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13. ABSTRACT *(Maximum 200 Words)*

THE EPIDERMAL GROWTH FACTOR RECEPTOR (EGFR) IS A RECEPTOR PROTEIN KNOWN TO PROMOTE GROWTH AND DIFFERENTIATION OF EPITHELIAL CELLS. INCREASED EXPRESSION OF EGFR HAS BEEN ASSOCIATED WITH POOR PROGNOSIS AND MORE AGGRESSIVE BREAST TUMORS. THIS RESEARCH WAS UNDERTAKEN TO UNCOVER A LINK BETWEEN CHEMOTHERAPEUTIC EXPOSURE AND INCREASED EXPRESSION OF EGFR IN BREAST CANCER CELLS. WE HAVE SHOWN THAT EXPOSURE OF MCF-7, MDA-MB-453, T-47D, AND ZR-75-1 BREAST CANCER CELLS TO THE ANTI-METABOLITE COMPOUND METHOTREXATE (MTX) CAUSES AN UP-REGULATION OF EGFR EXPRESSION AT BOTH THE MRNA LEVEL (2-8 FOLD) AND CELL-SURFACE PROTEIN LEVEL (2-3 FOLD). WE ARE CURRENTLY PERFORMING EXPERIEMNTS TO DEMONSTRATE THAT THE MTX-INDUCED EGFR EXPRESSION IN THESE CELLS ALTERS EGF-MEDIATED ACTIVATION OF THE ERK AND AKT SIGNALING PATHWAYS, BOTH OF WHICH ARE INVOLVED IN SUPPRESSION OF APOPTOSIS. FURTHERMORE, WE ARE WORKING TO DEMONSTRATE A DIRECT LINK BETWEEN EGFR ACTIVITY AND APOPTOSIS SUPPRESSION IN MTX-TREATED BREAST CANCER CELL LINES CELLS.

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INTRODUCTION:

The epidermal growth factor receptor (EGFR) is a cell-surface protein that relays signals from the extracellular environment into the cell by binding specific polypeptide hormones followed by activation of intracellular signal transduction pathways. Although rarely an oncogene, the ability of EGFR-mediated signaling to generate diverse responses including growth, differentiation, stress response, apoptosis suppression, and altered mobility makes this protein a potentially powerful tumor promoter. The association between higher EGFR expression and poorer prognosis in breast cancer and the frequency of higher EGFR levels in more aggressive/metastatic breast tumors reinforce this possibility. Our research aims to uncover a link between chemotherapeutic exposure and increased expression of EGFR in breast cancer cells with the hope of explaining why higher levels of EGFR are common to more advanced breast tumors. We have shown that exposure of MCF-7, T-47D, and ZR-75-1 breast cancer cells to the anti-metabolite compound methotrexate causes an up-regulation of EGFR receptor expression. Our work demonstrates that the EGFR up-regulation usually occurs at both the mRNA level and protein level (with increased expression on the cell surface) and that this may be accompanied by changes in the expression of EGFR ligands. We are currently performing experiments to determine whether methotrexate-induced EGFR expression in these cells alters EGF-mediated phosphorylation of ERK and AKT (causing changes in the specificity, timing and intensity of EGFR signaling through these pathways in a cell specific manner). ERK and AKT signaling pathways have been shown to mediate anti-apoptotic effects. In addition, we are investigating the potential role for increased EGFR expression and signaling in cell survival by suppression of chemotherapy induced apoptosis.

Task 1: **Characterize the chemotherapy-induced changes in EGFR expression in breast cancer cell lines. (months 1-12)**

- Screen different cell line/compound combinations using a fluorescent reporter gene placed under the control of the EGFR promoter. (months 1-6)
- Confirm the results from the GFP-screening method using RNase protection assays to demonstrate increases in EGFR mRNA under the same conditions (months 2-6)
- Determine if the observed changes in EGFR mRNA result from altered message stability using actinomycin-D and RNase protections assays to compare EGFR mRNA half life in treated and untreated cells. (months 6-9)

- Assess requirements for *de novo* protein synthesis by combining cyclohexamide pre-treatments with RNase protection assays. (months 6-9)
- Correlate changes in protein levels with changes in EGFR mRNA levels using western blot and immunohistochemistry methods. (months 6-12)

Task 2: **Determine the effects EGFR-signaling in the growth and/or survival of breast cancer cells challenged with chemotherapeutic compounds. (Months 12-24)**

- Determine the effects of EGFR-signaling on growth rate and re-plating efficiency in chemotherapy-treated breast cancer cells. (months 12-18)
- Assess to role of EGFR over-expression in promoting the survival of breast cancer cells challenged with different chemotherapeutic compounds through use of MCF-7 derived stable transfectant cell lines. (months 12-18)
- Study possible connections between chemotherapy-induced EGFR signaling and apoptosis by annexin staining and Hoescht staining protocols (in combination with conditions established in prior experiments). (months 18-24)
- Assess the role of EGFR signaling in regulating expression of the EGFR gene by observing the impact of a specific EGFR-inhibitor on the chemotherapy-induced changes in EGFR expression. (months 18-24)

Figures referred to in the following section are presented in the appendix.

Progress, Task 1:

The experiments for Task 1 were significantly redesigned in order to achieve the main objective: determination of chemotherapeutic induction of EGFR expression. Three breast cancer cell lines were chosen for this project based on these criteria: MCF-7, T-47D, and ZR-75-1. A fourth cell line that does not express ER, MDA-MD-453, was added in order to address (or eliminate) a role for ER in our experimental model. The ER and EGFR status for the selected cell lines are listed in **TABLE 1**.

To measure changes in EGFR expression in the MCF-7, MDA-MB-453, T-47D, and ZR-75-1 cell lines, it was necessary to develop a protocol with which we could reliably detect changes in EGFR content in cells that express the receptor at very low levels. Several conventional procedures were attempted with limited

success, including immunoprecipitation, Western blotting and immunohistochemistry. We finally settled on an immunofluorescence / flow cytometry protocol that yielded consistent, reproducible results. This method involved the binding of EGFR specific-antibodies to intact living cells. The levels of antibody binding, as detected through the use of a fluorescent secondary antibody, were measured by flow cytometry. Data are presented as fluorescent units per cell, with mean peak values calculated per sample population. (This protocol was substitued for the fluorescenct reporter gene protocol described in Task 1).

FIGURE 1 represents the comparative level of EGFR expression in the MCF-7, MDA-MB-453, T-47D, and ZR-75-1 cell lines as determined through the application of this method. As shown in **FIGURE 1a**, MCF-7, MDA-MB-453, and ZR-75-1 cells exhibit very low baseline EGFR expression levels. Mean peak fluorescent values for these cell lines were consistently below 10 fluorescent units. In contrast, T-47D cells, which express a modestly higher amount of EGFR, had a mean fluorescent peak value of approximately 100 (about ten-fold greater that the other cells lines). For broader sense of context, this method was applied to the EGFR over-expressing MDA-MB-231 cell line. The mean fluorescence peak value for these cells was close to 600 fluorescent units (**FIGURE 1b**).

Several chemotherapeutic options were available for study in this project. Methotrexate (MTX), a folic-acid analogue that is classified as an antimetabolite, was the chemotherapeutic agent selected for use in our research. This choice was made for several reasons. First, the compound has a long history of use in the treatment of breast cancer, providing clinical context for this study. Second, MTX was toxic to the cells only after prolonged exposures (four days and longer). This allowed a window of time for studying the effects MTX-exposure on EGFR expression . Other chemotherapeutic agents which were more acutely toxic (adriamycin, cyclophosphomide) were more limiting in this regard. Finally, MTX is stable, easily stored (at –80 °C) and has no inherent fluorescence (unlike adriamycin, for example) that would interfere with flow cytometric measurements.

As shown in **FIGURE 2**, 72-hour MTX exposure (at nM to □M concentrations) resulted in an elevation of EGFR mRNA levels in three of the four cell lines tested. MCF-7 cells exhibited a 5-10 fold increase in EGFR mRNA (**FIGURE 2a**), the strongest change observed among the four cell lines. ZR-75-1 cells displayed a 2 to 3-fold rise in EGFR mRNA (**FIGURE 2d**) following the same treatment and MDA-MB-453 cells displayed a 1.5 to 2-fold rise in EGFR mRNA (**FIGURE 2b**). In these three cell lines, the MTX concentration ranges that induced changes in EGFR mRNA corresponded to those that produced a cytostatic effect in each cell line (see **FIGURE 1**). Thus, equipotent MTX doses were found to induce an increase in EGFR mRNA

among MCF-7, MDA-MB-453 and ZR-75-1 cells. In contrast, T-47D cells did not alter EGFR mRNA levels (**FIGURE 2c**) following a 72-hour MTX treatment.

FIGURE 3 depicts the changes in EGFR cell surface protein expression detected in MCF-7, MDA-MB-453, T-47D, and ZR-75-1 cells after 72-hours MTX treatment using this method. For MCF-7, ZR-75-1 and MDA-MB-453 cells, MTX doses that induced elevation of EGFR mRNA also produced a corresponding increase in EGFR protein expression at the cell surface. For MCF-7 cells (**FIGURE 3a**) a 3-fold increase in mean peak fluorescence was observed, while for ZR-75-1 cells (**FIGURE 3c**) the fold increase in mean peak value was approximately 2-fold. [In **FIGURE 4**, representative histograms from 72-hour MTX-dose response experiments are presented for each cell line.]

At this point, our focus has shifted from mechanistic aspects to clinical.translational aspects. Thus, we did not pursue the actinomycin D/ cyclohexamide experiments outlined in Task 1 during year one.

Task 2: **Determine the effects EGFR-signaling in the growth and/or survival of breast cancer cells challenged with chemotherapeutic compounds.**

Experiments designed to achieve the goals outlined in **Task 2** are currently underway but not complete at this time.

KEY RESEARCH ACCOMPLISHMENTS

Our research demonstrated the following:

- Exposure of MCF-7, MDA-MB-453, T47-D and ZR-75-1 breast cancer cells to methotrexate (MTX) induces an increase in EGFR mRNA expression within 72 hours
- Under the same MTX-treatment conditions, MCF-7, MDA-MB-453, T47-D and ZR-75-1 breast cancer cells also exhibit increased EGFR expression at the cell surface as detected by immuno-fluorescence assay
- The MTX-induced EGFR expressed at the cell surface is functional as demonstrated by increased EGF-stimulated ERK and AKT pathway activation following MTX-exposure (preliminary data, not shown).

REPORTABLE OUTCOMES:

ABSTRACTS:

Welch, JN, Chrysogelos, SA, Clarke, R. Epidermal Growth Factor Expression In Response To Chemotherapy In Breast Cancer Cells. The Susan G. Komen Breast Cancer Foundation 4th Annual Mission Conference, Washington, DC, 2000.

Expression and Function of the Epidermal Growth Factor Receptor in Breast Cancer Cells Exposed to Chemotherapy. Proc. American Association for Cancer Research 92nd Annual Meeting, New Orleans, 2000, **Welch JN,** Chrysogelos, SA, Clarke, R.

PUBLICATIONS:

Clarke, R Leonessa, F, **Welch, JN,** Skaar, TC. Cellular and Molecular Pharmacology of Antiestrogen Action and Resistance. *Pharmacological Reviews* 53:25-71, 2001

Hilakivi-Clarke L, Cho E, deAssis S, Olivo S, Ealley E, Bouker KB, **Welch JN**, Khan G, Clarke R, Cabanes A. Maternal and Prepubertal Diet, Mammary Development and Breast Cancer Risk. *Journal of Nutrition* 131: 154S-157S, 2001.

Clarke R, Skaar TC, Bouker KB, Davis N, Lee RY, **Welch JN**, Leonessa F. Molecular and Pharmacological Aspects of Antiestrogen Resistance. *Journal of Steroid Biochemistry and Molecular Biology.* (In press)

Welch, JN, Chrysogelos, SA. "Positive Mediators of Cell Proliferation in Neoplastic Transformation" In: The Molecular Basis of Human Cancer (Coleman, WB and Tsongalis, GJ, eds), Humama Press, Towata, NJ. (in press)

Gu Z, Lee RY, Skaar TC, Bouker KB, **Welch JN**, Lu J, Liu A, Davis N, Leonessa F, Brunner N, Wang Y, Clarke R. Molecular Profiles of Antiestrogen Resistance Implicate NFkB, cAMP Response Element Binding, Nucleophosmin and Interferon Regulatory Factor-1. (submitted)

CONCLUSIONS:

Our findings suggest that exposing breast cancer cells to cytotoxic drugs leads to an increase in EGFR levels, which renders the cells more responsive to EGF-stimulation and promotes cell survival through the suppression of apoptosis.

Our data demonstrates a connection between MTX-treatment and both greater EGFR-mediated signaling through ERK/AKT pathways, as well as an anti-apoptotic/survival effect. The role of EGFR signaling in ERK/AKT activation has long been established. However, our results connecting EGFR signaling with decreased apoptosis adds new data to an area of study that has only recently emerged. Whether the increased ERK/AKT signaling is directly connected to the EGF-mediated anti-apoptotic effects remains to be determined.

Based on the data presented in a number of recently published studies (see references), the association between EGFR expression and apoptosis suppression is becoming clear EGFR signaling can affect the expression and post-translational modification of pro- and anti-apoptotic proteins to influence apoptosis.

EGFR-mediated activation of AKT can suppress apoptosis through the interaction of AKT with different apoptotic signaling proteins. EGFR-mediated activation of ERK can also result in the phosphorylation and down-regulation of Bad in a manner similar to that produced by AKT. Our data demonstrate both increased capacities for EGFR-mediated ERK and AKT signaling, and anti-apoptotic effects of EGFR stimulation in MTX-treated cell lines. These results, viewed in the context of the recently published research described above, suggest a connection between ERK/AKT signaling and anti-apoptotic EGFR-mediated effects. However, more experiments will have to be performed to confirm this relationship.

Although prior research has shown that exposure of breast cancer cells to chemotherapy can result in increased EGFR and that EGFR can suppress apoptosis in a number of cancer cell lines, there is very little published research directly relating these events. In other words, no one has yet published data showing chemotherapy induced EGFR expression as a direct precursor to EGFR-mediated suppression of apoptosis. Our research appears to be unique in demonstrating that connection. In addition, we show that subtle changes in the level EGFR expression can have measurable effects on cell survival. This indicates that EGFR-targeted interventions may have a wider use in the treatment of breast cancer, including the treatment of tumors that express low but functional levels of the receptor.

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APPENDIX: (Tables and Figures)

TABLE 1: Essential characteristics of the breast cancer cell lines selected for this research.

CELL LINE	ORGANISM	TISSUE	MORPHOLOGY
MCF-7	human	adenocarcinoma; mammary gland; breast; pleural effusion	epithelial
MDA-MB-231	human	adenocarcinoma; mammary gland; breast; pleural effusion	epithelial
MDA-MB-453	human	mammary gland; breast; pleural/pericardial effusion	epithelial
T-47D	human	ductal carcinoma; mammary gland; breast; pleural effusion	epithelial
ZR-75-1	human	ductal carcinoma; mammary gland; breast; ascites; epithelial; metastatic site: ascites	epithelial

Information collected from the 2001 American Type Culture Collection (ATCC) online catalog. (http://phage.atcc.org)

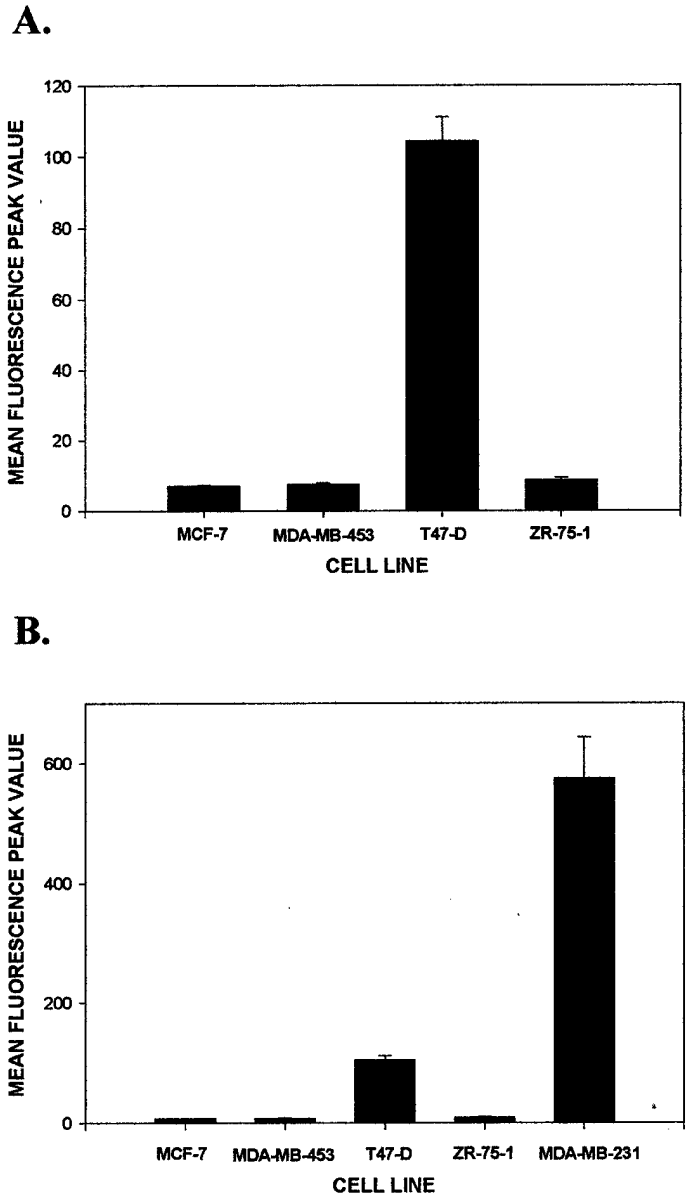

FIGURE 1: Comparison of normal-EGFR expression levels in MCF-7, MDA-MB-453, T-47D and ZR-75-1 breast cancer cell lines by immunofluorescence / flow cytometry. MCF-7, MDA-MB-435, T-47D and ZR-75-1 breast cancer cells were seeded in T-75 tissue culture flasks and grown to approximately 70% confluence. The cells were trypsinized, rinsed twice in PBS and pelleted (1000 rpm, 5 minutes). EGFR surface expression was assessed by immunofluorescence / flow cytometry. PRIMARY ANTIBODY: MAb 528 mouse monoclonal anti-EGFR (NEOMARKERS), 1:50 PBS / one hour room temperature; SECONDARY ANTIBODY: Goat anti-mouse PE conjugated antibody (JACKSON IMMUNOCHEMICALS), 1:50 PBS / 20 minutes room temperature (dark); FIXATION: 1% formaldehyde. Fluorescence per cell was assessed by flow cytometry and data for each cell line is presented as mean fluorescent peak values per cell population. **(a)** Baseline mean fluorescence peak values for untreated MCF-7, MDA-MB-453, T-47D and ZR-75-1 cells; **(b)** the same data presented in part **(a)** with the EGFR over-expressing MDA-MB-231 cell line added for comparison.

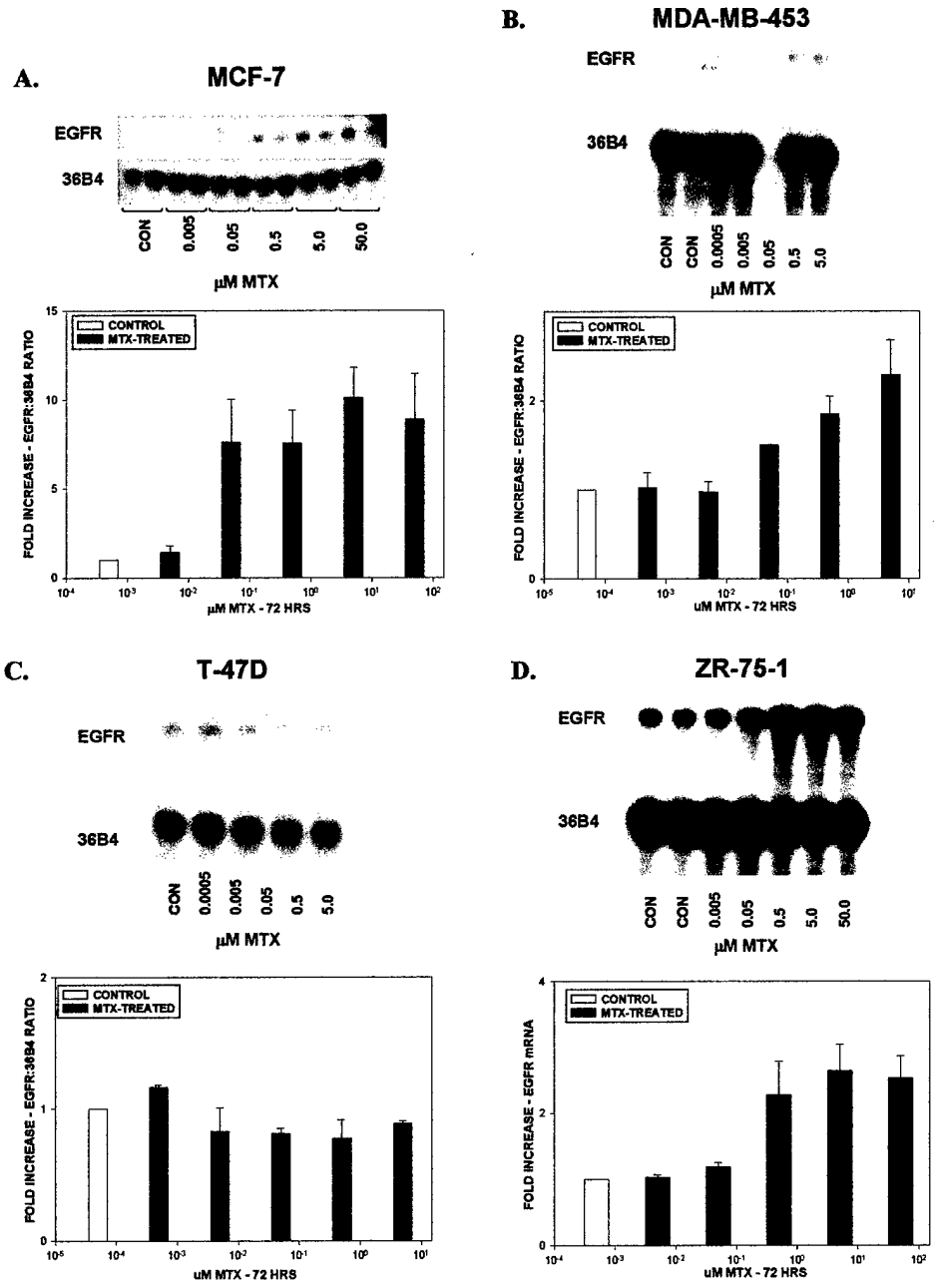

FIGURE 2: Comparison of normal-EGFR expression levels in MCF-7, MDA-MB-453, T-47D and ZR-75-1 breast cancer cell lines by immunofluorescence / flow cytometry. MCF-7, MDA-MB-435, T-47D and ZR-75-1 breast cancer cells were seeded in T-75 tissue culture flasks and grown to approximately 70% confluence. The cells were trypsinized, rinsed twice in PBS and pelleted (1000 rpm, 5 minutes). EGFR surface expression was assessed by immunofluorescence / flow cytometry. PRIMARY ANTIBODY: MAb 528 mouse monoclonal anti-EGFR (NEOMARKERS), 1:50 PBS / one hour room temperature; SECONDARY ANTIBODY: Goat anti-mouse PE conjugated antibody (JACKSON IMMUNOCHEMICALS), 1:50 PBS / 20 minutes room temperature (dark); FIXATION: 1% formaldehyde. Fluorescence per cell was assessed by flow cytometry and data for each cell line is presented as mean fluorescent peak values per cell population. **(a)** Baseline mean fluorescence peak values for untreated MCF-7, MDA-MB-453, T-47D and ZR-75-1 cells; **(b)** the same data presented in part **(a)** with the EGFR over-expressing MDA-MB-231 cell line added for comparison.

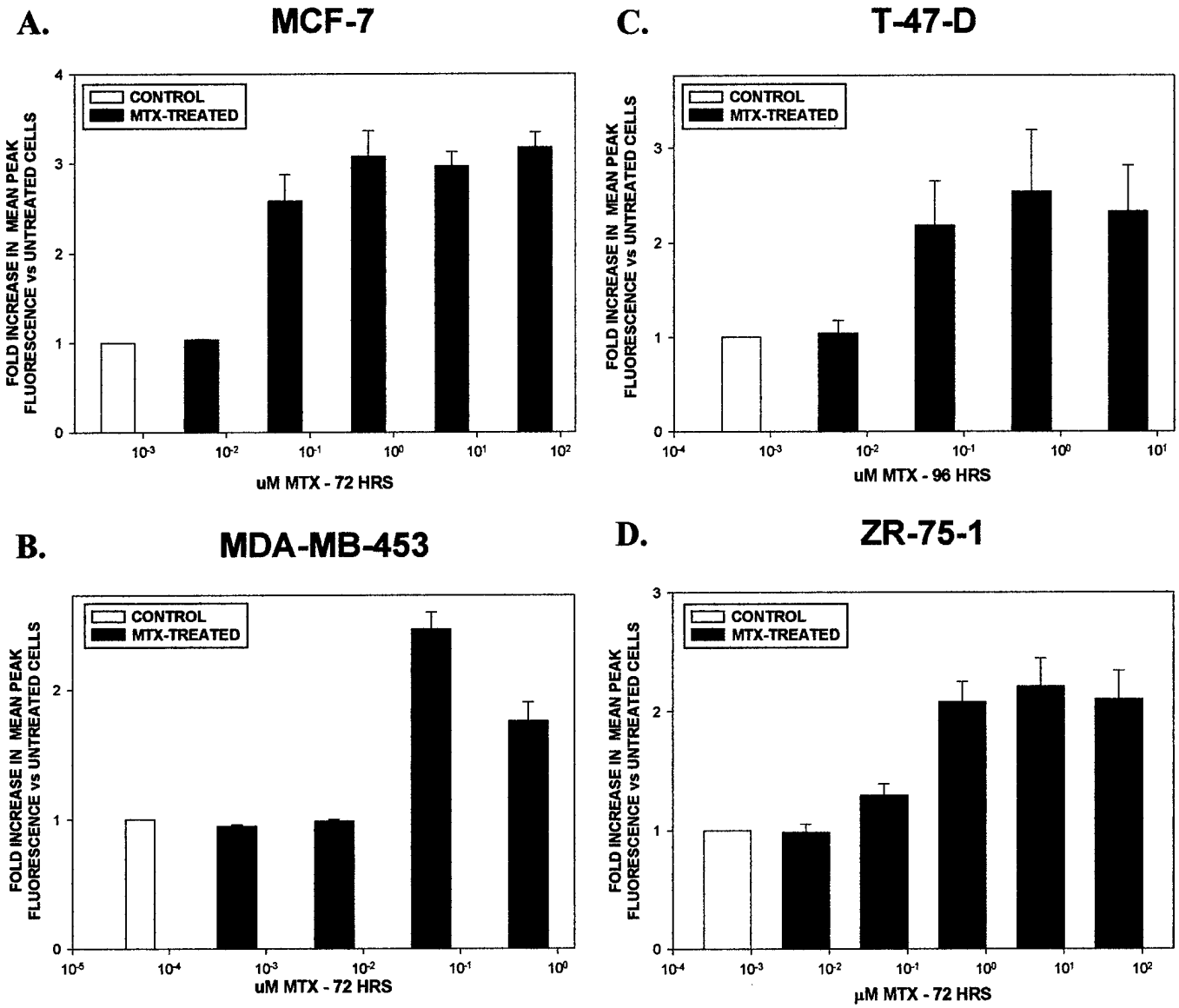

FIGURE 3: MTX-induced changes in EGFR surface expression in MCF-7, MDA-MB-453, T-47D and ZR-75-1 cells. (a) MCF-7, **(b)** MDA-MB-453, **(c)** T-47D, and **(d)** ZR-75-1 breast cancer cells were seeded in T-75 tissue culture flasks and grown to approximately 70% confluence. Methotrexate (MTX) was added to the cultures (concentration range 0.005 to 50.0 µM) and the cells were grown for 72 hours. The cells were trypsinized, rinsed twice in PBS and pelleted (100 rpm, 5 minutes). EGFR surface expression was assessed by immunofluorescence. PRIMARY ANTIBODY: MAb 528 mouse monoclonal anti-EGFR (NEOMARKERS), 1:50 PBS / one hour room temperature; SECONDARY ANTIBODY: Goat anti-mouse PE conjugated antibody (JACKSON IMMUNOCHEMICALS), 1:50 PBS / 20 minutes room temperature (dark); FIXATION: 1% formaldehyde. Fluorescence per cell assessed by flow cytometry. Data is presented as fold increases in mean peak fluorescence per sample for each MTX treatment (blue) vs. untreated control cells (white). Data from five separate experiments are combined. Error bars represent mean ± standard error; $n \geq 3$.

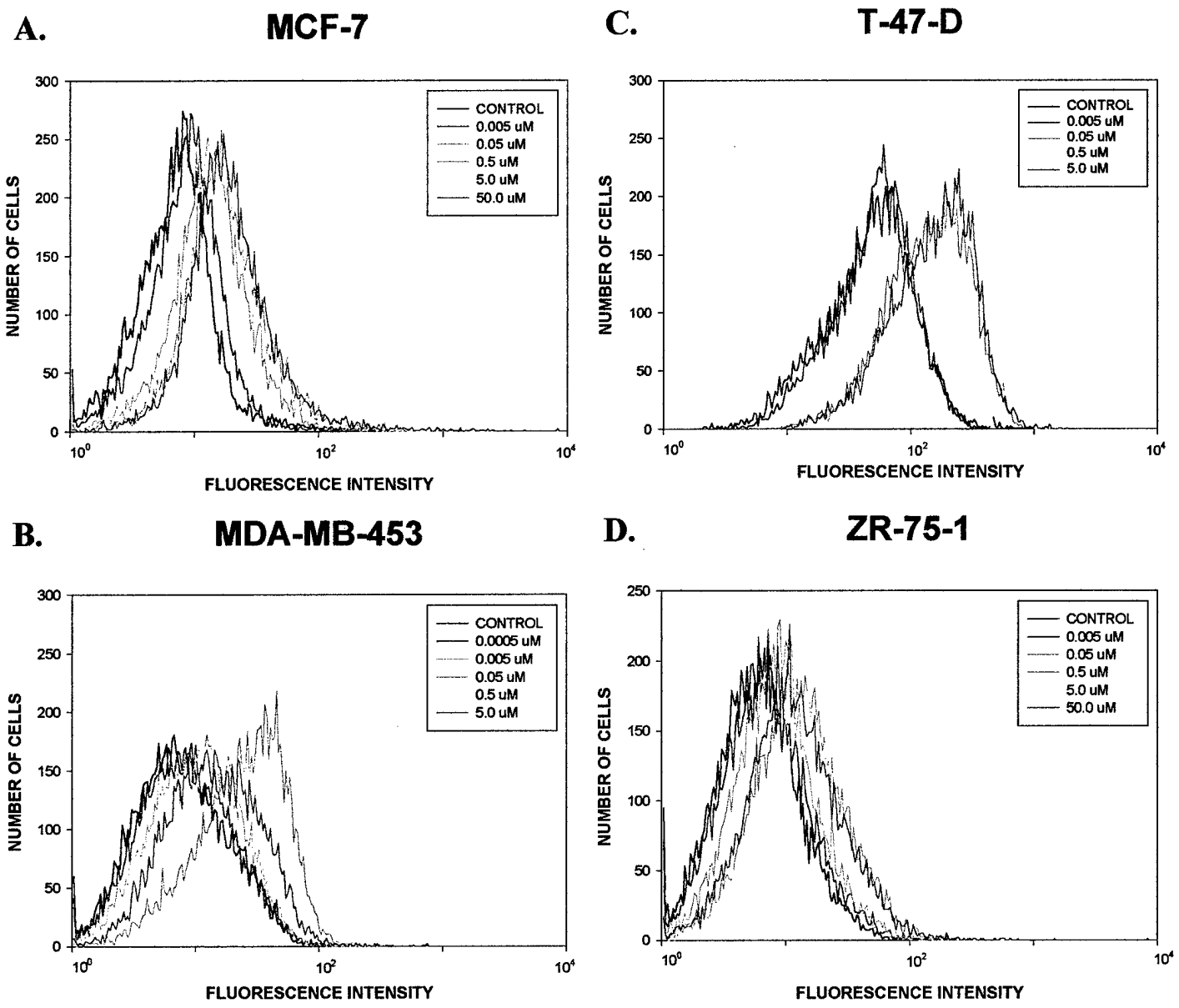

FIGURE 4: Representative histograms for the immunofluorescence / flow cytometry data regarding MTX-changes in EGFR surface expression. Representative histograms generated by the immunohistochemistry / flow cytometry protocol used to detect EGFR surface expression in **(a)** MCF-7, **(b)** MDA-MB-453, **(c)** T-47D and **(d)** ZR-75-1 cells. Details about this experiment method are presented in the legend for **FIGURE 3**.