RESEARCH COMMUNICATION

# Luteolin Inhibits Proliferation Induced by IGF-1 Pathway Dependent ERα in Human Breast Cancer MCF-7 Cells

**Li-Meng Wang, Kun-Peng Xie, Hong-Nan Huo, Fei Shang, Wei Zou, Ming-Jie Xie***

## Abstract

**The growth of many breast tumors is stimulated by IGF-1, which activates signal transduction pathways inducing cell proliferation. ERα is important in this process. The aim of the study was to investigate relationships *in vitro* among inhibitory effects of luteolin on the growth of MCF-7 cells, IGF-1 pathway and ERα. Our results showed that luteolin could effectively block IGF-1-stimulated MCF-7 cell proliferation in a dose- and time- dependent manner and block cell cycle progression and induce apoptosis evidenced by the flow cytometric detection of sub-G1DNA content. Luteolin markedly decreased IGF-1-dependent IGF-1R and Akt phosphorylation without affecting Erk1/2 phosphorylation. Further experiments pointed out that ERα was directly involved in IGF-1 induced cell growth inhibitory effects of luteolin, which significantly decreased ERα expression. Knockdown of ERα in MCF-7 cells by an ERα-specific siRNA decreased the IGF-1 induced cell growth inhibitory effects of luteolin. ERα is thus a possible target of luteolin. These findings indicate that the inhibitory effect of luteolin on the growth of MCF-7 cells is via inhibiting IGF-1 mediated PI3K-Akt pathway dependent of ERα expression.**

**Keywords:** Breast cancer - IGF-1 - ERα - luteolin - MCF-7

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## Introduction

Breast cancer is one of the most serious problems in oncology and is also the second leading cause of cancer-related death in women in the Western world, after lung cancer (Jemal et al., 2011), with about 132,000 deaths each year and a 5-year overall survival of approximately 79% (Berrino et al., 2007). The etiology of breast cancer is not understood clearly. There is accumulating evidence implicating IGF-1 system in development and/or progression of breast cancer (Sachdev et al., 2007). The insulin-like growth factor-1 (IGF-1) system (also called IGF-1 axis), including IGFs (IGF-1and IGF-2), IGF-1R (IGF-1receptor) and IGFBPs (IGF binding proteins), has been shown to play an important role in regulating normal and malignant cell growth (Sachdev et al., 2001).

Molecular targeting drugs are hotspot in cancer therapy. Because there is clear risk to treat breast cancer with chemotherapy, researchers worldwide started to search for natural products that have better anticancer activity and reduced side effect. Luteolin is a representative of natural flavonoids existing in many natural herbs, vegetables and fruits. Recent studies have shown that luteolin elicits significant inhibitory activity against breast cancer (Ren et al., 2003; Du et al., 2008). This effect is mainly exerted through blocking signal transduction in cancer cells (Lin et al., 2008; Dong et al., 2009; Hou et al., 2009). The IGF/IGF-1R downstream signaling pathways have been recently shown also to be involved in the metastatic cascade in breast cancer cells (Zhu et al., 2011). During tumorigenesis, IGF-1 mediated mitogen-activated protein kinase (MAPK) pathway and phosphatidylinositol 3-kinase (PI3K) pathway are important (Sachdev et al., 2001). For example, Fang et al. (2007) demonstrated that luteolin inhibited prostate cancer through inhibiting IGF-1 mediated MAPK and PI3K pathways. Xia et al. (2007) demonstrated that curcumin exhibited a potent ability to reverse the IGF-1-induced cell growth and apoptosis resistance involving IGF-1 mediated MAPK and PI3K pathway. In addition, it was previously believed that IGF-1 mediated signal transduction pathway is linear, as do most growth factor receptor signal transduction cascades. However, recent studies revealed that IGF-1 pathway is also regulated by hormones and intracellular proteins. For example, estrogen positively regulates IGF-1R by promoting IGF-1R and IRS-1 expression, resulting in the downstream activation of IGF-1 pathway (Kahlert et al., 2000). Castoria et al. (2001) reported that estrogen stimulates IGF-1R expression, which activates PI3K-Akt pathway, leading

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to increased mastocarcinoma mitosis. The biological effect of estrogen is primarily mediated through estrogen receptor α (ERα). IGF-1R can translocate to the nucleus and auto-regulate the activity of IGF/IGF-1R pathway in breast cancer cells depending on the estrogen receptor status (Sarfstein et al., 2012). Estrogen binds with estrogen receptor α (ERα), which in turn regulates the transcription of multiple estrogen responsive genes (for example IGF-1R), leading to the change of related protein levels in IGF-1 pathway. Therefore, ERα is important in IGF-1 signal transduction pathway. However, the relationship between the inhibitory effect of luteolin on the growth of MCF-7 cells and IGF-1 pathway or ERα has not been systematically studied. To elucidate the target of the inhibitory effect of luteolin on the growth of MCF-7 cells, our current study investigated relationships among IGF-1 pathway, ERα and the inhibitory effect of luteolin on the growth of MCF-7 cells.

## Materials and Methods

*Reagents and chemicals*

MCF-7 cells were provided by cell center of Institute of Basic Medical Science (CAMS). Newborn calf serum and RPMI-1640 were purchased from GIBCO. Trypsin, Methyl thiazolyl tetrazolium (MTT) and propidium iodide (PI) were from Ameresco. Human IGF-1 was from Peprotech. Antibodies against p-IGF1R, IGF1R, p-Akt, Akt, p-Erk1/2, Erk1/2, ERα and β-actin were purchased from Santa Cruz Biotechnology. Horseradish peroxidase conjugated goat anti-rabbit IgG and goat anti-mouse IgG were from Beijing Zhongshan Jinqiao Co. ECL was from Amersham Biosciences. ICI182780 was purchased from Sigma. L ipofectamineTM 2000 was from Invitrogen. OPTI-MEMI was from GIBCO. Expression vector psiRNA-ERα was constructed by Dalian Baoshengwu Co.

*Cell culture*

MCF-7 was cultured in RPMI-1640 supplemented with 10% newborn calf serum, penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37 °C in 5% $CO_2$ incubator. During test, cells in log phase were used.

*MTT cell viability assay*

MCF-7 cells at log phase were seeded in 96-well plate at $4\times10^3$ cells/well, cultured for 24 h, then switched to serum-free medium for 24 h. After that, cells were treated respectively with luteolin (0, 10, 20, 40 μmol/L), IGF-1 (0, 5, 10, 20, 40, 50, 60 μg/L ) and 50 μmol/L of antiestrogen ICI182780 in serum-free medium. Cells were cultured for 24, 48 and 72 h. Then, MTT (20 μl at 5 mg/ml) was added to each well and culture continued for 4 h at 37 ºC. Medium was then replaced with 150 μl of DMSO. Absorption was measured at 490 nm with reference wavelength at 570 nm. The assay was repeated 3 times (n=3) unless noted otherwise.

*Cell cycle and apoptosis analysis*

MCF-7 cells at log phase were seeded in 6-well plate at $2\times10^5$ cells/well using complete medium overnight for attachment, then switched to serum-free medium for 24 h. After that, cells were treated respectively with of 40 μmol/L of luteolin and 50 μg/L of IGF-1 in serum-free medium for 24 h. Adherent cells were released by trypsinization, combined with nonadherent cells, and recovered by centrifugation at 500 g for 5 min at 4 °C and washed with PBS. Then, cells were fixed with 70% ethanol over 24 h. Finally, cells were treated with 50 μg/ml of RNase A and 50 μg/ml of propidium iodide for 30 min. Stained cells were monitored by flow cytometry.

*Western blot analysis*

When cells became attached in 6-well plate, medium was replaced with serum-free medium and the cells were cultured for 24 h. After treatment with luteolin for 5 h, cells were treated with inhibitors (LY-294002 for PI3K, PD-98059 for MEK or ICI182780 for ERα) for 1 h. Finally, cells were treated with IGF-1 for 30 min. After luteolin treatment growth medium was removed and cells were washed with ice-cold PBS and lysed in a modified RIPA buffer (in mM: 50Tris–Cl, 150NaCl, 1EDTA, 1EGTA, 1phenylmethylsulfonyl fluoride, 1 NaF, 1 Na3VO4, and 1% v/v NP-40, 0.35% w/v sodium-deoxycholate, 10 μg/mL each of aprotinin, leupeptin, and pepstatin A, pH adjusted to 7.4) for 20 min at 4 °C. After centrifugation at 14,000g for 15 min at 4 °C, the supernatant was collected and regarded as whole cell extract. Samples containing 30–50 μg of protein were separated by SDS–polyacry-lamide gel electrophoresis and then transferred onto nitrocellulose membranes (0.45μm, Bio-Rad). Blots were incubated overnight at 4 °C with (a) anti-pIGF-1R antibody (1:1000) (Santa Cruz Biotechnology) (b) anti-IGF-1R antibody (1:1000) (Santa Cruz Biotechnology) (c) anti-pAkt antibody (1:1000) (Santa Cruz Biotechnology) (d) anti-Akt antibody (1:1000) (Santa Cruz Biotechnology) (e) anti-pERK1/2 antibody (1:1000) (Santa Cruz Biotechnology) (f) anti-ERK1/2 antibody (1:1000) (Santa Cruz Biotechnology) (g) anti-ERα antibody (1:3000) (Santa Cruz Biotechnology) and (h) anti-β-actin antibody (1:2000) (Santa Cruz Biotechnology), then incubated at 37°C in shaker with HRP-conjugated secondary antibody for 1 h. Upon color development with ECL, X-ray film was used to detect light. The film was developed at room temperature, fixed, and scanned with scanner. Transillumination light density was measured with gel image system.

*siRNA transfection*

Transfection was conducted using Lipofectamine TM2000 according to the manufacturer's recommendation. 24 h before transfection, log phase MCF-7 cells were seeded in 6-well plate. Transfection was done at 90% confluence. Cells were allotted to three groups: (1) blank control (cells without any treatment); (2) negative control (transfected with negative plasmid psiRNA-Con); (3) trasnfection with psiRNA-ERα. After transfection at certain time, cells were cultured in $CO_2$ incubator at 37 ºC for 4 h, then switched to normal RPMI-1640 medium containing 10% serum. Culture continued for a period indicated, then harvested. Total protein was collected and analyzed with Western blot.

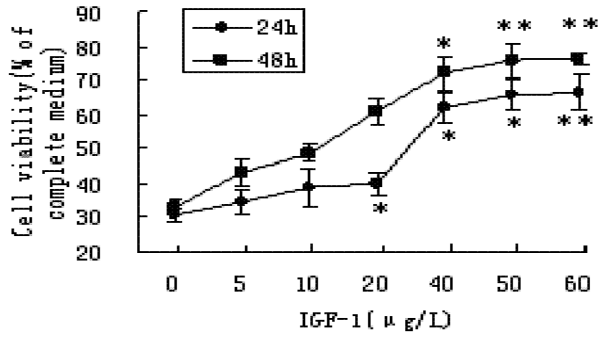


**Figure 1. The Effect of IGF-1 on MCF-7 Cell Proliferation in Vitro.** Cells were plated and cultured as described in Materials and methods. Cell viability was determined using the MTT assay. Each graph represents data from triplicate separate experiments. Values were expressed as means ±SD. a significant (p<0.05 and p<0.01) increase (compared with 0 μg/L of IGF-1) was indicated by * and **, respectively

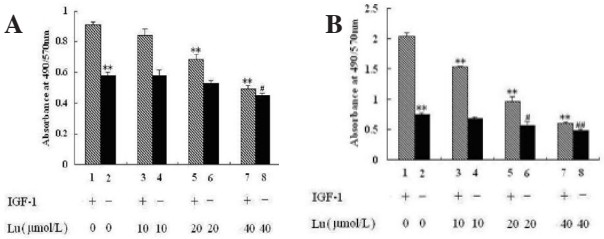


**Figure 2. Effect of Luteolin on IGF-1-induced Breast Cancer Cell Proliferation.** MCF-7 cells were plated and cultured as described in Materials and methods. After serum starvation, cells were cotreated with luteolin and IGF-1 in serum-free medium for 24 and 48 h. Cell viability was determined using the MTT assay. A: Luteolin abrogated IGF-1-stimulated cell proliferation for 24 h in MCF-7 cells. B: Luteolin abrogated IGF-1-stimulated cell proliferation for 48 h in MCF-7 cells. Each values were expressed as means ± SD. from three replicates in each treatment group. a significant (p<0.05 and p<0.01) increase (compared with serum-free control) was indicated by # and ##, respectively. a significant (p<0.01) decrease (compared with IGF-1) was indicated by ** at 24 h and 48 h. Lu, Luteolin

*Statistical analysis*

Values were expressed as mean ± SD. SPSS16.0 and Excel XP were used for t-test. Differences with p<0.05 were considered to be statistically significant.

## Results

*Effect of IGF-1 on MCF-7 cell proliferation*

At 24 h, cells survial rate in serum-free medium achieved only (30.06±2.18)% (p<0.05) of that found in complete medium (containing 10% newborn calf serum). When IGF-1 was added, cell survial rate was enhanced in a dose-dependent manner. After stimulation with IGF-1 at 20, 40 and 50 μg/L, the cell survial rate was (39.90±3.25)%, (62.10±4.13)% and (65.62±4.28)% (p<0.05) of that observed in complete medium, respectively. When the cells were not treated with IGF-1, there was no significant cell growth between the cultures for 24 h and 48 h. When cells were treated with different concentrations of IGF-1 for 48 h in serum-free medium, cell survial rate was significantly increased, compared with 24 h (Figure

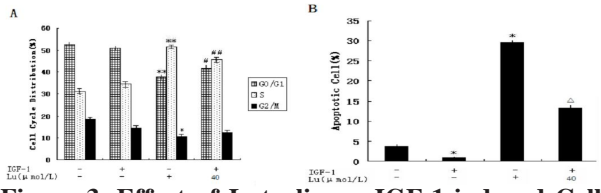


**Figure 3. Effect of Luteolin on IGF-1-induced Cell Cycle Progression and Apoptosis in MCF-7 Cells.** MCF-7 cells were treated with luteolin and IGF-1 as described in Materials and methods. Cell cycle distribution and cell apoptotic rate were determined by flow cytometry using PI staining. A: Quantification of cell cycle distribution from three independent experiments. B: Sub-G1 phase cell populations from three independent experiment were quantified. The intensity of the different bands were determined by densitometry and plotted as means±S.D.a significant (p<0.05 and p<0.01) difference (compared with control) was indicated by * and **, respectively. A significant (p<0.05 and p<0.01) decrease (compared with IGF-1) was indicated by # and ##, respectively. a significant (p<0.01) difference (compared with IGF-1or Lu) was indicated by△. Lu, Luteolin

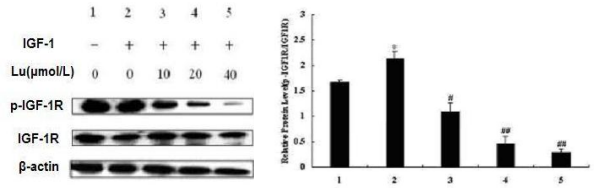


**Figure 4. Effects of Luteolin on IGF-1 Induced IGF-1R Phosphorylation.** MCF-7 cells were treated with luteolin and IGF-1 as described in Materials and methods. The amount of proteins was assessed by immunoblotting. The intensity of the different bands were determined by densitometry and plotted as means±S.D.from three replicates in each treatment group. a significant (p<0.05) increase in (compared with serum-free control) was indicated by *, a significant (p<0.05 and p<0.01) decrease in protein level (compared with IGF-1 ) was indicated by # and ##, respectively. Lu, Luteolin

1). We further observed that the increasing rate of cell proliferation was slowed down when high concentrations of IGF-1 (50 μg/L and 60 μg/L) were added. Based on these results, we selected IGF-1 at 50 μg/L in serum-free conditions for the subsequent experiments.

*Effect of luteolin on IGF-1-induced breast cancer cell proliferation*

Concurrent treatment with luteolin (10, 20 or 40 μmol/L) and IGF-1 (50 μg/L) for 24 h reduced IGF-1-stimulated cell proliferation by (8.21±4.33)%, (25.19±3.52)% and (46.48±2.50)%, respctively, compared with IGF-1 alone (Figure 2A). When treated for 48 h, compared with IGF-1 treatment, luteolin at (10, 20 or 40 μmol/L) treatment decreased cell viability by (25.19±2.10)%, (52.63±5.04)% and (71.05±2.61)% (p<0.01) (Figure 2B). This data suggests luteolin could inhibit IGF-1-induced cell proliferation effect in a dose- and time- dependent manner.

*Effect of luteolin on IGF-1-induced cell cycle progression and apoptosis*

Flow cytometric analysis showed that cell cycle progression of PI-stained MCF-7 cells. Cells were

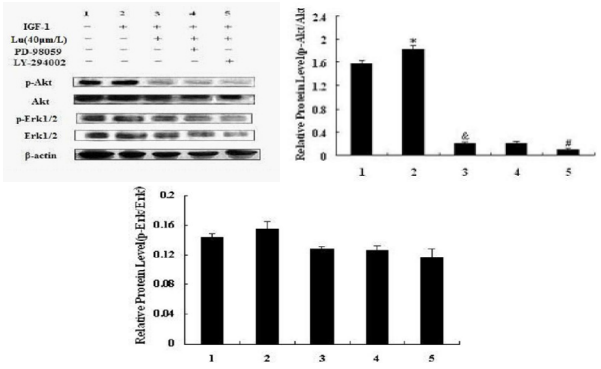


**Figure 5. Effects of Luteolin on IGF-1 Induced Akt and Erk1/2 Phosphorylation.** MCF-7 cells were treated with luteolin, IGF-1, LY294002 and PD98059 as described in Materials and methods. The amount of proteins was assessed by immunoblotting. Each value represents the mean of three experiments; bars, ± SD. a significant ($p<0.05$) increase in p-Akt (compared with serum-free control)protein level was indicated by *, a significant ($p<0.01$) decrease in p-Akt (compared with IGF-1 )protein level was observed (&) and a significant ($p<0.05$) decrease in p-Akt (compared with IGF-1+Lu) protein level was indicated by #. Lu, Luteolin

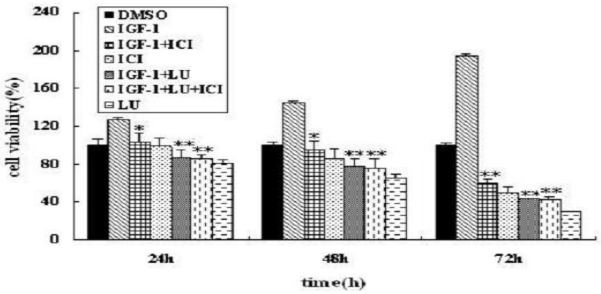


**Figure 6. Relationship Between ERα and the Inhibitory Effect of Luteolin on IGF-1 Induced Breast Cancer Proliferation.** Cells were treated with luteolin, IGF-1 and ICI182780 as described in Materials and methods. Cell viability was determined using the MTT assay. Each value represents the mean of three experiments; bars, ± SD. a significant ($p<0.05$ and $p<0.01$) decrease (compared with IGF-1) was indicated by * and **, respectively. Lu, Luteolin; ICI, ICI182780

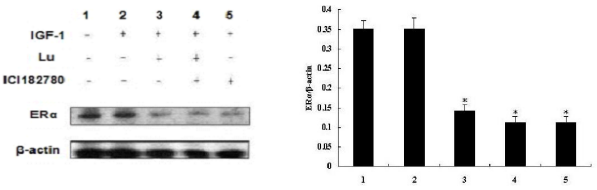


**Figure 7. Effect of Luteolin on ERα Expression. MCF-7 Cells were Treated with Luteolin, IGF-1 and ICI182780 as Described in Materials and Methods.** The amount of protein was assessed by immunoblotting. The intensity of the different bands were determined by densitometry and plotted as means ± SD. from three replicates in eachtreatment group. a significant ($p<0.01$) decrease in band intensity (compared with IGF-1) was indicated by *. Lu, Luteolin

cultured 24 h under the indicated conditions. 40 $\mu$mol/L of luteolin and 50 μg/L of IGF-1 combined treatment reduced the number of cells in the G0/G1 phase and caused a significant increase of S phase cells, compared with IGF-1 alone ($p<0.01$) (Figure 3A). In addition, analysis showed that cell apoptosis rate in serum-free control group and IGF-1 group was (3.81±0.42)% and (0.95±0.25)%, respectively, suggesting it was statistically significant ($p<0.01$) and IGF-1 inhibited cell apoptosis. However, luteolin and IGF-1 combined treatment increased cell apoptotic rate by 11.28 fold, compared with IGF-1 alone ($p<0.01$) (Figure 3B). This data suggests that luteolin inhibited IGF-1-induced cell cycle progression and resistance of breast cancer cells against apoptosis.

*Effects of luteolin on IGF-1 induced IGF-1R phosphorylation*

Compared with serum-free control, the group of the cells treated with IGF-1 increased the amount of p-IGF-1R by 27.38%, respectively ($p<0.05$), suggesting IGF-1 was able to induce IGF-1R phosphorylation. When cells were cotreated with luteolin (10, 20 and 40 μmol/L) and IGF-1 in serum-free medium, the amount of p-IGF-1R was decreased by 48.83% ($p<0.05$) 77.57% and 86.20% ($p<0.01$), compared with IGF-1 treatment (Figure 4). This suggests that luteolin could inhibit IGF-1 induced IGF-1R phosphorylation, which acted through IGF-1 mediated signal transduction pathway to exerts its anti-breast cancer effect.

*Effects of luteolin on IGF-1 mediated MAPK/Erk1/2 and PI3K-Akt pathways*

Western blot analysis showed that the amount of p-Akt in IGF-1 group was 1.15 fold that of serum-free control ($p<0.05$). The amount of p-Erk1/2 was increased, though it was not statistically significant ($p>0.05$). Treatment with luteolin at 40μmol/L deacreased the amount of p-Akt by 88.93% ($p<0.01$) without an effect on p-Erk1/2 level ($p>0.05$), suggesting that luteolin inhibited IGF-1 induced Akt phosphorylation. When PI3K inhibitor LY-294002 was added, the level of p-Akt was declined by 50.10% when compared with treatment with luteolin and IGF-1 combined ($p<0.05$). Yet when MEK inhibitor PD-98059 was added, p-Akt level did not change ($p>0.05$) (Figure 5). This suggests that the inhibitory effect of luteolin on IGF-1 induced breast cancer cell proliferation was mainly mediated by PI3K-Akt signal transduction pathway, not by MAPK/Erk1/2 pathway.

*Relationship between ERα and the inhibitory effect of luteolin on IGF-1 induced breast cancer proliferation*

Both luteolin and antiestrogen ICI182780 are able to inhibit IGF-1 induced MCF-7 proliferation in a time-dependent manner. When treated for 48 h, compared with IGF-1 treatment, IGF-1 and luteolin treatment decreased cell survival rate by 46.18% ($p<0.01$), IGF-1 and ICI182780 treatment decreased that by 34.06% ($p<0.05$). However, IGF-1, luteolin and ICI182780 combined treatment decreased cell survival rate by 47.64% ($p<0.01$), which was not significantly different from treatment with either IGF-1 and luteolin or IGF-1 and ICI182780 treatment ($p>0.05$) (Figure 6). These data suggest that the inhibitory effect of luteolin on IGF-1 induced breast cancer proliferation is mainly related to ERα.

*Effect of luteolin on ERα expression*

Concurrent treatment with luteolin and IGF-1 for 6 h

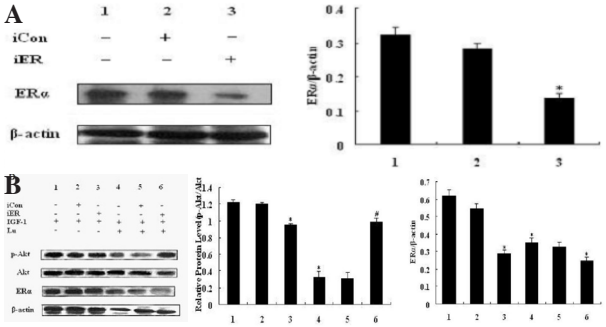


**Figure 8. Effect of Luteolin on IGF-1 Induced Akt Phosphorylation with and Without ERα Knockdown.** A: MCF-7 cells were transfected with ERα-siRNA and Con-siRNA (as control siRNA) as described in the Materials and methods. The amount of proteins was assessed by immunoblotting. B: MCF-7 cells transfected with ERα-siRNA and Con-siRNA treated with 40 $\mu$mol/L of luteolin or with 50 μg/L of IGF-1 together for the indicated time points. Protein extracts were analyzed with Western blot analysis. The experiment was carried out in triplicate and results from densitometric analysis are plotted as means ± SD. from three replicates for each treatment group. a significant ($p<0.01$) decrease in band intensity (compared with IGF-1) was indicated by *, and a significant ($p<0.01$) increase in band intensity (compared with IGF-1+Lu) was indicated by #. Lu, Luteolin

reduced ERα expression by 59.52% ($p<0.01$), compared with IGF-1 alone. However, ERα expression did not change by either of ICI182780 or ICI182780 and luteolin combined ($p>0.05$) (Figure 7). These observations suggest that luteolin decreased ERα expression level, which had an effect on IGF-1-induced MCF-7 cells growth.

*Effect of luteolin on IGF-1 induced Akt phosphorylation with and without ERα knockdown*

To verify whether ERα plays a role in IGF-1 induced cell growth inhibitory effect of luteolin, we tested the effect of luteolin in cell of which ERα expression was down regulated by transient transfection of ERα-specific siRNA. Forty-eight hours after siRNA transfection ERα knockdown was verified with Western blot analysis (Figure 8A). Before transfection, treatment with luteolin decreased p-Akt level by 73.07% ($p<0.01$), compared with IGF-1 treatment. After transfection, treatment with luteolin had little effect on p-Akt level ($p>0.05$) (Figure 8B). Our results showed that verified ERα-knockdown cells were more resistant to luteolin inhibition. These suggest that that the inhibitory effect of luteolin on the growth of MCF-7 cells via inhibiting IGF-1 mediated PI3K-Akt pathway is dependent on, at least in part, ERα expression.

## Discussion

The identification and further modulation of molecular targets with central role in breast carcinogenesis represent a rational approach forprevention and treatment (Sogno et al., 2010; Vera-Ramirez et al., 2011; Baselga et al., 2012). Data published during the last decade has implicated insulin-like growth factor (IGF) and its signaling cascade in the development and progression of breast cancer (Pollak 2008). Studies recently demonstrated that overexpression of IGF-1 in cancer cells is associated with tumor growth (Werner et al., 2000; Bustin et al., 2002). The current experiment showed that when cells were treated with IGF-1, cell viability was significantly enhanced, compared with serum-free control group. our results indicated that IGF-1 was actually one of the important growth factors for MCF-7 cell growth. These results are consistent with previous reports (Kappel et al., 1994; Resnicoff et al., 1995).

Recently, researchers worldwide started to search for flavonoids that have better anticancer activity existing in many natural herbs. Recent studies revealed the inhibitory effect of most flavonoids is mainly exerted through blocking IGF-1system in cancer cells. Luteolin, the 3', 4', 5, 7-Tetrahydroxyflavone, is a common dietary flavonoid and has been found to have a wide spectrum of anti-tumor activities. As expected, we documented that luteolin did inhibit the stimulation effect of IGF-1 on MCF-7 cell proliferation and inhibitory effect on apoptosis. These inhibitory effects were associated with a S cell cycle blockage and a sub-G1 apoptotic peak induction. This data suggests that luteolin inhibited IGF-1-induced cell cycle progression and resistance of breast cancer cells against apoptosis. Our data are supported by previous studies that showed luteolin exerted its anticancer effects via proliferation inhibition and apoptosis induction in prostate cancer cells.

IGF-1 mediated signal transduction pathway is critical in oncogenesis and tumor development (Surmacz 2002; Carboni et al., 2007). Its biological effect is primarily mediated through MAPK/Erk1/2 and PI3K-Akt pathways (Dong et al., 2007). The binding of IGF-1 with the extracellular domain of IGF-1R causes phosphorylation of IGF-1R and downstream substrate, ultimately phosphorylate MAPK and Akt. Thus IGF-1 signal is transmitted to nucleus, initiating gene expression to promote cell proliferation and to inhibit apoptosis. IGF-IR was required for oncogenic transformation and has an established role in breast cancer tumorigenesis (Buck et al., 2010). Because of its critical importance, the IGF-IR pathway has been intensively studied as a cancer therapeutic target and multiple agents are in clinical development (Yuen et al., 2008; Baserga 2009; Gualberto et al., 2009; Buck et al., 2011). The current experiment demonstrated that luteolin markedly decreased IGF-1R and Akt phosphorylation without affecting Erk1/2 phosphorylation. The group treated with PI3K inhibitor LY-294002 had lower p-Akt level, compared with the group treated with luteolin. Yet, the group treated with MEK inhibitor PD-98059 had no significant difference in p-Akt level compared with the groups treated with luteolin. This suggests that the inhibitory effect of luteolin on IGF-1 induced breast cancer cell proliferation was mainly mediated by PI3K-Akt signal transduction pathway, not by MAPK/Erk1/2 pathway.

It was previously believed that IGF-1 mediated signal transduction pathway is linear, as do most growth factor receptor signal transduction cascades. However, recent studies revealed that IGF-1 pathway is also regulated by hormones and intracellular proteins. IGF-IR expression level has been associated with ER positivity in both breast

cancer cell lines and patients'tumor specimens. SiRNA-mediated knockdown of the ER reduced the expression level of IGF-IR (Surmacz et al., 2004; Weroha et al., 2008; Zha et al., 2009). Studies recently demonstrated that ERα could associates with IGF-1R leading to activation of downstream PI3K-Akt and MAPK signaling cascades. Although ERα does not contain a membrane localization sequence, it was found to co-immunoprecipitate with IGF-1R in the membrane. Klotz et al. (2002) demonstrated that knockout of ERα diminished IGF-1-stimulated normal mouse uterine cell proliferation. Oesterreich et al. (2001) showed that a nanomolar level IGF-1 could not stimulate ER- MCF-7 but ER+ MCF-7 proliferation. However, when ER- MCF-7 is rescued with ERα, IGF-1 stimulated proliferation effect was recovered. In our experiment, When cells were treated for 48 h, cell survival rates were the same after treatment with either luteolin or ICI182780 or luteolin and ICI182780 combined. And ERα expression was also decreased. This evidence suggests that ERα is directly involved in luteolin inhibiting IGF-1 induced MCF-7 proliferation. As to the relationship between ERα expression and IGF-1 induced MCF-7 proliferation, we determined inhibition effects of luteolin on IGF-1 induced Akt phosphorylation before and after ERα-siRNA treatment. The current experiment showed that blocking of ERα expression markedly inhibited IGF-1 induced p-Akt expression. Before transfection, compared with IGF-1 control, treatment with luteolin markedly reduced p-Akt level. After transfection, luteolin treatment had a p-Akt level similar to the control group. In other words, the inhibitory effect of luteolin on IGF-1 disappeared upon the decrease in ERα expression. These suggest that the inhibitory effect of luteolin on the growth of MCF-7 cells via inhibiting IGF-1 mediated PI3K-Akt pathway dependent on ERα expression. ERα is possibly one of the targets of luteolin.

Although IGF-IR signaling activation has been detected in both ER+and ER-breast cancers with associated poor clinical outcome (Law et al., 2008), multiple studies have demonstrated a functional relationship and extensive cross-talk between IGF-IR and ER pathways (Fagan et al., 2008). ERα is the single most powerful predictor of breast cancer prognosis as well as an important contributor to the biology of carcinogenesis. ERα may interact with IGF-1 signaling at multiple levels and in multiple fashions, ultimately resulting in increased signaling through IGF-1R. ERα belongs to the nuclear receptor superfamily and is one of the transcription factors. A number of studies demonstrated that ERα can drive IGF-1-mediated biology via transcriptional up-regulation of key IGF pathway genes [e.g. IGF-I receptor (IGF-1R), insulin receptor substrate (IRS)] (Salerno et al., 1999). Moreover, loss of ERα expression and/or function results in decreased IGF-induced growth and survival (Zhang et al., 2005). Our results suggest luteolin decreased ERα expression level, which had an effect on IGF-1-induced MCF-7 cells growth. Studies recently demonstrated that flavonoids could bind to DNA duplexes by electrospray lonization mass spectrometry (Wang et al., 2008). Further studies would be needed to elucidate whether luteolin exerted its effects through interacting with ERα or through inhibiting transcription of ERα gene, ultimately resulting in decreased IGF-1 signaling pathway.

Taken together, data presented here demonstrate that luteolin exhibited a potent ability to blunt IGF-1-stimulated MCF-7 cell growth in a dose- and time-dependent manner. ERα was directly involved in luteolin inhibiting IGF-1 induced MCF-7 cell proliferation. Luteolin significantly decreased ERα expression level. Knockdown of ERα-specific siRNA decreased the anti-proliferation activity of luteolin in MCF-7 cells. These findings indicate that the inhibitory effect of luteolin on the growth of MCF-7 cells via inhibiting IGF-1 mediated PI3K-Akt pathway dependent on ERα expression. ERα is possibly one of the targets of luteolin. Therefore, we suggest luteolin as a novel drug that may be effective in the treatment of breast cancer.

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