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13. SUPPLEMENTARY NOTES

14. ABSTRACT

During this grant period, we found that growth factor inhibitors as well as nutrient depletion activated autophagy in human beast cancer cells, and the increased activity of autophagy was associated with a decrease in cellular ATP and an increase in activities of AMP kinase and eEF-2 kinase. Silencing of eEF-2 kinase relieved the inhibition of protein synthesis, led to a greater reduction of cellular ATP, and blunted autophagic response. We further demonstrated that suppression of eEF-2 kinase-regulated autophagy impeded cell growth in serum/nutrient-deprived cultures and handicapped cell survival, and enhanced the efficacy of the growth factor inhibitors such as trastuzumab, gefitinib, and lapatinib. The results of this study provide new evidence that activation of eEF 2 kinase-mediated autophagy plays a protective role for cancer cells under metabolic stress conditions, and that targeting autophagic survival may represent a novel approach to enhancing the effectiveness of growth factor inhibitors such as trastuzumab, gefitinib, and lapatinib

15. SUBJECT TERMS

elongation factor-2 kinase; autophagy; metabolic stress; growth factor inhibitor; breast cancer

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INTRODUCTION

The members of the EGFR family such as EGFR/HER1 and HER2/erB2 represent attractive targets for therapeutic intervention in treatment of cancer, due to the roles of these receptor tyrosine kinases in stimulating oncogenic signaling pathways and in the development and progression of cancers (1-3). Aberrant expression or activity of the EGFR family receptor tyrosine kinases is encountered in many types of malignancies including breast cancers. Indeed, the EGFR tyrosine kinase inhibitors such as lapatinib and gefitinib, and the HER2/neu-targeted agent trastuzumab, have been shown to possess notable antitumor activity in several types of cancers (4). These drugs can specifically bind to the receptors with high affinity, resulting in blockade of the downstream signaling pathways and inhibition of tumor growth. Nevertheless, refractoriness to these growth factor inhibitors is common (5, 6). For instance, in patients with HER2-positive metastatic breast cancers, the response rate of trastuzumab is only ~26 % (7). Thus, understanding of the mechanisms underlying the insensitivity to the growth factors inhibitors and developing approaches to sensitizing tumor cells will make these drugs more valuable in treating patients with cancer. In this study, we sought to determine whether activation of eEF-2 kinase-mediated autophagy altered sensitivity of human breast cancer cells to inhibition of growth factor-initiated signaling, and whether modulating autophagy via targeting eEF-2 kinase would render tumor cells more susceptible to the effect of growth factor inhibitors. Our study shows that the eEF-2 kinase-mediated autophagy plays a cytoprotective role in breast cancer cells treated with growth factor inhibitors, and inhibiting autophagic survival can modulate sensitivity to these therapeutic agents.

BODY

Task 1 To determine the importance of autophagy in the life and death of breast cancer cells.

Task 2 To determine the role of autophagy in the sensitivity of breast cancer to treatment.

To determine the effect of metabolic stress on autophagy, we first treated the human breast cancer cells MCF-7 with DPBS, and then examined autophagic activity in the treated cells. As shown in Fig. 1A, nutrient deprivation increased the level of LC3-II, a specific marker of autophagic activity. Autophagosome formation was confirmed by GFP-LC3 puncta localization (Fig. 1B). We further found that MCF-7 and MDA-MB-468 breast cancer cells treated with the growth factor inhibitors, gefitinib and lapatinib, showed an increased in LC3-II amount in a dose-dependent manner, as determined by Western blot (Fig. 1C and D). The effects of gefitinib and lapatinib on autophagy were verified using GFP-LC3 cleavage assay (Fig. 1E). These observations suggest that activation of autophagy may represent a cellular response to metabolic stress, including treatment with growth factor inhibitors.

To ascertain whether a causal relationship exists between activation of autophagy and metabolic stress in breast cancer cells, we measured protein synthesis activity and ATP level in MCF-7 cells subjected to nutrient starvation. As shown in Fig. 2, treatment of the cells with DPBS (Dulbecco's Phosphate-Buffered Saline) caused a marked decrease in protein synthesis

(Fig. 2A) and ATP content (Fig. 2B). Cellular response to energy stress was also evidenced by an increased phosphorylation on Thr172 of AMPK, an intracellular energy sensor that is activated when cellular energy level decreases (Fig. 2C).

We next determined the effects of nutrient deprivation on the activity of eEF-2 kinase, a unique calmodulin-dependent enzyme that inhibits protein synthesis and activates autophagy, and on the signaling molecules associated with the regulation of the activity of this kinase. Fig. 3A shows that treatment of MCF-7 cells with DPBS activated eEF-2 kinase, as measured by the phosphorylation of EF-2, the substrate for the kinase. Activation of eEF-2 kinase was also manifested in an increased auto-phosphorylation on Ser398, which is known to positively regulate the activity of this kinase, and in a decreased phosphorylation of the kinase on Ser366, a site known to negatively regulate the activity of this enzyme (Fig. 3A). The activity of S6 kinase, a key translation controller downstream of mTOR, was decreased, as evidenced by a decrease in the phosphorylation on Thr389 of S6 kinase (Fig. 3B). The activity of 4EBP1, a translation repressor, was increased, as shown by a decrease in the phosphorylation of this protein (Fig. 3B).

To analyze whether eEF-2 kinase plays a regulatory role in nutrient starvation-induced reduction of protein synthesis and ATP content and in activation of autophagy in breast cancer cells stressed with nutrient depletion, we silenced EF-2 kinase expression using siRNA, and then measured ATP level, protein synthesis and autophagy activity following treatment with DPBS. As shown in Fig. 4A, inhibition of eEF-2 kinase by siRNA decreased starvation-induced autophagy. Inhibition of eEF-2 kinase also resulted in mitigation of the nutrient depletion-induced inhibition of protein synthesis (Fig. 4B) and led to further reduction of ATP levels (Fig. 4C). These results further support a role for eEF-2 kinase in activating autophagy in metabolically stressed tumor cells.

To further test our hypothesis that autophagy plays a pro-survival role in response to a compromised supply of cellular nutrients and growth factors during breast cancer development and progression, we knocked down eEF-2 kinase, beclin-1 or ATG5 (two of the key autophagy-related genes) in MCF-7 cells (Fig. 5A), and then compared the growth and survival of these autophagy-deficient cells with that of the cells transfected with a non-targeting RNA in serum-free medium or HBSS. As shown in Fig. 5B, suppression of autophagy by knockdown of those autophagy-regulated genes hindered the tumor cell growth in the absence of serum. Knockdown of eEF-2 kinase, beclin-1 or ATG5 also caused more death of MCF-7 cells cultured in HBSS (Fig. 5C). The autophagy inhibitor, 3-MA, was used as a control and showed a similar inhibitory effect on cell growth and survival in the absence of serum or nutrients (Fig. 5B and C).

To determine whether suppression of the eEF-2 kinase-mediated autophagy alters sensitivity of tumor cells to growth factor inhibitors that are in clinical use, we first transfected MCF-7 cells with an eEF-2 kinase-targeted siRNA or a non-targeting RNA, and then treated the transfected cells with a series of concentrations of gefitinib or lapatinib. Fig.6 A and B show that silencing of EF-2 kinase expression increased sensitivity of MCF-7 cells to gefitinib and lapatinib. Similar results were observed with the human breast cancer cells MDA-MB-468 (Fig. 6C and D). Inhibition of autophagy by an eEF-2 kinase-targeted siRNA also enhanced the cytotoxic effects of a small molecule EGFR/ErbB-2 inhibitor (EEi) and trastuzumab, an anti-Her2 therapeutic antibody (Table 1). Combined use of the inhibitors of growth factor and autophagy produced combination indexes (CIs) smaller than 1 (Table 1), indicating a synergism between the actions of those treatments.

KEY RESEARCH ACCOMPLISHMENTS

• We found that growth factor inhibitors activated autophagy in human breast cancer cells.

• We showed nutrient deprivation caused a reduction of protein synthesis and cellular ATP.

• We showed that nutrient deprivation activated eEF-2 kinase through the mTOR/S6 kinase pathway.

• We demonstrated that eEF-2 kinase was involved in autophagy induction and ATP reduction in response to nutrient depletion.

• We observed that suppression of autophagy decreased growth and survival of metabolically stressed breast cancer cells.

• We found that inhibition of eEF-2 kinase sensitized breast cancer cells to growth factor inhibitors.

REPORTABLE OUTCOMES

Manuscript
Cheng Y, Li H, Ren XC, Niu TK, Hait WN, Yang JM: Cytoprotective Effect of the Elongation Factor-2 Kinase-mediated Autophagy in Breast Cancer Cells Subjected to Growth Factor Inhibition. *PLoS ONE*   Accepted.

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CONCLUSIONS

The results of this study provide new evidence that activation of eEF-2 kinase-mediated autophagy plays a protective role for cancer cells under metabolic stress conditions, including treatment with those growth factor antagonists that are in clinical use, and that targeting autophagic survival may represent a novel approach to enhancing the effectiveness of growth factor inhibitors.

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APPENDIX
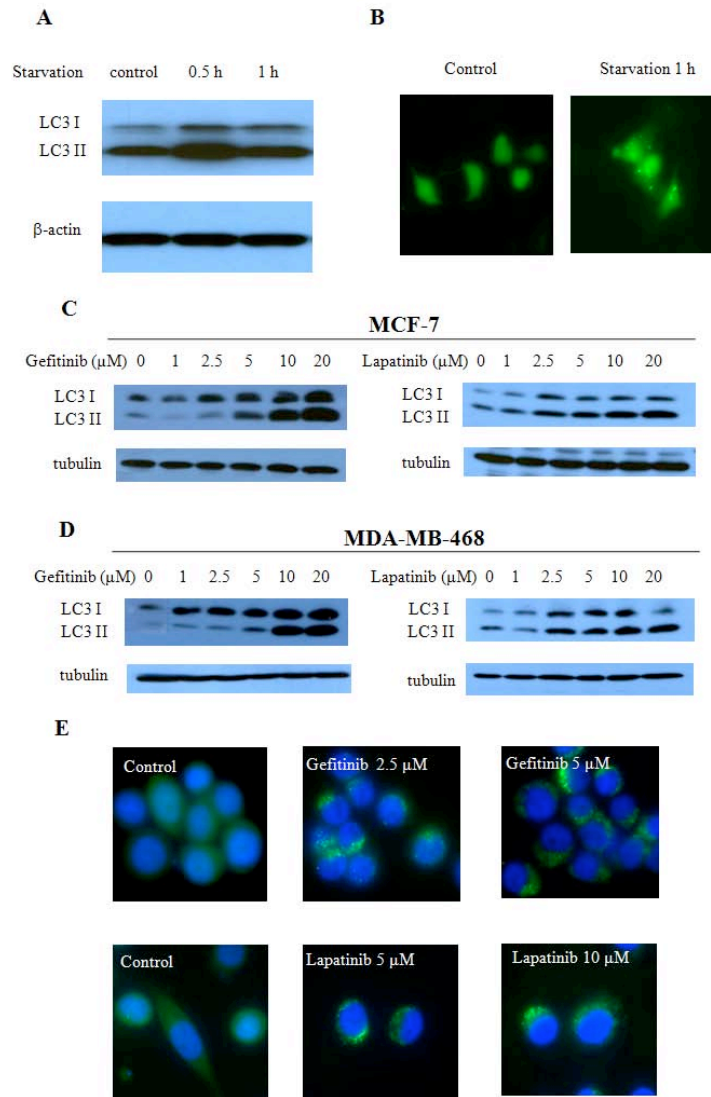
Figure 1. Effect of nutrient deprivation and growth factor inhibitors on autophagy in human breast cancer cells. (**A**) MCF-7 cells were treated with DPBS for the indicated times. At the end of treatment, formation of the autophagy marker LC3-II was detected by immunoblotting with an anti-MAP-LC3 antibody. (**B**) MCF-7 cells transfected with 3μg of GFP-LC3 plasmid were treated or untreated with DPBS for 1h, and then observed under a fluorescent microscope. A representation of GFP-LC3 positive cells was shown. (**C, D**) MCF-7 (C) or MDA-MB-468 (D) cells cultured in medium containing 10% FBS were treated with 0.5, 1, 2.5, 5 or 10μM gefitinib or lapatinib for 24h, and the LC3-II level was examined by immunoblotting. (**E**) MDA-MB-468 cells were transfected with a GFP-LC3-expressing vector, and then treated with the indicated concentration of gefitinib or lapatinib in the presence of the lysosomal protease inhibitors E64d (10 μg/ml) and pepstain A (10 μg/ml). At the end of treatments, cells were fixed with 4% formaldehyde for 15 min. To determine the autophagic response, cells were inspected at 60x magnification for numbers of GFP-LC3 puncta.
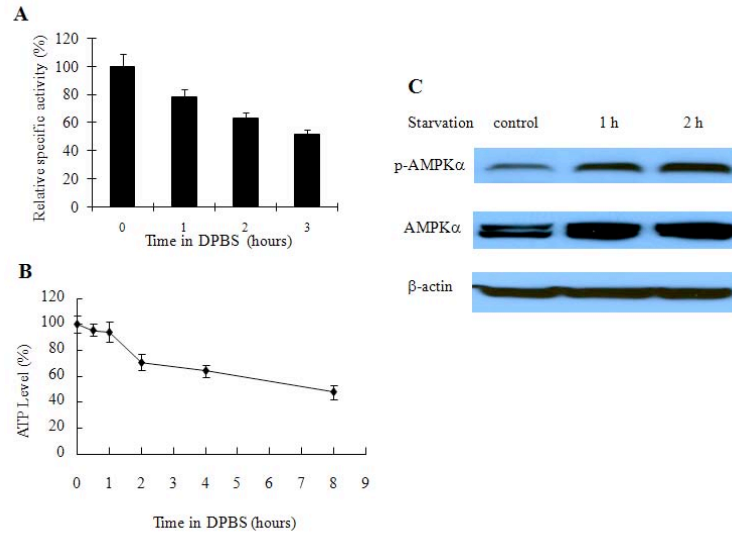
Figure 2. Effect of nutrient deprivation on protein synthesis and cellular ATP in MCF-7 cells.
(**A**) MCF-7 cells were treated with DPBS for the indicated times and the rate of protein synthesis was measured by labeling the cells with 25 µCi/ml of EasyTag EXPRESS [^{35}S] protein labeling mix and liquid scintillation counting, as described in "Material and Methods". The specific activity of protein synthesis was determined by the amount of incorporated ^{35}S-methionine/cysteine per mg of total protein per min, and relative activities at indicated times of starvation were calculated as percent of control. (**B**) MCF-7 cells were treated with DPBS for the indicated times and ATP content was measured using the ATPlite™ Luminescence Assay Kit. (**C**) AMPK activity was determined by Western blot analysis of phospho-AMPK using an anti-phospho-AMPK antibody, as described in "Material and Methods". Actin was used as a loading control. Results shown are the representative of three similar experiments; each bar or point represents mean ± SD of quadruplicate determinations.
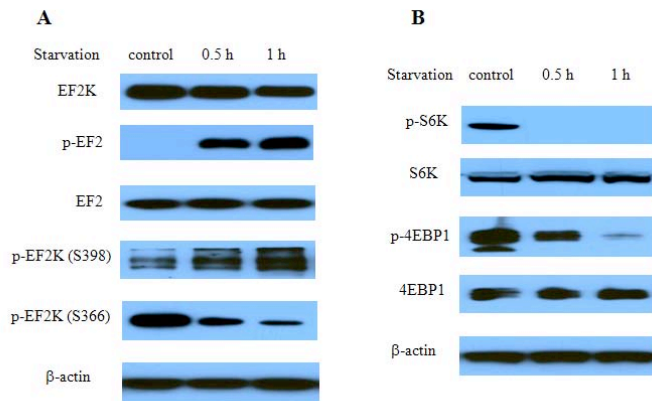
Figure 3. Effect of nutrient deprivation on eEF-2 kinase activity and the associated signaling molecules. (**A**) MCF-7 cells were treated with DPBS for the indicated times, and the levels of eEF-2 kinase, p-EF2, EF2, p-eEF2 kinase (S398) and p-eEF2 kinase (S366) were examined by Western blot using the respective antibodies. (**B**) MCF-7 cells were treated with DPBS for the indicated times, and p-S6 kinase, S6 kinase, p-4EBP1, and 4EBP1 were examined by Western blot using the respective antibodies. β-actin was used as a loading control. Results shown are the representative of three similar experiments.
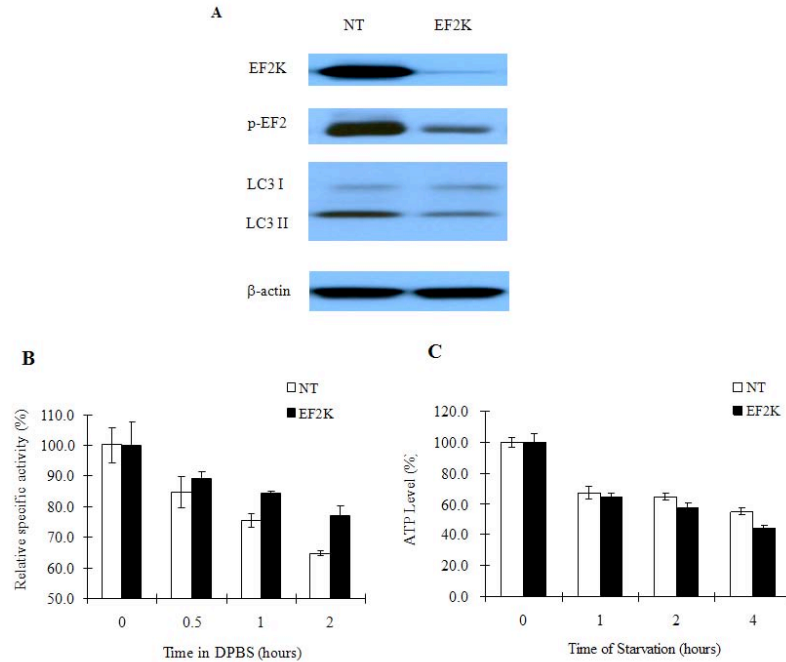
Figure 4. Inhibition of eEF-2 kinase blunts autophagy, mitigates inhibition of protein synthesis and hastens reduction of cellular ATP. (**A**) MCF-7 cells were transfected with a non-targeting RNA or an eEF-2 kinase-targeted siRNA (100 nM) for 72 h, and then treated with DPBS for 1 h. eEF-2 kinase, phosphor-EF-2, and the autophagy marker, LC3-II, were detected by Western blot. (**B**) MCF-7 cells with or without silencing of eEF-2 kinase were treated with DPBS; at the indicated times, cells were harvested for protein synthesis assay. (**C**) MCF-7 cells transfected with 50 nM of NT RNA or an eEF-2 kinase siRNA were seeded in 96-well tissue culture plates (1 x10^4 cells per well). Forty-eight h later, the cells were starved in DPBS for 1 h, 2 h and 4 h. Cells were collected at the end of starvation for ATP assay. Results shown are the representative of three similar experiments; bars represent mean ± SD of quadruplicate determinations.
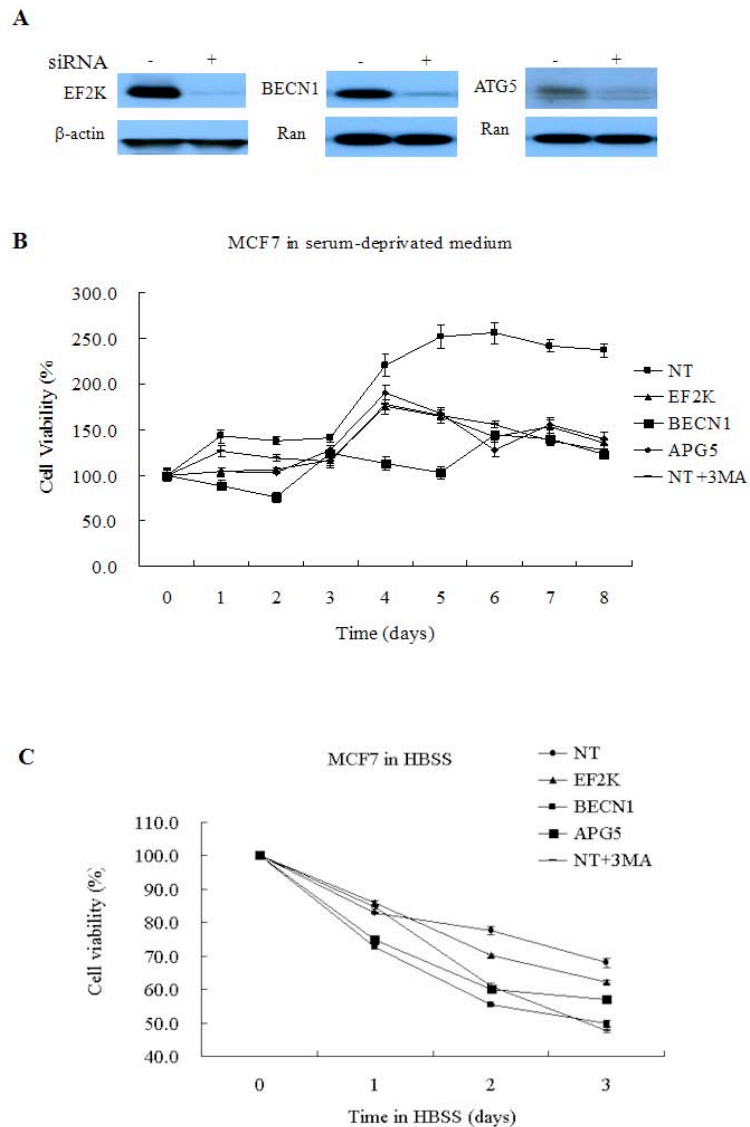
Figure 5. Effects of autophagy suppression on growth and survival of human breast cancer cells.
(**A**) MCF-7 cells were transfected with a non-targeting RNA or a siRNA targeting eEF-2 kinase, beclin1, or ATG5. Expressions of eEF-2 kinase, beclin1, or ATG5 were determined by Western blot using the respective antibodies. β-actin or Ran was used as a loading control. (**B**) MCF-7 cells treated with 3-MA or with siRNA targeting eEF-2 kinase, beclin1, ATG5 or a non-targeting RNA were seeded with 10% FBS RPMI 1640 medium in 96-well culture plates (3×10^3 cells per well). After overnight incubation, medium was changed to serum-free medium. Cell viability was determined at the indicated times using MTT assay. (**C**) MCF-7 cells treated with 3-MA or with siRNA targeting eEF-2 kinase, beclin1, ATG5 or a non-targeting RNA were seeded with 10% FBS RPMI 1640 medium in 96-well culture plates (3×10^3 cells per well). After overnight incubation, medium was changed to HBSS. Cell viability was determined at the indicated times using MTT assay. Results shown are the representative of three similar experiments; each point represents mean ± SD of quadruplicate determinations.
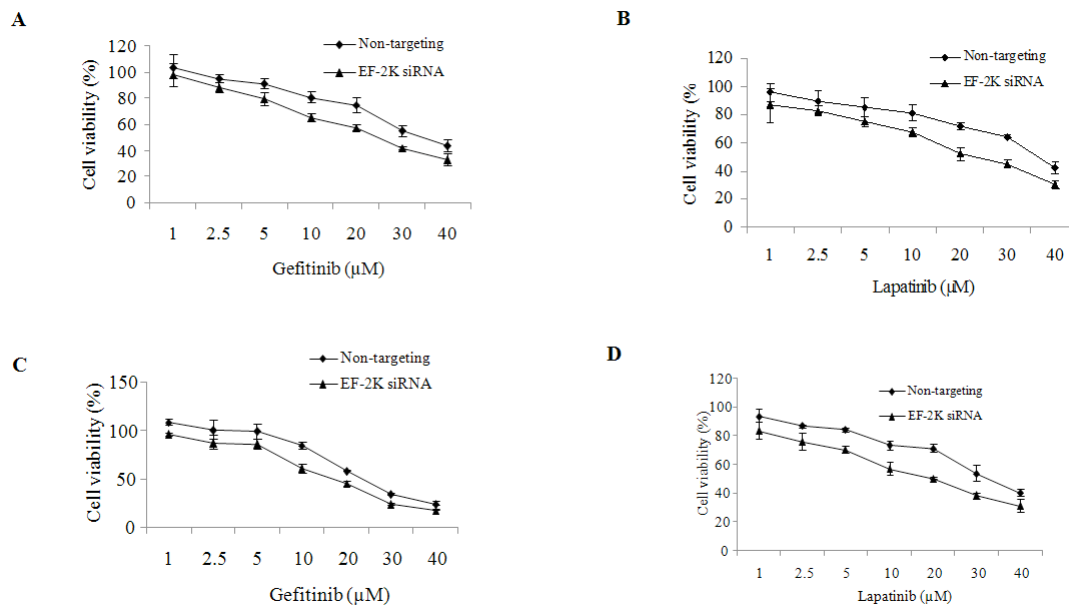
Figure 6. Effects of eEF-2 kinase silencing on sensitivity of human breast cancer cells to growth factor inhibitors. MCF-7 (**A, B**) and MDA-MB-468 (**C, D**) cells transfected with an siRNA targeting eEF-2 kinase or a non-targeting RNA were cultured in RPMI 1640 or DMEM media supplemented with 10% fetal bovine serum at 37 °C in a humidified incubator (5% CO_2 and 95% air), and then treated with a series of concentrations of gefitinib (**A, C**) or lapatinib (**B, D**) for 48h. At the end of treatment, cell viability was determined using MTT assay. Results shown are the representative of three similar experiments; each point represents mean ± SD of quadruplicate determinations.