科技部補助專題研究計畫成果報告
期末報告

以4T1產生乳癌之小鼠為實驗模式，重新評估並探討DHEA對乳癌之產生作用機制

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中 文 摘 要 ： 根據本研究目的，我們設置三大研究方向 ： 1)將只表現ERβ之4T1乳癌細胞植入小鼠的背側以建立乳癌自動轉移的動物模式。藉由公鼠，母鼠，去睪丸，或去卵巢等技術， 於宿主體內建置不同賀爾蒙環境，進行連續二至五周之冷光測定，觀測小鼠體內原位癌的生長及乳癌細胞的轉移，並分析4T1乳癌組織於不同宿主體內不同賀爾蒙組成下對脫氫表雄酮之反應，2) 利用DPN (ERβ刺激劑) ，PHTPP(ERβ拮抗劑) 與ERβ shRNA 改變脫氫表雄酮影響原位乳癌發展，觀察4T1乳癌組織之生長及乳癌細胞的轉移，與在細胞實驗中，偵測脫氫表雄酮活化ERβ對細胞型態， 爬行，與入侵之影響。利用太平洋紫杉醇 (Taxol)、秋水仙素(colchicine)探討乳癌細胞內的微管 (microtubule)對ERβ加速爬行之作用機轉， 3) 瞬態電壓感受器陽離子通道刺激劑或抑制劑探討脫氫表雄酮影響細胞型態， 爬行，入侵與原位乳癌發展.動物實驗結果顯示，E2和DHEA皆可活化ERβ導致癌細胞擴散並提升癌細胞轉移率。細胞實驗結果顯示活化TRPV1使得癌細胞型態從圓形變為紡錘狀、細胞內鈣離子濃度提高以及癌細胞爬行數目增加。接著探討ERβ和TRPV1 兩者之間的關係。細胞實驗結果顯示，TRPV1拮抗劑 (CapZ和RHC) 會抑制E2和DHEA活化ERβ導致的細胞型態改變、細胞內鈣離子濃度以及癌細胞爬行數目。ERβ拮抗劑 (PHTPP) 不會影響OAG和Cap活化TRPV1導致的細胞型態改變、細胞內鈣離子濃度以及癌細胞爬行數目。動物實驗結果顯示，TRPV1拮抗劑 (CapZ)會抑制E2和DHEA活化ERβ導致的癌細胞擴散和轉移率。本研究實驗結果推論DHEA在停經前後45-55歲的乳癌患者中扮演一個很重要的角色。E2和DHEA活化ERβ導致的惡性乳癌細胞擴散可能經由TRPV1的臨床藥物獲得改善。

中 文 關 鍵 詞 ： 脫氫表雄酮, 雌激素貝他接受器, 乳癌轉移, 細胞爬行, 微管, 瞬態電壓感受器陽離子通道

英 文 摘 要 ： It is known that cortisol and dehydroepiandrosterone (DHEA) are increased with urban stress. Prolonged elevation of cortisol causes drug resistance of breast tumor cells. However, it is not clear whether the prolonged elevations of DHEA affect breast tumor formation.
Since DHEA is a precursor of testosterone and an endogenous ligand for ERβ, we hypothesized that the activation of ERβ+ by DHEA accelerates the development of ERβ+ breast cancer. Today, it is known that the activation of TRPV1 increases the formation of tyrosinated tubulin, and accelerates dynamics of tubulin networking. Since dynamics of tubulin nextworking facilitates cell migration, our working model in this proposal is that activation of ERβ+ by DHEA in a unique host compartment facilitates cell migration and accelerate spontaneous metastasis of ERα?/ERβ+ breast cancer via the interaction between TRPV1 and tubulin. Accordingly, three specific aims are proposed:
AIM 1: to explore the involvement of estrogen and ERβ in DHEA-induced tumor metastasis of 4T1 breast tumor
AMI 2: to investigate the mechanism of ERβ-induced

migration, tubulin dynamics, and TRPV1 activation, AIM 3: to determine the involvement of TRPV1 in DHEA-mediated metastasis of 4T1-containg breast cancer.The first aim was to assess whether DHEA played a role in hormone imbalance as in the perimenopausal period which accelerated the formation of ERβ+ breast cancer in chapter 2. The second aim was to determine whether activation of ERβ by DHEA caused the migration of 4T1/Luc+ breast tumor cells via TRPV1.

英文關鍵詞：DHEA, estrogen receptor beta, tumor metastasis, cell migration, microtubules, TRPV1

附件一

科技部補助專題研究計畫成果報告

（□期中進度報告/■期末報告）

以 4T1 產生乳癌之小鼠為實驗模式，重新評估並探討 DHEA 對乳癌之產生作用機制（計畫名稱）

計畫類別：■個別型計畫　□整合型計畫

計畫編號：MOST　104-2629-B-006-001

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計畫主持人：蔡美玲

共同主持人：

計畫參與人員：郭虹萱

本計畫除繳交成果報告外，另含下列出國報告，共 ___ 份：

□執行國際合作與移地研究心得報告

□出席國際學術會議心得報告

□出國參訪及考察心得報告

中文摘要

已知糖皮質酮長期增加使乳癌細胞產生抗藥性。但脫氫表雄酮對乳癌生成之作用仍不清楚。已知脫氫表雄酮是雄性激素前驅物，也是 ERβ 刺激劑。因此本研究的目的是探討是否脫氫表雄酮於特定宿主環境可促進 ERβ 乳癌的發展並研究脫氫表雄酮活化 ERβ 導致加速乳癌發展中所調控的機制。根據本研究目的，我們設置三大研究方向 ：1)將只表現 ERβ 之 4T1 乳癌細胞植入小鼠的背側以建立乳癌自動轉移的動物模式。藉由公鼠，母鼠，去睪丸，或去卵巢等技術， 於宿主體內建置不同賀爾蒙環境，進行連續二至五周之冷光測定，觀測小鼠體內原位癌的生長及乳癌細胞的轉移，並分析 4T1 乳癌組織於不同宿主體內不同賀爾蒙組成下對脫氫表雄酮之反應，2) 利用 DPN (ERβ 刺激劑) ， PHTPP(ERβ 拮抗劑) 與 ERβ shRNA 改變脫氫表雄酮影響原位乳癌發展，觀察 4T1 乳癌組織之生長及乳癌細胞的轉移，與在細胞實驗中，偵測脫氫表雄酮活化 ERβ 對細胞型態， 爬行，與入侵之影響。利用太平洋紫杉醇 (Taxol)、秋水仙素(colchicine)探討乳癌細胞內的微管 (microtubule)對 ERβ 加速爬行之作用機轉， 3) 瞬態電壓感受器陽離子通道刺激劑或抑制劑探討脫氫表雄酮影響細胞型態， 爬行，入侵與原位乳癌發展。動物實驗結果顯示，E2 和 DHEA 皆可活化 ERβ 導致癌細胞擴散並提升癌細胞轉移率。細胞實驗結果顯示活化 TRPV1 使得癌細胞型態從圓形變為紡錘狀、細胞內鈣離子濃度提高以及癌細胞爬行數目增加。接著探討 ERβ 和 TRPV1 兩者之間的關係。細胞實驗結果顯示，TRPV1 拮抗劑 (CapZ 和 RHC) 會抑制 E2 和 DHEA 活化 ERβ 導致的細胞型態改變、細胞內鈣離子濃度以及癌細胞爬行數目。ERβ 拮抗劑 (PHTPP) 不會影響 OAG 和 Cap 活化 TRPV1 導致的細胞型態改變、細胞內鈣離子濃度以及癌細胞爬行數目。動物實驗結果顯示，TRPV1 拮抗劑 (CapZ) 會抑制 E2 和 DHEA 活化 ERβ 導致的癌細胞擴散和轉移率。

Abstract

Both incidence and mortality of breast cancer are much lower in Asia than those in the West. However, the onset of peak age for Asian women between 40 and 50 years old is much earlier than that for Western women between 60 and 70 years old. Although more estrogen receptor (ER)-positive breast tumors (about 70% out of breast tumor cases) are found in the Taiwan's patients than that in American women, conventional breast cancer therapies are more effective for Western women in controlling the mortality rate than those for Taiwan's women. Therefore, the mortality rates of breast cancer in Taiwan are continuously increased in the past decades. Current studies show a positive correlation between urbanization and breast cancer mortality. It is known that cortisol and dehydroepiandrosterone (DHEA) are increased with urban stress. Prolonged elevation of cortisol causes drug resistance of breast tumor cells. However, it is not clear whether the prolonged elevations of DHEA affect breast tumor formation.

Since DHEA is a precursor of testosterone and an endogenous ligand for ERβ, we hypothesized that the activation of ERβ+ by DHEA accelerates the development of ERβ+ breast cancer. Today, it is known that the activation of TRPV1 increases the formation of tyrosinated tubulin, and accelerates dynamics of tubulin networking. Since dynamics of tubulin nextworking facilitates cell migration, our working model in this proposal is that activation of ERβ+ by DHEA in a unique host compartment facilitates cell migration and accelerate spontaneous metastasis of ERα−/ERβ+ breast cancer via the interaction between TRPV1 and tubulin. Accordingly, three specific aims are proposed:

AIM 1: to explore the involvement of estrogen and ERβ in DHEA-induced tumor metastasis of 4T1 breast tumor

AMI 2: to investigate the mechanism of ERβ-induced migration, tubulin dynamics, and TRPV1 activation,

AIM 3: to determine the involvement of TRPV1 in DHEA-mediated metastasis of 4T1-containg breast cancer. This study will demonstrate the importance of ERβ in accelerating cell migration and invasion which contributes to the aggressiveness of ERα-/ERβ+ breast cancer. In vivo imaged indicated that E2 and DHEA-induced metastasis is ERβ-dependent. Analysis of both cell morphology and intensity of calcium fluorescence showed, a positive association with Cap (TRPV1 activator)-increased migrated cells. CapZ (TRPV1 blocker) and RHC (DAG lipase inhibitor) decreased the number of migrated cells by E2 and DHEA. However, PHTPP did not inhibit the number of migrated cells by Cap and OAG (TRPV1 endogenous activator). The10-day treatment with E2 and 14-day treatment with DHEA promoted the breast cancer metastasis and enhanced the relative abundance of ERβ protein. CapZ inhibited the increase of cancer metastasis and ERβ expression by an ERβ activator. Taken together, these results suggest that alterations of both cell morphology and intracellular calcium concentrations are involved in ERβ-mediated and TRPV1-induced breast cancer progression. Abnormal elevation of DHEA in perimenopausal period may activate ERβ, open TRPV1, and accelerate calcium–dependent tumor development.

Introduction

Breast cancer is the leading cause of cancer death in females worldwide (Jemal et al., 2011) but its risk profiles are different between Western and Asian women. Both incidence and mortality of breast cancer are much lower in Asia than those in the West. The onset of peak age for Asian women between 40 and 50 years old is much earlier than that for Western women between 60 and 70 years old (Leong et al., 2010). Current therapies for breast cancer decrease the mortality rate in Western countries but do not effectively control the rate in Asian countries. The differences in risk

profiles and therapeutic outcome between Asian and Western women suggest a unique pathogenic mechanism responsible for the development of breast cancers in Asian women at the age of 40-50 years old.

Since breast cancer mortality is positively correlated with urbanization, it is of importance to review current studies of prolonged elevations of cortisol and DHEA on cancer growth. Activation of glucocorticoid receptor by dexamethason (a long-lasting cortisol) enhances the expression of genes related to the suppression of chemotherapy-induced apoptosis in tumor cells (Herr et al., 2003; Wu et al., 2004; Melhem et al., 2009). The increase of glucocorticoid receptor for cortisol is positively correlated with rapid recurrence of ER-independent breast cancers (Pan et al., 2011). These reports suggest the indirect effect of cortisol in accelerating the recurrence of breast cancers by interfering with chemotherapy-induced tumor cell death. Relatively, the role of DHEA in cancer development is not explored in depth. DHEA inhibits spontaneous formation of breast cancers in female C3H mice (Schwartz, 1979), ZR-75-1-containing breast tumors in nude mice (Couillard et al., 1998), and 1,2-dimethylhydrazine (DMH)-induced tumors in Balb/c mice (Nyce JW et al., 1984) but causes hepatocarcinogenesis in F-344 rats (Hayashi et al., 1994). More interestingly, DHEA induces the growth of hepatic tumor to a greater extent in male than female mice (Rao et al., 2002). The question is why DHEA induces hepatic cancer in a gender-dependent manner.

DHEA derived from pregnenolone is an important precursor for both testosterone and estrogen. The physiological concentration of DHEA is about 30 nM. Physiological concentrations of DHEA are higher in males than females. Today, two ERs (ERα and ERβ) have been identified. ERα is mainly expressed in female sex organs (such as breast and uterus), whereas ERβ is expressed in both sex organs (such as lung and adrenal cortex) and male sex organs (such as prostate) (3). The EC50 of DHEA to ERβ was about 200 nM (Chen et al., 2005). DHEA stimulated reporter activity in ERβ transfected HepG2 cells (human liver carcinoma cells). The increased activities by DHEA was reversed by an ERβ-selective antagonist (R,R-THC (R,R-cis-diethyl tetrahydrochrysene) (Miller et al., 2013). Since ERβ exists in non-nuclear compartments of hepatic tumor cells, higher levels of DHEA in males may trigger a unique mechanism other than cell proliferation to induce a gender-dependent effect on hepatocarcinogenesis.

It is well documented that 4T1 cells do not contain ERα. Estrogen cannot induce 4T1 proliferation. Although elevation of estrogen for two weeks accelerates lung

metastasis of 4T1 breast tumors (Banka et al, 2006), accelerated metastasis of 4T1-containing tumor cannot be suppressed by ICI 182, 780, an antiestrogen receptor antagonist (Yang et al., 2013). It becomes clear that activation of ERβ by DHEA causes tumor formations through the mechanism other than cell proliferation. Migration, essential for tumor expansion (Geho et al., 2005), may be responsible for DHEA-induced tumor formation. Therefore, our Aim II is to determine whether DHEA-induced tumor formation is though activation of cytoskeleton rearrangement. It is known that the driving force for cell migration is thought to be provided by dynamic re-arrangement of actin and microtubules (Ballestrem et al., 2000). How can dynamic changes of cytoskeleton (actin and microtubules) be regulated? Elevation of intracellular calcium oscillation accelerates dynamic changes of cytoskeleton. Activation of capsaicin receptor (TRPV1), a non-selective cation channel, increases intracellular calcium, accelerates disassembly of tubulin, and then enhances dynamic networking of tubulin without altering the dynamic networking of actin in neurons (Goswami et al., 2006). Storti et al. in 2012 showed a direct association between TRPV1 and tubulin (Storti et al., 2012). Without altering ion flow and electrical current, estrogen activates TRPV1 via a PKCε-dependent pathway, destabilizes tubulin networking via the interaction of TRPV1 with tubulin, and then sensitizes pain sensation (Goswami et al., 2011). If DHEA is an endogenous ligand for ERβ, our Aim II is to further investigate whether activation of ERβ by DHEA enhances cell migration and tubulin dynamics through a TRPV1-dependent pathway and Aim III is to determine the involvement of TRPV1 in DHEA-mediated metastasis of 4T1-containg breast cancer in vivo.

Material and methods

Reagents and antibodies

Reagents used were: 17-Estradiol (E2; Sigma Aldrich, MO, USA); 4-[2-phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]pyrimidin-3-yl]phenl (PHTPP; Tocris Bioscience, Ellisville, MO) ; diarylpropionitrile (DPN; Cayman Chemical, Ann Arbor, MI) ; Paclitaxel (Taxol; Sigma Aldrich, MO, USA) ; Colchicine (Sigma Aldrich, MO, USA). Antibodies used were: β-actin (Chemicon, CA) ; α-tubulin (Santa Cruz, CA) ; β-tubulin (Epitomics, CA) ; Pyruvate kinase (AbD Serotec, UK) ; ERα(Santa Cruz, CA) ; ERβ (Upstate, NY) ; Acetyl α-tubulin (Sigma Aldrich, MO, USA).

Cell line and cell culture

MCF7 cells, a human breast tumor cell, were maintained in phenol red-free Dulbecoo's Modified Eagle's Media-F12 (Sigma-Aldrich, MO, USA) supplemented

with 1.3 g/L sodium bicarbonate, 10 % fetal bovine serum (Gibco, CA, USA) and 100 units/ml penicillin-streptomycin (Gibco, CA, USA). MDA-MB-231 cells, a human breast tumor cells, were obtained from Ph.D. Chun Hei Antonio Cheung in National Cheng Kung University, Taiwan. 4T1 cells, a mouse breast tumor cell transfected with luciferase (4T1/Luc+), were obtained from Dr. Ming-Lung Guo in National Taiwan University, Taiwan. MDA-MB-231 and 4T1 cells were maintained in phenol red free RPMI-1640 medium (Sigma-Aldrich, MO, USA.) with 2 g/L sodium bicarbonate, 10% fetal bovine serum (Hyclone, CA, USA), 100 units/ml of penicillin and streptomycin (Gibco, CA, USA). All cells were incubated at 37 °C, 5 % CO2.

Animal care

BALB/c mice (18-20 g) at the age of 4 weeks were obtained from the National Cheng-Kung University Laboratory Animal Center, Tainan, Taiwan, in a controlled environment (temperature 24±1°C, humidity 50% at a photoperiod of 12 light : 12 dark, from 7 A.M. to 7 P.M.), and fed ad libitum with standard rat chow (PMI Nutrition International, Inc., Missouri, U.S.A.). Experimental protocols of animal studies in this study were proved by Animal Committee and animal experiments were performed according to the “Guide for the Care and Use of Laboratory Animals” of National Cheng-Kung University.

In vivo tumor xenograft model

4T1/Luc+ cells (1x105 cells/mouse) were transplanted into the subcutaneous region of dorsal flask of intact male, intact female, and ovariectmoized female mices at the age of 4-5 weeks .One day after transplantation, all mice studied received daily injections of E2 (20 μg/kg/day) or E2 with PHTPP (400 μg/kg/day), an ERβ antagonist intraperitoneally for 2 weeks.

In vivo imaging

One day after the last injection, the mice were anesthetized with 4% isoflurane (AErrane®, Guayama, USA) inhaled through the Soft Lander® (Shin-Ei Industry Co., Taiwan) at a flow of 2 L/min (mixed with air). Luciferin (100 mg/kg, Cold Spring Harbor Laboratory, USA) was injected intraperitoneally. The mice were scanned using an IVIS Spectrum (Caliper Life Sciences, Alameda, CA). Bioluminescent signals were quantified using Living Image 3.0 (Caliper Life Sciences, Alameda, CA).Quantified bioluminescence consisted of averaged photon radiance on the animal and was expressed as photons/sec/cm2/sr (sr = steradian). Quantified bioluminescence covered area on the animal and was expressed as tumor area (cm2).

Cell counting assay

2x104 cells were seeded onto a 24-well plate and incubator for 24 hr. Cells were treated with E2 at different dose in serum-free medium for 24 hr. after they were starved for 12 hr. DMSO was a vehicle control. After treatment, cells were trypsinized and harvested. Cell population was determined by trypan blue exclusion assay and the number of cell were counting by using hemocytometer.

Counting of cell with morphological changes and measuring the cell area

Cells in serum free medium were treated with vehicle (0.1 % DMSO) or E2 (10 nM) for 3 or 24 hr. A cell was counted as “morphological changes” with more than 2 polarized protrusions or counted as the elongated cell which the ratio of long/wide was more than 2 were measured by using Motic Image Plus 2.0 software (Motic Instruments Inc., Canada). The number of protrusion cells and elongated cells were calculated and expressed as percent of total cells. The area of cells was measured by using Motic Image Plus 2.0 software.

MTT assay

5x103 cells were seeded onto a 96-well plate and treated with 5g/μl of 3-(4,5)-dimethylthiahiazo (-z-y1)-3,5-di- phenytetrazoliumromide (MTT, Sigma-Aldrich, MO, USA) in a final volume of 200 μl for 4 h. After the conversion of MTT to formazan by mitochondrial dehydrogenases in living cells, the purple precipitates were dissolved in dimethyl sulfoxide and detected by ELISA reader (Sunrise, Tecan, Switzerland) at 490 nm.

Migration assay

Migration of tumor cells was analyzed by Boyden chamber (48 well, 8 mm2 area, Neuro probe Inc, MD, USA). Trypsinized cells were washed with PBS, resuspended in RPMI, and placed into the upper chamber with a polycarbonate filter (8 μm pore size, Neuro probe Inc, MD, USA). The lower chamber was filled with medium containing 10% FBS as a chemoattractant. After a 12-h treatment, cells in the upper chamber were removed with a cotton swab. Cells present on the lower surface of the filters were fixed in methanol for 10 min. Filters were then stained with hemataxylen (Vector Laboratories Inc, CA, USA). These images were obtained with the 20x objective lens. For statistical purposes, the total number of cells migrated in 20 microscopic fields per well were counted.

Data analysis and statistical evaluation

Data are expressed as the mean ± SEM (standard errors). Survival rate and necrosis

percentage of mice were analyzed by chi-squire tests on SPSS 17.0 (SYSTAT 17.0, SPSS Science, CA, USA). Time-dependent change in tumor development for multiple comparisons was analyzed by two-way ANOVA analysis of variance followed by Bonferroni's test or Dunnett's T3 test for post hoc comparison on SPSS 17.0. Statistical analysis for one factor was performed by T'test or one-way ANOVA analysis and Bonferroni's or Dunnett's T3 test hoc test for multiple comparisons or with t-tests for two-group comparisons on SPSS 17.0. $P < 0.05$ was considered to be statistically significant.

Results

To fully characterize the role of ERβ in the metastasis of 4T1-containing tumor, we studied the effect of E2 and DHEA on 4T1 cell proliferation and migration in vitro first. Furthermore, we examined the effect of E2 and DHEA on 4T1-containing tumor metastasis in vivo. E2 and DHEA from 10-6-10-9 M did not affect cell number and cell viability. PHTPP from 10-7-10-9 M did not affect the cell number and cell viability whereas 10-7 M decreased the cell number (Fig. 7). To examine the role of ERβ activation on 4T1 cell migration, an ERβ blocker, PHTPP, were used in Boyden chamber migration assay. Migration assay showed that 10-8 M E2 and 10-8 M DHEA increased the number of migrated cells. 10-7 M PHTPP (Fig. 8) and knockdown of ERβ (Fig. 9) inhibited the E2- and DHEA-increased number of migrated cell. The results suggested that E2 and DHEA induced 4T1 breast tumor cell migration through ERβ but was not involved in cell proliferation.

To further examine the effects of E2 and DHEA on cancer metastasis from primary to secondary sites, tumor tissues in the primary site (breast tissue) and secondary site (liver) were isolated for luminescence detection 10 days and 14 days after transplantation. A 10-day treatment with E2 (20 μg/mouse/day) (Fig. 10) and 14-day treatment with DHEA (20 μg/mouse/day) (Fig. 11) increased the total density and metastasized ratio of 4T1 breast tumors at the primary sites, which was inhibited by PHTPP (10 μg/mouse/day). These results indicated that the induction of 4T1-containing tumor metastasis by E2 and DHEA.

3.3.3. Activated TRPV1 causes cell migration via tumor cell morphological changes and the elevation of intracellular calcium

Tumor cell morphological changes and the elevation of intracellular calcium contribute to cell migration. To fully characterize the role of TRPV1 on 4T1 cell migration, we studied the effects of Cap (TRPV1 activator) on 4T1 cell proliferation, morphological changes, intracellular calcium levels and migration. Cap and CapZ (TRPV1 blocker) from 10-6-10-9 M did not affect the cell number and cell viability (Fig. 12). 10-9 M Cap increased the ratio of length / width, the intensity of calcium

fluorescence and number of migrated cells. 10-8 M CapZ inhibited the ratio of length / width, the intensity of calcium fluorescence and number of migrated cells by Cap (Fig. 13).

To further confirm the incrase of intracellular Ca2+ concentrations induced cell migration, cell morphology and intracellular calcium wereassessed. EGTA (extracellular Ca2+ chelator) and ionomycin (Ca2+ transporter across cell membranes) were used to modulate intracellular calcium concentrations. 10-8 M ionomycin increased the migrated cell number and intensity of calcium fluorescence (Fig. 14). 1 mM EGTA inhibited the increased number of migrated cells and intensity of calcium fluorescence by ionomycin and Cap (Fig. 15).

To exclude the possibilities that the increase of migrated cells is due to inonomycine-induced cell proliferation, MTT assay was used. 10-8 M ionomycin did not affect the cell viability (Fig. 16). The migrated cell number did not change (Fig. 17) although 1 mM EGTA decreased the cell viability. The results suggested that Cap induced 4T1 breast tumor cell migration through TRPV1 induced-cell morphological changes and extracellular Ca2+ influx but did not involved in cell proliferation.

3.3.4. Activated ERβ accelerates cell migration via TRPV1

To determine the role of TRPV1 in E2- and DHEA- induced cell migration, calcium concentration image and Boyden chamber migration assay were used. 10-8 M E2 and 10-8 M DHEA increased the ratio of length / width, the intensity of calcium fluorescence and number of migrated cells. 10-7 M CapZ inhibited the ratio of length / width, the intensity of calcium fluorescence and number of migrated cells by 10-8 M E2 (Fig. 18) and 10-8 M DHEA (Fig. 19).

To further determine whether activation of TRPV1 by E2 or DHEA requires the presence of diacylglucerol (DAG) which activates protein kinse C, a DAG analogue (OAG) and diacylglucerol lipase inhibitor (RHC) were used. 10-9 M OAG increased the ratio of length / width, the intensity of calcium fluorescence and number of migrated cells. 10-8 M RHC inhibited the ratio of length / width, the intensity of calcium fluorescence and number of migrated cells by OAG treatment (Fig. 20). To exclude the possibility that the increased of migrated cells is due to cell proliferation, MTT assay was used. 10-9 M OAG did not affect the cell viability (Fig. 21). Moreover, 10-7 M RHC lowered the ratio of length / width, the intensity of calcium fluorescence and number of migrated cells by E2 (Fig. 22) and DHEA (Fig. 23). 10-8 M PHTPP did not inhibit the ratio of length / width, the intensity of calcium fluorescence and number of migrated cells by OAG (Fig. 24) and Cap (Fig. 25) treatments. Knockdown of ERβ did not inhibit the number of migrated cells by OAG and Cap (Fig. 26). These results suggested that activated ERβ accelerates cell migration via TRPV1.

3.3.5. Acceleration of tumor metastasis by activated ERβ is TRPV1-dependent

To confirm the role of TRPV1 in E2-and DHEA-induced 4T1 spontaneous metastasis from primary to secondary sites, tumor tissues in primary and liver were isolated for luminescence detection 10 days or 14 days after transplantation. A 10-day treatment with E2 (20 μg/mouse/day) and a 14-day treatment with DHEA (20 μg/mouse/day) increased the total density and metastasized ratio of 4T1 breast tumor at primary sites, which was inhibited by CapZ (10 μg/mouse/day) (Fig. 27). In addition, E2 and DHEA increased ERβ expression in tumor tissues. CapZ inhibited the increased expression of ERβ by E2 and DHEA (Fig. 28). These results indicated that acceleration of tumor metastasis by activated ERβ is TRPV1-dependent.

Discussion

In line with previous study, activation of ERβ alone is positively associated with tumor aggressiveness; the present study demonstrated that activated ERβ by DHEA facilities breast cancer metastasis. In this study, daily injection of E2 for 10 days promoted the breast cancer development, increasing the size of uterus and ERα expression in uterus on mature OVX female mice. However, DHEA treatment for 14 days just accelerated the breast cancer development which did not affect the uterus size and ERα expression in uterus. One possible explanation is that E2 activates both ERα and ERβ and DHEA may activate only ERβ alone because of different binding affinity. E2 shows similar physiological binding affinity to ERα and ERβ ; whereas DHEA shows 2 folds greater physiology binding affinity of ERβ compare to ERα. Moreover, it is known that E2 enlarged the uterus and increased the uterus ERα mRNA expression in ERβ knockout mice. Thus, E2 may activate both ERα, which resulted in increasing the uterus size and ERα expression and ERβ, which induced breast cancer spreading. While DHEA may just activate ERβ alone, thus just facilitating the breast cancer progression. In summary, DHEA is a better candidate to activate ERβ to enhanced breast cancer cell migratory ability and breast cancer metastasis.

Previous studies indicated that TRPV6 channels are positively associated with aggressive breast cancer and TRPV1 channels regulate non-invasive MCF-7 breast tumor cell proliferation. However, whether TRPV1 plays a role in aggressive 4T1cells remain unknown. According our data, TRPV1 expressed in 4T1 cells and regulated the migratory ability. Activation of TRPV1 induced 4T1 cell migration. Cell morphological changes and extracellular Ca2+ influx contribute to cell migration. Although the morphological changes and Ca2+ pattern vary from different cell line, they still share familiar points. The cytoskeleton is associated with protruding cellular structures that form morphological changes of migrating cells. Ca2+ influx facilitate focal adhesion turnover and actomyosin contractility. In brief, our data indicated that

expression and functionality of TRPV1 channels in 4T1 cells.

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科技部補助計畫衍生研發成果推廣資料表

日期:2016/10/31

科技部補助計畫	計畫名稱: 以4T1產生乳癌之小鼠為實驗模式，重新評估並探討DHEA對乳癌之產生作用機制
	計畫主持人: 蔡美玲
	計畫編號: 104-2629-B-006-001- 學門領域: 性別主流科技計畫

無研發成果推廣資料

104年度專題研究計畫成果彙整表

計畫主持人：蔡美玲	**計畫編號：**104-2629-B-006-001-
計畫名稱：以4T1產生乳癌之小鼠為實驗模式，重新評估並探討DHEA對乳癌之產生作用機制	

成果項目					量化	單位	質化 （說明：各成果項目請附佐證資料或細項說明，如期刊名稱、年份、卷期、起訖頁數、證號...等）
國內	學術性論文	期刊論文			0	篇	
		研討會論文			1		生醫年會報告
		專書			0	本	
		專書論文			0	章	
		技術報告			0	篇	
		其他			0	篇	
	智慧財產權及成果	專利權	發明專利	申請中	0	件	
				已獲得	0		
			新型/設計專利		0		
		商標權			0		
		營業秘密			0		
		積體電路電路布局權			0		
		著作權			0		
		品種權			0		
		其他			0		
	技術移轉	件數			0	件	
		收入			0	千元	
國外	學術性論文	期刊論文			0	篇	
		研討會論文			0		
		專書			0	本	
		專書論文			0	章	
		技術報告			0	篇	
		其他			0	篇	
	智慧財產權及成果	專利權	發明專利	申請中	0	件	
				已獲得	0		
			新型/設計專利		0		
		商標權			0		
		營業秘密			0		
		積體電路電路布局權			0		
		著作權			0		
		品種權			0		
		其他			0		

	技術移轉	件數	0	件	
		收入	0	千元	
參與計畫人力	本國籍	大專生	0	人次	
		碩士生	1		
		博士生	0		
		博士後研究員	0		
		專任助理	0		
	非本國籍	大專生	0		
		碩士生	0		
		博士生	0		
		博士後研究員	0		
		專任助理	0		
其他成果 （無法以量化表達之成果如辦理學術活動、獲得獎項、重要國際合作、研究成果國際影響力及其他協助產業技術發展之具體效益事項等，請以文字敘述填列。）					

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1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估
   ■達成目標
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2. 研究成果在學術期刊發表或申請專利等情形（請於其他欄註明專利及技轉之證號、合約、申請及洽談等詳細資訊）
   論文：□已發表　□未發表之文稿　■撰寫中　□無
   專利：□已獲得　□申請中　■無
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3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性，以500字為限）
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4. 主要發現
   本研究具有政策應用參考價值：■否　□是，建議提供機關
   （勾選「是」者，請列舉建議可提供施政參考之業務主管機關）
   本研究具影響公共利益之重大發現：■否　□是
   說明：（以150字為限）