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Tanshinone IIA Inhibits Breast Cancer Stem Cells Growth In Vitro and In Vivo Through Attenuation of IL-6/STAT3/NF-kB Signaling Pathways

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ABSTRACT

Cancer stem cells (CSCs) are maintained by inflammatory cytokines and signaling pathways. Tanshinone IIA (Tan-IIA) possesses anti-cancer and anti-inflammatory activities. The purpose of this study is to confirm the growth inhibition effect of Tan-IIA on human breast CSCs growth in vitro and in vivo and to explore the possible mechanism of its activity. Human breast CSCs were enriched and expanded under serum-free mammosphere culture condition, and identified through mammosphere formation, toluidine blue staining, immunofluorescence staining, and flow cytometry analysis of stemness markers of CD44/CD24 and ALDH, and tumorigenecity in vivo; the growth inhibition effect of Tan-IIA on human breast CSCs in vitro were tested by cell proliferation and mammosphere formation assays; inflammatory signaling pathway related protein expression in response to Tan-IIA, IL-6, STAT3, phospho-STAT3 (Tyr705), NF-κBp65 in cytoplasm and nucleus and cyclin D1 were evaluated with Western blotting; the growth inhibition effect of Tan-IIA on human breast CSCs growth were tested in vivo. A useful model of human breast CSCs for researching and developing the agents targeting CSCs was established. After Tan-IIA treatment, cell proliferation and mammosphere formation of CSCs were decreased significantly; the expression levels of IL-6, STAT3, phospho-STAT3 (Tyr705), NF-κBp65 in nucleus and cyclin D1 proteins were decreased significantly; the tumor growth and mean tumor weight were reduced significantly. Tan-IIA has the potential to target and kill CSCs, and can inhibit human breast CSCs growth both in vitro and in vivo through attenuation of IL-6/STAT3/NF-kB signaling pathways. J. Cell. Biochem. 114: 2061–2070, 2013.

KEY WORDS: BREAST CANCER; CANCER STEM CELLS; TANSHINONE IIA; GROWTH INHIBITION; IL-6/STAT3/NF-kB SIGNALING PATHWAYS

Breast cancer is the most frequent malignant cancer and the leading cause of cancer-related deaths in women worldwide [Ray et al., 2010; Yan et al., 2012]. Although much progress has been made in breast cancer treatment modalities and improvement of patients' survival and quality of life, the patients with breast cancer continue to die of the diseases [Mitra and Faruque, 2004; Mitra, 2011]. Increasing scientific evidence suggested that tumors, including breast cancer contain a heterogeneous population of cells, in which a specific subgroup, the cancer stem cells (CSCs) are chemo-resistant, radio-resistant, and implicated in tumor recurrence, metastasis, and high patient mortality [Hu and Fu, 2012; Omene et al., 2012]. Pharmacological targeting of CSCs might be a highly promising modality for the treatment of breast cancer [Zhu et al., 2012], but to date, there are no specific medicines targeting CSCs [Xu et al., 2011].

Recent studies indicate that inflammatory signaling pathways are important for self-renewing and maintenance of breast CSCs [Hinohara and Gotoh, 2010]. Pro-inflammatory cytokines play important roles in maintaining the stemness of breast CSCs [Papi et al., 2012]. IL-6 secreted from breast CSCs and non-CSCs can transform non-CSCs into CSCs, maintaining their dynamic equilibrium with non-CSCs [Iliopoulos et al., 2011]. It can generate CD44+ cells with stem-like properties through induction of the EMT in epithelial-like T47D breast cancer cells [Xie et al., 2012] and in turn

Caiyu Lin and Li Wang contributed equally to this work.

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activate Stat3/NF-κB pathways in both tumor and stromal cells, driving CSC self-renewal by positive feedback loops [Korkaya et al., 2011b]. Hence, agents that inhibit inflammatory cytokines may have the potential to target and damage CSCs. Blocking inflammatory signaling pathways might be an effective strategy for breast cancer treatment.

Tanshinone IIA (Tan-IIA) is an important lipophilic diterpene isolated from *Salvia miltiorrhiza* BUNGE (Danshen), a widely used Chinese herbal medicine [Yin et al., 2012]. Previous studies demonstrated that Tan-IIA posses anti-cancer [Kapoor, 2009], anti-inflammatory [Fan et al., 2009; Ren et al., 2010; Yin et al., 2012], anti-oxidative activities [Chen et al., 2012b], etc. in vitro and in vivo. Numerous investigations proved that Tan-IIA suppressed breast cancer cells growth both in vitro and in vivo through a variety of mechanisms [Wang et al., 2005; Su and Lin, 2008; Kapoor, 2009; Gong et al., 2012; Su et al., 2012; Yan et al., 2012]. The anti-inflammatory of effect Tan-IIA was associated with inhibition of inflammatory cytokines, such as IL-6 [Jang et al., 2003; Fan et al., 2009; Sun et al., 2012; Yin et al., 2012], and NF-κB [Su and Lin, 2008; Wang et al., 2010; Tang et al., 2011; Sun et al., 2012; Wu et al., 2012; Xu et al., 2012]. Down-regulation or attenuation expressions of IL-6 and NF-κB is a potential therapeutic strategy for cancers [Guo et al., 2012; Hafeez et al., 2012; Korkaya et al., 2011b], including breast cancer [Leslie et al., 2010; Liu et al., 2010; Jiang et al., 2011; Shostak and Chariot, 2011; Su et al., 2012].

As the small population of CSCs in breast cancer appears responsible for tumor initiation, progression, resistance to conventional treatment, recurrence, and high death rate of cancer [Hu and Fu, 2012; Omene et al., 2012; Zhu et al., 2012]. CSCs are maintained by inflammatory signaling pathways and microenvironment [Hinohara and Gotoh, 2010; Papi et al., 2012; Iliopoulos et al., 2011; Korkaya et al., 2011b; Xie et al., 2012], in which inflammatory cytokines, IL-6 [Iliopoulos et al., 2011; Xie et al., 2012] and NF-κB [Liu et al., 2010; Shostak and Chariot, 2011; Yip et al., 2011] play important roles. Tan-IIA exhibited potent potential of inhibiting of inflammatory cytokines, such as IL-6 and NF-κB expressions [Jang et al., 2003; Su and Lin, 2008; Fan et al., 2009; Wang et al., 2010; Tang et al., 2011; Sun et al., 2012; Wu et al., 2012; Xu et al., 2012; Yin et al., 2012]. It could be assumed that Tan-IIA may likewise inhibit breast CSCs growth through inhibiting inflammatory cytokine and blocking inflammatory signaling pathways. However, there is no such a study reported. In order to confirm this hypothesis, this study was therefore designed to investigate whether Tan-IIA could inhibit breast CSCs growth in vitro and in vivo, and to characterize its partial mechanism of anticancer activity.

MATERIAL AND METHODS

REAGENTS

DMEM, DMEM/F12 medium, Accutase, and B27 supplement were purchased from Gibco/BRL Invitrogen (Shanghai, China); Recombinant human basic fibroblast growth factors (bFGF) and human epidermal growth factor (EGF) were obtained from Peprotech (Shanghai, China); fetal bovine serum (FBS) was purchased from Lanzhou National HyClone Bio-Engineering Co., Ltd (Lanzhou, China). Trypsin, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), insulin, heparin, hydrocortisone, bovine serum albumin (BSA), DMSO, and other chemicals and reagents were obtained from Sigma–Aldrich (Shanghai, China). Tan-IIA was purchased from Chengdu Must Bioscience and Technology, Co., LTD (Chengdu, China, purity ≥98%, HPLC).

CELL LINES AND CULTURE

Human breast cancer cell lines (MCF-7) were obtained from Shanghai Cell Biology Institute of Chinese Academy of Sciences (ATCC® Number: HTB-22™, Shanghai, China). Adherent monolayer MCF-7 cells were maintained in DMEM supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml) in a humidified atmosphere of 5% CO_2 at 37°C.

MAMMOSPHERE CULTURE

Floating cells (MCF-7) were collected from 70% confluent adherent monolayer MCF-7 culture dishes by gentle washing with PBS and cultured with serum-free DMEM/F12 medium [Cioce et al., 2010]. Briefly, single cell suspensions were resuspended and expanded at a density of $1–2 \times 10^4$ cells/ml with DMEM/F-12 containing 5 μg/ml insulin, 0.5 μg/ml hydrocortisone, 20 ng/ml EGF, 10 ng/ml bFGF, 4 μg/ml heparin, and 1% BSA in ultralow attachment plates (Corning). Culture medium was changed twice a week, and mammosphere formation was monitored weekly. To prepare single (MCF-7M) cells for further use, mammospheres were disaggregated by Accutase treatment and mechanical disaggregation with a sterile Pasteur pipette, and filtered with a 40 μM cell strainer.

CANCER STEM-LIKE CELL CHARACTERIZATION ASSAYS

Toluidine blue staining assay. To evaluate the light cell (LC) and dark cell (DC) populations in adherent monolayer MCF-7 cells and floating mammospheres (MCF-7M), both cell suspensions were stained with toluidine blue staining buffer containg 10 mM HEPES buffer (pH 7.4), 2 mM EDTA, 0.5% BSA, 0.4% toluidine blue (Sigma–Aldrich) for 5′ at RT [Cioce et al., 2010]. Images of the cells were captured with photocamera-equipped light microscope, and an average of six fields/sample was analyzed. Three independent experiments were performed.

Detection of CD24/CD44 and ALDH1by immunofluorescence. Adherent monolayer MCF-7 cells and MCF-7M cells were fixed with 4% paraformaldehyde for 10 min, permeabilized with 0.01% Triton X-100 and then incubated with mouse anti-human CD44-FITC, CD24-PE monoclonal antibodies (BD PharMingen) and rabbit anti-human ALDHA1-FITC polyclonal antibody (Beijing Biosynthesis Biotechnology, Co., LTD, Beijing, China), respectively in 1% BSA in PBST in a humidified chamber for 1 h at RT. Images were acquired using a Leica DMI400B inverted fluorescence microscope linked to a DFC340FX camera. Three independent experiments were performed.

ALDH1 expression assay by flow cytometry. Adherent monolayer MCF-7 cells and MCF-7M cells were detached with 0.5% BSA and 2 mM EDTA in PBS, counted and washed in 0.1% BSA in PBS, $1–2 \times 10^5$ adherent monolayers MCF-7 and MCF-7M cells were incubated with rabbit anti-human ALDHA1-FITC polyclonal

antibody at 4°C for 30 min in the dark. After washing with PBS, labeled cells were analyzed by flow cytometry using a FACScan flow cytometer (BD Biosciences). Three independent experiments were performed.

BREAST CANCER STEM-LIKE CELL GROWTH INHIBITION OF TAN-IIA IN VITRO

Cell proliferation assay. The cell proliferation inhibition activity of Tan-IIA was determined by MTT assay [van Meerloo et al., 2011]. Both adherent monolayer MCF-7 cells and MCF-7M cells (2×10^3/well) were seeded in 100 μl of medium/well in 96-well plates (Costar Corning, Rochester, NY). After incubation overnight, Tan-IIA was added at various concentrations (0.125, 0.25, 0.5, 1.0, and 2.0 μg/ml), five wells at each concentration. After incubation with Tan-IIA for 72 h, 20 μl of 5 mg/ml MTT (pH 4.7) was added to each well and cultivated for another 4 h, supernatant fluid was then removed, 100 μl DMSO was added per well and plates were shaken for 15 min. The absorbance at 570 nm was measured with microplate reader (Bio-Rad, Richmond, CA), using wells without cells as blanks. The effect of Tan-IIA on the viabilities of adherent monolayer MCF-7 cells and MCF-7M cells were presented as the % cytoviability using the following formula: % cytoviability = A_{570} of treated cells/A_{570} of control cells × 100% [Kim et al., 2006]. Three independent experiments were performed.

Mammosphere formation assay. The inhibition of mammosphere colony formation of Tan-IIA on MCF-7M cells was determined by seeding approximate 50 and 100 cells per well in ultralow attachment 24-well plates, cells were incubated for approximately 24 h, and then treated with 0.25, 0.25, and 1.0 μg/ml Tan-IIA, respectively. After 12 days of incubation, colonies with >50 cells were counted under microscope. Three independent experiments were conducted, each in triplicate.

WESTERN BLOT ANALYSIS

The control MCF-7M cells and Tan-IIA treated MCF-7M cells (0.25, 0.5, 1.0 μg/ml) were used for Western blot analysis. Cell lysates were prepared in NP-40 lysis buffer (2 mM Tris–Cl pH 7.5, 150 mM NaCl, 10% glycerol, and 0.2% NP-40 plus protease inhibitor cocktail). For NF-κBp65 detection, proteins of both untreated and treated MCF-7M cells were prepared with the nuclear and cytoplasmic protein extraction kit (Beijing ComWin Biotech, Co., Ltd., Beijing, China); protein concentrations were determined using BCA protein assay kit (KeyGen BioTECH, Nanjing, China). Twenty micrograms protein of each sample was mixed with Laemmli buffer, and boiled for 5 min. Proteins were separated by 12% SDS–PAGE, transferred PVDF membranes using a semi-dry blotting apparatus (Bio-Rad, Hercules, CA), and blocked in 5% non-fat milk. The membranes were subsequently incubated with the corresponding primary antibodies (Beijing Biosynthesis Biotechnology, Co., LTD) with different dilution: rabbit anti-β-actin, anti- tubulin β, anti-IL-6, anti-NF-κBp65, anti-phospho-STAT3(Tyr705), and anti-cyclin D1, 1:300, anti-STAT3, 1:400, and mouse anti-BUF (Upstream Binding Factor; Santa Cruz Biotechnology), 1:500. Antibody recognition was detected with peroxidase-conjugated goat anti-rabbit IgG (H + L) secondary antibody (Zhongshan Goldenbridge Biotechnology, Co., LTD, Beijing, China) used at 1:3,000 dilutions. Antibody-bound proteins were detected by BeyoECL Plus kit (Beyotime Institute of Biotechnology, Shanghai, China) and Western blotting analysis system (Universal Hood II, Bio-Rad, USA), and normalized to β-actin and quantified using the ChemiDocTM XRS (Bio-Rad).

TUMORIGENICITY ASSAY AND GROWTH INHIBITION OF TAN-IIA OF BREAST CSCs IN VIVO

To evaluate the tumorigenicity of MCF-7M cells, 10^3, 10^4, 10^5, 10^6 of adherent monolayer MCF-7 cells and 10^2, 10^3, 10^4, 10^5 of MCF-7M cells were suspended in 50 μl matrigel (BD Biosciences) and 50 μl PBS, respectively, were then inoculated in the bilateral mammary pads of each Balb/C nude mouse. To investigate growth inhibition effect of Tan-IIA on breast CSCs in vivo, 10^4 MCF-7M cells suspended in 50 μl matrige and 50 μl PBS were inoculated in the bilateral mammary pads of 20-eight-week-old female nude mice. Animals were maintained under standard conditions according to the guidelines of the Institutional Animal Care and Use Committee of Sichuan University. After 1 week of inoculation, the tumor bearing mice were randomized into four groups, of five mice each. In three treatment groups, mice were injected with 10, 20, and 40 mg/kg of Tan-IIA i.p., respectively, three times a week for 4 weeks. In control group, mice were injected with saline. Mice were weighted weekly. Tumor volume were assessed by measuring two perpendicular dimensions (length and width) using a caliper and their volumes were calculated using a standard formula (width^2 × length × 0.5) [Tsai et al., 2009]. At the end of the experiment, mice were killed by carbon dioxide asphyxiation; tumor masses were dissected, and weighed. Tumorigenicity of breast CSCs and growth inhibition effect of Tan-IIA on breast CSCs was determined by gross and pathological examination conventionally. Tumor inhibitory rates were calculated using the following formula: tumor inhibitory rate (%) = (mean tumor weight of the control mice − mean tumor weight of the treated mice)/average tumor weight of the control mice × 100%.

STATISTICAL ANALYSIS

Data were expressed as mean ± standard deviation (mean ± SEM). All data were analyzed using analysis of variance (ANOVA), followed by Dunnett's test for pairwise comparison. Statistical significance was defined as $P < 0.05$ for all tests.

RESULTS

CHARACTERIZATION OF MCF-7M CELLS BY MORPHOLOGY, TOLUIDINE BLUE STAINING, CD44/CD24, AND ALDH1 EXPRESSION

Parental MCF-7 cells cultured under DMEM supplemented with 10% FBS grew as adherent monolayer (Fig. 1A), while MCF-7M cells formed typical mammospheres (Fig. 1B); adherent monolayer MCF-7 and MCF-7M cells contain distinct cell LC and DC populations (Fig. 1D,C). The LCs in floating mammospheres (73.9%) were more than those in adherent monolayer MCF-7 cells (4.2%, Fig. 1E, $P < 0.01$); Adherent monolayer MCF-7 cells contain 13.9 ± 2.7% of CD44+/CD24− cells (Fig. 2A), floating mammospheres contain 92.3 ± 6.9% of CD44+/CD24− cells (Fig. 2B), which is higher than those in adherent monolayer MCF-7 cells (Fig. 2C, $P < 0.01$); little ALDH1 expression was detected in adherent monolayer MCF-7 cells

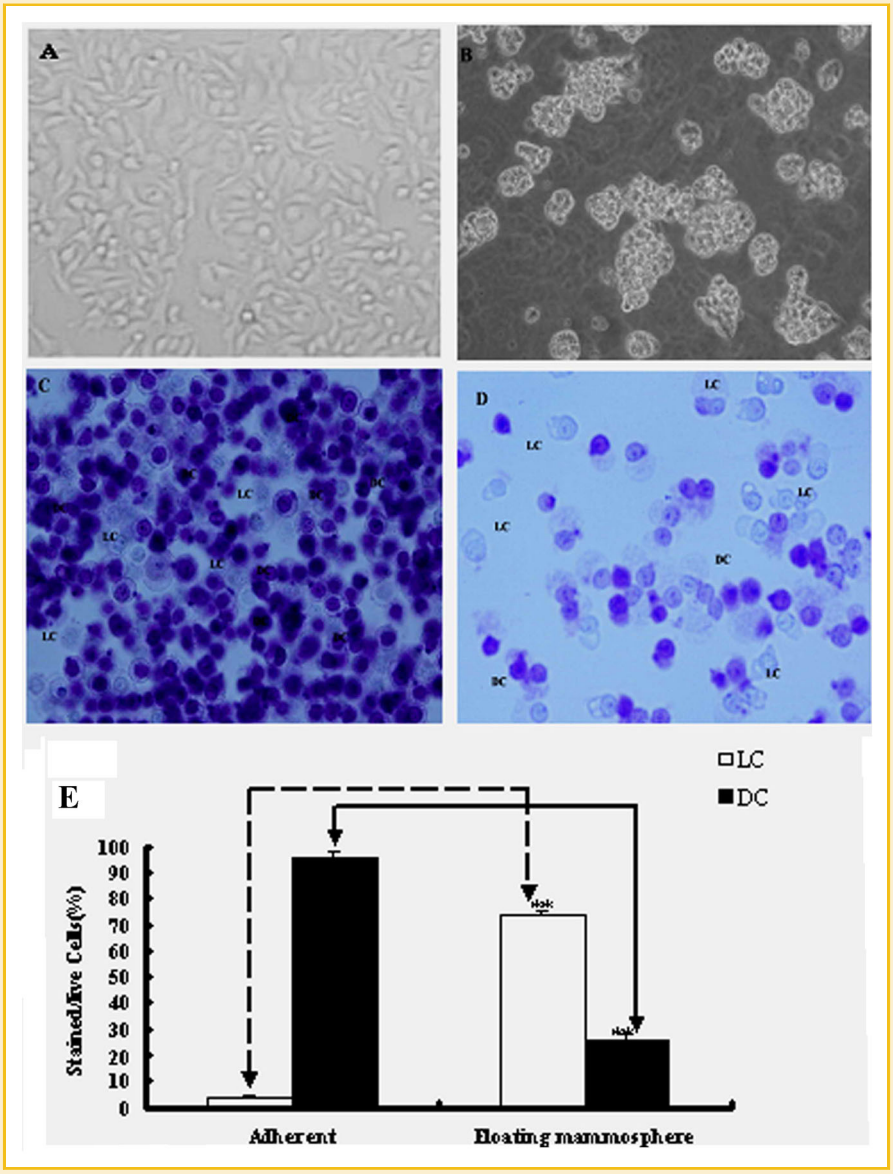

Fig. 1. Morphological characterization of parental MCF-7 cells and mammosphere cells. A: Parental MCF-7 cells cultured under DMEM + 10% FBS grew as adherent monolayer. B: Floating MCF-7 cells cultured under serum free mammosphere culture condition formed typical mammospheres (MCF-7M). C: Toluidine blue pale (LC) and dark (DC) cell subpopulations in MCF-7 cells. D: Toluidine blue pale (LC) and dark (DC) cell subpopulations in MCF-7M cells. E: The histogram shows that there was significant increase of toluidine blue pale (LC) cell subpopulation and decrease of dark (DC) cell subpopulation in MCF-7M cells compared with adherent monolayer MCF-7 cells, the single asterisk (*) indicates a significant difference from the control ($P < 0.05$); the double asterisk (**) indicates a very significant difference from the control ($P < 0.01$). Results are mean values ± SD of independent experiments performed in triplicate.

(Fig. 3A,C), but evident ALDH1 expression was detected in mammospheres (Fig. 3B). Flow cytometry analysis showed that ALDH1+ cells in mammospheres were 67.9 ± 1.8% (Fig. 3B,D), significantly higher than those in adherent monolayer MCF-7 cells (10.5 ± 1.6%, Fig. 3E, $P < 0.01$).

TUMORIGENICITY OF MCF-7M CELLS IN VIVO

MCF-7M cells and adherent monolayer MCF-7 cells were inoculated into immunodeficiency mice, 1×10^2 MCF-7M cells generated tumors of 3/4 inoculations, 1×10^4 parental MCF-7 cells generated tumors of 1/4 inoculations (Table I), the tumor take rate or tumorigenicity of MCF-7M cells was higher than parental MCF-7 cells (Table I). Histopathological examination revealed no obvious morphological difference of tumors generated from parental MCF-7 and from CSCs.

GROWTH INHIBITION EFFECT OF TAN-IIA ON HUMAN BREAST CANCER CELLS (MCF-7) AND CSCs (MCF-7M)

Cell proliferation inhibition effect of Tan-IIA on parental MCF-7 and MCF-7M cells is shown in Figure 4A. The percentages of growth inhibition of Tan-IIA at various concentrations on human breast cancer cells (MCF-7) and breast CSCs (MCF-7M) were determined as

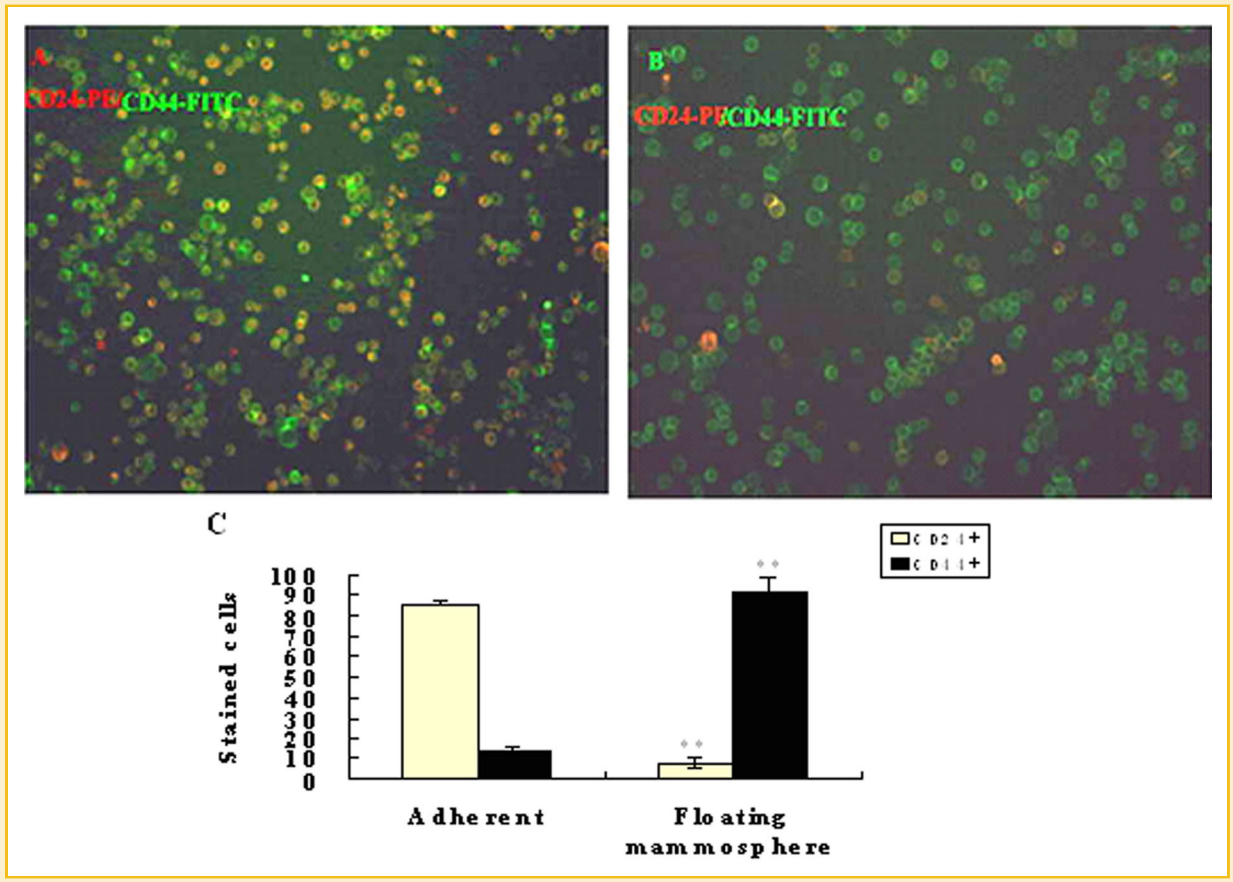

Fig. 2. Immunofluorescence detection of CD44−/CD24+ and CD44+/CD24− cell populations in parental MCF-7 and MCF-7M cells. A: CD44−/CD24+ and CD44+/CD24− cell populations in parental MCF-7 cells. B: CD44−/CD24+ and CD44+/CD24− cell populations in MCF-7M cells. C: The histogram shows that there was significant increase of CD44+/CD24− cell subpopulation and decrease of CD44−/CD24+ cell subpopulation in MCF-7M cells compared with MCF-7 cells, the single asterisk (*) indicates a significant difference from the control ($P < 0.05$); the double asterisk (**) indicates a very significant difference from the control ($P < 0.01$). Results are mean values ± SD of independent experiments performed in triplicate.

the percentage of viable treated cells in comparison with viable cells of untreated controls. Tan-IIA exhibited a dose-dependent inhibitory effect on human breast cancer cells (MCF-7) and MCF-7M cells ($P < 0.01$). IC_{50} was 0.65 μg/ml for MCF-7 cells and 0.40 μg/ml for MCF-7M cells, the maximal inhibition of breast CSCs growth (>80%) was obtained at 2.0 μg/ml.

INHIBITION EFFECT OF TAN-IIA ON MAMMOSPHERE FORMATION OF MCF-7M CELLS

Untreated MCF-7M cells seeded at 50 and 100 cells per well generated 39 ± 4.9 and 74 ± 9.1 mammospheres, respectively. Mammosphere formation was suppressed significantly in a dose-dependent manner after treated with 0.25, 0.5, and 1.0 μg/ml of Tan-IIA (Fig. 4B, $P < 0.01$).

INFLAMMATORY SIGNALING PATHWAY RELATED PROTEIN EXPRESSIONS IN RESPONSE TO TAN-IIA

To explore the potential signaling pathways underlying the growth inhibition effect of Tan-IIA on breast CSCs in vitro, inflammatory signaling pathway related proteins, IL-6, STAT3, phospho-STAT3 (Tyr705), NF-κBp65 in both cytoplasm and nucleus, and cyclin D1 were evaluated with Western blotting. The expression levels of IL-6 (Fig. 5.1), STAT3, phospho-STAT3 (Tyr705; Fig. 5.2), NF-κBp65 in nucleus (Fig. 5.3) and cyclin D1 (Fig. 5.1) proteins were decreased in dose-dependent manner ($P < 0.01$ Fig. 5), after treated with 0.25, 0.5, 1.0 μg/ml of Tan-IIA.

GROWTH INHIBITION OF TAN-IIA ON HUMAN BREAST CSCs (MDF-7M) IN VIVO

The result of tumor growth inhibition of Tan-IIA on human breast cancer generated from human breast CSCs is shown in Figure 6. To evaluate the growth inhibition effect of Tan-IIA on human breast CSCs in vivo, human breast cancer bearing-mice were injected with 10, 20, and 40 mg/kg of Tan-IIA i.p., three times a week for 4 weeks. Mean tumor weight of the control mice was 1.02 ± 0.26 g, and those of the mice injected with 10, 20, and 40 mg/kg of Tan-IIA were 0.62 ± 0.17 g, 0.53 ± 0.18 g, and 0.43 ± 0.12 g, respectively (Fig. 6A). Tumor inhibitory rates were 39.22% ($P < 0.05$), 48.04% ($P < 0.05$), and 57.84% ($P < 0.01$), respectively (Fig. 6B). Lower tumor cell density and more focal necrosis were observed in the xenografts of Tan-IIA treated mice, compared with those of untreated mice by

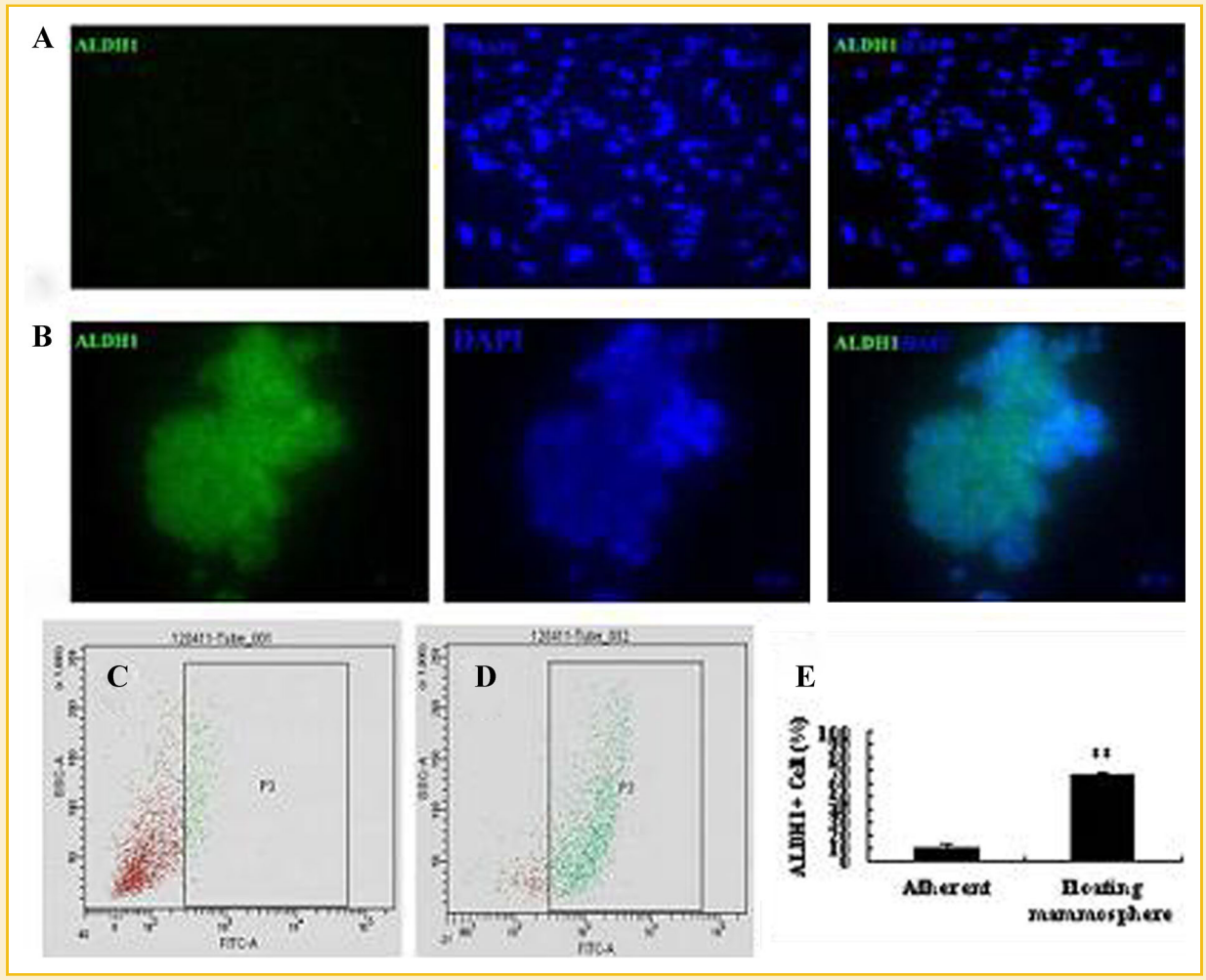

Fig. 3. Immunofluorescence detection and flow cytometry analysis of ALDH1+ cell populations in parental MCF-7 and MCF-7M cells. A: ALDH1+ cell population in parental MCF-7 cells. B: ALDH1+ cell population in MCF-7M cells. C: ALDH1+ cell population in parental MCF-7 cells by flow cytometry analysis. D: ALDH1+ cell population in MCF-7M cells by flow cytometry analysis. E: The histogram shows that there was significant increase of ALDH1+ cell population in MCF-7M cells compared with parental MCF-7 cells by flow cytometry analysis, the single asterisk (*) indicates a significant difference from the control ($P < 0.05$); the double asterisk (**) indicates a very significant difference from the control ($P < 0.01$). Results are mean values ± SD of independent experiments performed in triplicate.

histopathological examination. No evidence of toxicity was observed in treated animals by comparing the body weight increase, histopathological changes of major organs, and blood biochemistry analysis of both the control and treated animals (data not shown).

DISCUSSION

TABLE I. The Tumor Takes of MCF-7 and MCF-7M Cells

	Cell number inoculated				
Cell type	10^2	10^3	10^4	10^5	10^6
MCF-7	ND	0	1/4	4/4	4/4
MCF-7M	3/4	3/4	4/4	4/4	ND

MCF-7, adherent monolayer cells; MCF-7M, floating mammosphere cells.
ND, not done.

Breast cancer, a prevalent disease around the world, is considered the most common malignant cancer and the most common cause of cancer-related death in women worldwide [Mitra and Faruque, 2004; Ray et al., 2010; Aalaoui-Jamali et al., 2011; Mitra, 2011; Yan et al., 2012]. Its high recurrence, metastasis and mortality after conventional therapy, led the patients with breast cancers continue to die of the disease [Mitra and Faruque, 2004; Mitra, 2011]. Previous studies indicated that small population of CSCs appears responsible for tumor initiation and progression as well as resistance to conventional treatment. Substances impairing CSC activity could be invaluable as novel cancer therapeutics. However there are no such substances targeting CSCs as they are resistant to most conventional therapies [Xu et al., 2011; Hu and Fu, 2012; Omene et al., 2012; Zhu et al., 2012].

To research and develop substances targeting CSCs, a reliable model of CSCs becomes crucial for basic and preclinical studies, but currently there are no universal markers to be used for the

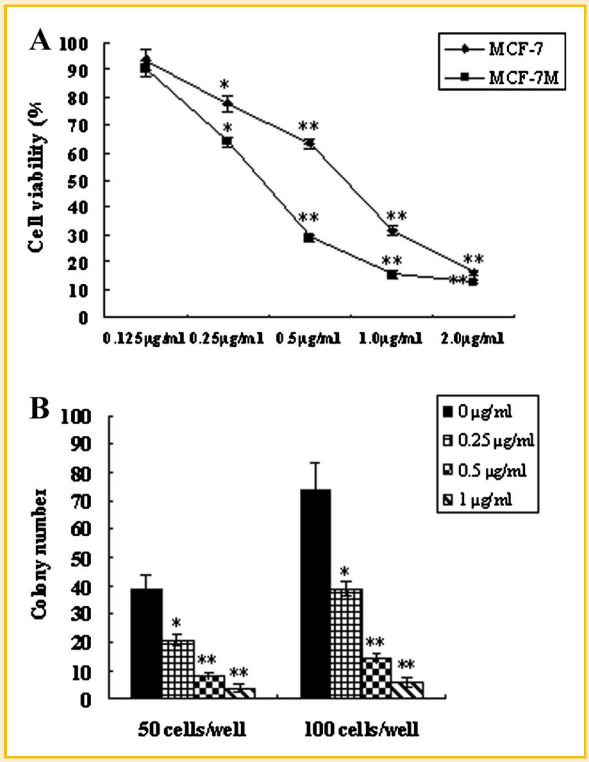

Fig. 4. Growth and mammosphere formation inhibition effect of Tan-IIA on parental MCF-7 and MCF-7M cells in vitro. A: Growth inhibition effect of Tan-IIA on parental MCF-7 and MCF-7M cells in vitro. Both parental MCF-7 cells and MCF-7M cells (2×10^3/well) were seeded in 100 μl of medium/well in 96-well plates and treated with Tan-IIA at different concentrations for 72 h, and percentage of cell viability was determined by MTT assay. A dose dependent growth inhibition of Tan-IIA on parental MCF-7 and MCF-7M cells was observed. OD values of each treated group were compared with that of the control at the same time point, the single asterisk (*) indicates a significant difference from the control ($P < 0.05$); the double asterisk (**) indicates a very significant difference from the control ($P < 0.01$). Results are mean values ± SD of independent experiments performed in triplicate. B: Mammosphere formation inhibition effect of Tan-IIA on parental MCF-7 and MCF-7M cells in vitro. Approximate 50 and 100 cells per well were seeded in ultralow attachment 24-well plates and treated with Tan-IIA at different concentrations, the mammosphere number was counted under microscope. A dose-dependent mammosphere formation inhibition was found. The mammosphere number of each treated group were compared with that of the control, the single asterisk (*) indicates a significant difference from the control ($P < 0.05$); the double asterisk (**) indicates a very significant difference from the control ($P < 0.01$). Results are mean values ± SD of independent experiments performed in triplicate.

isolation and identification of CSCs in any particular cancer [Chen et al., 2012a].

The use of cell lines and defined serum-free culture conditions is a powerful method to select one particular cell population from mixed populations and has been particularly important in establishing in vitro models for CSCs expansion [Mather, 2012]. Breast CSCs can be enriched and propagated in vitro by culturing cells in suspension as mammospheres/tumorspheres [Cioce et al., 2010; Ao et al., 2011].

In this experimental study, human breast CSCs were enriched and expanded under serum-free mammosphere culture condition. Most cells formed typical mammospheres (Fig. 1B); and CSCs from mammospheres (MCF-7M) are stained pale to toluidine blue (LCs, Fig. 1D) [Cioce et al., 2010]. The populations of CD44+/CD24− and ALDH1+ cell populations were increased significantly (Figs. 2 and 3). As few as 1×10^2 MCF-7M cells generated tumors of 3/4 inoculations; however 1×10^4 parental MCF-7 cells generated only one tumor of four inoculations (Table I). The tumor-initiating potential of CSCs from mammospheres increased about 100-folds compared with parental breast cancer cells. Positive stemness markers, capability of forming spheres, and increased potential to form new tumors in mice are thought to be hallmarks of CSCs [Cioce et al., 2010; Ao et al., 2011; Chen et al., 2012a; Tirino et al., 2012]. These findings in the present experiment suggested that CSCs (MCF-7M) enriched and expanded under this experimental condition might be useful for studying and developing the substances targeting CSCs [Cioce et al., 2010; Chen et al., 2012a; Mather, 2012; Ao et al., 2011; Tirino et al., 2012].

CSCs are responsible for resistance to conventional therapeutics, tumor relapse and progression of breast cancer [Hu and Fu, 2012; Omene et al., 2012]. Inflammatory signaling pathways and microenvironment maintain the phenotype and stemness of CSCs [Hinohara and Gotoh, 2010; Iliopoulos et al., 2011; Korkaya et al., 2011; Papi et al., 2012; Xie et al., 2012].

Tan-IIA has anti-cancer [Kapoor, 2009], anti-inflammatory activities [Fan et al., 2009; Ren et al., 2010; Yin et al., 2012], inhibits breast cancer growth in vitro and in vivo [Wang et al., 2005; Su and Lin, 2008; Kapoor, 2009; Gong et al., 2012; Su et al., 2012; Yan et al., 2012]. Its anti-inflammatory activity was associated with inhibition of inflammatory cytokine expressions [Jang et al., 2003; Su and Lin, 2008; Fan et al., 2009; Wang et al., 2010; Tang et al., 2011; Sun et al., 2012; Wu et al., 2012; Xu et al., 2012; Yin et al., 2012]. However, there is no study on the growth inhibition effect of Tan-IIA on human breast cancer stem-like cells in vitro and in vivo through modulation inflammatory signaling pathways.

In this study, human breast cancer cells (MCF-7) and CSCs (MCF-7M) were exposed to Tan-IIA, the proliferation and mammosphere formation of CSCs (MCF-7M) were suppressed in dose-dependent manner (Fig. 4). CSCs seemed to be more sensitive to Tan-IIA ($IC_{50} = 0.40$ μg/ml) than parental MCF-7 breast cancer cells (($IC_{50} = 0.65$ μg/ml). In vivo experiment, Tan-IIA slowed down the growth of human breast cancer initiated from CSCs (MCF-7M) and reduced the weight of tumor masses ($P < 0.05$; Fig. 6) significantly without any untoward toxicity. These findings suggest that Tan-IIA might have potential growth inhibition effect on human breast cancer stem-like cells both in vitro and in vivo.

It was well-recognized that inflammatory cytokines and signaling pathways plays pivotal roles in maintaining the stemness phenotype, self-renewal, and proliferative activities of breast CSCs [Hinohara and Gotoh, 2010; Korkaya et al., 2011b; Iliopoulos et al., 2011; Papi et al., 2012; Xie et al., 2012].

IL-6 secreted from breast cancer stem-like cells, non-CSCs, and tumor microenvironment activates STAT3/NF-κB signaling pathway, leading to self-renewal of breast CSCs [Sansone et al., 2007; Iliopoulos et al., 2009; Hinohara and Gotoh, 2010; Korkaya et al., 2011a; Iliopoulos et al., 2011]. Most inflammatory signals affect tumorigenesis by activating STAT3 and NF-κB [Li et al., 2011]. STAT3 is required for the viability of CD44 + CD24-breast CSCs [Zhou

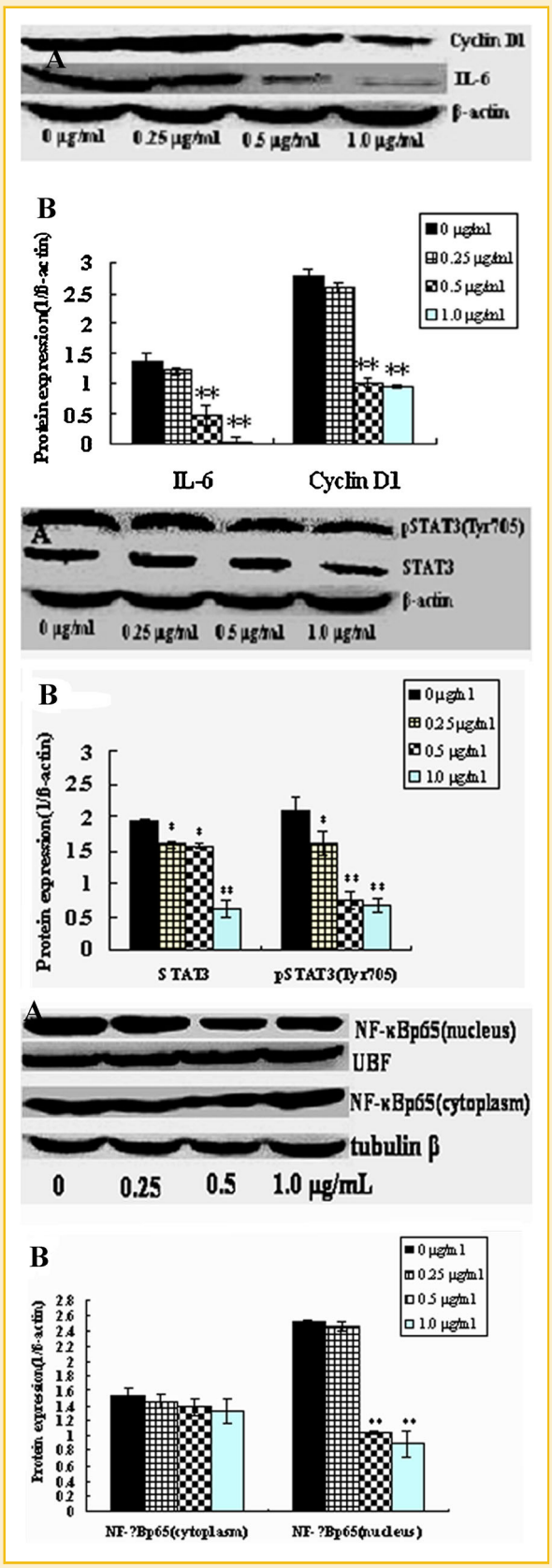

Fig. 5. Inflammatory signaling pathway related protein expression of MCF-7M cells in response to Tan-IIA. Human breast cancer stem-like MCF-7M cells were treated with Tan-IIA (0.25, 0.5, 1.0 μg/ml) for 72 h, respectively, the protein expressions of IL-6, STAT3, phospho-STAT3 (Tyr705), NF-κBp65 in cytoplasm and nucleus and cyclin D1 were evaluated with Western blotting as indicated in material and methods. 5.1. IL-6 and cyclin D1 protein expressions pre and post Tan-IIA treatment. A: Images of IL-6 and cyclin D1 antibody-bound proteins. B: The histogram shows that there were significant decrease of IL-6 and cyclin D1 protein expressions after Tan-IIA treatment in a dose-dependent manner. 5.2. STAT3 and phospho-STAT3 (Tyr705) protein expressions pre and post Tan-IIA treatment. A: Images of STAT3 and phospho-STAT3 (Tyr705) antibody-bound proteins. B: The histogram shows that there were significant decrease of STAT3 and phospho-STAT3 (Tyr705) protein expressions after Tan-IIA treatment in a dose-dependent manner. 5.3. NF-κBp65 protein expression in cytoplasm and nucleus pre and post Tan-IIA treatment. A: Images of NF-κBp65 expression in cytoplasm and nucleus antibody-bound proteins. B: The histogram shows that there was significant decrease of NF-κBp65 retention in nucleus after Tan-IIA treatment in a dose-dependent manner. The single asterisk (*) indicates a significant difference from the control ($P < 0.05$); the double asterisk (**) indicates a very significant difference from the control ($P < 0.01$). Results are mean values ± SD of independent experiments performed in triplicate.

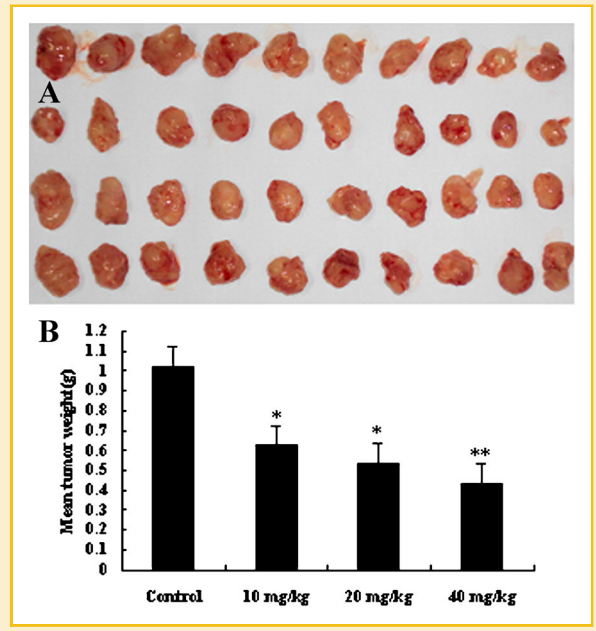

Fig. 6. Inhibition of Tan-IIA on human breast cancer growth initiated from MCF-7M cells in vivo. A: Tumor masses of each group. B: The histogram shows that there was a significant difference of mean tumor weight of each Tan-IIA treated group compared with the control. The single asterisk (*) indicates a significant difference from the control ($P < 0.05$); the double asterisk (**) indicates a very significant difference from the control ($P < 0.01$).

et al., 2007; Marotta et al., 2011]. NF-kB is upregulated in breast CSCs and its activity is required for mammosphere formation [Iliopoulos et al., 2009; Hinohara and Gotoh, 2010; Murohashi et al., 2010]. STAT3 is required for proper induction of IL6 by NF-κB and plays critical roles in binding to IL6 promoters as well as nuclear retention of NFκB [Yoon et al., 2012], IL-6/STAT3/NF-κB signaling

pathway work cooperatively, driving CSC self-renewal by positive feedback loops [Korkaya et al., 2011b; Yoon et al., 2012]. Thus, blocking IL-6/STAT3/NF-κB signaling pathway and its positive feedback loops might be a promising modality targeting CSCs for breast cancer therapeutics.

In this study, breast CSCs (MCF-7M) expressed high levels of IL-6, STAT3, NF-κBp65, and cyclin D1 proteins (Fig. 5), Tan-IIA not only down-regulated the expressions of proteins but also decreased phosphorylation of STAT3 (Tyr705) and nuclear translocation and/or retention of NF-κBp65 of the treated cancer stem-like cells (MCF-7M) in dose-dependent manner ($P < 0.01$; Fig. 5), which resulted in reduction of cyclin D1 protein expression and growth inhibition of breast cancer stem-like cells (MCF-7M) in vitro and in vivo.

Combined with findings in this study, it could be assumed that Tan-IIA has potent growth inhibition effect on human breast CSCs in vitro and in vivo; its partial mechanism of activity might be associated with inhibition of inflammatory cytokine production, blocking IL-6/STAT3/NF-κB signaling pathway and its positive feedback loops.

In summary, breast CSCs (MCF-7M) enriched and expanded under this mammosphere culture condition might be a useful model for studying and developing the substances targeting CSCs; Tan-IIA has the potential to target and kill CSCs, and can inhibit human breast CSCs growth in vitro and in vivo through attenuation of IL-6/STAT3/NF-kB signaling pathways. Tan-IIA might serve as a novel therapeutic agent targeting at CSCs in the treatment and/or prevention of human breast cancer, and deserve to be investigated further.

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