

AD _____

GRANT NUMBER: DAMD17-94-J-4074

TITLE: The Effects of Signaling Through the EGF Receptor System
Upon Regulation of Growth in Human Mammary Epithelial Cells

PRINCIPAL INVESTIGATOR: Becky Worthylake

CONTRACTING ORGANIZATION: University of Utah
Salt Lake City, UT 84112

REPORT DATE: June 1997

TYPE OF REPORT: Final

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
distribution unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

19980310 069

DTIC QUALITY INSPECTED 2

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE June 1997	3. REPORT TYPE AND DATES COVERED Final (15 May 94 - 14 May 97)	
4. TITLE AND SUBTITLE The Effects of Signaling Through the EGF Receptor System Upon Regulation of Growth in Human Mammary Epithelial Cells		5. FUNDING NUMBERS DAMD17-94-J-4074	
6. AUTHOR(S) Becky Worthylake			
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Utah Salt Lake City, UT 84112		8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) Commander U.S. Army Medical Research and Materiel Command Fort Detrick, MD 21702-5012		10. SPONSORING/MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES			
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited		12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200) Previous reports showed that transactivation of the human epidermal growth factor receptor 2 (HER2) by epidermal growth factor (EGF) leads to the downregulation of HER2. This report presents evidence that overexpression of HER2 nearly abolished its downregulation. Unexpectedly, downregulation of the epidermal growth factor receptor (EGFR) was also inhibited by overexpression of HER2. This suggests that the EGFR and HER2 share a trafficking component which becomes limiting when HER2 is overexpressed. There are two trafficking steps which kinetic analyses predict to be regulated by a saturable component: internalization and lysosomal targeting. We show that the internalization rate of the EGFR is independent of the level of HER2 expression. Thus, we speculate that the molecule responsible for lysosomal targeting is shared between EGFR and HER2. Further, this implies that overexpression of one of the EGFR family members can cause misregulation of the receptors expressed at normal levels.			
14. SUBJECT TERMS Breast Cancer		15. NUMBER OF PAGES 17	
		16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

HW Where copyrighted material is quoted, permission has been obtained to use such material.

HW Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

HW Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

____ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

HW For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

HW In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

HW In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

HW In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Benny Worthington 6/14/97
PI - Signature Date

Table of contents

<u>page</u>	<u>content</u>
1	Front cover
2	SF298
3	Foreword
4	Table of contents
5	Introduction
6	Abbreviations/Methods
7-8	Body
9	Conclusions
10	References
11-17	Appendix

Introduction

Localization of HER2 as affected by EGF-R trafficking, and HER2 overexpression is examined. Immunolocalization, flow cytometric analysis and Western blot techniques are used. We directly observe the localization of HER2, quantitate the fraction of receptors inside vs. on the surface of cells, and monitor degradation of receptors by lysosomal targeting. Unexpectedly, we found HMEC possess a large fraction of HER2 inside the cells. EGF-R mutants that are not properly trafficked do not mediate appropriate EGF induced HER2 trafficking. Overexpression of HER2 causes mislocalization of HER2. In addition, EGF induced HER2 trafficking is saturated in the HER2 overexpressing cells.

ABBREVIATIONS

EGF	epidermal growth factor
EGF-R	EGF receptor
HER2	receptor related to EGF-R
HMEC	human mammary epithelial cells
MTSV	a particular HMEC line (3)
ce2	derivative of MTSV that overexpresses HER2
ce1	another derivative of MTSV that overexpresses HER2
184A1L5	a particular HMEC line, also abbreviated "A1" (4)

METHODS

Downregulation:

Cells were given 100ng/ml EGF and incubated at 37° in humidified 5% CO₂ incubator. At different times of incubation, cells were subjected to western blot using standard techniques (1). Blots were probed with HER2 antibody, 1917 which was graciously provided by Dr. Gordon Gill. Bands were quantitated with BioRad phosphorimager.

Internalization:

Cells are incubated with 10ng/ml radiolabeled EGF for a 5 minute time course. Every minute cell surface and internalized counts are measured. The slope of the line yield the internalization rate constant (K_e) as described previously (2).

Localization:

Cells are fixed with 2% paraformaldehyde +/- 0.1% saponin. The fixed cells are incubated with monoclonal antibodies against the EGF-R or HER2 (Oncogene Science), followed by secondary antibodies that are conjugated to fluorescein. Secaondary antibody alone was used as a control. Fluorescence intensity is measured by flow cytometry. The mean fluorescent intensity was used as the value for each sample. For a more description see figure 5.

BODY

Specific aim II. was to be completed in year 3 of the granting period.

II. Explore the relationship between misregulation of the EGF receptor system and growth properties of human mammary epithelial cells.

- Overexpress the EGF-R or HER2 in HMEC and determine if trafficking of these receptors is altered. These cells will then be used to assess the effects upon HMEC growth and organization.

As discussed in last year's report, we have obtained another HMEC cell line, MTSV, and its derivative, ce2, which overexpresses HER2. In the parental cell line, HER2 is downregulated following EGF treatment. However, in the HER2 overexpressing cell line, downregulation of HER2 is nearly abolished. (Figure 1) We hypothesized that this is because some molecule that is required to direct HER2 trafficking to the lysosomes is present in limiting amounts. Thus, overexpression of HER2 results in a decrease in its downregulation following transactivation with EGF. Consistent with this idea, the number of HER2 molecules lost following 24 hour treatment with EGF is essentially identical in the MTSV and ce2 cell lines. (Figure 2) This implies that system responsible for degrading HER2 in MTSV cells is operating at maximum capacity.

Previously, I described experiments that demonstrated that appropriate trafficking of the EGF-R correlated with efficient downregulation of HER2. Thus, we reasoned that the limiting component for HER2 trafficking may be the EGF-R itself. To address this possibility, we examined the downregulation of EGF-R itself. Unexpectedly, we found that EGF-R downregulation was also significantly impaired by overexpression of HER2. (Figure 3) We propose a model in which both the EGF-R and HER2 share a common trafficking component. If HER2 is overexpressed, then the degradation of both HER2 and EGF-R is less efficient.

There are two trafficking steps for which kinetic analyses have predicted the existence of a saturable component: internalization and lysosomal targeting. We measured the rate of internalization of the EGF-R to determine if HER2 overexpression effected EGF-R internalization. (Figure 4) These results showed that the internalization rate of the EGF-R was the same in MTSV and ce2 cells. Thus, we propose that the component responsible for targeting receptors to the lysosomes is shared between the two receptors. Experiments are underway to determine if other receptors utilize this same trafficking component.

Unfortunately, these HMEC do not organize into mammary like structures when plated on the extracellular matrix material, matrigel. Hence, the investigations into the role of trafficking in organization on matrigel were not performed.

Instead, we have followed up on another exciting observation, namely that we find that HER2 there is a significant intracellular pool of receptors, instead of the expected exclusive cell surface expression.

Subcellular localization of HER2

In previous reports we showed a significant intracellular pool of HER2 in B82 fibroblasts, 184A1L5 mammary epithelial cells, and MTSV mammary epithelial cells. In order to more quantitatively examine the surface vs. intracellular distribution of receptors in the mammary epithelial cells, I designed a technique utilizing fluorescent flow cytometry. (Figure 5) Cells are either fixed or fixed and permeabilized, then stained with fluorescent antibodies against either the EGF-R or HER2. Mean fluorescent intensity is compared between fixed samples (representing cell surface receptors) and fixed/permeabilized samples (representing internal receptors). These techniques demonstrated that 184A1L5 cells have approximately 60% of both EGF-R and HER2 inside the cell, while MTSV have approximately 30% of both receptors located internally. (Figure 6)

Next we sought to determine if overexpression of HER2 changed the subcellular localization of HER2. Figure 7 shows that the distribution of HER2 was unchanged in ce2 compared to MTSV. Thus, the regulation of HER2 localization is not a saturable process, at least not at the level of overexpression present in ce2. Further experiments are underway to measure this novel finding by other complementary techniques.

Conclusions

Explore the relationship between misregulation of the EGF receptor system by overexpressing HER2.

This report presents evidence demonstrating that overexpression of HER2 impairs the ability of the cell to downregulate transactivated HER2. Unexpectedly, the downregulation of the EGF-R itself was inhibited. This implies that these two receptors share a common trafficking component that is required for their efficient degradation, and that this component is present in limited quantities. Further investigation revealed that internalization of the EGF-R was not affected by HER2 overexpression, indicating that the limiting component is not regulating ligand induced internalization. From these data we propose a model in which both the EGF-R and HER2 are trafficked to the lysosome by a shared component. Importantly, when HER2 is overexpressed, this component becomes limiting and the result is less efficient degradation of these receptors.

Future experiments are planned to determine the range of receptors that interact with this limiting component. Frequently one or more members of the EGF-R family are overexpressed in breast tumor tissues. These findings are significant in understanding the molecular mechanisms in breast cancer because it suggests that overexpression of one member of this receptor family can affect not only the its own regulation, but the regulation of other family members as well.

Subcellular localization of EGF-R and HER2

This report builds on previous results demonstrating the existence of an intracellular pool of EGF-R and HER2 in mammary epithelial cells. Here we show that another method of measuring surface vs. internal pools of receptors (flow cytometry) reveals similar results. Approximately 60% of these receptors are located inside the cell in 184A1L5, while 30% in MTSV cells are in internal pools. Interestingly, overexpression of HER2 does not affect the localization of the receptor. We feel that this novel finding is important, and have several lines of investigation in progress to follow it up. These include other techniques to measure the amount of receptors located inside the cell, whether the internal pools are part of the endocytic pathway, and where the activation of the receptor is occurring.

REFERENCES

1. "Analysis of Proteins" in *Current protocols in molecular biology*; Vol.2, Chapter 10, John Wiley & Sons, Inc. 1997
2. "Receptors as Models for the Mechanisms of Membrane Protein Turnover and Dynamics" in *Current topics in membranes and transport*, Vol 24, Chapter 9, Academic Press Inc. 1985
3. J Bartek et al. (1991) "Efficient immortalization of luminal epithelial cells from human mammary gland by introduction of simian virus 40 large tumor antigen with a recombinant retrovirus" PNAS (88) 3520-3524
4. Stampfer, M. R. (1985) *J. Tissue Culture Mehtods* , 107-116

APPENDIX

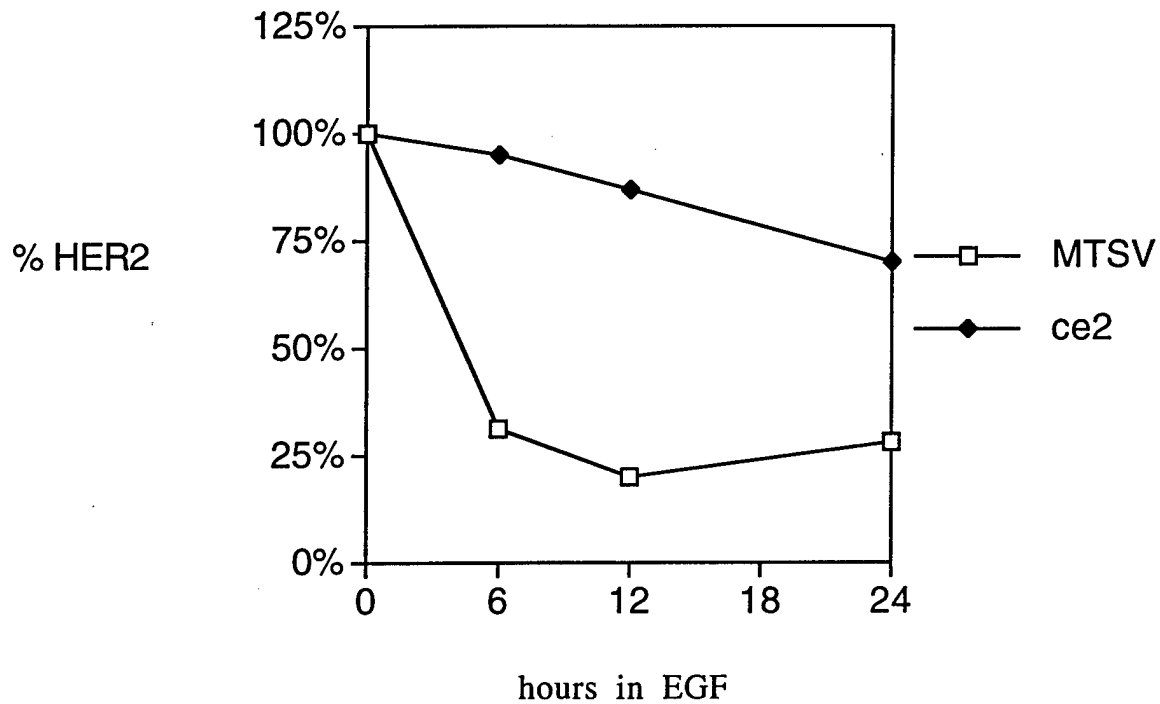


Figure 1. Overexpression of HER2 inhibits its downregulation. Both the parental, MTSV, and the HER2 overexpressing cell line, ce2, were treated with 100ng/ml EGF for the indicated times. Cell extracts were subjected to Western blot for HER2. Bands were quantitated by phosphorimager analysis and plotted as a percentage of control.

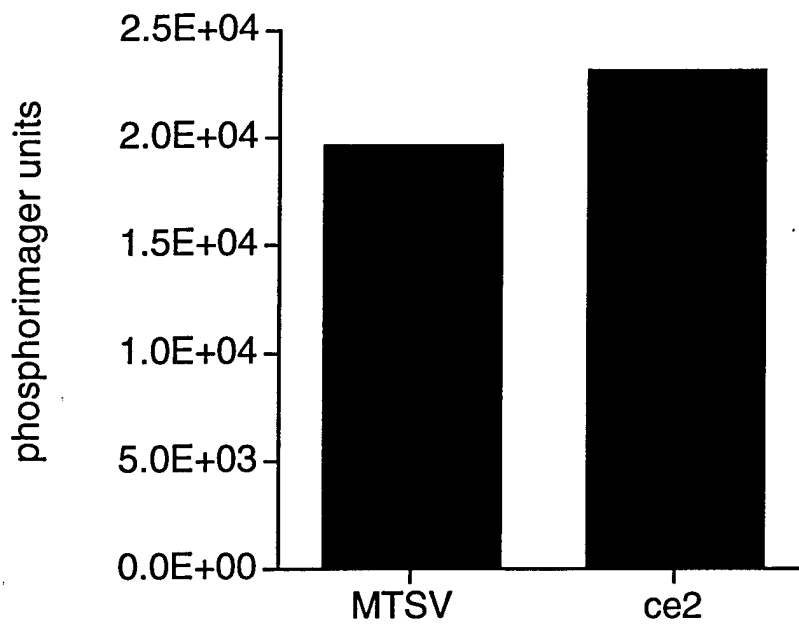


Figure 2. MTSV are degrading HER2 at maximal capacity
Absolute values of HER2 loss after 24 hours of EGF treatment in MTSV compared to ce2. Values were obtained from phosphorimager analysis of cells exposed to EGF for 0 and 24 hours in figure 1. ce2 cells degrade only a marginally greater number of HER2 in response to EGF.

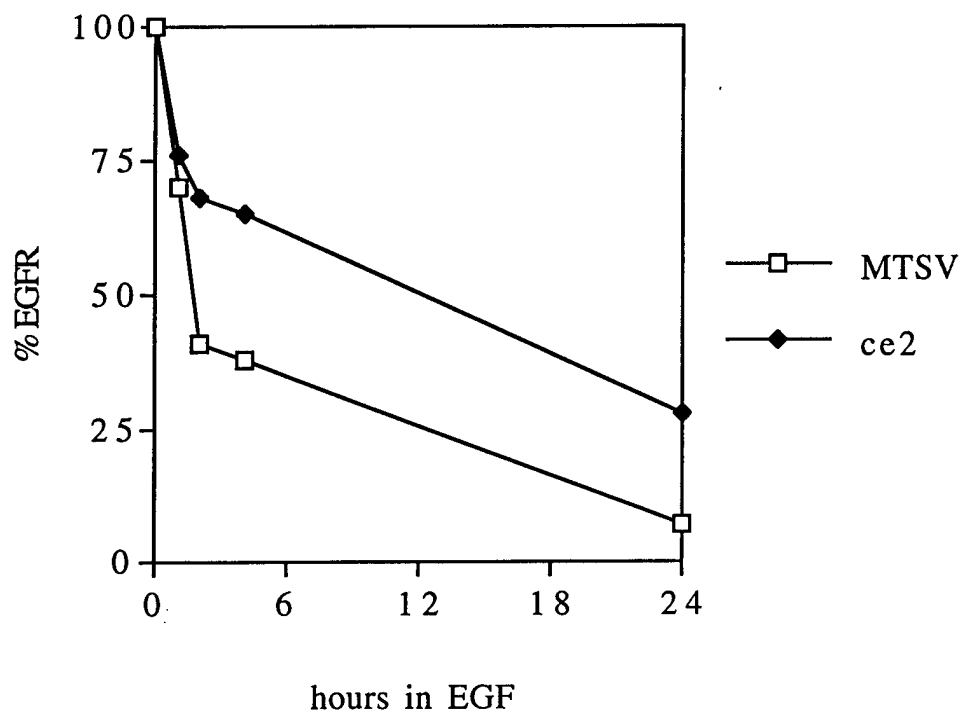


Figure 3. Overexpression of HER2 inhibits the downregulation of EGFR
Cells were treated with EGF for the indicated times. Cell extracts were analyzed by western blot for EGFR content. Bands were quantitated by phosphorimager analysis and plotted as a percentage of control.

Internalization Plot

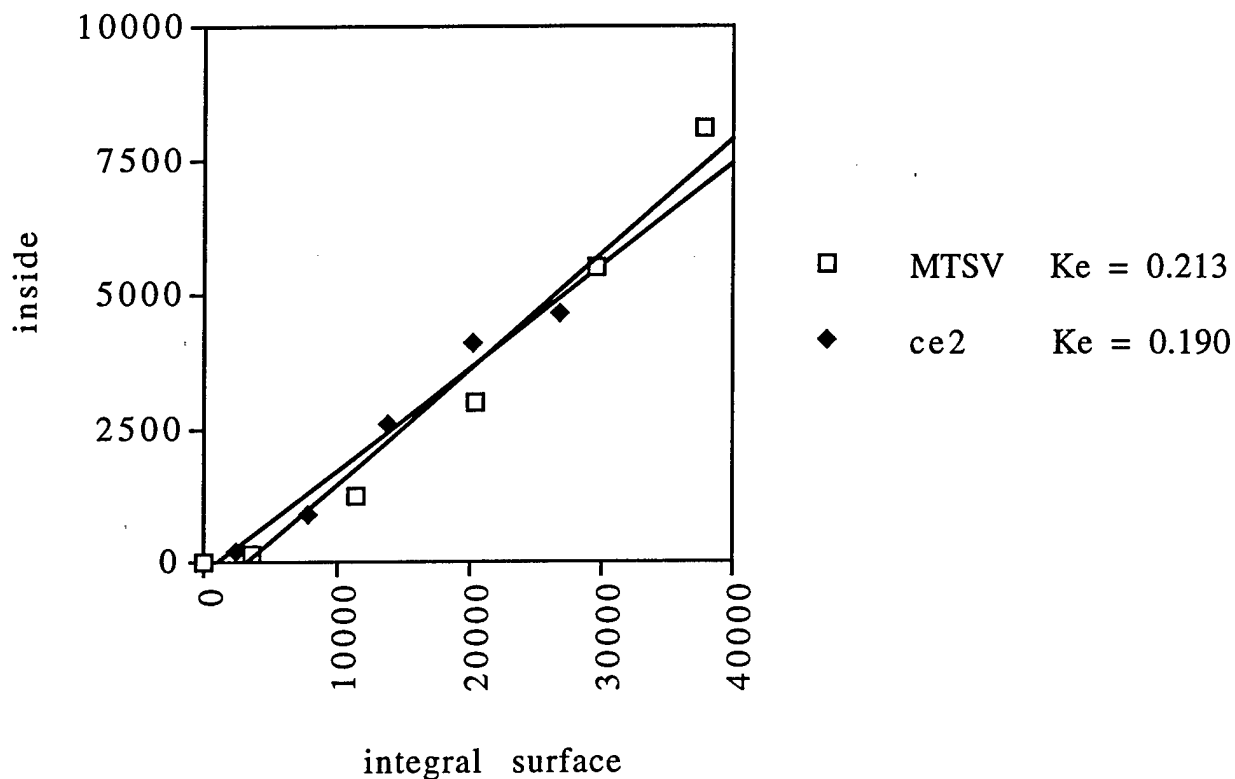


Figure 4. Internalization of the EGF-R is not affected by HER2 overexpression. Cells are incubated with 10ng/ml radiolabeled EGF for a 5 minute time course. Every minute cell surface and internalized counts are measured. The slope of the line yields the internalization rate constant (K_e).

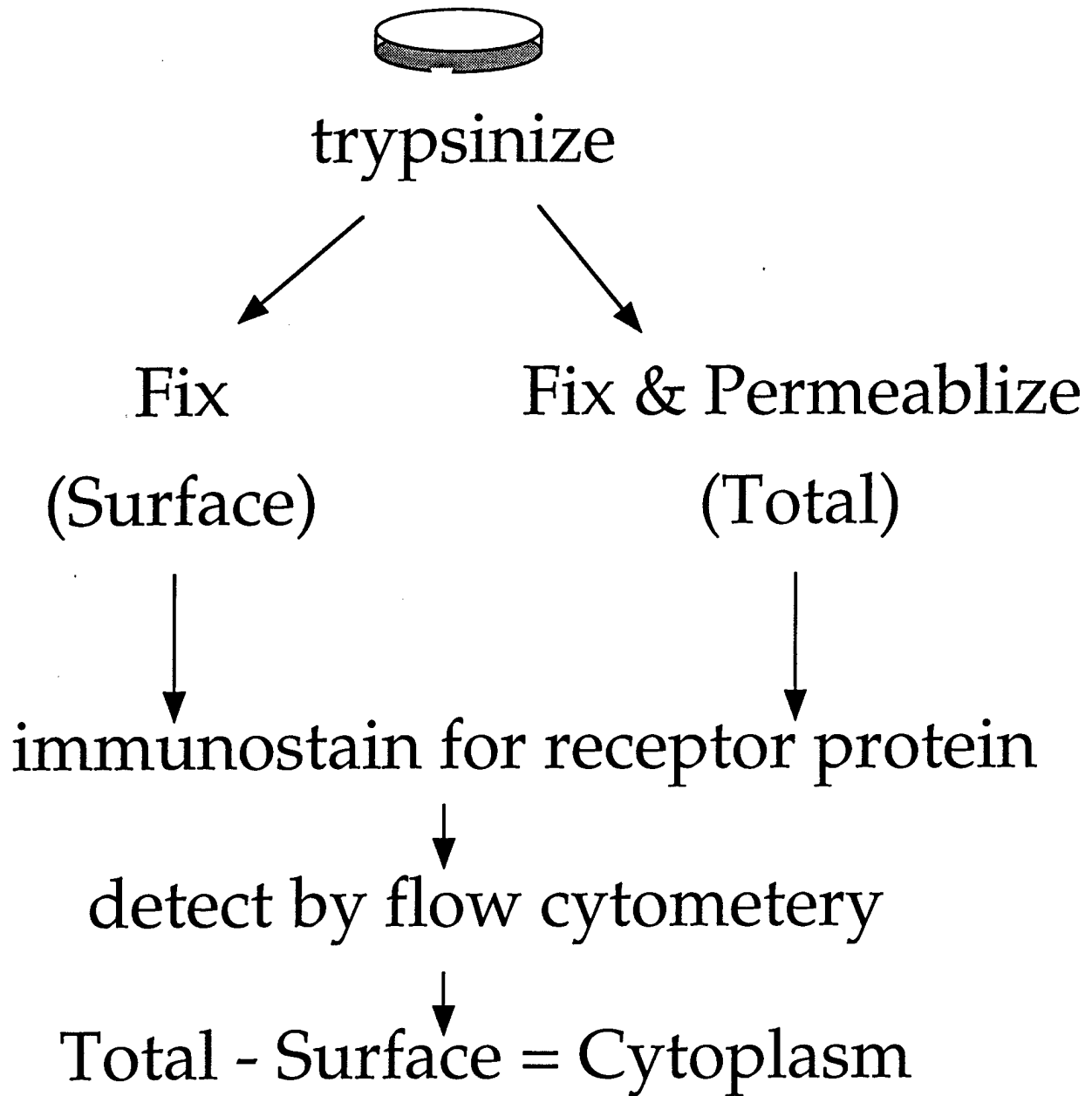


Figure 5. Experimental Schematic for measuring surface vs cytoplasmic distribution

Percent of Receptors in the Cytoplasm by Flow Cytometry

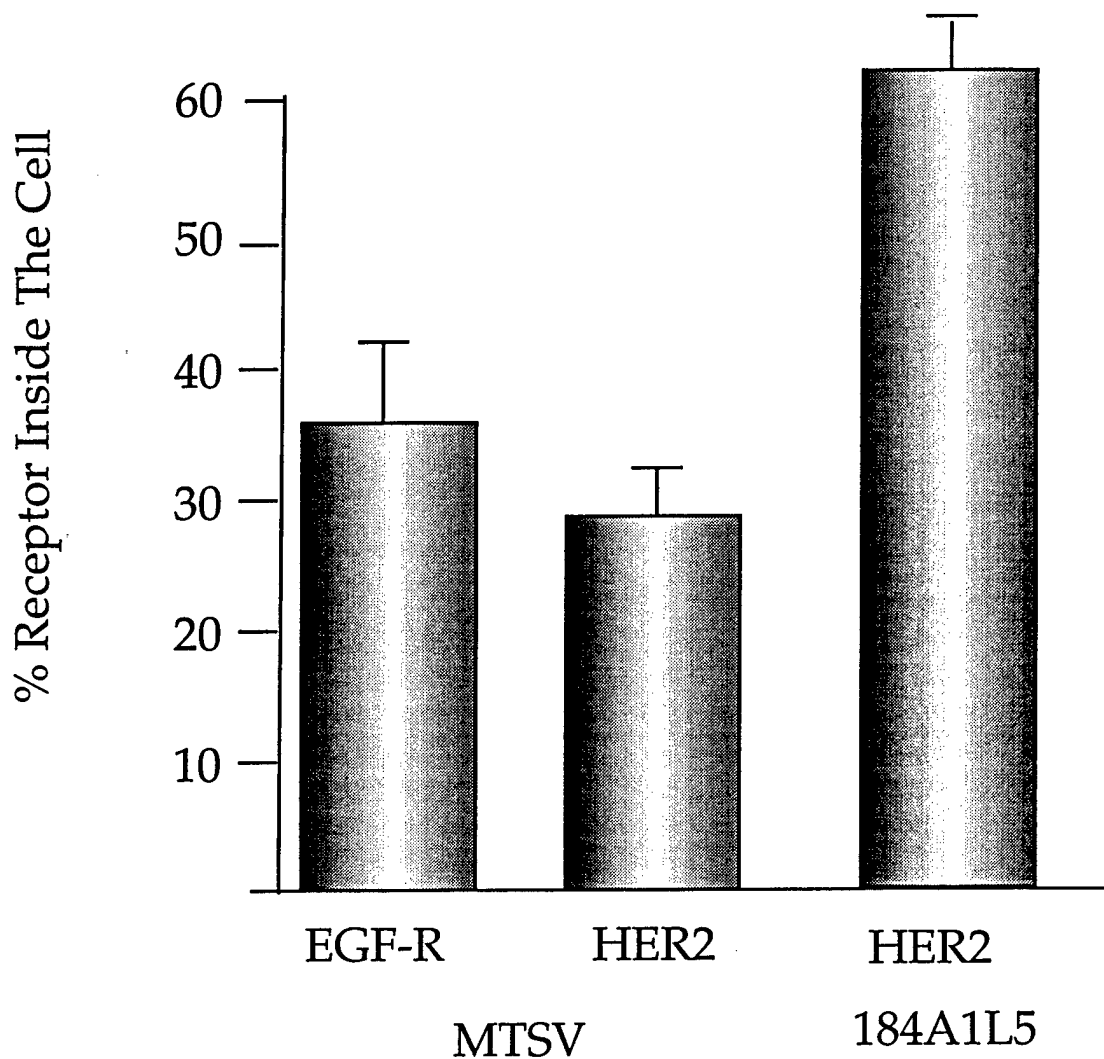


Figure 6. HMEC have a significant cytoplasmic pool of receptors

Cells were fixed or fixed and permeablized and bound with receptor antibodies as described in figure 5 and the methods section. The number of receptors present inside the cell is determined by subtracting the signal from the surface (fixed) from the total signal (fixed and permeablized).

Percent receptor on the surface in cells overexpressing HER2

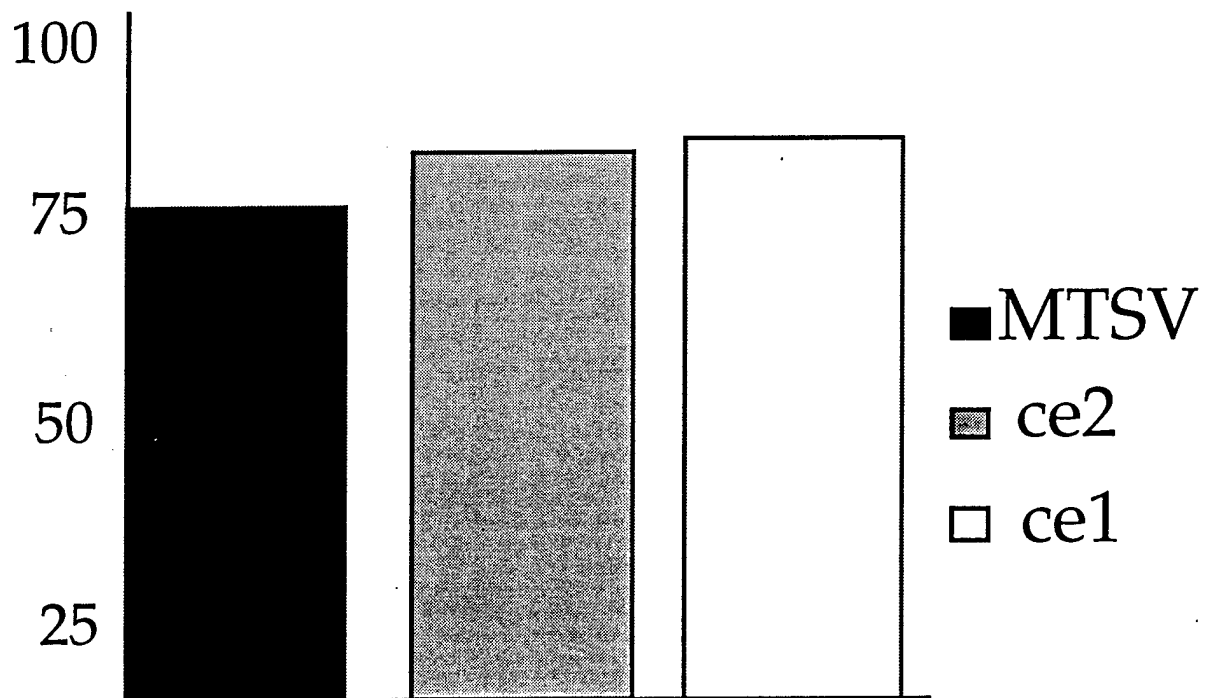


Figure 7. HER2 overexpression does not change the distribution of receptors
Cells were analyzed as in figure 6. ce2 and ce1 which overexpress HER2 do not have a different distribution than the parental MTSV cells.