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Proliferation and Polarity in Breast Cancer:

Untying the Gordian Knot

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Abstract

Epithelial cancers are associated with genomic instability and alterations in signaling pathways that affect proliferation, apoptosis, and integrity of tissue structure. Overexpression of a number of oncogenic protein kinases has been shown to malignantly transform cells in culture and to cause tumors *in vivo*, but the interconnected signaling events induced by transformation still awaits detailed dissection.

We propose that the network of cellular signaling pathways can be classified into functionally distinct branches, and that these pathways are rewired in transformed cells and tissues after they lose tissue-specific architecture to favor tumor expansion and invasion. Using three-dimensional (3D) culture systems, we recently demonstrated that polarity and proliferation of human mammary epithelial cancer cells were separable consequences of signaling pathways downstream of PI3 kinase. These, and results from a number of other laboratories are beginning to provide insight into how different signaling pathways may become interconnected in normal tissues to allow homeostasis, and how they are disrupted during malignant progression.

In the normal mammary gland, communication between the epithelium and the surrounding stromal cells generates and maintains cellular differentiation and epithelial polarity, and provides the organizing principle for complex tissue structures [1,2]. These interactions are adaptive, and can attenuate the effects of tumorigenic alterations, as demonstrated by the fact that epithelial cells harboring oncogene-activating mutations can exist within normal tissue but be prevented by normal contextual cues from becoming malignant [3-7]. Accordingly, evolution of a tumor to malignancy requires persistent alterations in both the epithelium and the stroma. Consistent with this expectation, genetic alteration of signaling receptors in stroma can lead to tissue-specific epithelial tumors [8,9], and alterations in the microenvironment can release the suppression of context-inhibited malignant cells, allowing tumor growth [10].

Polarity is a fundamental property of epithelium, allowing the surfaces of cells and tissues to divide into distinct apical and basolateral domains. The development and maintenance of this asymmetric architecture and polarity depends upon cell-cell contacts and cell-extracellular matrix (ECM) interactions [11], as well as intrinsic cellular properties [12]. Epithelial polarization gives directionality to protein localization processes necessary for appropriate organ function. Correct epithelial polarity also regulates intracellular and extracellular proliferative signaling inputs through spatial organization and segregation of signaling

effectors, restraining cell proliferation [13]. Temporary loss of epithelial polarity that occurs during tissue repair and remodeling in wound healing processes stimulates mitogenic and migration pathways [14]. However, normal wound healing is accompanied by restoration of polarity and suppression of proliferation, the permanent loss of polarity in tumors disrupts tissue structure, compromises the segregation of signaling effectors, and exacerbates the increased cell proliferation that is induced by other oncogenic signals.

Increased proliferation is one of the most prominent features of tumors, but whether aberrant proliferation *per se* can cause loss of tissue structure and polarity remains an outstanding question. It is thus important to see whether dysregulated proliferation and loss of polarity are distinguishable mechanistic events during tumor progression. The combination of these effects can be observed in transgenic mouse models, where overexpression of a single oncogenic kinase such as ErbB2/NEU in the mammary gland can induce tumor development and metastasis [15]. The downstream effectors of ErbB2/NEU regulate not only cell proliferation and apoptosis, but also probably polarity and tissue structure [16]. However, the precise role of oncogenic kinases in human cancers is obscured because the vast majority of human tumors are intrinsically genomically unstable, so that by the time cancer manifests, the numerous genetic lesions prevent strict definition of cause and effect [17]. Genetic studies of *Drosophila* and mice in which up-regulated proliferation or

disruption of polarity alone is not sufficient to cause tumors suggest that activated oncogenes control distinct downstream pathways that affect proliferation and polarity separately [13,18-21]. To dissect the oncogenic effects of polarity and proliferation, we used a genetically tractable model system of human breast cancer cells cultured in a physiologically relevant context, and found that two signaling pathways downstream of phosphatidylinositol 3-kinase regulate proliferation and tissue polarity separately [22].

Class I phosphatidylinositol 3-kinase (PI3K) is activated by growth factor-responsive tyrosine kinases such as epidermal growth factor receptors (EGFR) [23] and integrin-responsive kinases such as focal adhesion kinase (FAK) [24]. 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 such as Akt and SGK, which regulate cell proliferation and apoptosis [25]. PI3K regulates Rac1, a small GTPase that has numerous functions including polarity control [26], that is one of the key mediators in processes that regulate cell orientation and directionality of chemotaxis for both *Dictyostelium* and cultured human leukocytes [27-29]. Its pleiotropic effects make PI3K an ideal candidate signaling mediator to explore whether cellular proliferation and polarity can be uncoupled in bifurcating downstream signaling pathways.

PI3K is one of the major oncogenic kinases and is constitutively upregulated in a substantial fraction of human cancers [25]. Genetic studies in transgenic mice revealed that PI3K was essential for polyomavirus middle T-mediated mammary tumorigenesis [30] and upregulated PI3K signaling by overexpression of a constitutively active subunit of PI3K or by inactivation of the PIP3 phosphatase PTEN led to tumor formation in various tissues [25,31]. Moreover, overexpression of PI3K in cultured nonmalignant human mammary epithelial cells is sufficient to confer a malignant phenotype [32].

To address the question of whether proliferation and polarity are distinct phenotypes downstream of PI3K, we took advantage of a three dimensional (3D) laminin-rich basement membrane (lrBM) culture model developed in collaboration with Ole Petersen [33]. In this system, cells are embedded in 3D lrBM, a context that mimics the physiological environment and that provides cells with structural scaffolding and contextual information necessary for cell differentiation and polarity. Under these conditions, nonmalignant human mammary epithelial cells undergo growth arrest and differentiate into structures that resemble acini found in the mammary gland (well-organized spheres with correct apicobasal polarity); in contrast, malignant cells fail to respond properly to lrBM and proliferate into amorphous, disorganized structures [33]. Using this system and HMT-3522 T4-2 malignant human mammary epithelial cells, we have shown that inhibition of either EGFR or β1 integrin can

“revert” the cancer cells to a nonmalignant phenotype creating phenotypically normal acinar structures that nevertheless retain all of the mutations present in the parental breast cancer cells [34,35].

Our analysis showed that down-modulating PI3K signaling by treating with specific PI3K inhibitors LY294002 or wortmannin was sufficient to reduce both cellular proliferation and anchorage-independent growth, and to restore apicobasal tissue polarity in the context of 3D lrBM. Significantly, PI3K and its phospholipid product PIP3 were localized to the basal pole in the reverted spheres (Figure 1) [22]. These findings demonstrate that dysregulated PI3K activity can directly affect proliferation and polarity, and that these effects are reversible in the lrBM model system.

Akt is one of the central effectors of PI3K that mediate cellular proliferation and survival [25,36]. In contrast to the tumorigenetic effects of activated PI3K signaling [37], and targeted deletion of PTEN [31], ectopic expression of active Akt is not sufficient to disrupt tissue structure or stimulate tumor development, although it can enhance the potential for development of more massive [20,38-42]. Consistent with these findings, we observed that active Akt caused increased proliferation in the 3D lrBM reversion assay, but did not prevent the re-establishment of basal tissue polarity upon PI3 kinase inhibition [22] (Figure 2); similar effects were observed by treating cells with rapamycin (unpublished data), a specific inhibitor of mTOR (mammalian target of rapamycin), a major downstream

effector of Akt and a regulator of cell proliferation and growth [43]. Proliferation was significantly reduced by rapamycin, but tissue polarity could not be restored. A separate study showed that expression of active Akt in nonmalignant human mammary epithelial MCF-10A cells cultured on 3D lrBM increased the size and cell number of the acinar structures but did not disrupt basal polarity [44]. These results show that while active Akt can stimulate proliferation, it does not affect cell polarity, implying that additional pathway(s) downstream of PI3K and parallel to Akt must exist to control polarity.

The Rho family of GTPases have been extensively investigated for their function in cell organization and function [45]. Rac1 is a RhoGTPase that is a component of an evolutionarily conserved protein complex (PAR3/PAR3/aPKC) that is key for regulation of cell polarity [46]. Dysregulation of Rac1 activity in kidney epithelial cells cultured in 3D compromises cellular polarity [47]. It has been shown that PI3K affects Rac1 activity through control of its activators Tiam1 and Vav1 [48]. We found that Rac1 activity was decreased in T4-2 cells treated with PI3K inhibitors, and that while cell proliferation was unaffected, basal tissue polarity could not be restored in cells expressing constitutively active Rac1 (Figure 2)[22], showing that Rac1 acts downstream of activated PI3K to mediate the loss of tissue polarity without significant effect on cell proliferation. That Rac1-induced polarity disruption and Akt-induced proliferation enhancement were the principal downstream effects of activated PI3K in T4-2 cells was

demonstrated by the fact that cells coexpressing both active Akt and Rac1 were immune to reversion by PI3K inhibitors [22] (Figure 2). These results show that increased proliferation (by activated Akt) and loss of polarity (via up-regulated Rac1 activity) constitute the minimal signaling inputs required from PI3K to cause the tumor phenotype, and that disruption of polarity is a requirement for the malignant phenotype even if cell proliferation is highly enhanced.

Our results provide insight into the mechanisms of PI3K-induced tumorigenesis in breast cancers. In our model, signals from PI3K bifurcate into function-specific and independent pathways for control of proliferation and polarity. Activation of cell cycle-promoting or anti-apoptotic pathway(s) such as Akt or SGK increase tissue mass, while dysregulation of the cytoskeleton by Rac1 disrupts tissue polarity and architecture. The convergence of the two effects synergistically leads to tumor development [22]. We have shown that dysregulated cell proliferation and polarity defects are separable effects downstream of PI3K in mammary epithelial cells, but this principle might have general implications for other cell types and signaling pathways, as suggested by elegant analyses in *Drosophila* showing that the synergy of both defects is required for the development of the malignant phenotype [20,21,42]. Accordingly, it may be that separation of regulation of proliferation and polarity represents an evolutionarily conserved anticancer mechanism. Nevertheless, fundamental questions remain: how the defects in polarity and proliferation are coupled, to what

extent loss of polarity is sufficient to initiate cancer formation, and at which step the merge of the two defects is sufficient to lead to transformation during this progressive process. Unraveling the specific contributions of proliferation and polarity to tumor development through the use of 3D model systems and genetically tractable organisms will have a profound effect on our understanding of the nature of cancer development.

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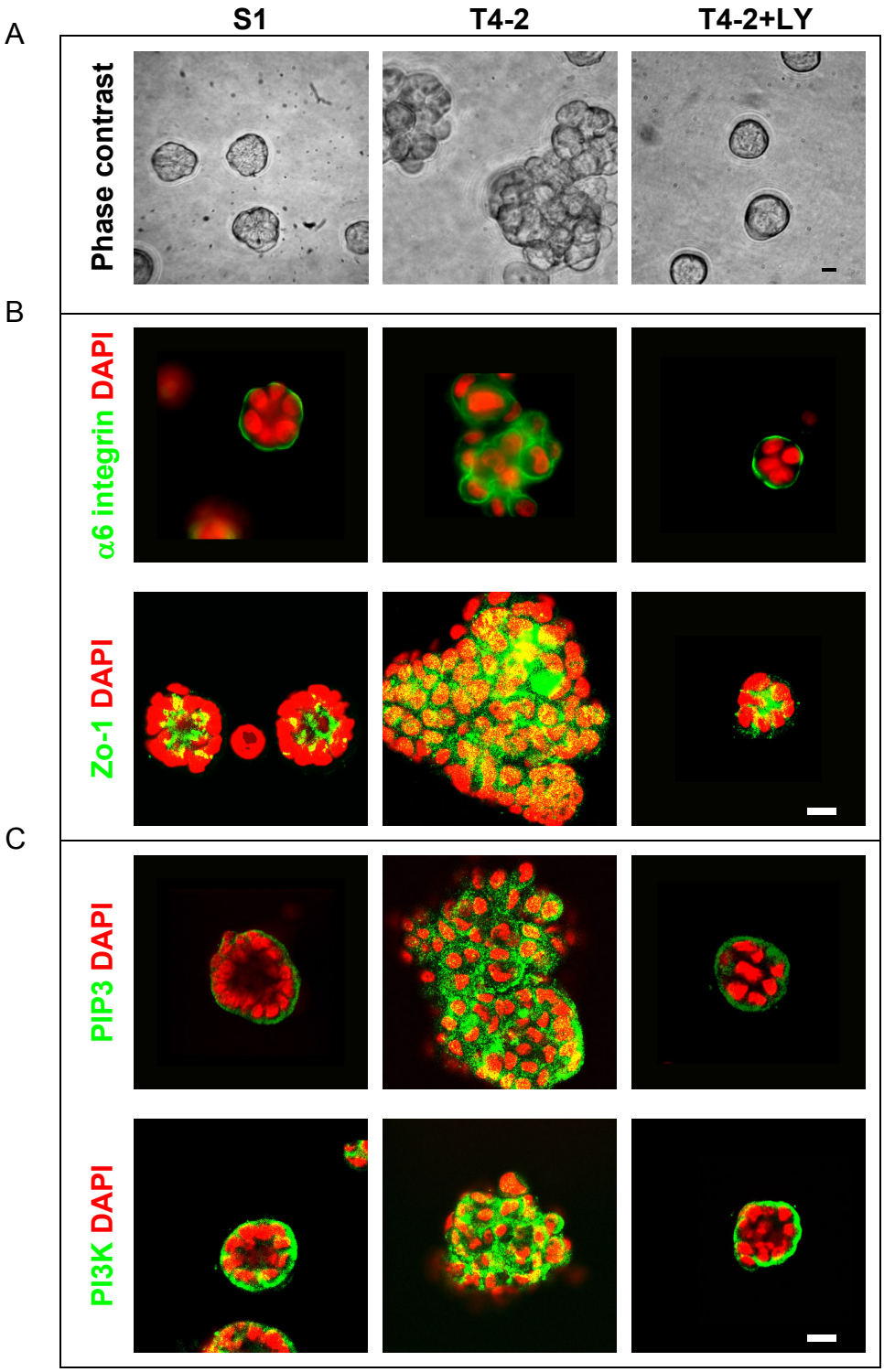
Figure legends

Figure 1. Down-modulation of PI3 kinase activity results in phenotypic reversion, re-establishment of tissue polarity, and repolarization of PI3-kinase, and its lipid product, phosphatidylinositol (3,4,5) trisphosphate (PIP3) in the acini. (A) Phase contrast micrographs of phenotypically normal (S-1), malignant (T4-2), and T4-2 cells treated with 8 μM PI3 kinase inhibitor, LY 294002 (T4-2+LY) cultured in 3D lrBM for 10 days; (B) tissue polarity is re-established in "reverted" T4-2 cells. Tight junction protein ZO-1 and the basal ECM receptor, α6 integrin, were used as apical and basal markers; (C) the basolateral localization of PI3-kinase and PIP3 in S-1 cells is lost in their T4-2 progeny, but attenuation of PI3-kinase leads to repolarization. Scale bar: 10 μm (Adapted from Liu et al., 2004, *J Cell Biol*. 164:603-12,).

Figure 2. Proliferation and tissue polarity are controlled separately by Akt and Rac1 downstream of PI3 kinase. Malignant T4-2 cells stably expressing constitutively active Akt, Rac1, or both were grown in 3D lrBM in the absence or presence of PI3 kinase inhibitor for 10 days. Scale bar: 10 μm (Adapted from Liu et al., 2004, *J Cell Biol*. 164:603-12,).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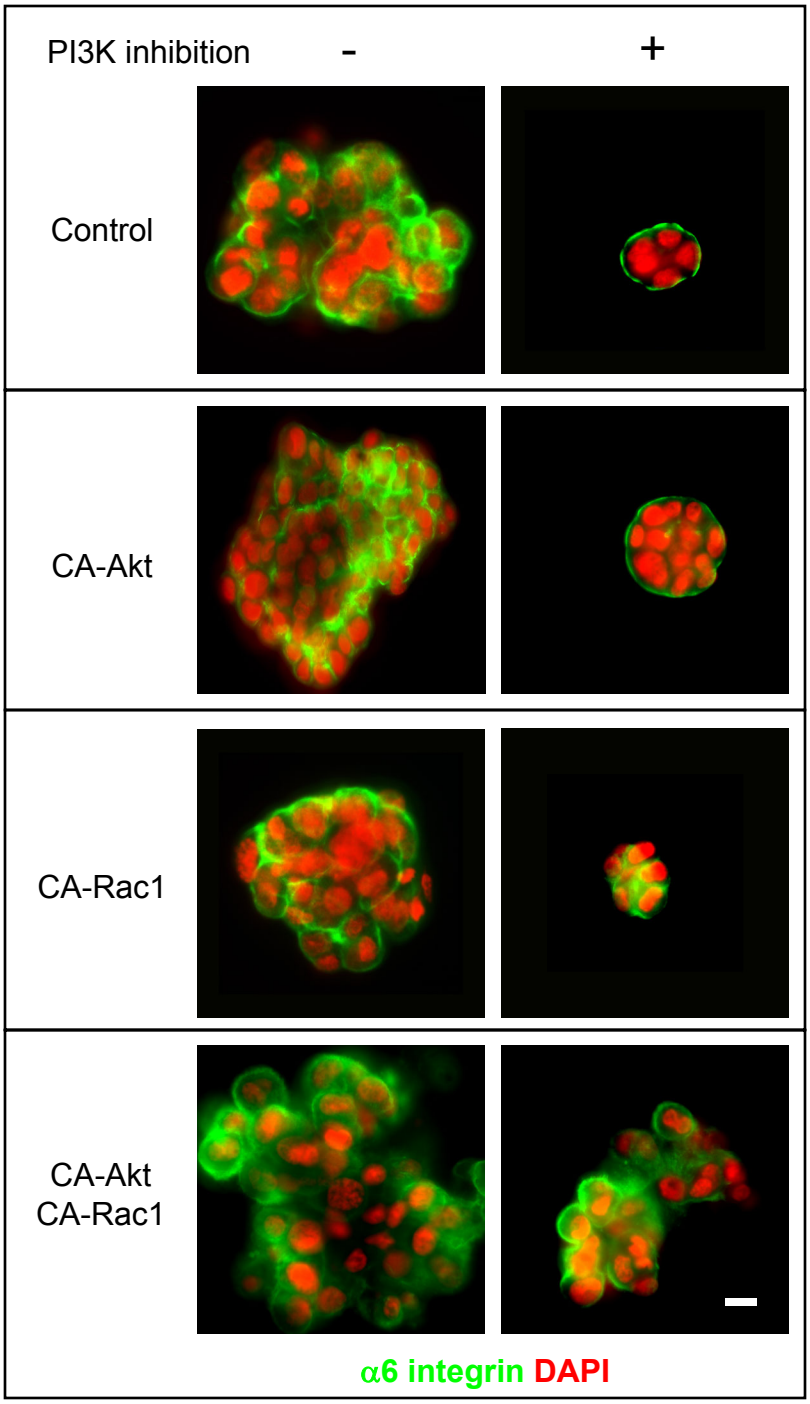
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