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Introduction: Malignant breast cancer appears to develop over time from mammary epithelial cells that have been transformed. These transformed cells form early lesions that are characterized by loss of epithelial architecture and uncontrolled proliferation. Currently, early lesions are defined using histological techniques and the molecular features that distinguish different histological types are not known. Although oncogenes implicated in breast cancer have been described, how they specifically interfere with tissue architecture giving rise to different histopathological patterns remain to be elucidated.

ErbB receptor network and breast cancer: ErbB receptors are a family of receptor tyrosine kinases (RTKs) that are important during normal development and tumorigenesis of the mammary gland. Many biological processes are regulated by ErbB receptors such as cell division, migration, adhesion, differentiation, morphogenesis, and prevention of apoptosis[1]. There are four members in this family, ErbB1 (EGFR, HER1), ErbB2 (HER2/Neu), ErbB3 and ErbB4. Over-expression of ErbB is associated with breast, ovary, brain and prostate cancers. ErbB2 is over-expressed in 25-30% of breast cancers. ErbB2 gene amplification and overexpression also correlates with a poor clinical prognosis[2, 3].

Epithelial cell polarity: Mammary epithelial cells line the ducts of the mammary gland and possess an apical-basal polarity as defined by their ability to localize their tight junctions, gap junctions and adherens junctions, assymetrically along the apical-basal axis. Establishment of apical-basal cell polarity requires the concerted effort of protein complexes and other localized proteins. Recent studies have identified three protein complexes namely, the Par complex, Scribble complex, and the Crumbs complex that act in a cooperative manner to establish apical-basal polarity[4-6]. Recent studies demonstrate that in addition to being part of the Par complex, Par6 also interacts with the crumbs complex and the Scribble complex suggesting that Par6 may play and important role in coordinating the interaction with other polarity complexes [7].

Rho GTPase family and cancer: Rho family members are known to play important roles in regulating epithelial cell polarity for instance, Rac and CDC42 are part of the Par protein complex and play critical roles during establishment of epithelial cell polarity. Rho proteins can cycle between GTP and GDP bound states, which are effected by a variety of different regulator molecules that either promote or inhibit GTP binding. They are categorized into three different subgroups based on similarity to RhoA, Rac1 and Cdc42, and proteins that lack GTPase activity. Rho GTPases are over-expressed in human breast, colon, lung and pancreatic cancers [8]. Specifically RhoA, Rac1 and Cdc42 are over-expressed in breast cancer [9]. Since then, there have been no reports mutated Rho proteins in tumors. The evidence suggests that it is cycling of GDP/GTP that is important for transformation [10, 11]. Other studies show that Rac activity is increased in breast cancer cell lines[8, 12]. The accumulating evidence suggests that it is the deregulation of the proteins that correlates with tumor progression and poor prognosis[8].

Cell culture system: Our lab combines a unique system to study the inducible effects of ErbB receptors with a three-dimensional cell culture system. ErbB receptors have the ability to homo and heterodimerize generating a complex signaling system. Our lab has circumvented this problem by using a chimeric ErbB receptor. The chimera can bind to synthetic ligand, dimerize, phosphorylate, and generate downstream signals. This system allows us to specifically activate the receptor of interest. [13]. We have also set up a culture system for mammary epithelial cells (MECs) on a matrix abundant in collagen and laminin (Matrigel). In this environment cells can grow from a single cell to into 3D acini-like structures with a single layer of epithelial cells surrounding a luminal space. These 3D acini structures have characteristics similar to the resting epithelium of mammary glands, such as low proliferation rate and organized architecture. MEC lines have been established that express chimeric ErbB1 (B1) and ErbB2 (B2) chimeras using a retroviral expression system. We have shown that activation of ErbB2 cells in the 3D system induces multilayering, changes in cell polarity, and re-

initiates proliferation [14]. This resembles the early stages of transformation in vivo and thus is a valuable model to study the important factors in the initiation of the oncogenic process.

Using both the traditional 2D and 3D cell culture approach in combination with ErbB receptor activating systems I propose to investigate how activation of ErbB2 cooperates with regulators of cell polarity and cell architecture. In particular, I am interested in investigating if the Par complex and small Rho GTPases play a role in ErbB2-induced transformation of human mammary epithelial cells.

Body:

To study the role of par complex and Rho GTPase in ErbB2 induced transformation we utilized viral mediated overexpression of wild type and mutant versions of these proteins our ErbB chimera and 3D model systems.

Activation of ErbB2 in conjunction with dominant negative RhoN19 expression in D12 structures: The Rho family of GTPases has been shown to be activated via Ras, which is a primary effector pathway of ErbB2. Activation of ErbB2 re-initiates proliferation and induces disruption of 3D acini-like structures. We have previously observed a cooperative effect of RhoN19 expression and ErbB2 activation on acini morphology (Figure1). When RhoN19 was co-expressed with activated ErbB2, the disrupted acini form protruding blebs (Figure 2). ErbB2 is still able to re-initiate proliferation in the presence of RhoN19 expression (determined by Ki-67 stain) and formation of multistructures is not affected. Since RhoN19 expression does not affect proliferation or multistructure formation we decided to focus on acinar morphology. Normal acini secrete a polarized layer of extracellular matrix (ECM) such as collagen and laminin. In RhoN19 and ErbB2 activated structures the ECM that surrounds the acini is altered. The cooperative effect of RhoN19 and ErbB2 activation disrupts organized laminin (Figure3A and B). Since breakdown of ECM is one characteristic of invasive cells, we utilized an invasion assay where the acini are embedded in a mixture of collagen and matrigel. Our preliminary experiments (data not shown) suggest that these blebbing protrusions have the ability to invade the surrounding matrix. We plan to do RNAi experiments to verify this cooperative phenotype and the study the downstream pathways.

Full-length Par6 expression induced EGF independent growth on 2D: Previously we made a serendipitous observation when Par6 was expressed in MCF-10A cells as a control for our chimeric system. Under normal cell culture conditions MCF-10A cells require EGF to proliferate. We found that Par6 expression allowed cells to proliferate rapidly in EGF free media when compared to control vector, Par6 C-Terminus, and parental MCF-10A cells (Figure 4). We decided to verify this observation by generating multiple populations of various expression levels and by varying the location of the flag tag. In all cases Par6 expression promotes EGF independent proliferation regardless of expression level or location of the epitope tag. Growth factor independence is a hallmark for transformation so we proceeded to characterize how Par6 can play a role in promoting proliferation. We have taken two approaches: Structure function analysis of Par6 and signaling pathway characterization by chemical inhibitors.

Structure function analysis to determine what are the effector proteins among the different binding partners of par6: One readout that we have established is to monitor the cell cycle by S-phase using propidium iodide staining and subsequent flow cytometry. We have determined that par6 expression confers a two-fold increase in cells undergoing s-phase (Figure 5). A series of mutations are being generated to interfere with Par6 and it's effector proteins. Par6 is a major component of the par complex (Par3, aPKC and cdc42). New information suggests that it plays an even more essential role interacting with other components of polarity complexes such s crumbs, pals and lgl proteins. The proposed mutations will affect cdc42 (Δpro136), aPKC (K19A) and Pals/lgl (M235W) binding.

Truncations are also being generated to interfere with the effector pathways (Figure 6). The generation of these mutations will alow us to dissect out which effector pathway of par6 is responsible for EGF independent proliferation and will allow us to design future experiments to verify the effectors involvement. Preliminary data suggests that expression of C-terminus or PDZ domain alone to do not confer EGF independent proliferation. A summary of constructs and subsequent phenotypes are depicted in Table 1.

What signaling pathways are involved in Par6 EGF independent proliferation? We are utilizing chemical inhibitors to help us quickly determine the pathways involved in EGF independent proliferation. We chose commercially available inhibitors of MAPK (U0126), PI3K (LY294002), Src kinase (PP2), JNK (SP600125) and MTOR (rapamycin) signaling pathways. We also chose two receptor kinase inhibitors specific for EGFR (AG-1478) and ErbB2 (AG-825). (Figure 7A, 7B). Inhibition of MAPK and Src abrogated Par6's ability to promote proliferation (Figure 7A). Since both of these pathways are downstream of receptor tyrosine kinase such as EGFR and ErbB2 we decided to do a more detailed inhibition experiment (Figure 7B). We observed that another downstream signal of RTK activity (PI3K) is also required for EGF independent proliferation. Controls, such as rapamycin and JNK II inhibitor were used to help us determine if our inhibition is specific. Only inhibition of EGFR, not ErbB2, abolishes the proliferation phenotype (Figure 7B). From these experiments we hypothesized that EGFR might be activated in these cells. We attempted to determine if some exogenous EGFR ligand was being secreted in an autocrine fashion by exchanging media and co-culture experiments. However, these experiments proved to be inconclusive. Most of our data suggests that there is no secreted factor promoting cell growth. We need to do more sensitive assays for growth factor secretion such as concentrating the media of Par6 cells or performing ELISA assays for EGFR ligands. Since we thought EGFR could be involved we did a time course analysis of EGFR expression and regulation.

EGFR phosphorylation and regulation in Par6 overexpressing cells: Experiments were designed to determine the phosphorylation state of EGFR in Par6 expressing cells. Total phosphorylated tryrosine immunoblot did not produce any consistent result. However, we observed a decrease in expression level of total EGFR in Par6 cells. In order to follow this result a time course experiment was preformed to determine if EGFR was activated in Par6 expressing cells. EGFR activation and internalization was observed growing cells (day3), growth arrested cells (day 4) no longer activate and internalize EGFR and it is expressed in control cells. Expression levels of EGFR in Par6 cells suggest continued internalization or less total expression (Figure 8), reprobe with phosphotyrosine didn't produce any differential phosphorylation (data not shown). We thought we could stabilize EGFR in Par6 cells by using the proteosome inhibitor MG-132. Again we were unable to observe a robust regulation. (Figure 8 B). We are continuing to determine if EGFR is differently phosphorylated by EGFR IP, phosphotyrosine IP and EGFR phosphotyrosine specific antibodies. These experiments are ongoing.

Full-length Par6 expression induced EGF independent growth on 3D: Since Par6 expression confers EGF independent in 2D we decided to culture these cells in our 3D cell culture system. MEC require EGF to develop into polarized growth arrested acini. Par6 overexpressing cells need EGF during the first 4 days of morphogenesis. Once EGF is removed they continue to develop until about day 8, whereas control acini remain at day 4. We assayed acini size using phase contrast imaging and zeiss software to generate the area of an acini (Figure 9). We are in the process of S-phase analysis to determine if the cell cycle profile of control cells differs in par6 expressers. Currently we are characterizing this phenotype and will utilize the same tools that are being used in 2D studies.

Activation of ErbB2 in conjunction with par6 expression in acini structures: We want to determine if there is an interaction between ErbB2 and the par complex. Previously we have determined that overexpression of Par3 in 3D acini results in acid production and change in media color. We also determined that the small GTPase Cdc42 when inhibited or activated disrupts acini structure without ErbB2 activation. Therefore we wanted to determine if overexpression of Par6 in conjunction with ErbB2 activation generates further disruption. We noticed that acini structures that had been activated for 8-16 days (4-8 days after multiacinar formation) had subtle blebbing phenotype that progresses with extended activation of ErbB2. In Figure 10, acini structures have been stimulated for 16 days, blebbing began to appear subtly after 4 days of stimulation. Blebbing was also observed in par6 cells with ErbB2 off. This can be explained by basal activation of ErbB2, since in 10A-Par6 cells no blebbing has been observed even in late day structures. Experiments are ongoing to determine the extent of this cooperation and if this blebbing can also be characterized as invasion by changing the matrices.

Future Directions: The observed Par6 induced EGFF independent growth phenotype may be of great relevance to understand the transformation process in polarized cells such as the mammary gland and thus we plan to focus on the characterization of this pathway. We will continue the structure function analysis to find the regions of par6 required for EGF independent growth. The binding partners of these regions may be good candidates to be downstream transducers of Par6-mediated proliferative signaling and thus will be studied to dissect what cellular pathways par6 uses for EGF independent proliferation. We will continue to explore the possible link between Par6 and the EGFR pathway to determine if Par6 mimics EGF signaling by studying the EGFR status in Par6 overexpressed cells. More detailed studies will be carried our in both 2D and 3D to determine the possible cooperation of par6 with EGRF and ErbB2. We will also study the phenotype of other oncogenes such as myc and src as well as in cooperation with par6. These studies will help answer questions proposed in task 1 and 2.

Protein Expressed	**Successful Virus Generation**	**Phenotypic observation in 2D**	**Phenotypic observation in 3D**	**Activation of ErbB2 phenotype in 3D**
Flag Par6 FL	Yes	EGF independent proliferation	EGF independent proliferation	Not known
Par6 FL Flag	Yes	EGF independent proliferation	EGF independent proliferation	Blebbing in late day structures
FL Par6	No	Not known	Not known	Not known
Flag Par6 C-Terminal	Yes	None observed	None observed	Not known
Flag Par6 N-Terminal	No	Not known	Not known	Not known
Flag Par6 PDZ	Yes	None observed	None observed	Not known
Flag Par6 K19A	Yes	Not known	Not known	Not known
Flag Par6 M235W	No	Not known	Not known	Not known
Flag Par6 ΔPro136	No	Not known	Not known	Not known
Flag FL Par6 β	Yes	Not known	Not known	Not known
Mcy ER	Yes	Not known	Not known	Not known

Table 1. Summary of retroviral constructs generated, phenotypic observations in 3D cell culture system and traditional 2D cell culture system.

Key Research Accomplishments:

- Generation of retrovirus.
    - C-Terminal Flag tag Par6 full length MSCV IRES GFP
    - N-Terminal Flag tag Par6 full length MSCV IRES GFP
    - N-Terminal Flag tag Par6 C-terminal MSCV IRES GFP
    - N-Terminal Flag tag Par6 PDZ domain MSCV IRES GFP
    - N-Terminal Flag tag Par6 K19A MSCV IRES GFP
    - Myc-ER
- Generation of MCF-10A cell lines expressing Par proteins (see below)
- Identified the ability of Par6 to promote EGF independent cell proliferation in 2D
- Identified the ability of Par6 to promote EGF independent cell proliferation in 3D
- Identified cooperation of Par6 and ErbB2 in 3D

Reportable Outcomes:

Academic:

Oral presentation: Cold Spring Harbor Laboratory Student seminar: Biological effects of Par6 over-expression in mammary epithelial cells- Nolan M, Muthuswamy SK, Feb 2005

Stony Brook University Student Seminar: Biological effects of Par6 over-expression in mammary epithelial cells- Nolan M, Muthuswamy SK - Jan 2005

Poster presentation: Stony Brook University Genetic Program Symposium: Biological effects of Par6 over-expression in mammary epithelial cells- Nolan M, Basu S, Muthuswamy SK - January 2005

Era of Hope Department of Defense Breast Cancer research Program Meeting: Biological effects of activating distinct ErbB receptor dimers in polarized growth arrested epithelia Nolan M, Muthuswamy SK- June 2005

Development of reagents and cell lines:

Cell lines:

MCF-10A expressing C-Terminal Flag tag Par6 full length, high/medium/low expression
MCF-10A expressing C-Terminal Flag tag Par6 full length and ErbB2 chimera
MCF-10A expressing N-Terminal Flag tag Par6 full length high/medium/low expression
MCF-10A expressing N-Terminal Flag tag Par6 C-Terminal truncation
MCF-10A expressing N-Terminal Flag tag Par6 C-Terminal truncation and ErbB2 chimera
MCF-10A expressing N-Terminal Flag tag Par6 full length and ErbB2 chimera
MCF-10A expressing N-Terminal Flag tag Par6 PDZ truncation
MCF-10A expressing N-Terminal Flag tag Par6 PDZ truncation and ErbB2 chimera
MCF-10A expressing N-Terminal Flag tag Par6 K19A mutation
MCF-10A expressing N-Terminal Flag tag Par6 K19A mutation and ErbB2 chimera
MCF-10A expressing N-Terminal Flag tag Par6 β
MCF-10A expressing Mcy-ER

Conclusions:

Previously we have determined that ErbB2 activation re-initiates proliferation and disrupts cell polarity. We nobserved a cooperation between ErbB2 and dominant negative Rho, which further disrupts 3D acinar architecture. Second, overexpression of Par6 in mammary epithelial cells confers EGF independent proliferation in both 2D and 3D cell culture systems. Further characterization of Par6 expression suggests EGFR signaling involvement. Par6 expression and ErbB2 activation also cooperate to further disrupt acini architecture. The generation of these reagents and cell lines will help us determine how Erbb2 transforms mammary epithelial cell acini structures. These studies may identify a novel signaling pathway by which oncogenes regulate cell polarity.

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Appendix:

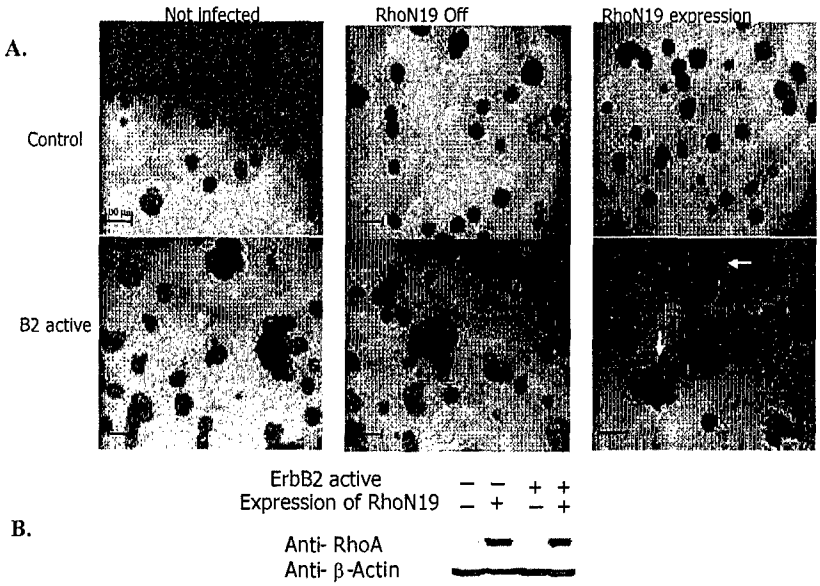

Figure 1. A) Typical presentation of multi-structure phenotype in dimerized ErbB2 without RhoN19 expression (Bottom,left panel). "Blebs" of cells extending from the main body are observed upon expression of RhoN19 RhoN19 expression and ErbB2 activation cooperate to further disrupt acini architecture. B) RhoN19 expression in 3D acini lysate.

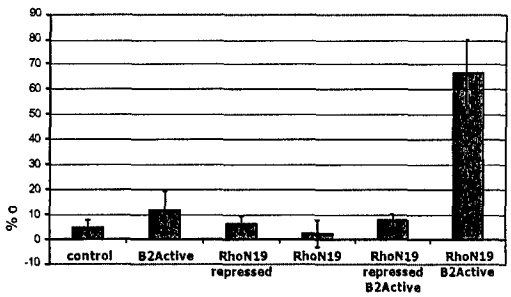

Figure 2. Quantification of dimerized ErbB2 and RhoN19 induced blebbing phenotype in mammary epithelial cells. Neither stimulus alone is sufficient to cause the majority of structures to have the blebbing phenotype.

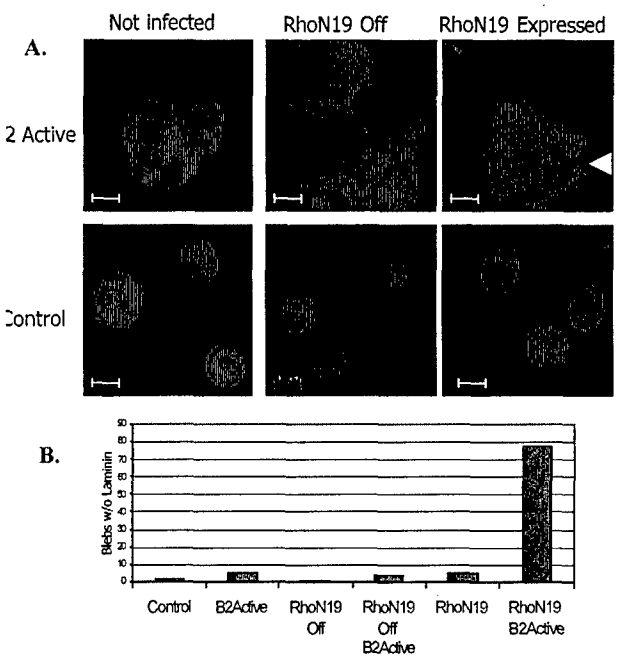

Figure3. Acini expressing RhoN19 with activated ErbB2 lack organized laminin A) Immunofluorecent staining of acini with DAPI (blue) and laminin (red). There is a lack of a laminin border around acini upon ErbB2 activation and RhoN19 expression. . B) Quantification of "blebbing" phenotype without organized laminin under ErbB2 dimerization and RhoN19 expression conditions.

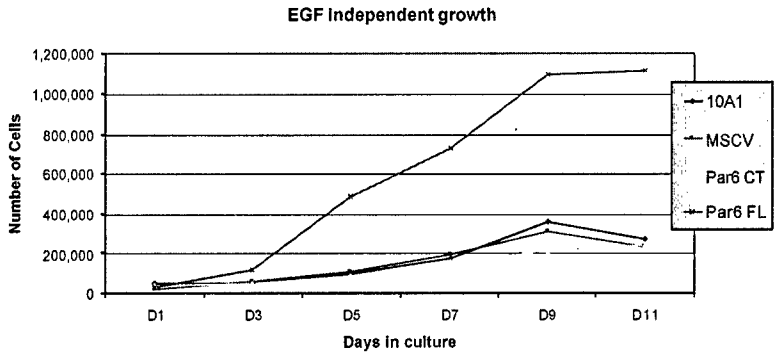

Figure 4. EGF independent growth of MCF-10A cells expressing Par6 compared to parental, MSCV control and Par6 C-Term.

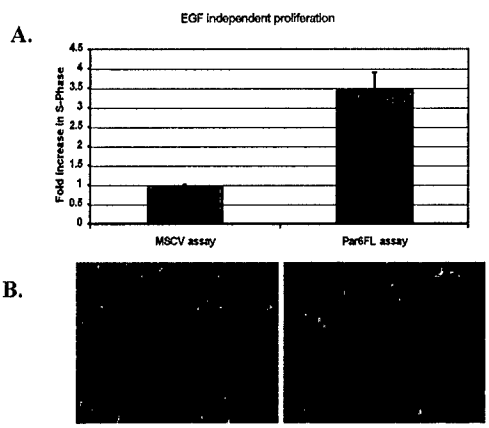

Figure 5. EGF independent proliferation. A) S-phase analysis, by flow cytometry shows over 2-fold increase in S-phase. B) Phase contrast image of control vs Par6 expressing cells in EGF free media

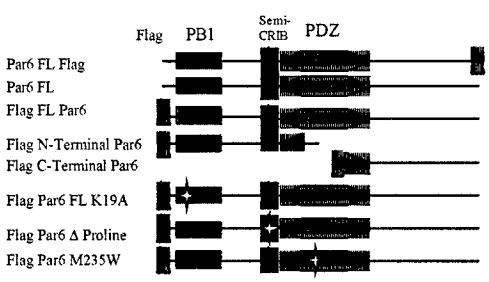

Figure 6. Par6α Constructs in MSCV IRES GFP

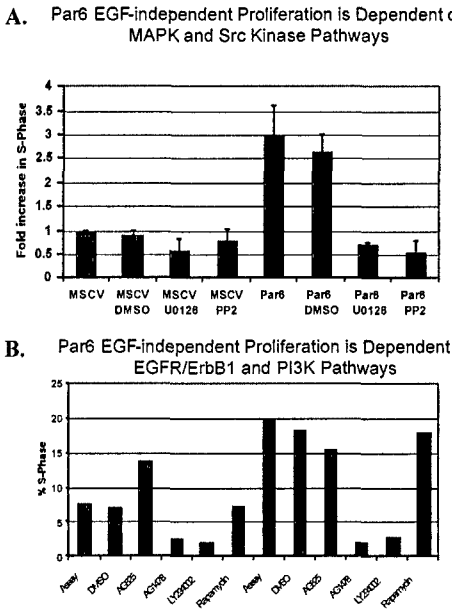

Figure 7. A) Inhibition of Src, MAPK and B) PI3K signal transduction pathways prevents EGF independent proliferation in Par6 cells. B) Inhibition EGFR kinase activity inhibits proliferation but inhibition of ErbB2 and MTOR doesn't affect increase in S-phase.

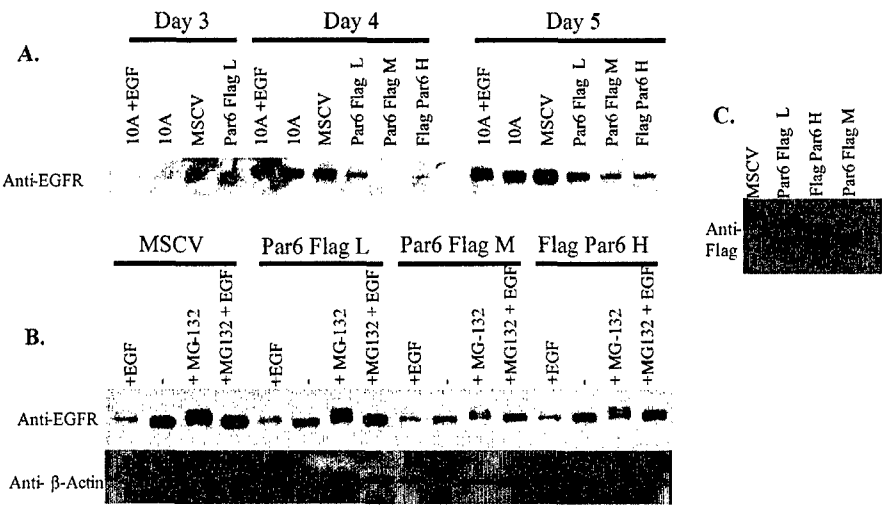

Figure 8. A). Time course showing downregulated EGFR in par6 overexpressing lysates, suggesting EGFR turnover similar to EGF stimulation. B) Proteosome inhibitor experiment showing possible stabilization of EGFR in low expressor. C) Par6 Flag low, medium and Flag Par6 high expression.

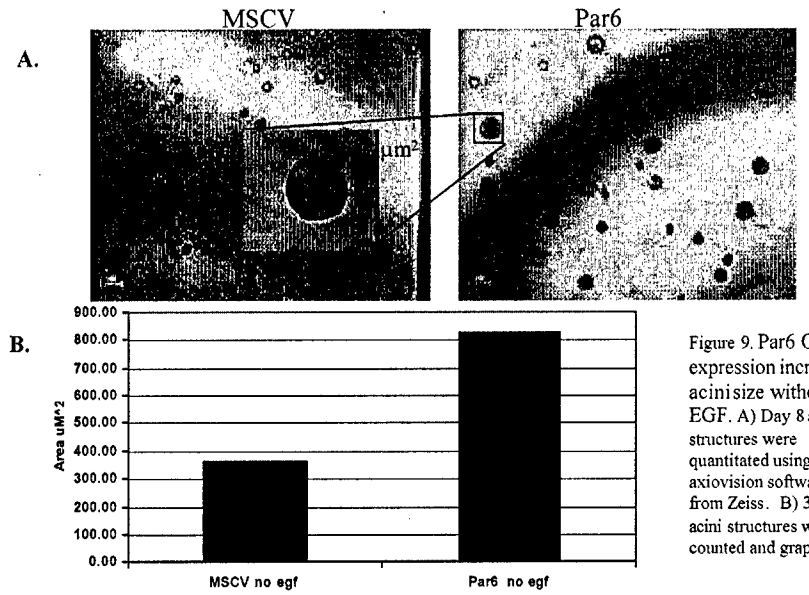

Figure 9. Par6 Over-expression increases acini size without EGF. A) Day 8 acini structures were quantitated using axiovision software from Zeiss. B) 300 acini structures were counted and graphed.

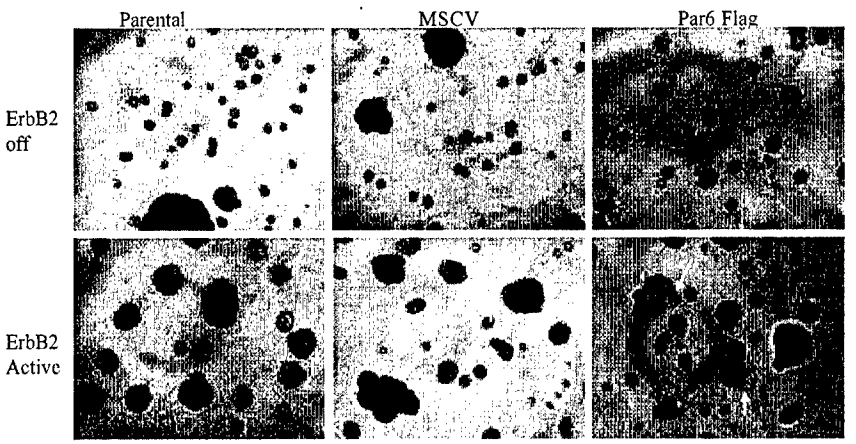

Figure 10. ErbB2 acini structures were activated from day 4 and imaged on day 20. Blebs started to subtly appear in Par6 overexpressing cells by day 8 and became more pronounced throughout the experiment. Other experiments were performed with stimulation on day 8 and day 12, blebs also appeared after 8 days of stimulation in Par6 expressing cells.