Loss of tissue polarity and increased proliferation are the characteristic alterations of the breast tumor phenotype. To investigate these processes, we have used a three-dimensional (3D) culture system in which malignant human breast cells can be reverted to a normal phenotype. Exposure to inhibitors of phosphatidylinositol 3-kinase (PI3K) leads to decreased proliferation and restored tissue polarity. We show that Akt and Rac1 act as downstream effectors of PI3K and function as control points of cellular proliferation and tissue polarity, respectively. Expression of active Akt causes increased proliferation without affecting basal tissue polarity, whereas active Rac1 prevents the restoration of tissue polarity but does not affect proliferation; when activated in combination, these two signaling effectors are sufficient to prevent restoration of normal phenotype. Our results also show that the entire PI3K signaling pathway is an integral component of the overall signaling network induced by growth in 3D, as reversion effected by inhibition of PI3K signaling also down-modulates the endogenous levels of upstream modulators of PI3K, β1 integrin and EGFR, and upregulates the antagonist of PI3K, PTEN. Our results reveal key events downstream of PI3K that act synergistically to maintain tissue polarity and that when disrupted produce the malignant phenotype.
INTRODUCTION

Epithelial cancers are caused by multiple genetic lesions that eventually lead to both aberrant proliferation/apoptosis and loss of polarity. Although a variety of oncogenes, especially oncogenic kinases have been shown to transform normal cells in vitro and to result in tumor formation in vivo, the complexity of their signaling networks implicated that their downstream effectors could be subgrouped into functionally distinct branches at certain level, which might controls various activities, such as proliferation and polarity respectively. By using a 3 dimensional rBM culture system and human mammary epithelial cell progression series, we previously showed that polarity could be restored and proliferation reduced in human mammary epithelial cancer cells when PI3 kinase (PI3K) overactivation was diminished. In order to further dissect the molecular mechanisms of the reversion and to distinguish if proliferation and polarity are two separable events downstream of PI3K signaling pathways that eventually integrate and synergistically contribute to tumor development, the activities of effectors, such as Akt and Racl that regulate proliferation and polarity, were genetically manipulated and their effects on reversion phenotypes were tested in 3D rBM cell culture system.

BODY

In my proposal, we have shown that PI3K activity was up-regulated in T4-2 cells comparing with nonmalignant S1 cells, and that down-modulated PI3K signaling by the treatment of specific PI3K inhibitors LY294002 or wortmannin is sufficient to reduce both cell proliferation and anchorage-independent growth as expected. Interestingly, the apicobasal tissue polarity was also restored in the context of 3D ECM. In addition, polarized, basolateral localization of PI3K and its phospholipid product PI3P was re-established in the reverted spheres. The evidence that inhibition of PI3K can effect cross-modulation of a number of distinct signaling pathways is a demonstration that pathways downstream of PI3K are integrated into transduction networks when cells are grown in the physiological 3D rBM; consistent with this model, we found that reversion of the tumor cells to a normal phenotype was associated with down-modulation of EGFR and β1 integrin and increased expression of PTEN, the PI3K antagonist (Figure 1A, B). These observations suggest that dysregulated PI3K activity has profound effects
on both proliferation and polarity. We tried to determine if tumorigenic T4-2 cells could be also reverted if the expression of PTEN, a phospholipids phosphatase and antagonist of PI3K signaling (Vazquez and Sellers, 2000), was up-regulated. However, we could not obtain stable population because cells died once they overexpressed PTEN-GFP construct.

The primary consequence of PI3K activation is the generation of PIP3 in the membrane, which functions as a second messenger to activate downstream pathways that involve Akt and other proteins such as SGK, RSK, atypical PKC, and Rac1 (Vivanco and Sawyers, 2002). Akt has been shown to be one of the most important effectors that mediates PI3K’s effects on cellular proliferation and apoptosis (Scheid and Woodgett, 2001). PI3K can also affect Rac1 activity by regulating the activity of its GTP/GDP exchange factors (GEF) such as Tiam1 and Vav1 (Vanhaesebroeck et al., 2001). Rac1 is one of major components of an evolutionarily conservative protein complex (PAR3/PAR3/aPKC) that plays an important role in tight junction formation and regulation of polarity development (Kim, 2000). Dysregulated Rac1 activity leads to a loss of polarity owing to the failure to deposit the ECM component laminin asymmetrically (O’Brien et al., 2001). In addition, an alternative splice variant of Rac1 (Raclb) with increased GTP/GDP exchange rate is highly expressed in breast and colon carcinoma (Schmelzer et al., 2000).

As the effectors downstream of PI3K could regulate both proliferation and probably cell polarity, we hypothesized that the signaling pathways of PI3K might bifurcate into function-specific signaling groups. In order to address the question if proliferation and polarity are two distinct phenotypes controlled by different pathways downstream PI3K, we established stable populations of T4 cells overexpressing myrisolated Akt (active Akt), Rac1 L61, Rac1 N17, and both constructs (active Akt+Racl L61 or active Akt+Racl N17) and determined their effects on the reversion phenotypes mediated by PI3K-specific inhibitors, such as LY294002 or wortmannin, in 3D rBM culture system. We also tried to knock down endogenous Akt by RNAi or use adenoviral constructs of
Racl V12 and N17. However, the cells could not survive or die as the result of cytotoxic effects of adenovirus.

We observed that active Akt endowed tumorigenic T4-2 cells with greater resistance to proliferation reduction induced by PI3K down-modulation in 3D rBM assay. Intriguingly, active Akt did not prevent the re-establishment of basal tissue polarity (Figure 2) although it drastically augmented cell's ability to grow in anchorage-independent assays (Figure 3). Our results are consistent with another recent study in that active Akt, when overexpressed, increased the size and cell number of the cysts formed by MCF-10A cells, a transformed, nonmalignant mammary epithelial cell line, but their basal polarity was maintained in 3D rBM assay (Debnath et al., 2003). All these observations provide strong evidences suggesting that alteration of proliferation through Akt pathway is not tied to polarity control and that PI3K signaling pathways regulating proliferation and polarity might branch off at the level of Akt, implying that another pathway(s) parallel to Akt but downstream of PI3K might exist to control polarity.

Now came the question as to which effector downstream PI3K controlled polarity. We found that GTP-loaded Rac1 level was substantially higher in malignant T4-2 cells than their nonmalignant counterpart S1 cells and did decrease when PI3K activity was down-modulated (Figure 4) and that in contrast to the cells expressing active Akt mutant, normalization of PI3K activity could substantially reduce cell proliferation to the level as treated vector control or parental cells, however, basal tissue polarity was totally lost if the decreased level of Rac1 activity was prevented by expressing constitutively active Rac1 (Figure 2). Furthermore, if the cells were provided with the potentials of increased proliferation and loss of polarity by coexpressing both active Akt and Rac1, PI3K inhibition could no longer revert tumor phenotypes, which cell proliferation became insensitive to the treatment and basal tissue polarity could not be restored (Figure 2). These observations have three significant implications. First, Rac1 acts as one of the mediators downstream of dysregulated PI3K signaling to mediate the loss of tissue polarity...
without significant effect on cell proliferation and this pathway of polarity regulation is separated from the pathway that controls cell proliferation; second, aberrant proliferation (through increased Akt activity) and loss of polarity (via up-regulated Rac1 activity) constitute the minimal signaling inputs required from PI3K to cause tumor phenotypes; third, disruption of polarity is one of the major requirements or enhancers for epithelial tumorigenesis once cell proliferation becomes uncontrollable. To further test this model, we injected those transfectants (vector control, active Akt, Rac1 L61, Rac1 N17, active Akt+Rac1 L61, and active Akt+Rac1 N17) into nude mice (n=6 for each group) and observed the tumor growth in vivo. We found that active Akt but not Rac1 L61 greatly increased tumor size and that double transfectants of active Akt and Rac1 L61 synergistically led to much larger tumor than active Akt alone (Figure 5).

Our results suggest a model for the mechanisms of PI3K-induced tumorigenesis in at least mammary epithelial cancers. PI3K acts as one of the signaling nodes to be activated by and relay the signals from overactivated cell surface receptor such as receptor/protein tyrosine kinases and integrins. The signals from PI3K then bifurcate into function-specific and probably mutually independent pathways: proliferation and polarity controls. Activation of cell cycle-promoting or anti-apoptosis pathway(s) such as Akt or SGK increase cell proliferation, whereas, dysregulated polarity pathway(s) such as Rac1 interrupt tissue polarity or architecture. The convergence of the two phenotypical effects induced by those different pathways synergistically and ultimately leads to tumor development. Whether or not this model could be generalized to other oncogenes or multiply functioning kinases remains to be tested.

Figure 5. Myr-Akt and Rac1 L61 double transfectants synergistically promote tumor formation in nude mice. Transfectants of T4-2 cells were injected subcutaneously into rear flanks of nude mice and tumor growth were monitored twice a week by measuring tumor size (L×W×H). *, Akt+Rac1 L61 vs Akt alone or Akt+Rac1 N17 (p<0.02); #, Akt alone, Akt+Rac1 L61, or Akt+Rac1 N17 vs. Rac1 L61, Rac1 N17, or vector control (p<0.01).

KEY RESEARCH ACCOMPLISHMENT

1. Subclone Rac1 mutants (constitutively active L61 and dominant negative N17) into retroviral vector (pLXSN).
2. Establishment of stable populations of cells expressing Rac1 L61, Rac1 N17, myrisolated Akt (Myr-Akt), and both Myr-Akt and Rac1 mutants.

3. Determine the role of active Rac1 signaling in the prevention of polarity restoration in PI3K attenuation-mediated phenotypical reversion in 3D rBM assay without effect on cell proliferation.

4. Determine the role of active Akt in the partial prevention of the reduction of cell proliferation in PI3K attenuation-mediated reversion in 3D rBM assay without effect on polarity restoration.

5. Determine that loss of the control of both polarity (by overactive Rac1) and proliferation (by overactive Akt) is sufficient to totally prevent the phenotypical reversion by PI3K down-modulation in 3D rBM.

6. Determine that active Akt signaling endows the cells with much greater ability to grow in anchorage-independent assay and that double transfectants of active Akt and active Rac1 but not inactive mutant synergistically promotes the growth in the same assay.

7. Accomplish in vivo experiments in mice to confirm the observations in vitro.

REPORTABLE OUTCOMES

Part of the results was summarized and published in the Journal of Cell Biology on February 16, 2004 (see the original copy on APPENDICES).

CONCLUSIONS

In conclusion, we have used the 3D rBM assay and genetic manipulation of Akt and Rac1 activity to determine the role of PI3K signaling in the tumorigenic phenotype, signaling reorganization, and tissue polarity of mammary epithelial cells. Our elucidation that the polarity and proliferation are controlled independently by separable events downstream of PI3K sheds light on the molecular mechanisms by which increased proliferation and loss of tissue polarity act as function-specific pathways to affect various cell phenotypes even under one overactive kinase signaling pathway and the aberration of both phenotypes are required to produce the malignant phenotype, which might be generalized to other signaling pathways or models to dissect the process of tumor development.


APPENDICES

2. Curriculum vitae of Hong Liu.
Polarity and proliferation are controlled by distinct signaling pathways downstream of PI3-kinase in breast epithelial tumor cells

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Loss of tissue polarity and increased proliferation are the characteristic alterations of the breast tumor phenotype. To investigate these processes, we used a three-dimensional (3D) culture system in which malignant human breast cells can be reverted to a normal phenotype by exposure to inhibitors of phosphatidylinositol 3-kinase (PI3K). Using this assay, we find that Akt and Rac1 act as downstream effectors of PI3K and function as control points of cellular proliferation and tissue polarity, respectively. Our results also demonstrate that the PI3K signaling pathway is an integral component of the overall signaling network induced by growth in 3D, as reversion affected by inhibition of PI3K signaling also down-modulates the endogenous levels of β1 integrin and epidermal growth factor receptor, the upstream modulators of PI3K, and up-regulates PTEN, the antagonist of PI3K. These findings reveal key events of the PI3K pathway that play distinct roles to maintain tissue polarity and that when disrupted are instrumental in the malignant phenotype.

Introduction

Class I phosphatidylinositol 3-kinase (PI3K) is activated by growth factor-responsive tyrosine kinases such as epidermal growth factor receptor (EGFR; Grant et al., 2002) and integrin-responsive kinases such as focal adhesion kinase (FAK; Chen and Guan, 1994). Activated PI3K leads to the production of membrane-associated phosphatidylinositol 3,4,5-trisphosphate (PIP3), which in turn causes the recruitment to the cell membrane and subsequent activation of a number of signaling molecules (Vivanco and Sawyers, 2002). PI3K is a key mediator in processes that regulate cell orientation; for both *Dictyostelium* and cultured human leukocytes, directionality of chemotaxis is controlled by polarization of PIP3 to the leading edge of the cell (Servant et al., 2000; Funamoto et al., 2002; Wang et al., 2002b). PI3K has been found to be constitutively up-regulated in a substantial fraction of human breast cancers (Vivanco and Sawyers, 2002), and overexpression of PI3K in cultured nonmalignant human mammary epithelial cells is sufficient to confer a malignant phenotype (Zhao et al., 2003).

During tumor progression, tissue polarity is lost and control of proliferation is compromised (Fish and Molitoris, 1994; Reichmann, 1994; Bissell and Radisky, 2001), and although these two phenomena have been suggested to be linked, previous investigations have not revealed the extent to which the increased cellular proliferation in tumors can directly produce tissue disorganization, and to what extent loss of polarity is an independent function of deregulated signaling pathways downstream of the oncogenic signal(s). To dissect the molecular mediators of these processes we have used an assay (Petersen et al., 1992) in which human mammary epithelial cells from the HMT-3522 tumor progression series are cultured in a physiologically relevant, three-dimensional (3D) laminin-rich basement membrane (IrBM). When cultured in 3D IrBM, the phenotypically normal, nonmalignant HMT-3522 S-1 (S-1) cells undergo growth arrest, produce an endogenous basement membrane, and form polarized acinus-like structures, very similar to primary cells from reduction mammoplasty. In contrast, the
malignant HMT-3522 T4-2 (T4-2) cells continue to proliferate into apolar, amorphous structures, similar to structures formed by primary tumor cells in this assay (Petersen et al., 1992). In comparison to S-1 cells, expression levels of EGFR and β1 integrin in T4-2 cells are greatly increased, and down-regulation of these signaling pathways in T4-2 cells grown in 3D IrBM can restore the formation of polarized acinus-like structures, resulting in a reversion similar to the normal phenotype of the S-1 cells (Weaver et al., 1997; Wang et al., 1998).

As PI3K is activated downstream of both EGFR and β1 integrin (Chen and Guan, 1994; Lee and Juliano, 2000; Grant et al., 2002), we hypothesized that the phenotypic reversion affected by down-modulation of EGFR/β1 integrin signaling in T4-2 cells was due to attenuation of PI3K activity. We showed previously that even highly malignant metastatic cancer cells, cultured in 3D IrBM, could be reverted to a normal phenotype by inhibition of PI3K, if treatment with PI3K inhibitors was performed in combination with appropriate manipulation of other signaling pathways (Wang et al., 2002a). Here we use inhibition of PI3K alone to dissect the signaling pathways that control proliferation and polarity in breast tumor cells. Our results reveal a new functional link between extracellular signaling mediators and tissue function that provides insight into processes that control the malignant phenotype if imbalanced. We also show that the PI3K and its lipid product, PIP3, are relocalized to the basal surface of the acini when the malignant cells are reverted in IrBM, a process that may play a role in integration of signaling pathways in reformation of polarity.

Results

Down-modulation of PI3K activity results in phenotypic reversion of human mammary tumor cells

We found previously that malignant T4-2 cells showed increased signaling from EGFR and β1 integrin, relative to their nonmalignant forerunners, and that down-modulation of either EGFR or β1 integrin activity in cells grown in 3D IrBM caused the cells to form growth-arrested, polarized acinus-like structures (Weaver et al., 1997; Wang et al., 1998). As PI3K is an effector of both of these signaling pathways (Chen and Guan, 1994; Lee and Juliano, 2000; Grant et al., 2002), we hypothesized that inhibition of PI3K signaling would also revert these cells. We found that treatment of T4-2 cells with 8 μM of the PI3K inhibitor LY294002 (which prevented phosphorylation of downstream Akt at serine 473, as well as other downstream signaling mediators glycosyn kinase kinase-3β [GSK-3β] and p70S6K; Fig. 1 B) did cause phenotypic reversion, as characterized by inhibition of proliferation, decreased colony size, and reduced growth in soft agar cultures (Fig. 1, A and C). Using indirect immunofluorescence, we found that the LY294002-reverted T4-2 cells regained the polarization of the apicolateral tight junction marker ZO-1, the basal marker α6 integrin, and the reorganization of the actin cytoskeleton (Fig. 2 A). Similar data were obtained in cells treated with the alternative PI3K inhibitor, wortmannin (unpublished data). These results demonstrate that down-regulation of the PI3K pathway in T4-2 mammary tumor cells restores an intrinsic property of forming polarized, growth-arrested structures in response to a physiologically relevant microenvironment.

Phenotypic reversion is accompanied by repolarization of PI3K and its phospholipid product

Proper interpretation of extracellular signaling cues requires asymmetric distribution of intracellular signaling molecules (Comer and Parent, 2002; Wedlich-Soldner and Li, 2003). Recently, signaling asymmetry of PI3K and its lipid product, PIP3 has been shown to control the directionality of chemotactic migration in human neutrophils and in single Dictyostelium cells (Servant et al., 2000; Funamoto et al., 2002; Wang et al., 2002b), and PIP3 has been found to become polarized to the basal surface of MDCK cells grown as monolayers on filters or in 3D collagen gels (Waterton and Downward, 1999; Yu et al., 2003). We found that both PI3K (p85 subunit) and PIP3 are polarized to the basal surface of phenotypically normal S-1 cells grown in 3D IrBM, and that this asymmetric distribution is lost in T4-2 cells (Fig. 2 B). However, phenotypic reversion of the T4-2 cells through attenuation of PI3K signaling led to repolarization of these signaling components (Fig. 2 B). Given that the S-1 and reverted T4-2 cells show correct tissue polarity, whereas the untreated T4-2 cells are apolar, these results provide the
first evidence that polarized distribution of PI3K and PIP3 is an intrinsic property of phenotypically normal acini that is lost during tumor progression.

Phenotypic reversion of T4-2 cells by treatment with LY294002 results in cross-modulation of multiple signaling pathways

We had shown previously that inhibition of either EGFR or β1-integrin results in phenotypic reversion of T4-2 cells associated with down-modulation of the total levels of both signaling molecules, and that this activity influences and is influenced by the MAPK signaling pathway (Wang et al., 1998). We now show that PI3K signaling is also an integral component of this cross-modulated signaling network. T4-2 cells treated with LY294002 show reduced levels of EGFR and β1 integrin (Fig. 3 A). This effect depended upon 3D IrBM as it is not observed in cells cultured on two-dimensional (2D) plastic substrata (it should be noted that inhibition of PI3K activity, as measured by activation of downstream mediators Akt and GSK-3β, was equally effective in cells on 2D or in 3D; Fig. 3 A). In addition, our results revealed that PTEN, the antagonist of PI3K that acts to de-phosphorylate PIP3 and which becomes down-regulated in many carcinomas (Simpson and Parsons, 2001; Yamada and Araki, 2001), is also a component of the cross-modulated signaling network, as treatment of T4-2 cells with LY294002 resulted in an increase of PTEN to the level of the nonmalignant cells; this modulation, too, was seen only in cells cultured on 3D IrBM (Fig. 3 B). Taken together, these results demonstrate the existence of a retrodirectional control network that exists only when cells are cultured in a proper tissue context.

Increased proliferation and loss of tissue polarity are functionally separable consequences of increased PI3K signaling

PI3K has been found to control a wide variety of downstream signal transduction pathways, the number and composition of which vary according to cell and tissue type (Chan et al., 1999; Vanhaesebroeck et al., 2001). The best-studied effector of PI3K is Akt, a regulator of cellular proliferation and apoptosis (Scheid and Woodgett, 2001). We examined the
A 3D 2D
despite their considerably larger size (Fig. 4, E and F). This tural repolarization and reversion to a nonmalignant pheno-
of colony polarity revealed that the LY294002-treated T4- phenotype. We find that down-modulation of P13K due to reduction of Akt activity by expressing a dominant ac-
does not change under these conditions (Weaver et al., 1997).

Discussion

A number of studies have shown that the P13K signaling pathway becomes dysregulated in many types of carcinoma (Vivanco and Sawyers, 2002). The results presented here define mechanisms by which the high activity of P13K in malignant T4-2 human breast cells contributes to their malignant phenotype. We find that down-modulation of P13K activity in the T4-2 cells grown in 3D IrBM causes structural repolarization and reversion to a nonmalignant phenotype (Fig. 1) similar to the effects observed previously from inhibition of EGFR and β1 integrin (Weaver et al., 1997; Wang et al., 1998), and we additionally show that both the normal and the normalized reverted acinus-like structures had basal polarization of P13K and its lipid signaling product, PIP3 (Fig. 2 B). The evidence that inhibition of P13K can affect cross-modulation of a number of distinct signaling pathways is a demonstration that pathways downstream of P13K are integrated into transduction networks when cells are grown in the physiological 3D IrBM; consistent with this model, we found that reversion of the tumor cells to a normal phenotype was associated with increased expression of PTEN, the P13K antagonist (Fig. 3 B). Looking for the signaling effectors that controlled the increased proliferation and decreased polarity downstream of P13K in the T4-2 cells led to identification of Akt as a mediator of increased proliferation (Fig. 4) and Rac1 as an inhibitor of polarization (Fig. 5), and we found that these two genes, when expressed in combination, were sufficient to overcome the inhibition of

possibility that the P13K inhibitor-mediated reversion was due to reduction of Akt activity by expressing a dominant active Akt construct (Myr-Akt) in the T4-2 cells (Fig. 4 A). Expression of this construct blocked the effect of P13K inhibitor on phosphorylation of Akt and downstream mediators of Akt activity (Fig. 4 B), and substantially increased the proliferation of T4-2 cells (Fig. 4, C and D). However, examination of colony polarity revealed that the LY294002-treated T4- Myr-Akt cell spheroids largely retained basal tissue polarity despite their considerably larger size (Fig. 4, E and F). This result revealed that increased proliferation alone was not sufficient to disrupt tissue polarity in the reverted T4-2 cells (which retain all of their phenotype-altering genetic mutations), and suggested that other effectors of P13K might be responsible for disruption of tissue polarity.

Recent investigations of acinus-like cysts of MDCK cells grown in 3D collagen gels have demonstrated a role for Rac1 in control of cellular polarity (O'Brien et al., 2001). When we examined the activity of Rac1 in S1, T4-2, and T4-2+LY grown in 3D IrBM or on 2D plastic substrata for 10 d were analyzed for expression of (A) EGFR, β1 integrin, phosphorylated Akt (serine 473)/total, phosphorylated GSK-3β (serine 9)/
total, and (B) PTEN (n = 3); E-cadherin was used as the loading con- control. It was shown previously that the total level of E-cadherin does not change under these conditions (Weaver et al., 1997).

this construct in the target cell population (assessed by immuno- fluorescence; unpublished data). As a complementary method to validate this result, we also found that infection with an adenovirus containing Rac1V12, which was expressed in a much higher proportion of the cells, showed much greater resistance to LY294002-mediated reversion of polarity (Fig. S1 C, available at http://www.jcb.org/cgi/content/full/jcb.200306090/DC1).

These results demonstrated that the increased P13K signal- in T4-2 cells, relative to the same cells treated with LY294002 or to the nonmalignant S-1 cells, leads to activation of both Rac1 and Akt, and that these effectors signal to two functionally distinct phenotypes: activation of Rac1 causes the loss of cellular polarity, and activation of Akt causes increased proliferation. To determine whether together these two effectors were sufficient to recapitulate the effects of P13K, T4-2 cells were infected with both constructs (Fig. 6 A). Colonies derived from cells transfected with these constructs were considerably larger than from control vector-infected cells. Treatment with P13K inhibitor reduced the increased proliferation only to the level of the malignant vector-transfected cells (Fig. 6, B and C), but it had no repolarizing effect (Fig. 6 D). To determine whether these effects were also manifested in a different surrogate tu-
mor malignancy assay, the four cell types (T4-2, T4+Myr- Akt, T4+Rac1L61, and T4+MyrAkt+Rac1L61) were cultured in 3D methylcellulose in the absence or presence of LY294002 (Fig. 6 E, −LY and +LY). In this assay as well, we found that combined expression of both constructs completely abrogated the effects of LY294002 treatment.
PI3K (Fig. 6). These results suggest a model in which the key aspects of the early malignant phenotype, growth and disorganization, can be controlled through disruption of signaling pathways that become interconnected and integrated in 3D IrBM (Fig. 7).

It is an important consideration, however, that the progressively increased proliferation and disorganization that typify acquisition of malignancy in mammary epithelial cells is due to more than just PI3K-dependent activation of Rac1 and Akt (although combined activation of these two pathways has been shown to be sufficient to confer malignancy in an experimental model of premalignant human mammary epithelial cells; Zhao et al., 2003). During tumor progression there are many genetic and epigenetic alterations that altogether contribute to produce malignancy; for the progression of S-1 to T4-2 cells, these include induction of many pathways likely not directly related to PI3K signaling. Reversion of the T4-2 tumor cells grown in 3D suppresses most (if not all) of these other pathways, reducing the proliferation and restoring polarity, but does not change the fact that the reverted cells still retain all of the same genetic alterations and abnormalities. Thus, reversion (whether by LY294002 or by any other T4-2 reversion reagent) results in a cell system that is genetically malignant but biochemically normal, and the consequences of manipulating a single signaling pathway in reverted cells provides information directly relevant to basic characteristics of malignant cell behavior. Using this model, we now show how the altered signaling pathways in T4-2 tumor cells may contribute to the tumor phenotype, how the functions of master regulators (such as PI3K) may be dissected in terms of downstream signaling pathways (in this case, Rac1 and Akt), and how manipulation of these pathways can cause the tumor cells to adopt a normal phenotype.

Normalization of signaling pathways in T4-2 cells in response to inhibition of PI3K is dependent upon culture in 3D IrBM, as T4-2 cells grown on 2D tissue culture plastic do not show the dramatic downmodulation of β1 integrin and EGFR (Fig. 3 A), up-regulation of PTEN (Fig. 3 B), or the alterations in cellular morphology in response to treatment with inhibitors of PI3K (Figs. 2 and 3). Also, for T4-2 cells grown in 3D IrBM, the reduction in PI3K signaling is paralleled by a reorganization of signaling orientation, as both PI3K and its phospholipid product, PIP3, became repolarized to the basolateral surface of the reorganized T4-2 cell.
structures (Fig. 2). This basolateral distribution of PI3K and PIP3 might indeed reflect the localization of active cell surface receptors, e.g., integrins and receptor tyrosine kinases, many of which have particular functions when localized to the basal or basolateral surfaces (Playford et al., 1996; Weaver et al., 1997; Vermeert et al., 2003). Basolateral polarization of PI3K has been suggested to be a critical determinant of differentiated tissue behavior in polarized MDCK cells grown as monolayers on filters (Watton and Downward, 1999) or as cysts in 3D collagen gels (Yu et al., 2003), and PIP3 becomes apolarly distributed in the plasma membrane during branching morphogenesis (Yu et al., 2003), a process believed to involve the transitory dedifferentiation to a migratory and invasive state that is highly reminiscent of the malignant phenotype. PI3K signaling polarization is also an essential component of chemotactic migration in neutrophils and Dictyostelium (Servant et al., 2000; Funamoto et al., 2002; Wang et al., 2002b), and the directionality of neuronal axon growth is controlled by spatially localized PI3K activity (Shi et al., 2003). Our observations in mammary epithelial cells do not reveal the extent to which the polarized distribution of PI3K and PIP3 causes, or is the consequence of, tissue polarity, but previous observations with these cells in 3D IrBM and with MDCK cysts have suggested that formation of cell–cell contacts is an essential component of ECM-induced cell polarity (Weaver et al., 1997, 2002; Yeaman et al., 1999). If so, then formation of tight junctions at points of cell–cell contact may provide boundaries for localization of PI3K and other signaling effectors such as integrins and growth factors that then provide the polarizing principle. These possibilities are under investigation.

We have found that the 3D presentation of IrBM is essential for coupling the expression levels and activity of EGFR and β1 integrin in cultured mammary epithelial cells (Wang et al., 1998), and evidence in other systems also implicates reorganization of signaling pathways in cells cultured in 3D IrBM (Cukierman et al., 2001, 2002; Muthuswamy et al., 2001). We now show that components of the PI3K signaling pathway are involved in this cross-modulation process, as phenotypic reversion by inhibition of PI3K is associated with, and presumably, supported by, up-regulation of the PI3K antagonist, PTEN (Fig. 3). This also requires the establishment of organized structures in 3D IrBM, as treatment of T4-2 cells with PI3K inhibitors does not result in up-regulation of PTEN when cells are grown on 2D plastic substrata (Fig. 3B). Given that the signaling reorganization associated with reversion of T4-2 cells is associated with global repolarization of signaling molecules, we suggest that di-
The roles of Akt and Rac1 in tumor phenotype | Liu et al. 609

Simultaneous expression of both Akt and Rac1 is sufficient to prevent reversion induced by attenuation of PI3K activity. (A) Expression of both constitutively active Akt (Myr-Akt) and Rac1 (Racl L61) in T4-2 cells was detected by probing Western blots of cell lysates with anti-HA antibody. (B) PI3K inhibition fails to decrease proliferation and to restore tissue organization in Myr-Akt and Rac1 L61-double transfectants, as assessed by phase contrast microscopy and (C) by increased nuclei per spheroid cross section (mean ± SD, P values calculated using Student’s t test; more than 300 colonies were analyzed for each condition from three independent experiments). Bar in B, 20 μm. (D) Restoration of basal tissue polarity is disrupted in double transfectants, as assessed by aberrant location of α6 integrin in LY294002-treated T4-2 Myr-Akt+Racl L61 cells grown in 3D IrBM for 10 d. Representative images from three independent experiments are shown. Bar, 10 μm. (E) Myr-Akt and Rac1 L61 double transfectants collaborate to increase anchorage-independent cell growth and overcome the inhibitory effects of treatment with PI3K inhibitor. Transfectants were grown in methyl cellulose for 3 wk in the absence or presence of PI3K inhibitor. Representative images are shown from duplicate experiments.

Rectional orientation of signaling is an essential component of the cross-modulation process. In this regard, it is tempting to speculate that the 3D IrBM–directed basal localization of PI3K and PIP3 may explain why the cells in the outer layer of acini are more resistant to apoptosis than those not in contact with 3D IrBM (Debnath et al., 2002).

High expression of PI3K is commonly found in cancers and cancer cell lines (Vivanco and Sawyers, 2002; Wang et al., 2002a), and there is considerable evidence that the activity of this enzyme is a key component of the tumorigenic process. Cowden syndrome (an autosomal-dominant cancer predisposition syndrome caused by inherited mutations in PTEN) causes elevated risk of breast, thyroid, and skin tumors (Liaw et al., 1997); mice made heterozygous for expression of PTEN develop cancers at multiple sites (Di Cristofano et al., 1998), and transgenic mice deficient for PTEN expression in the mammary gland developed tumors at early stage (Li et al., 2002). Recent experiments using immortalized human mammary epithelial cells has shown that early passage cells require transfection of additional oncogenes along with PI3K (or Rac1/Akt) to become malignant, whereas late passage cells (which presumably accumulate more alterations) can be transformed with only PI3K (or Rac1/Akt) (Zhao et al., 2003); these results are also consistent with our model in which additional abnormalities must exist in addition to PI3K activation in order for HMT3522 mammary epithelial cells to become tumorigenic. Overexpression of constitutively active Akt in T lymphocytes, pancreatic cells, and the mammary gland increases cellular proliferation and promotes survival but does not induce cellular transformation or increase tumor incidence (Vivanco and Sawyers, 2002), suggesting that additional factors must be required. Rac1 isoforms are overexpressed in cancers of the breast and other organs, and increased activity of Rac1 or Rac3 has been found in breast carcinoma cell lines and Ras-transformed breast epithelial cells (Mira et al., 2000; Sahai and Marshall, 2002). Here, we have unified the roles of elevated Rac1 and Akt activities in a simple mechanistic framework: we have found that the high levels of PI3K in T4-2 cells contribute to the loss of polarity and increased proliferation through Akt and Rac1, and we find that these pathways and phenomena are functionally separable. We find that PI3K-Akt signaling is responsible for an appreciable increase in cell proliferation (Fig. 4), whereas the PI3K-Rac1 signaling is responsible for the loss of basal tissue polarity (Fig. 5), and that expression of both can completely prevent reversion by LY294002. These results show that overactive PI3K signaling activates these two effectors for separate but
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Ligands
RTK
Integrins
MAPKK
MAPK
PI3K
PTEN
Rac-GEFs
Rac-GTP

ECM attachment

Proliferation
Survival
Loss of Polarity

Malignant Phenotype

★ Cross-modulation point
→ Cross-modulation
+ Genetic mutants in this study

Figure 7. Scheme of proposed tumor cell signaling network to control polarity, proliferation, and apoptosis. T4-2 signaling network. PI3K activity is increased in tumor cells as a result of aberrant signaling from cell–ECM and cell–growth factor receptor interactions. The consequent activation of Akt contributes to the increased cellular proliferation through downstream pro-proliferation and anti-apoptotic pathways. Increased PI3K also leads to up-regulation of Rac1-specific GEF activity, resulting in altered organization of the actin cytoskeleton, formation and maintenance of tight junctions, and directionality of vesicle trafficking, effects that combine to disorganize the tissue structure. Thus, each pathway independently affects cellular behavior, but the synergistic effect leads to the tumor phenotype. (★) Cross-modulation in 3D IrBM. Inhibition at any of these pressure points results in normalization of the expression and activity at each of the other points, with concomitant normalization of downstream signaling pathways.

In conclusion, we have used the 3D IrBM assay to determine the role of PI3K signaling in the tumorigenic phenotype, signaling reorganization, and tissue polarity of mammary epithelial cells. Our discovery of asymmetric distribution of PI3K and PI3P in these polarized acinus-like structures strongly implies that they might act as spatial determinants to regulate mammary epithelial polarity. Our elucidation of the events downstream of PI3K sheds light on the process by which increased proliferation and loss of tissue polarity act collaboratively to produce the malignant phenotype.

Materials and methods

Reagents and cell culture

IrBM from Englebreth-Holm-Swarm tumors (matrigel), Vitrogen (rat tail collagen type I), and the antibodies for E-cadherin, β1-integrin, EGF, α6 integrin, and Ki-67 were described previously (Weaver et al., 1997; Wang et al., 1998). The other antibodies used in this study are: PTEN (clone 2), Akt (clone 7), Rac1 (clone 102), PI3K p85 (clone 4), and p110 subunit (clone 19) (Transduction Laboratory), HA (12CA5; Roche), Phospho-Akt (serine 473 or threonine 308), total and phospho-GSK-3β (serine 9), total and phospho-p70 S6 kinase (threonine 389; Cell Signaling Technology), PI3K monoclonal antibody (Chen et al., 2002; clone RC6F8; Echelon Corp.), and ZO-1 antibody (gift of Shoichiro Tsukita, Kyoto University Faculty of Medicine, Kyoto, Japan). HMT-3522 mammary epithelial cells were cultured as described previously (Weaver et al., 1997; Wang et al., 1998). The PI3K inhibitor LY294002 or wortmannin (Calbiochem) was dissolved in DMSO and added to culture medium at the final concentration of 8 μM or 2.5 nM after cells were plated; control cultures were treated with vehicle only. Cells were treated with inhibitor or vehicle inside matrigel or on tissue culture plates for 10 d and inhibition was maintained by replacing with fresh medium containing inhibitor or vehicle every 2 d. No toxicity was found during the 10-d treatment with LY294002 or wortmannin.

DNA constructs and gene transfection

A construct containing Rac1 L61-HA (gift of Tung C. Chan, Thomas Jefferson University, Philadelphia, PA) was digested with EcoRI and BamHI and cloned into the pLXSN-Neo retroviral construct (CLONTEC Laboratories, Inc.). The myr-Akt pWZL retroviral construct myrA4-129 (Kohn et al., 1998) was provided by Richard Roth (Stanford University, Stanford, CA). Transfection of Phoenix packaging cells (gift of Gary P. Nolan, Stanford University) and production of retroviral stock were carried out according to standard protocols. The HMT-3522 mammary epithelial cells were infected at 40–50% confluence. Myr-Akt and Rac1 L61-double transfectants were produced by sequentially infecting cells with each construct. The stably expressing cells were selected in the presence of neomycin (500 μg/ml) or hygromycin B (50 μg/ml) and surviving clones were pooled.

Immunoblotting, immunoprecipitation, and indirect immunofluorescence

Immunoblotting and indirect immunofluorescence were performed as described previously (Weaver et al., 1997; Wang et al., 1998). For immunoprecipitation, cells were lysed in IP buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, 2 mM NaF, 1 mM sodium orthovanadate, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 10 μg/ml aprotinin, 10 μg/ml E 64, and 1 mM Pefabloc) and centrifuged at 16,000 g at 4°C. Equal amounts of protein lysates were preclotted with 30 μl of protein G plus-conjugated agarose beads (Santa Cruz Biotechnology, Inc.) before the addition of 1 μg of primary antibody. Samples were incubated at 4°C with gentle rotation for 1 h. Subsequently, samples were incubated with 30 μl of protein G plus–conjugated beads for 1 h at 4°C. The beads were washed three times with IP buffer before being heated with sample buffer at 95°C for 5 min and analyzed by SDS-PAGE and Western blotting.

For Rac1 activity assay, the cells from 10-d culture in 3D BM were lysed in GST-Fish buffer (10% glycerol, 50 mM Tris, pH 7.4, 100 mM NaCl, 1% NP-40, 2 mM MgCl2, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 10 μg/ml aprotinin, 10 μg/ml E 64, and 1 mM Pefabloc) and centrifuged at 16,000 g at 4°C. Equal amounts of protein supernatants were incubated with recombinant GST-PAK-CD fusion protein (containing the Rac and Cdc42 binding region from human PAK1), Sander et al., 1998), bound to glutathione-coupled Sepharose beads (Amersham Biosciences) at 4°C for 30 min. The beads were washed with an excess of lysis buffer, eluted in sample buffer, and then analyzed by SDS-PAGE and Western blotting using antibody against Rac1.

For PI3K immunofluorescence, the isolated colonies were fixed with 3.7% formaldehyde, washed with CSK buffer (10 mM Hepes, 138 mM
KCI, 3 mM MgCl2, 1 mM EDTA), permeabilized by 0.1% Triton X-100 in CSK buffer, and blocked with 3% skim milk in blocking buffer (50 mM Tris/ HCl, pH 7.5, 1 mM CaCl2). Primary antibody diluted 1:100 in blocking buffer was incubated with samples for 1 h in room temperature followed by FITC-conjugated secondary antibody.

Nuclei were counterstained with DAPI (Sigma-Aldrich). Control sections were stained with secondary antibodies only. The slides were sealed with VectaShield (Vector Laboratories). The images were collected with Zeiss 410 LSM confocal microscope (Zeiss Plaun 40× oil objective lenses; Carl Zeiss MicroImaging, Inc.) or RT SLIDER SPOT digital camera (SPOT KCI, 3 mM MgCl2, 1 mM EDTA), permeabilized by 0.1% Triton X-100 in Cukierman, E., R. Pankov, Comer, Chen, Chen, I., Bissell M.J., and Radisky. 2002. The role of apoptosis in creating and maintaining luminal space within normal and oncogene-expressing mammary acini. Cell. 111:29–40. Di Cristofano, A., B. Pesce, C. Cordon-Cardo, and P.P. Pandolfi. 1998. Pten is essential for embryonic development and tumour suppression. Nat. Genet. 19: 348–355.


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Cell Biology Mammalian cell culture, three-dimensionnal cultures in various matrix, soft agar and methyl cellulose culture, cell invasion, wound-healing and migration assays, Brdu and Tunel assay, Facs analysis, fluorescent immunostaining
Biochemistry Protein purification, SDS-Page electrophoresis, 2-dimension gel electrophoresis, GST-pulldown assay, immunoprecipitation, Coomassie Blue and silver staining, Western blot, zymograph

Histology Processing, embedding, and sectioning of various tissue samples, H&E staining, immunohistochemistry, in-situ hybridization

Animal Study Animal care and maintenance, mouse injection

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