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13. ABSTRACT (Maximum 200) <p>184A1 human mammary epithelial cells (HMECs) form organotypic structures when plated on Matrigel; however, after three weeks on matrigel 184A1 cells do not form polarized, glandular epithelial structures, nor do they polarize if plated onto Transwell tissue culture inserts. Therefore, 184A1s can not be used to address the primary aim of my proposal: to determine if the loss of the correct spatial organization or inappropriate expression of the EGF-R system provides a growth advantage, enhances motility, or changes the differentiated state of normal cells. MCF-7 cells do express a phenotype consistent with polarized epithelia when plated on Transwells, however, MCF-7 are so loosely adherent to transwells that they are refractory to typical experimental manipulation. HB2 cells are a normal, non-transformed mammary epithelial cell line that expresses high numbers of EGF-R, are tightly adherent to transwells, and polarize on transwells as determined by immunofluorescence localization of ZO-1, the EGF-R and β-1 integrin. HB2 may provide an experimental system with which to address the primary of my proposal. Preliminary experiments comparing 184A1s and HB2 have revealed an interesting and novel finding--rapid internalization of EGF-R can be uncoupled from the normal negative regulatory process of down-regulation.</p>			
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INTRODUCTION:

Spatial Distribution of the EGF Receptor System in the Regulation of Breast Epithelial Cell Growth and Organization

The epidermal growth factor receptor (EGF-R) system is necessary for the motility, proliferation and differentiation of human mammary epithelial cells (HMECs) *in vitro*. Additionally, the EGF-R system displays a highly organized spatial distribution *in vivo*. Because the EGF-R system plays a central role in HMEC proliferation, it is reasonable to suspect that any defects in its regulation could lead to the clonal expansion of 'pre-malignant' cell populations. Such expanding clonal populations could give rise to cancerous clones. The spatial distribution of the EGF-R system is highly organized *in vivo*. *In vivo*, the receptor and one of its ligands, transforming growth factor alpha (TGF- α), are localized to the basolateral surface of mammary alveolar structures; on the other hand, epidermal growth factor (EGF) is synthesized and secreted from the apical side into the lumen of the alveoli. Regulation of the EGF-R system could be disrupted by removing the spatial restrictions which segregate one of the ligands from the receptor and/or the receptor from second messenger systems. I will investigate the spatial distribution of both the EGF-R and its ligands during the organization of HMECs on basement membranes (BM) *in vitro* and determine the consequences of disorganizing this distribution.

SPECIFIC AIMS:

1. Define the spatial distribution and expression levels of the EGF-R system in proliferating and spatially organized normal HMECs.
2. Determine whether a loss of the correct spatial organization or inappropriate expression of the EGF-R system provides a growth advantage, enhances cell motility, or changes the differentiated state of normally organized epithelial cells.

STATEMENT OF WORK:

PART I

Define the spatial distribution and expression levels of the EGF-R system in proliferating and spatially organized normal HMECs. (Months 1-18).

- a. Determine the expression levels of the EGF-R and its ligands in normal HMECs. (Months 1-6).
- b. Determine the spatial distribution of the EGF-R and its ligands in the organotypic

structures of normal HMECs. (Months 7-18).

PART II

Determine whether a loss of the correct spatial organization or inappropriate expression of the EGF-R system provides a growth advantage to normally organized epithelial cells. (Months 19-36)

- a. Disrupt The Polarization Of The EGF-R with two approaches. (Months 19-27).
 - 1) Overexpression of the wild type receptor.
 - 2) Transfection of c'958 truncation mutant which localizes to the apical surface of epithelial cells.
- b. Assess motility, morphological organization, and growth disrupted cell types. (Months 27-36).

BODY OF REPORT:

The HMECs that I chose to use in this work are designated 184A1 and were isolated by Martha Stampfer as an immortalized derivative from the parental 184 cell line (1). They exhibit the ability to organize into organotypic structures when plated onto the extracellular matrix material Matrigel (extracted from Englebreth-Holm-Swarm murine sarcoma), as shown in proposal. As discussed in the previous report, 184 differentiate into squamous rather than glandular epithelia. Also as mentioned in previous report, 184A1s fail to polarize when plated onto tissue culture membranes; therefore it is unlikely that they will provide a useful experimental system for pursuit of my specific aims. However, because these cells express large numbers of receptors, 2×10^5 receptors/cell (fig. 1), and are absolutely dependent upon EGF-R stimulation for growth (2), they do provide an interesting tissue culture system with which to explore other relationships between EGF-R regulation and epithelial cell physiology. My work with 184A1 cells has revealed two interesting and novel EGF-R regulatory mechanisms.

EGF-R levels, in 184A1s, have been examined during the squamous differentiation process and appear to decrease rapidly upon contact with the basement membrane and continue to decrease over a seven day period (fig. 2). Initial experiments that documented this finding were difficult to interpret with confidence because of technical problems with sample normalization. However, I now have an experiment design that allows me to conclude with much greater

confidence that EGF-R levels do decrease upon exposure of cells to Matrigel (fig. 3). Additionally, I have tested specific Matrigel components for their ability to influence EGF-R levels and found that fibronectin and collagen type I may be able to influence EGF-R levels in 184A1s (fig. 4). Other experiments indicate that the drop in EGF-R levels on Matrigel is, at least in part, the result of decreased EGF-R transcript levels; RT-PCR results in two different experiments show that the EGF-R/G3PDH transcript ratio decreases in the presence of matrigel (fig. 5). I am hesitant to say that these results are conclusive; I will compare EGF-R transcript levels to another internal control before publishing this finding. It is also possible that a component of this regulatory mechanism is post-transcriptional; other experiments are planned to test this.

Experiments with 184A1s have demonstrated that rapid internalization of EGF-R can be uncoupled from the negative regulatory mechanism of down-regulation. It has been shown that independent domains of the EGF-R, separable from the kinase domain, are responsible for ligand induced internalization and lysosomal targeting. In fibroblasts and L cells the kinase domain of an occupied EGF-R "activates" these different domains, alters the spatial distribution of the receptor and subsequently causes receptor degradation or down-regulation. This is a classic example of negative spatial regulation. I have demonstrated in 184A1L5 (a subclone of 184A1) that rapid internalization of endogenous EGF-R can be occupancy independent and that ligand occupancy in the 184A1L5 cell line is only necessary for lysosomal targeting. Unoccupied EGF-Rs in other human mammary epithelial cells (HB2 described below), fibroblasts, and transformed cells are internalized at a rate of only 0.02 min^{-1} (ie. 2% of total receptors are internalized/min) (fig. 6). 184A1L5 cells internalize unoccupied EGF-Rs at a rate of about 0.20 min^{-1} , approximately equivalent to the internalization rate of occupied EGF-R in HB2 cells and transformed cells (0.18 min^{-1}) (fig. 6). Additional experiments, including inside/surface ratios of unoccupied receptors and immunofluorescence, support the conclusion that empty receptors are being rapidly internalized (fig. 7 & 8). Despite the ten-fold more rapid internalization of empty EGF-R in 184A1L5, the half-life of EGF-R in the absence of EGF for 184A1L5 and HB2s is very similar (fig. 9). This indicates that the internalized empty receptors in 184A1L5 are being recycled. Occupancy of EGF-R in 184A1L5 cells rapidly targets receptors for degradation (fig. 9), additionally, high concentrations of exogenous EGF reduces receptor half-life in both HB2 and 184A1L5 cells similarly (fig. 9). These findings indicate that the individual steps of down-regulation can be uncoupled, and that intracellular trafficking of the EGF-R is regulated by signals other than receptor occupancy.

In order to address the original aims of my proposal I have been in search of the proper experimental cell system. Last year I reported that MCF-7 cells may serve as an effective model for studying the functional importance of a polarized distribution of the EGF-R system. Initial immunofluorescence results strongly suggested that MCF-7 cells polarize when plated onto tissue culture membranes (Costar, Transwells) as determined by the distribution of ZO-1 and

Beta-1 integrin (data previous report). However, MCF-7 are so loosely adherent to transwells that they are refractory to typical experimental manipulation. Multiple approaches were tried to make the cells more amenable to experimentation, including subcloning and plating onto basement membrane substratum, but no approach provided a satisfactory solution. Recently, we have obtained the more suitable HB2 cells from Dr. Joyce Taylor-Papadimitiou. These cells are human mammary epithelial cells derived from milk and immortalized with SV40 large T antigen (3). These cells possess a luminal phenotype (characteristic of malignant mammary epithelial cells), but are non-tumorigenic (3). Importantly, they express high numbers of EGF receptors, 1×10^6 receptors/cell (fig. 10), and form well polarized monolayers as determined by immunolocalization of EGF-R, $\beta 1$ -integrin, and ZO-1 (fig. 11). Unfortunately, the growth of HB2s is not sensitive to EGF-R stimulation (fig. 12). Unpublished observations of a collaborator, in another polarized cell type, indicate that EGF-R signaling patterns generated from apical or basolateral membrane domains are different. That type of analysis will be useful when assessing the functional significance of EGF-R polarity in HB2s.

Initial studies have been undertaken to assess the expression patterns and protein localization of EGF-R ligands in HB2 cells. RT-PCR indicates that all known EGF-R ligands are made by HB2 cells (fig. 13), in at least low levels. Since protein levels of the ligands are too low to detect by immunofluorescence, subcellular localization of the ligands has not been possible. This technical difficulty will not obstruct the investigation outlined in my proposal. Experiments are planned to over-express wild type EGF-R and $\Delta 2-7$ mutant receptors, which like the c'958, are expected to be apically as well as basolaterally located. The $\Delta 2-7$ mutant has been chosen because it has been published that it is a common mutated form of the EGF-R found in mammary carcinomas (4). Retroviral expression vectors have already been made and transduction competent virus collected.

CONCLUSIONS:

- 1.) EGF-R levels in 184A1 decrease upon exposure of cells to Matrigel.
- 2.) Exposure of 184A1 to fibronectin and collagen type I may decrease EGF-R levels.
- 3.) Drop in EGF-R levels on Matrigel is, at least in part, the result of decreased EGF-R transcript levels.
- 4.) Individual steps of receptor down-regulation can be uncoupled, and intracellular trafficking of the EGF-R is regulated by signals other than receptor occupancy.

- 5.) MCF-7 are so loosely adherent to Transwells that they are refractory to typical experimentation necessary to examine polarity and the functional significance of a polarized EGF-R system.
- 6.) HB2 cells express large numbers of EGF receptors, 1×10^6 receptors/cell.
- 7.) HB2s form well polarized monolayers as determined by immunolocalization of EGF-R, $\beta 1$ -integrin, and ZO-1.
- 8.) The growth of HB2s is not sensitive to EGF-R stimulation.
- 9.) RT-PCR indicates that all known EGF-R ligands are made by HB2 cells, at least low levels.
- 10.) In HB2 cells ligand levels are so low it has not been possible to detect their subcellular localization by immunofluorescence.

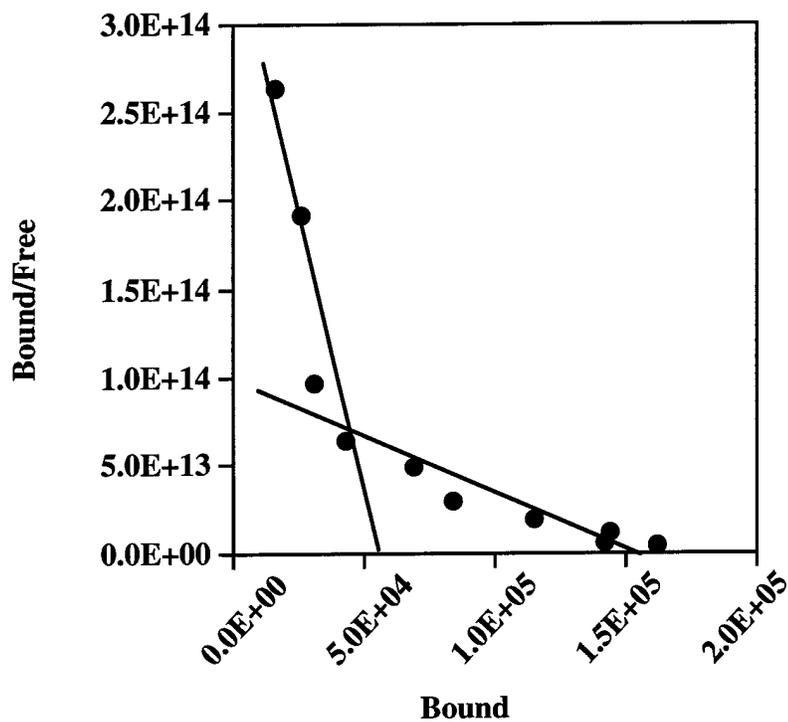
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2. Stampfer, M. R., Pan, C. H., Hosoda, J., Bartholomew, J., Mendelsohn, J., and Yaswen, P. 1993. *Exp. Cell Res.* **208**, 175-188.
3. Bartek, J., J. Bartkova, N. Kyprianou, E.N. Lalani, Z. Staskova, M. Shearer, S.Chang, and J. Taylor-Papadimitriou. 1991. *Proc. Natl. Acad. Sci. USA.* **88**: 3520-3524.
4. Moscatello, D.K., Holgado-Madruga, M., Godwin, A.K., Ramirez, G., Gunn, G., Zoltick, P.W., Biegel, J.A., Hayes, R.L., Wong, A.J. 1995. *Cancer Res.* **55**: 5536-5539.

Appendix

Figure 1

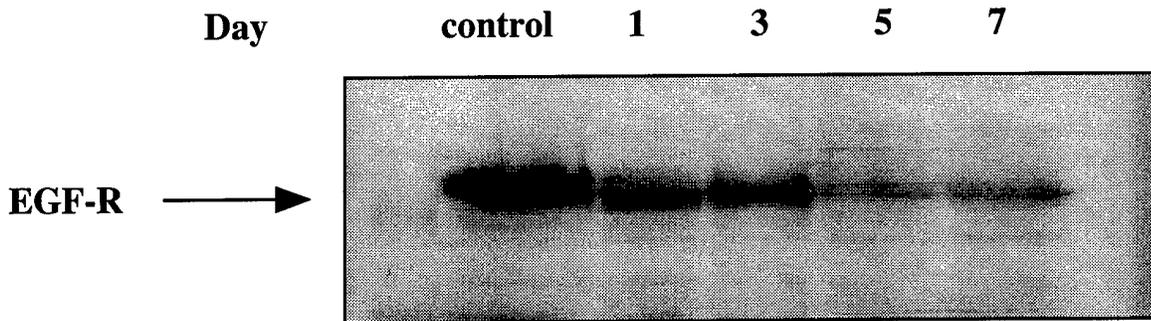
Scatchard Analysis of EGF-Rs in 184A1L5 Cells



184A1L5 cells, a subclone of the 184A1 cell line, was analyzed by Scatchard Plot using radiolabeled EGF. 184A1L5 cells express approximately $1.5\text{-}2.0 \times 10^5$ receptors/cell. The plot is curvilinear indicating high and low affinity receptor populations.

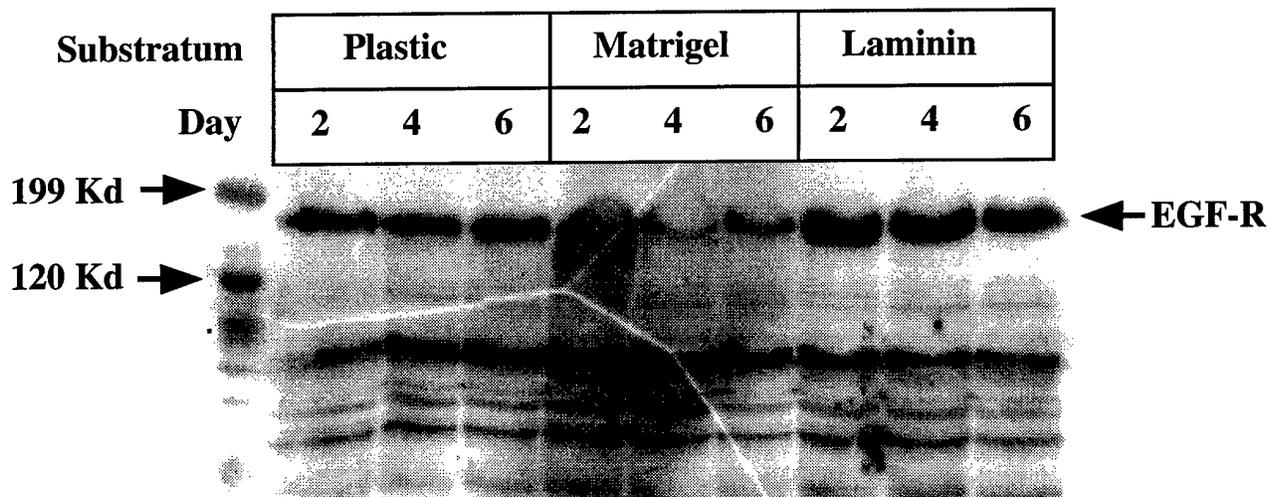
Appendix

Figure 2



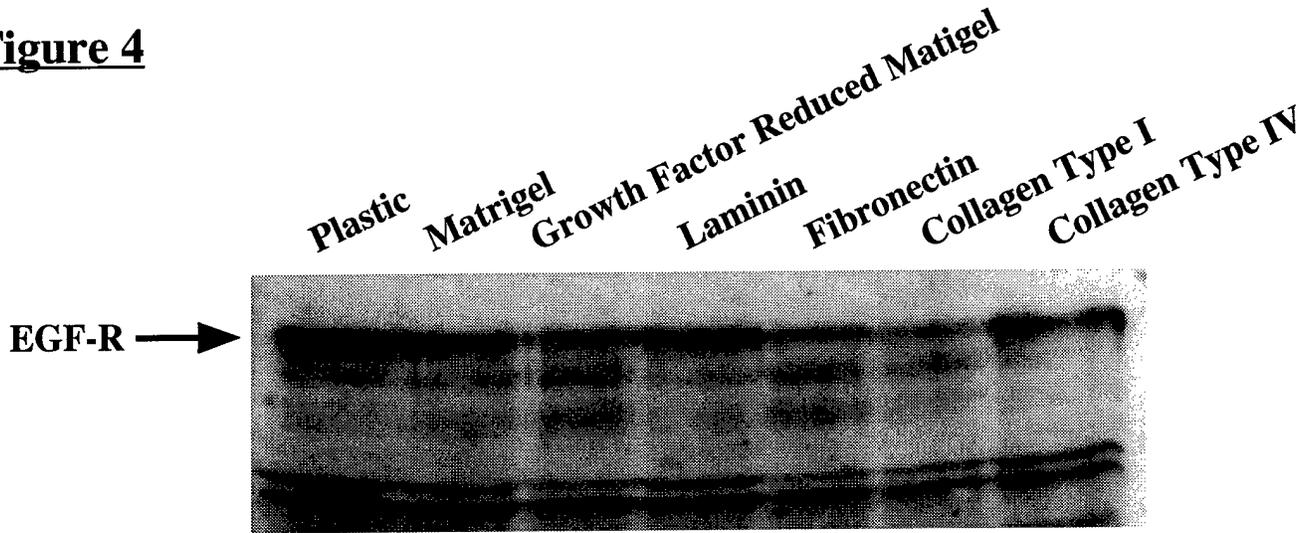
Decreased EGF-R expression during organization on matrigel as determined by western blot, equal amounts of protein were loaded in each lane; the control is cells grown on plastic.

Figure 3



Decreased EGF-R expression during organization on matrigel as determined by western blot, equal amounts of protein were loaded in each lane; the control is cells grown on plastic. Samples were also prepared from cells grown on pure laminin.

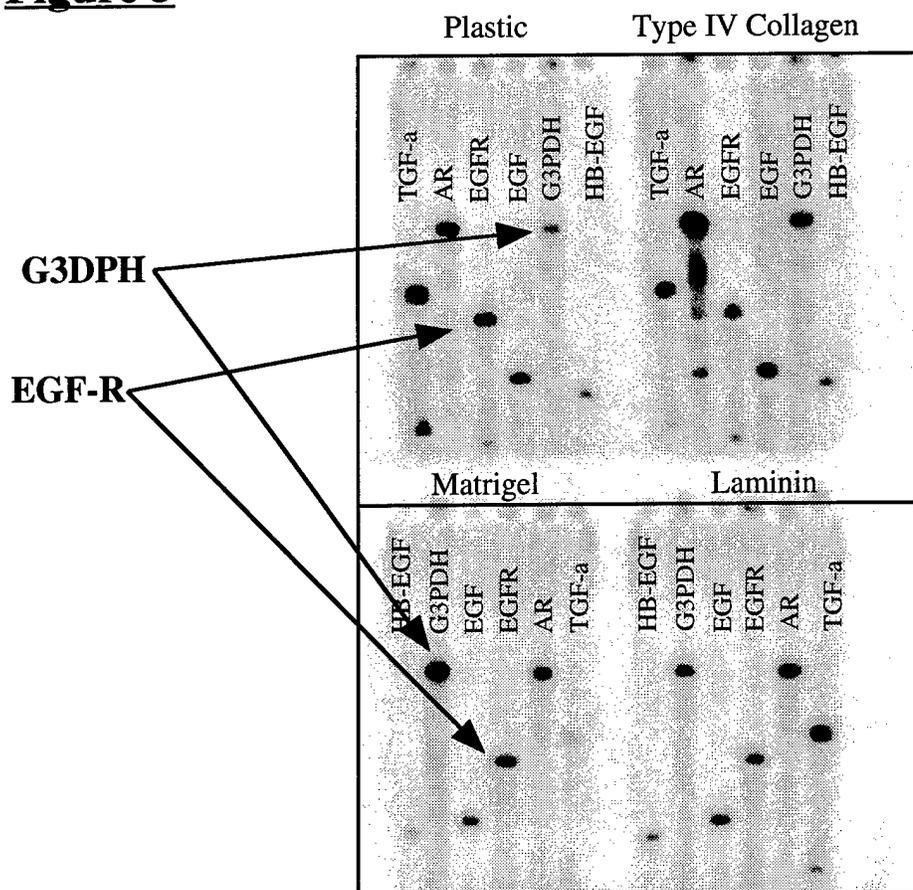
Figure 4



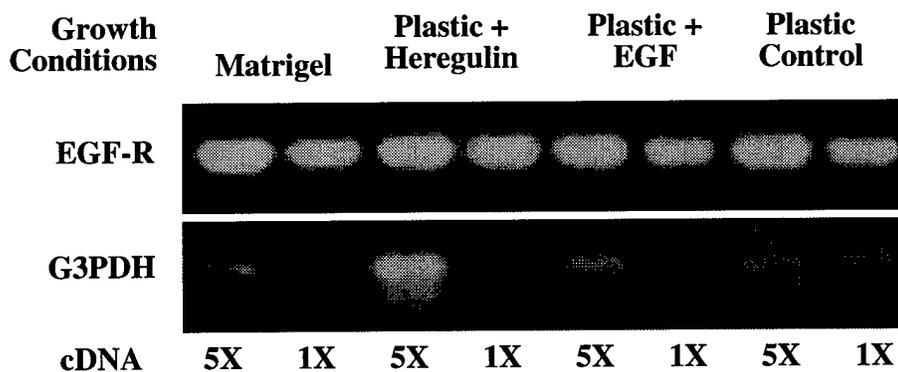
EGF-R expression in 184A1L5s grown on different ECM substrates for 2 days as determined by western blot, equal amounts of protein were loaded in each lane; the control is cells grown on plastic. All Purified extracellular matrix components used to coat the plastic plates were @ 1 mg/ml.

Appendix

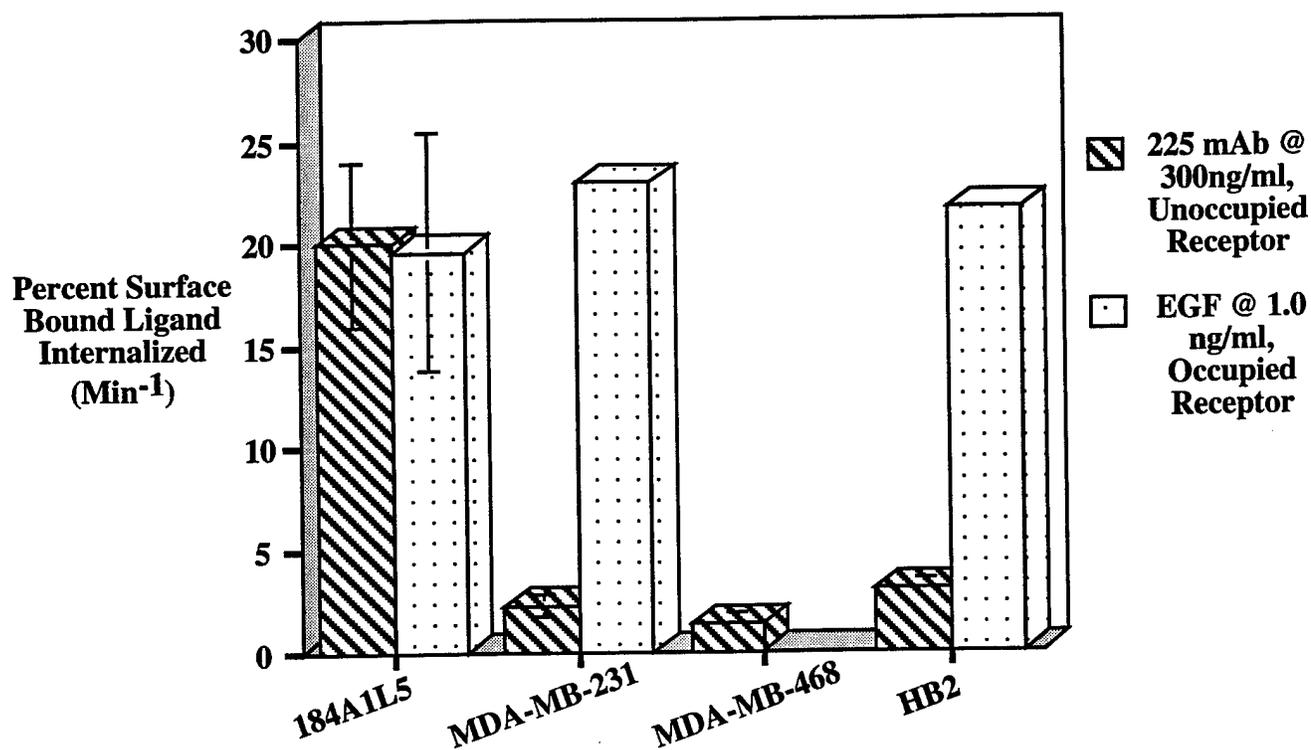
Figure 5



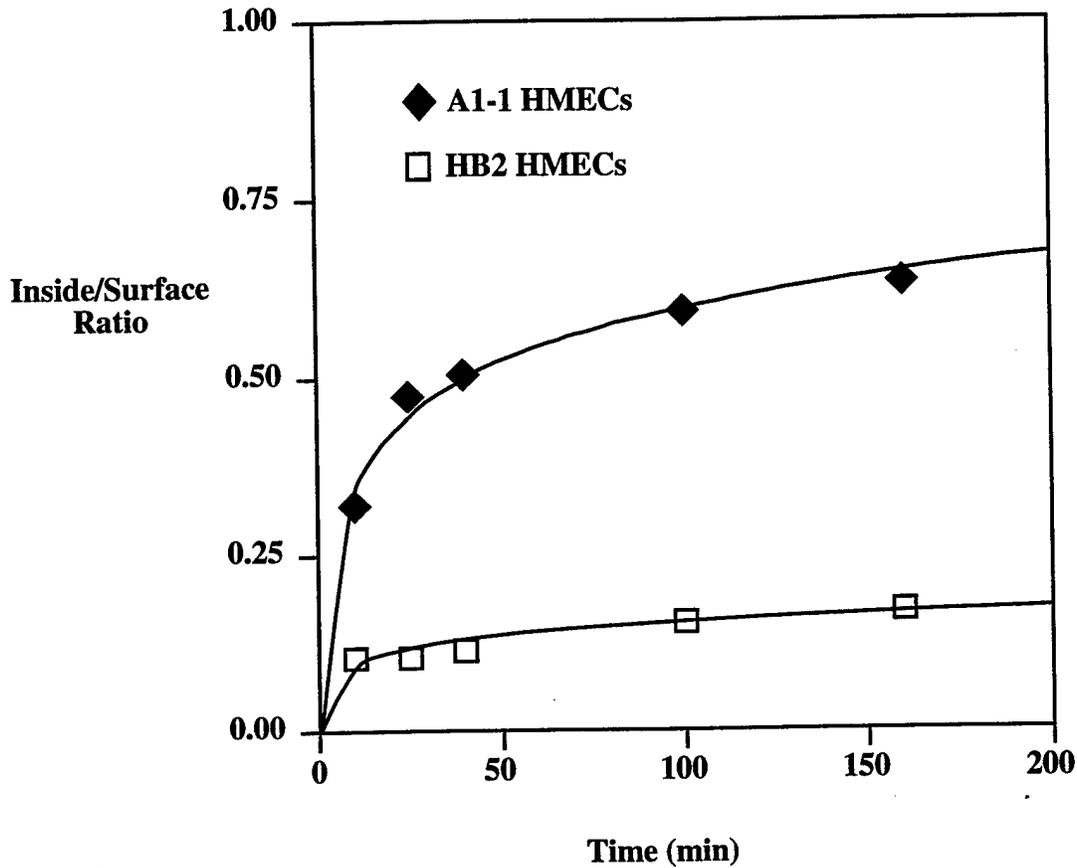
RT-PCR on RNA samples analyzing expression of EGF-R and EGF-R ligands. Samples were prepared from cells grown on plastic, type IV collagen, laminin, or Matrigel.



RT-PCR on RNA samples prepared from cells grown on plastic (control), on plastic + heregulin, on plastic + EGF, or on Matrigel.

Figure 6

Internalization rate constants for occupied and unoccupied EGF-Rs in various mammary epithelial cell lines. 184A1L5 and HB2 are normal, non-transformed cell lines; MDA-MB-231 and MDA-MB-468 are cell lines derived from mammary carcinomas.

Figure 7

Inside/surface ratio of unoccupied EGF-Rs in HB2s and 184A1L5s HMECs. Unoccupied EGFRs were labeled with radioactive 225 Mab on ice. The cells were warmed to 37 degrees centigrade and at various times after warming the cells the inside/surface ratio of unoccupied receptors was measured. This graph indicates that at steady state there is a significantly larger internal pool of unoccupied EGF-R in 184A1L5 cells than in HB2 cells.

Appendix

Figure 8

Distribution of EGF-Rs in HB2 Cells

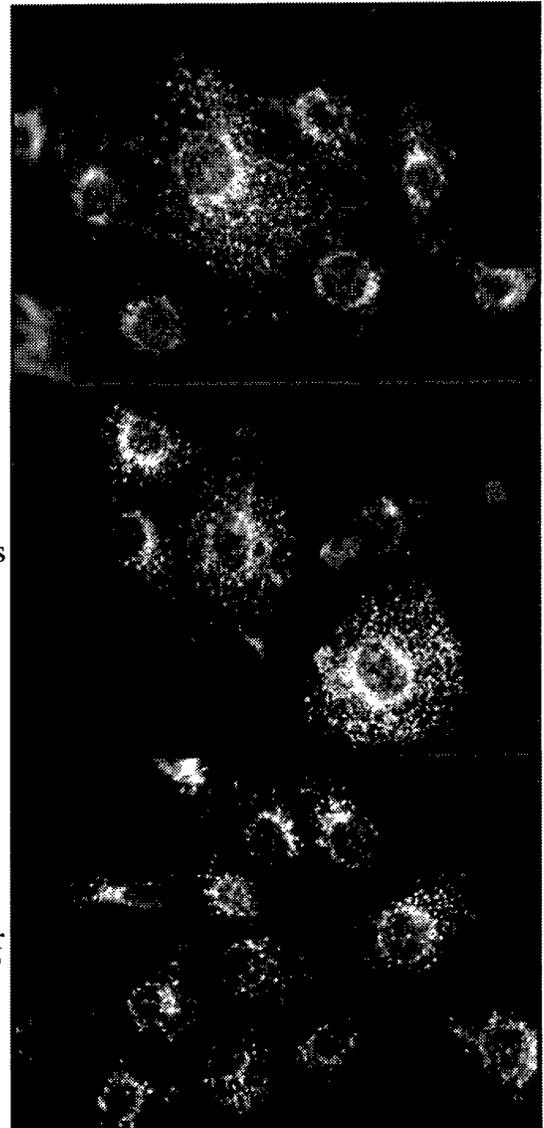


Steady-State
Distribution of All
EGF-Rs in
Serum Starved
Cells

Distribution of
Unoccupied EGF-Rs
Internalized in
2 hour Period

Distribution of All
EGF-Rs After 2 hour
Treatment with EGF

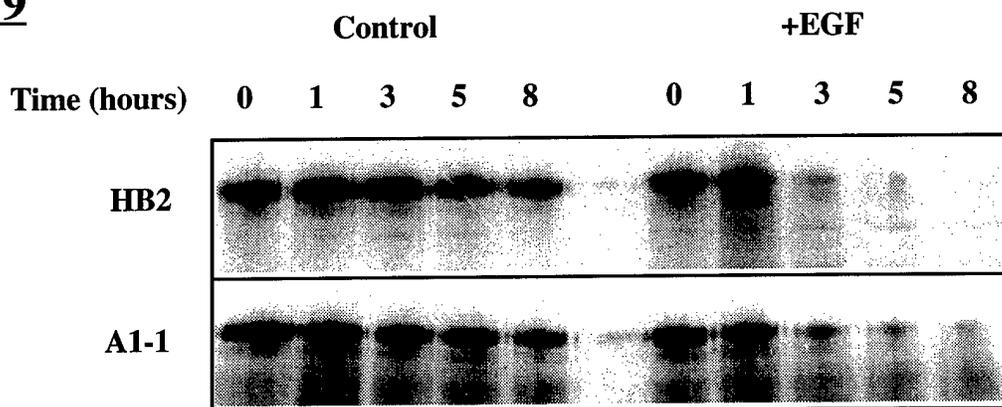
Distribution of EGF-Rs in 184A1L5 Cells



Cells were serum starved for 24 hrs prior treatment with either mAb 225 @ 500ng/ml or EGF @ 50 ng/ml or nothing (control) and kept at 37 degrees centigrade. Control cells and EGF treated cells were fixed and permeablized with 4% paraformaldehyde/0.1% Triton X-100. EGF-R was detected using primary antibodies mAb 225 and 13A9 (15 & 10 µg/ml respectively) followed by FITC goat anti-mouse secondary antibody. Cells treated with mAb 225 were handled similarly, but no primary antibodies was used post fixation. Distribution of the mAb 225 "fed" to cells was detected by FITC goat anti-mouse. The control HB2 cells are another non-tumorigenic mammary epithelial cell line of different origin; it was kindly provided by Dr. Joyce Taylor-Papadimitriou.

Appendix

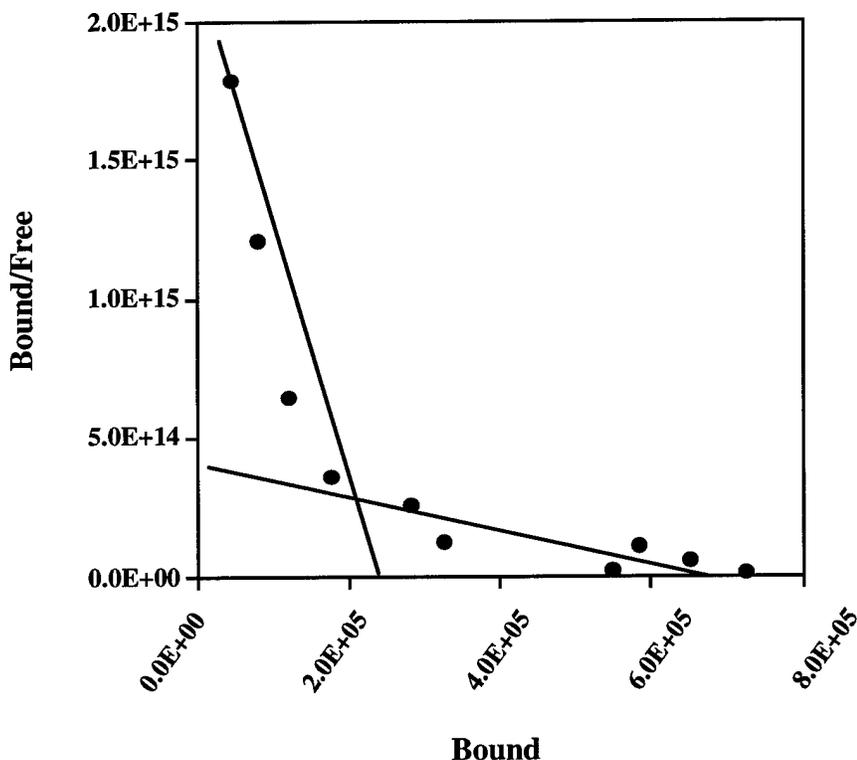
Figure 9



EGF-R trafficking in the absence and presence of EGF in 184A1L5 and HB2 cells. A standard pulse chase experiment was performed. Cells were labeled with radiolabelled with 35S met/cys for 24 hours and then chased for designated time periods with excess unlabeled met/cys. EGF-R was immunoprecipitated with mAb 225, run on a 5-15% gradient gel, exposed to a phosphoimager plate and the cpm /EGF-R band was quantitated.

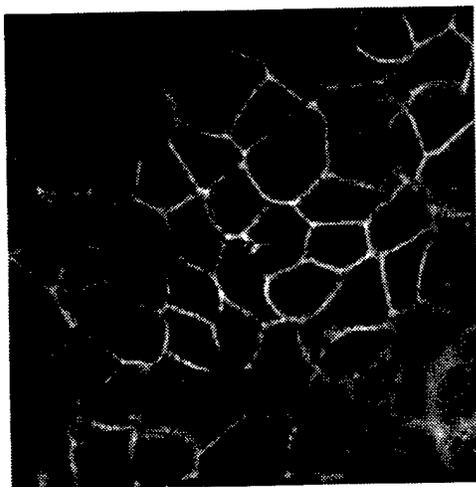
Figure 10

Scatchard Analysis of EGF-Rs in HB2 Cells

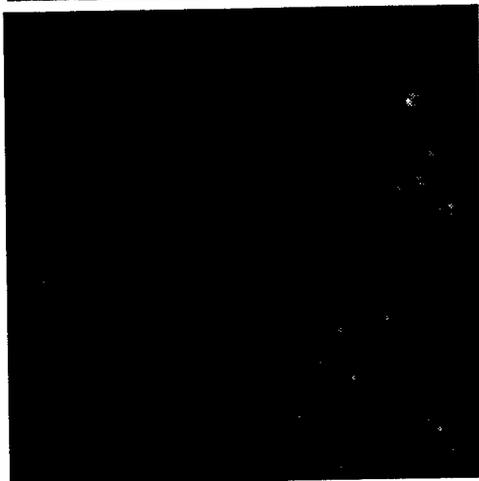


HB2 cells were analyzed by Scatchard Plot using radiolabeled EGF. HB2 cells express approximately $.8-1.0 \times 10^6$ receptors/cell. The plot is curvilinear indicating high and low affinity receptor populations.

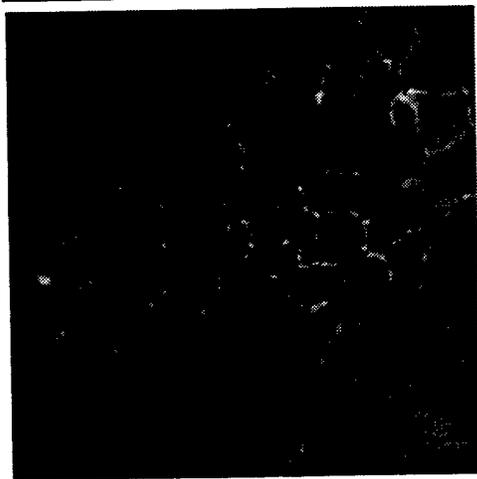
Figure 11



β -1 Integrin

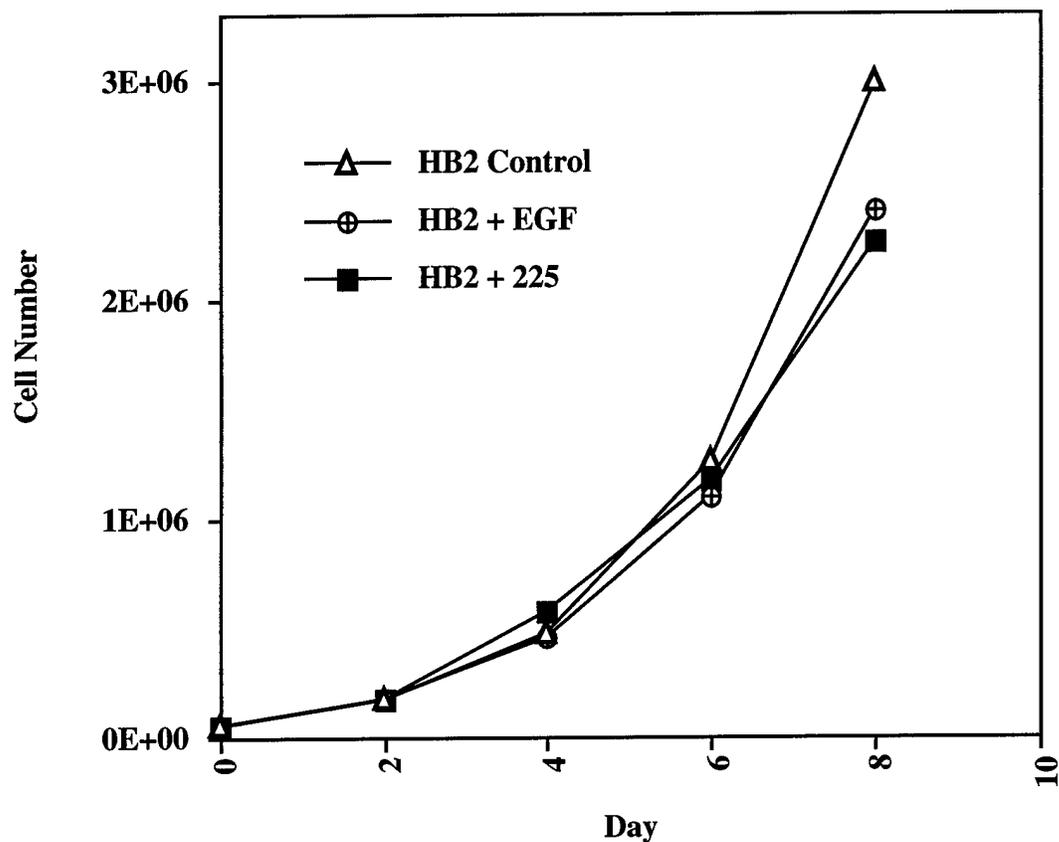


ZO-1 at the β -1 Integrin Plane



ZO-1 Apical to the β -1 Integrin Plane

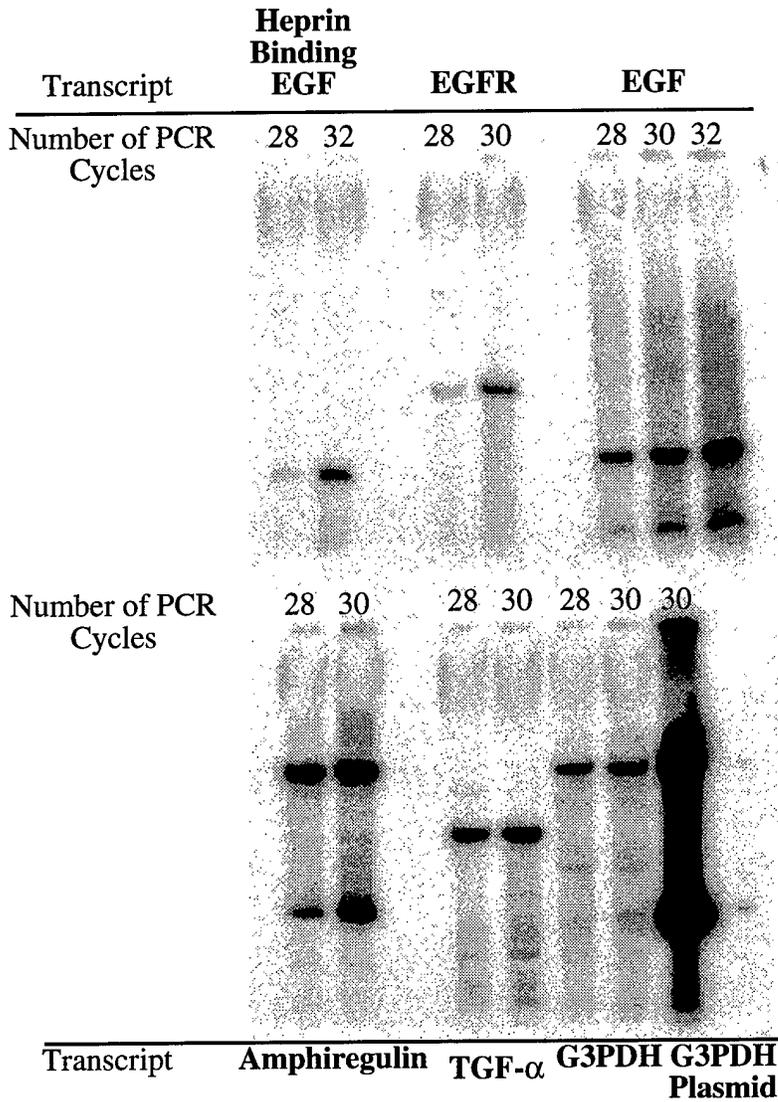
Subcellular localization of β -1 integrin and ZO-1 by indirect immunofluorescence in HB2 cells. The ZO-1 marker indicates the apical-basolateral boundary. The β -1 staining pattern is consistent with basolateral localization.

Figure 12

HB2 cell growth in the presence and absence of either EGF, @ 20ng/ml, or EGFR antagonistic antibody 225, @ 20 μ g/ml. Media was changed every two days and cell number was determined with a coulter counter. Although there appears to be a marginal effect of 225 on HB2 growth, it is not significant; repeat experiments have not been able to reproduce the effect.

Appendix

Figure 13



RT-PCR on RNA samples from HB2 cells grown on Transwells analyzing expression of EGF-R and EGF-R ligands.