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13. ABSTRACT *(Maximum 200 Words)*

Estrogen receptor (ER) negative breast cancer is associated with perturbations of growth regulatory pathways. Three in vitro models of ER+ and ER- breast cancer were evaluated for expression and activation of MAP kinase. Despite decreased activation of MAP kinase in ER- cells, these cells are still dependent on MAP kinase to maintain normal proliferation as evidenced by the lack of proliferation in the presence of UO126, a MAP kinase inhibitor. Data presented in this report demonstrates that inhibition of the PI-3 kinase pathway is only growth inhibitory for ER+ cells, further evidence of the importance of MAP kinase signaling for growth. A second MAP kinase family member, ERK 5, was evaluated for expression and activation by western blot. Two of the three ER- cell lines upregulate activation of ERK5, potentially through the erbB2 receptor, and ERK 5 activity was inhibited in the presence of UO126 and LY294002. These results demonstrate upregulation of growth factor signaling pathways is critical for proliferation of ER- breast cancer cells and further validate the MAP kinase pathway as a target for therapeutic intervention.

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Introduction

Progression of breast cancer from an estrogen-dependent, estrogen receptor (ER) positive status to an estrogen-independent, ER negative status is the focus of much current research. Observations made both from *in vitro* models and clinical research indicates that upregulation of growth factor signaling pathways is correlated with the progression to estrogen independence and loss of ER expression. Increased levels of MEK and Raf-a as well as increased MAP kinase activity has been observed in breast cancers compared to normal breast tissue, indicating that hyperactivation of growth signaling pathways both at the cell surface and intracellularly is an important factor in breast cancer progression (1,2). Increased expression of both the erbB2 and EGF receptors is observed in ER negative (ER-) breast cancer (3-6). *In vitro* models where these growth factor receptors have been over-expressed in the ER+ MCF-7 cell line suggest that increased signaling through growth factor receptors is an important step in the path to estrogen independence (7,8). More recently, increased MAP kinase activity has been shown to contribute to the loss of ER expression *in vitro*.

The T47D:A18, MCF-7 and ZR-75 breast cancer cell lines, along with their ER negative counterparts, all derived by selecting for cell growth in the absence of estrogen, provide models for further examining the status of growth factor signaling pathways in ER- breast cancer. The ER- 2W, LCC3 and ECMCF-7 (C4-12) cell lines have decreased MAP kinase activity compared to the parental ER+ cell lines, but maintain MAP kinase expression at levels similar to the parental cell lines (Fig 1). Conversely, activation of p70/S6 kinase, a component of the PI-3 kinase pathway that is necessary for cell cycle progression and growth control (9-11) was previously shown to be maintained in the ER-

cell lines. These observations suggested that the ER- cells may have a decreased dependence on MAP kinase signaling for growth and be more dependent on alternate growth pathways. An analysis of growth in the presence of U0126, an inhibitor that prevents MAP kinase activation and rapamycin, an inhibitor that prevents p70/S6 kinase activation was undertaken. This analysis demonstrated that the both the ER+ and ER- cells were dependent on MAP kinase activation for growth and were independent of p70/S6 kinase activation for growth. Further analysis was undertaken to evaluate the effect of PI-3 kinase inhibition on growth with the PI-3 kinase inhibitor LY294002. Since the MAP kinase pathway was critical for proliferation of the ER- cells, ERK5 expression and activity was evaluated. ERK5 participates in growth regulation in response to EGF and serum stimulation and has been shown to participate in breast cancer cell proliferation (12-15).

Results

The impact of PI-3 kinase activity on growth was determined for each of the ERα+ and ERα- cell lines in these models. Proliferation was monitored by quantifying cell number over time. The effect of PI-3 kinase inhibition by LY294002 was determined by maintaining cells in the continuous presence of LY294002 by adding fresh inhibitor every 24 hours.

The ER+ T47D:A18 cells were growth inhibited by 48 hours in the presence of LY294002 (Fig 2A). ER- 2W cells were initially growth inhibited but overcame this inhibition to achieve normal growth during the remainder of the assay (Fig 2B). Similar results were observed in the ZR-75 model. ER+ ZR-75 cells were growth inhibited in the

presence of LY294002 (Fig 2C) while the ER- LCC3 cells maintained a normal proliferative rate through the first 4 days of the assay (Fig 2D). A slight decrease in proliferation was observed in the ER- LCC3 cells by the end of the assay, however this may be due to inhibition of cell survival pathways rather than a direct effect on proliferation. These results demonstrate that the ER- cells in the T47D and ZR-75 models are not dependent on the PI-3 kinase pathway to maintain proliferation and further demonstrate that the decreased in MAP kinase activity previously reported in these ER- cells is critical for proliferation.

The effect of LY294002 on proliferation in the MCF-7 model did not reflect the results observed in the previous two models. Both ER+ MCF-7 and ER- ECMCF-7 cell lines were growth inhibited by LY294002 treatment with doubling times of greater than 100 hours for treated cells {Fig 2 E,F). These results demonstrate that the PI-3 kinase pathway is critical for sustained proliferation of the ERα+ cells and that both the Ras/Raf/MAPK pathway and PI-3 kinase pathway contribute to proliferation of the ERα- cells in the MCF-7 model.

Since MAP kinase activity was shown previously to be critical for proliferation of the ER- cells in these models, the MAP kinase family member ERK 5 was evaluated for expression and activation in the ER+ and ER- cells. ERK 5 expression and activity was evaluated by western blot, the presence of a shifted band indicates the presence of the phosphorylated activated form of ERK5. ERK 5 was shown to be expressed in all the ER+ and ER- cells evaluated (Fig 3A). Interestingly, ERK5 activation was only observed in the ER-2W and ER- LCC3 cells. ERK 5 was sensitive to the effects of the MEK inhibitor UO126 (Fig 3A) as well as to LY294002 in the ER-2W and ER- LCC3 cells

(data not shown). This suggests that ERK5 may be upregulated in the ER- cells to compensate for the decrease in MAP kinase activity observed in these cells. The observation that ErbB2 expression and activity is upregulated in the ER- 2W and ER- LCC3 cells (Fig 3B) supports this conclusion since erbB2 has recently been shown to activate the ERK5 pathway in breast cancer cells (15)

Conclusions

The results presented here demonstrate changes in the activity of growth factor regulatory pathways in ER- breast cancer cells. Multiple growth regulatory pathways are functional in ER+ cells, as the ER+ cells described here were shown previously to be sensitive to inhibition of MAP kinase and p70/S6 kinase. Signaling through the PI-3 kinase is shown here to be critical for proliferation of these ER+ cell lines. However, the progression from an ER+ to an ER- phenotype involves alterations in growth factor signaling pathways as evidenced by growth regulation of ER- cells in two of these models. ER- 2W cells and ER- LCC3 cells, are independent of PI-3 kinase signaling for growth, but maintain dependence on MAP kinase activity even though it is decreased in these cells. The observation that ERK5 is activated in these ER- cells and the increased activity of erbB2 demonstrates that ER- cells must upregulate activity of proliferative pathways. The results further demonstrate that MAP kinase signaling pathways are the critical pathways controlling growth of ER- breast cancer. This work further validates the MAP kinase pathway as a therapeutic target in breast cancer. Additionally, inhibition of this pathway in ER+ patients undergoing endocrine therapy may prevent the transition to an ER- phenotype.

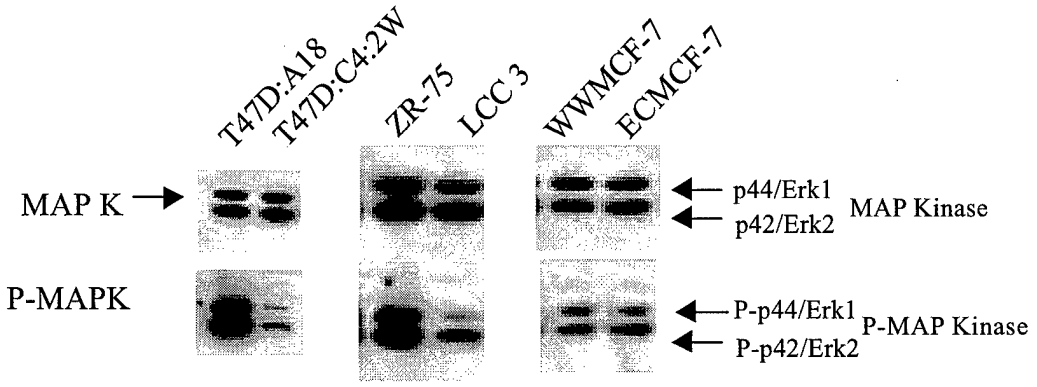
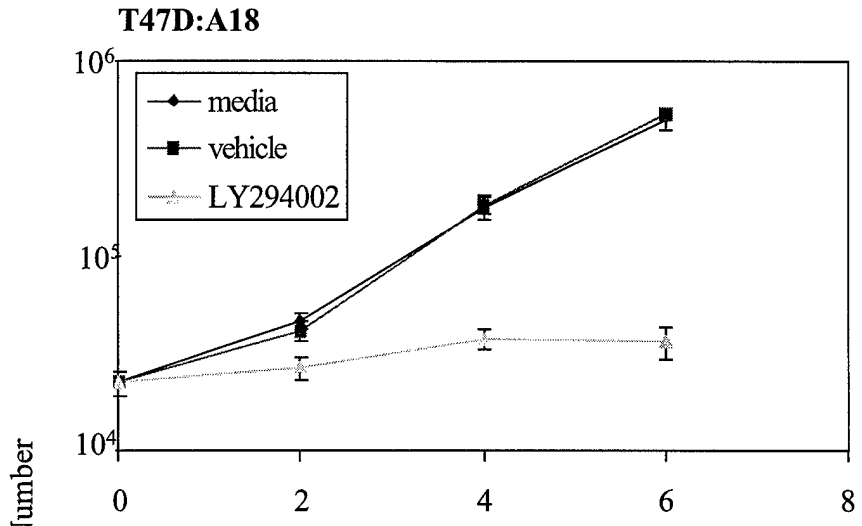


Figure 1. Expression and activation MAPK in ER+ and ER- breast cancer cells cells. Whole cell lysates (5 μg) from ER+ and ER- cells were separated on 10% polyacrylamide gels and probed for expression of MAPK (p44/ERK1, p42/ERK2) or for the phosphorylated, active forms of MAPK (pMEK1/2 respectively).

2A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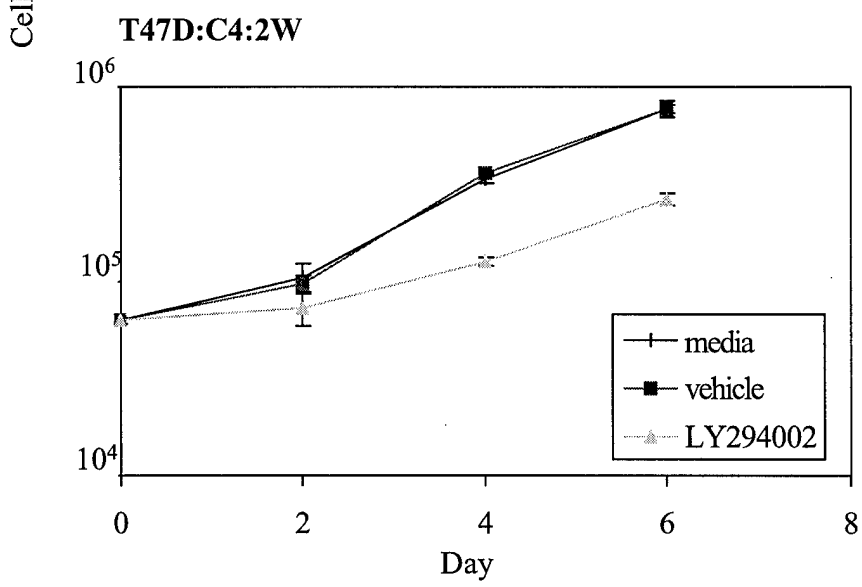
10^4
media
vehicle
LY294002
0
2
4
6
8
Cell Number

2B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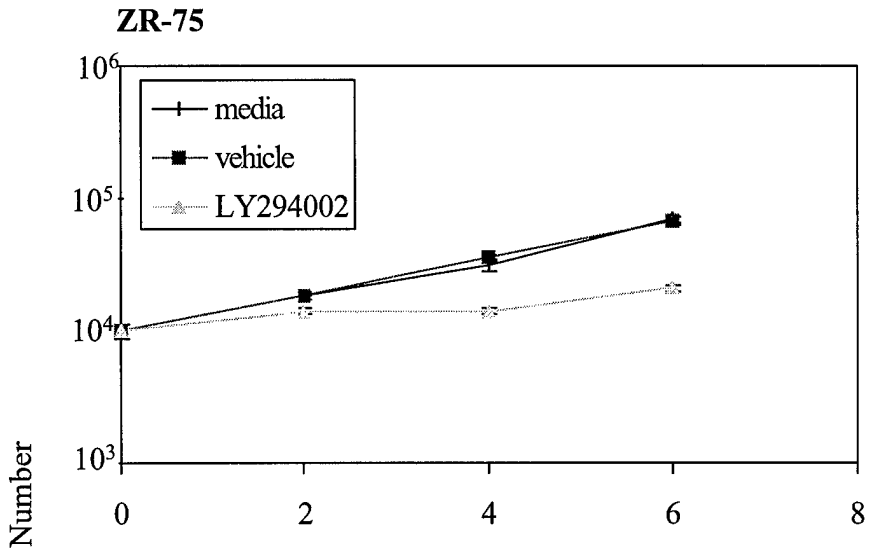
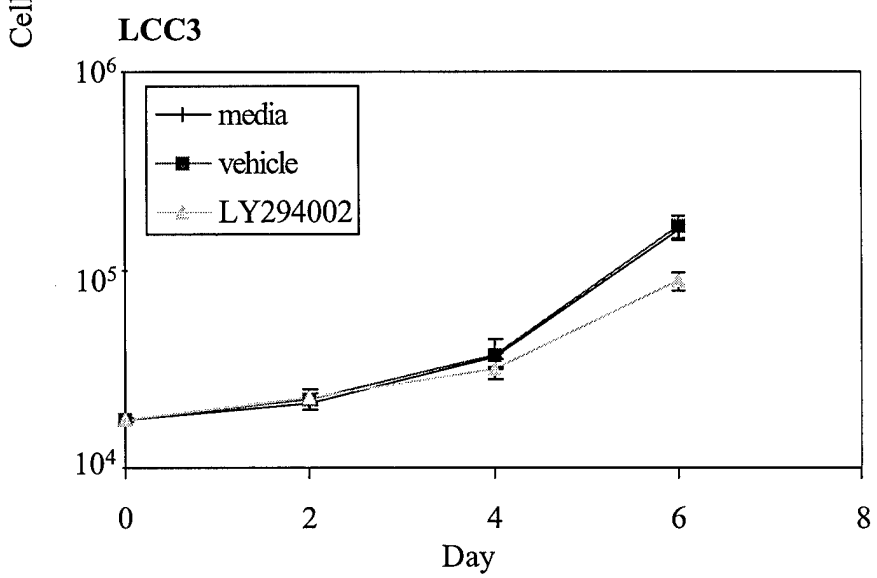
10^5
10^4
media
vehicle
LY294002
0
2
4
6
8
Day

2C

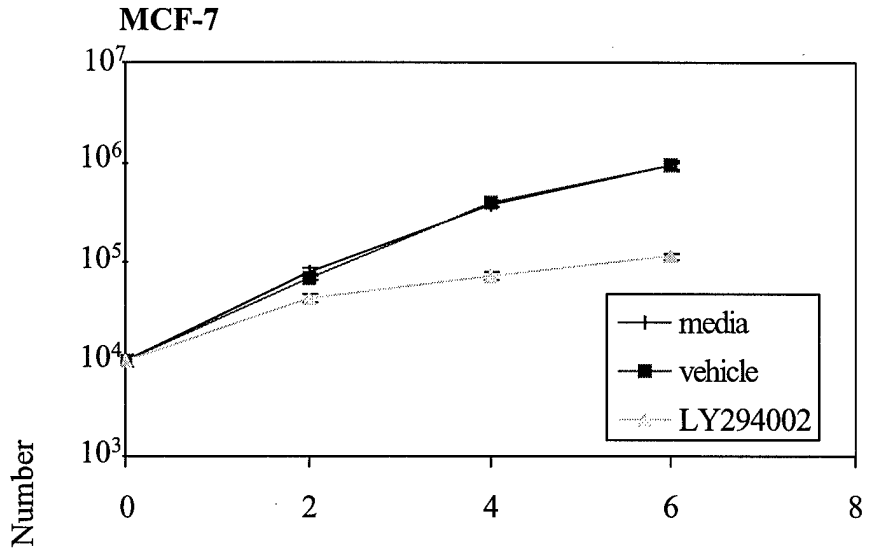


2D



Cell Number

2E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10^3
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2
4
6
8
media
vehicle
LY294002

2F

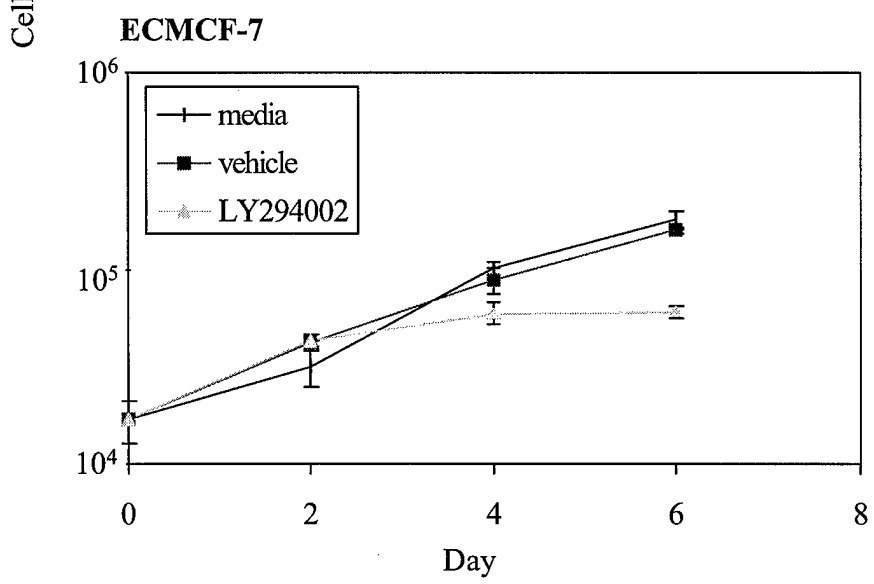
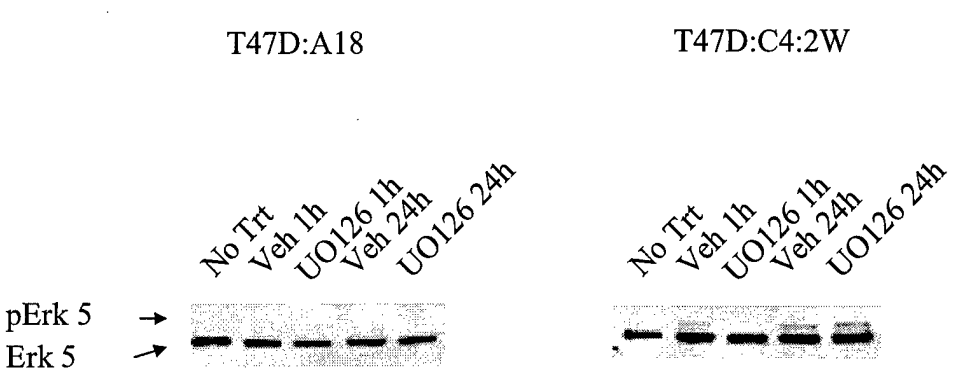

Cell Number
ECMCF-7
10^6
10^5
10^4
0
2
4
6
8
Day
media
vehicle
LY294002

Figure 2A-F ER+ cells are growth inhibited in the presence of LY294002. Proliferation of A) ER+ T47D:A18, B) ER- T47D:C4:2W, C) ER+ ZR-75, D) ER- LCC3, E) WWMCF-7 and F) ECMCF-7 cells was evaluated in the presence of LY294002. Cells were plated in 10% FBS- or CCS-supplemented IMEM in 24 well plates and allowed to attach for 24 hours. Media was removed and replaced with plating media containing 5 μM LY294002 or vehicle control on Day 0. LY294002 was then added every 24 hours to a final concentration of 5 μM, vehicle was added to vehicle control wells to the appropriate concentration. Triplicate cell counts were collected every 48 hours and doubling times were determined. Results are presented as the average of triplicate values at indicated time points, bars represent SD. Growth assays were performed twice with similar results.

3A


T47D:A18
T47D:C4:2W
No Trt
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UO126 1h
Veh 24h
UO126 24h
pErk 5
Erk 5
ZR-75
LCC3

3B

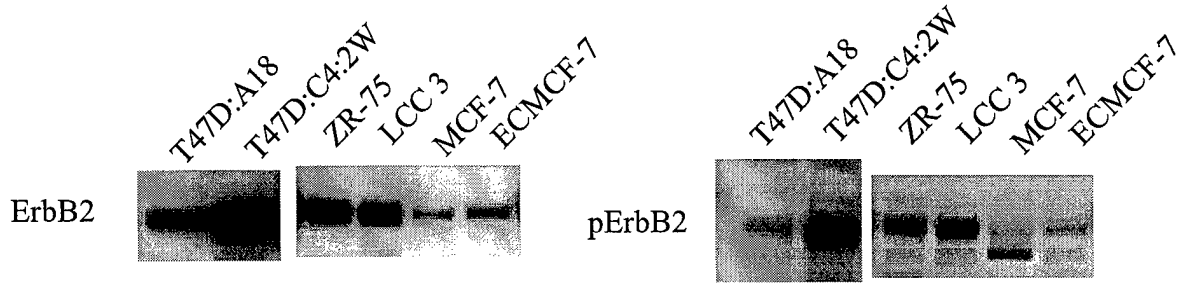
T47D:A18
T47D:C4:2W
ZR-75
LCC 3
MCF-7
ECMCF-7
ErbB2
pErbB2

Figure 3A ERK 5 Expression and activity in ER- and ER+ breast cancer cells. ER+ T47D:A18 or ZR-75 cells and ER- T47D:C4:2W or LCC3 cells were plated in 10% FBS- or CCS-supplemented IMEM in 6 well plates, allowed to attach for 48 hours and then treated for 1 hour or 24 hours with 10 μM UO126 or vehicle control (DMSO). Whole cell lysates were collected and normalized for protein concentration. Equal amounts of protein were loaded on two separate polyacrylamide gels for each kinase analyzed. Proteins were separated by SDS-page and transferred to nitrocellulose in parallel. Blots were probed for expression of ERK5 or for the phosphorylated, active form of ERK5 from UO126 or vehicle control treated cells.

Figure 3B. ErbB2 expression and activity in ER+ and ER- breast cancer cells. A) Whole cell lysates (35 μg) from ER+ T47D:A18, ZR-75 or WWMCF-7 cells and ER- T47D:C4:2W, LCC3 or ECMCF-7 cells were separated on 4-20% polyacrylamide gels and probed for expression of the erbB2 receptor or for phospho-erbB2 (p-erbB2) (pY1248)

Key Research Accomplishments

- Analyzed ER+ and ER- breast cancer cell lines for expression and activation of ERK5
- Analyzed ER+ and ER- breast cancer cell lines for expression and activation of ErbB2
- Determined the effect of PI-3 kinase inhibition on growth of ER+ and ER- breast cancer cell lines.

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