hCGβ-induced tumor suppression and apoptosis in 4T1 breast cancer

in hCGβ-induced tumor suppression and apoptosis in 4T1 breast cancer.

As the model described in Fig. 7, we hypothesize that hCGβ activates p53 expression by an unknown mechanism. As hCGβ expresses in cytoplasm, it cannot transactivate the p53 promoter; its action maybe indirect. Once activated, p53 would be expected to induce expression of p21 and Bax. The basis of the sustained growth arrest by p21 may depend on its ability to inhibit Cdk2 and prevent aberrant DNA replication, because Cdk2-dependent kinase activity is high in endoreduplicating cells [31]. Bax is an essential element of the p53-dependent apoptosis pathway. The enhancement of Bax would trigger the cell death. Another interesting observation is that hCGβ can reduce the angiogenesis through inhibition of MMP9 and VEGF production. It is reported that p53 can inhibit VEGF expression by regulating the transcriptional activity of Sp1 and also by down-regulating the Src kinase activity in mammary carcinoma [31].

To rule out the possibility that the repression of tumor growth induced by hCGβ is caused by the autocrine effect, the culture medium and the sera after 4T1-hCGβ tumor injection were detected with immunoradiometric assay; no significant hCGβ was measured. Moreover, no statistic differences between estrogen and progesterone in sera were observed between the mice that had been inoculated with breast cancer with hCGβ and those without. This implies that hCGβ reduces the breast cancer in mice is not relative to the endocrine function.

In summary, hCGβ suppresses the breast cancer growth both in vitro and in vivo. In addition, hCGβ triggers the cell apoptosis via Bax enhancement. These functions generated by hCGβ are associated with p53 activity. Also, this study may provide a potential therapeutic strategy for breast cancer therapy through induction of the anti-tumor activity based on hCGβ.

Acknowledgement This work was supported by the grants from "863" Project of China Ministry of Science and Technology (2004AA215182) and Key Innovation Research Programs of the Chinese Academy of Sciences (KSCX2-SW-201).

References

1. Parkin DM (2001) Global cancer statistics in the year 2000. Lancet Oncol 2(9):533–543
2. Gao RJ, Bao HZ, Yang Q, Cong Q, Song JN, Wang L (2005) The presence of serum anti-p53 antibodies from patients with invasive ductal carcinoma of breast: correlation to other clinical and biological parameters. Breast Cancer Res Treat 93(2):111–115
3. Taback B, Chan AD, Kuo CT, Bostick PJ, Wang HJ, Giuliano AE, Hoon S (2001) Detection of occult metastatic breast cancer cells in blood by a multimolecular marker assay: correlation with clinical stage of disease. Cancer Res 61(24):8845–8850
4. Span PN, Manders P, Heuvel JJ, Thomas CM, Bosch RR,Beex LV, Sweep CG (2003) Molecular beacon reverse transcription-PCR of human chorionic gonadotropin-beta-3, -5, and \-8 mRNAs has prognostic value in breast cancer. Clin Chem 49 (7):1074–1080
5. Srivastava P, Russo J, Russo IH (1997) Chorionic gonadotropin inhibits rat mammary carcinogenesis through activation of programmed cell death. Carcinogenesis 18(9):1799–1808
6. Rao ChV, Li X, Manna SK, Lei ZM, Aggarwal BB (2004) Human chorionic gonadotropin decreases proliferation and invasion of breast cancer MCF-7 cells by inhibiting NF-kappaB and AP-1 activation. J Biol Chem 279(24):22510–25503
7. Pulaski BA, Ostrand-Rosenberg S (1998) Reduction of established spontaneous mammary carcinoma metastases following immunotherapy with major histocompatibility complex class II and B7.1 cell-based tumor vaccines. Cancer Res 58(7): 1486–1493
8. Banka CL, Lund CV, Nguyen MT, Pakchoian AJ, Mueller BM, Eliceiri BP (2006) Estrogen induces lung metastasis through a host compartment-specific response. Cancer Res 66(7):3667–3672
9. Medina D (2004) Breast cancer: the protective effect of pregnancy. Clin Cancer Res 10(1 pt 2):380–384 (Suppl)
10. Grubbs CJ, Juliana MM, Whitaker LM (1988) Short-term hormone treatment as a chemopreventive method against mammary cancer initiation in rats. Anticancer Res 8(1):113–117
11. Guzman RC, Yang J, Rajkumar L, Thordarson G, Chen X, Nandi S (1999) Hormonal prevention of breast cancer: mimicking the protective effect of pregnancy. Proc Natl Acad Sci USA 962:2520–2525
12. Russo IH, Koszalka M, Russo J (1991) Comparative study of the influence of pregnancy and hormonal treatment on mammary carcinogenesis. Br J Cancer 64(3):481–484
13. Medina D, Kittrell FS (2003) p53 function is required for hormone-mediated protection of mouse mammary tumorigenesis. Cancer Res 63(19):6140–6143
14. Sivaraman L, Conneely OM, Medina D, O'Malley BW (2001) p53 is a potential mediator of pregnancy and hormone-induced resistance to mammary carcinogenesis. Proc Natl Acad Sci USA 98(22):12379–12384
15. Pati D, Haddad BR, Haegele A, Thompson H, Kittrell FS, Shepard A, Montagna C, Zhang N, Ge G, Otta SK, McCarthy M, Ullrich RL, Medina D (2004) Hormone-induced chromosomal instability in p53-null mammary epithelium. Cancer Res 64 (16):5608–5616
16. Levine AJ (1997) p53, the cellular gatekeeper for growth and division. Cell 88(3):323–331
17. Sherr CJ, Roberts JM (1999) CDK inhibitors: positive and negative regulators ofG1-phase progression. Genes Dev 13 (2):1501–1512
18. Modestou M, Puig-Antich V, Korgaonkar C, Eapen A, Quelle DE (2001) The alternative reading frame tumor suppressor inhibits growth through p21-dependent and p21-independent pathways. Cancer Res 61(7):3145–3150
19. Eischen CM, Roussel MF, Korsmeyer SJ, Cleveland JL (2001) Bax loss impairs Myc-induced apoptosis and circumvents the selection of p53 mutations duringmyc-mediated lymphomagenesis. Mol Cell Biol 21(22):7653–7662
20. Choudhuri T, Pal S, Agwarwal ML, Das T, Sa G (2002) Curcumin induces apoptosis in human breast cancer cells through p53-dependent Bax induction. FEBS Lett 512(1–3):334–340
21. Zhang L, Yang N, Carcia JR, Mohamed A, Benencia F, Rubin SC, Allman D, Coukos G (2002) Generation of a syngeneic mouse model to study the effects of vascular endothelial growth factor in ovarian carcinoma. Am J Pathol 161(16):2295–2309
22. Danna EA, Sinha P, Gilbert M, Clements VK, Pulaski BA, Ostrand-Rosenberg S (2004) Surgical removal of primary tumor reverses tumor-induced immunosuppression despite the presence of metastatic disease. Cancer Res 64(6):2205–2211
23. Siler-Khodr TM, Yu FQ, Wei P, Tao SX, Liu YQ (2004) Contraceptive action of a gonadotropin-releasing hormone II analog in the rhesus monkey. J Clin Endocrinol Metab 89 (9):4513–4520
24. Guo S, Russo IH, Lareef MH, Russo J (2004) Effect of human chorionic gonadotropin in the gene expression profile of MCF-7 cells. Int J Oncol 24(2):399–407
25. Mo FE, Muntean AG, Chen CC, Stolz DB, Watkins SC, Lau LF (2002) CYR61 (CCN1) is essential for placental development and vascular integrity. Mol Cell Biol 22(24):8709–8720
26. Stoeltzing O, Ahmad SA, Liu W, McCarty MF, Wey JS, Parikh AA, Fan F, Reinmuth N, Kawaguchi M, Bucana CD, Ellis LM (2003) Angiopoietin-1 inhibits vascular permeability, angiogenesis, and growth of hepatic colon cancer tumors. Cancer Res 63 (12):3370–3377
27. Williams TM, Medina F, Badano I, Hazan RB, Hutchinson J, Muller WJ, Chopra NG, Scherer PE, Pestell RG, Lisanti MP (2004) Caveolin-1 gene disruption promotes mammary tumorigenesis and dramatically enhances lung metastasis in vivo. Role of Cav-1 in cell invasiveness and matrix metalloproteinase (MMP-2/9) secretion. J Biol Chem 279(49):51630–51646
28. Gasco M, Shami S, Crook T (2002) The p53 pathway in breast cancer. Cancer Res 4(2):70–76
29. Jerry DJ, Kittrell FS, Kuperwasser C, Laucirica R, Dickinson ES, Bonilla PJ, Butel JS, Medina D (2000) A mammary-specific model demonstrates the role of the p53 tumor suppressor gene in tumor development. Oncogene 19(8):1052–1058
30. Ling XY, Arlinghaus RB (2005) Knockdown of STAT3 expression by RNA interference inhibits the induction of breast tumors in immunocompetent mice. Cancer Res 65(7):2532–2536
31. Stewart ZA, Leach SD, Pietenpol JA (1999) p21(Waf1/Cip1) inhibition of cyclin E/Cdk2 activity prevents endoreduplication after mitotic spindle disruption. Mol Cell Biol 19(1):205–215
32. Pal S, Datta K, Mukhopadhyay D (2001) Central role of p53 on regulation of vascular permeability vascular endothelial growth factor (VPF/VEGF) in mammary carcinoma. Cancer Res 61 (18):6952–6957