mTOR Signaling Feedback Modulates Mammary Epithelial Differentiation and Restrains Invasion Downstream of PTEN Loss

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Abstract

Oncogenic signaling pathways are tightly regulated by negative feedback circuits and relief of these circuits represents a common mechanism of tumor drug resistance. Although the significance of these feedback pathways for signal transduction is evident, their relevance for cellular differentiation and morphogenesis in a genetically defined context is unclear. In this study, we used isogenic benign mammary organotypic cultures to interrogate the role of mTOR-mediated negative feedback in the specific setting of PTEN inactivation. We found that mTOR signaling promoted basal-like differentiation and repressed nuclear hormone receptor expression after short-term PTEN loss in murine cell cultures analyzed ex vivo. Unexpectedly, we found that PTEN inactivation inhibited growth factor-induced epithelial invasion and that downstream mTOR-mediated signaling feedback was both necessary and sufficient for this effect. Mechanistically, using isogenic MCF10A cells with and without somatic PTEN deletion, we showed that mTOR inhibition promoted EGF-mediated epithelial invasion by derepressing upstream EGF receptor, SRC tyrosine kinase, and phosphoinositide 3-kinase signaling. In addition to offering new signal transduction insights, these results bring to light a number of important and potentially clinically relevant cellular consequences of mTOR inhibition in the specific context of PTEN loss, including modulation of hormone and growth factor responsiveness and promotion of epithelial invasion. Our findings prompt future investigations of the possibility that mTOR inhibitor therapy may not only be ineffective but even deleterious in tumors with PTEN loss. Cancer Res; 73(16); 5218–31. ©2013 AACR.

Introduction

PTEN is a tumor suppressor and lipid phosphatase that antagonizes oncogenic phosphoinositide-3-kinase (PI3K) activity. Increased net PI3K activity is a key event in breast tumorigenesis, occurring via activating PIK3CA mutations or with the loss of PTEN in as many as 75% of patients (1). The critical role of PTEN loss in promoting breast tumorigenesis is illustrated by Cowden syndrome, where germline PTEN inactivation is associated with an 85% lifetime incidence of breast carcinoma (2). One important result of PTEN loss is activation of AKT, which leads to phosphorylation and inactivation of the TSC1/2 tumor suppressor complex, and increased downstream mTORC1/2 signaling. The cellular effects of PTEN loss have been well-studied in two-dimensional (2D) monolayer culture systems and include increased proliferation and invasion and reduced apoptosis and cell–cell adhesion (3).

Importantly, the relevance of PTEN loss for epithelial differentiation and sensitivity to targeted therapeutics has only recently been elucidated. The association between PTEN loss and basal-like differentiation was initially reported for mammary carcinomas developing in PTEN⁻/− mice, which commonly express myoepithelial-type cytokeratins and are negative for estrogen receptor (ER; ref. 4). These murine tumors recapitulate human PTEN-null breast carcinomas, which cluster in the basal-like category by expression profiling and are commonly hormone receptor negative (4) and tamoxifen-resistant (5). In addition to hormone independence, loss of PTEN is also associated with independence from HER signaling, both in vitro and in vivo. PTEN-null MCF10A cells are EGF independent (6) and PTEN loss is a common genomic change underlying trastuzumab resistance (7, 8). However, the molecular mechanisms underlying these characteristic features of PTEN deletion remain unclear.

Recently, a number of studies have highlighted the importance of potent negative feedback loops from PI3K/AKT/mTOR signaling to upstream receptor tyrosine kinase (RTK) and hormone receptor signaling (9–16). As activated PI3K/AKT/mTOR signaling is a hallmark of PTEN loss, it is possible that this feedback may underlie resistance of PTEN-null tumors to hormonal and HER-targeted therapies. Experiments in genetically heterogeneous systems have shown that treatment with rapamycin (a potent inhibitor of mTORC1 signaling) or mTOR kinase inhibitors (which inhibit mTORC1 and mTORC2)
releases negative feedback to upstream HER, insulin growth factor receptor/insulin receptor (IGF/IR), and platelet-derived growth factor receptor (PDGFR) signaling, resulting in potent downstream activation of PI3K and mitogen-activated protein kinase (MAPK; refs. 11, 12, 17, 18). Furthermore, work on prostate cancer has shown that reciprocal negative feedback between PI3K/mTOR and androgen receptor (AR) signaling mediates hormonal resistance, although it remains unclear whether a similar mechanism regulates estrogen signaling in breast tissue (19, 20).

As targeted cancer therapeutics become the norm, defining the relevance of these negative feedback circuits in the context of specific genetic aberrations is increasingly important. Does PTEN loss, which promotes unfettered PI3K/AKT signaling, mitigates the effects of mTOR-mediated feedback on upstream RTK activity? A systematic comparison of the significance of this feedback pathway in isogenic PTEN-expressing and PTEN-null systems has not been conducted. A second important question is whether these potent signaling feedback pathways have relevance for cell behavior. To date, most work has elucidated the significance of this feedback at the signaling level. Although single-agent rapalog therapies are generally not effective and xenograft studies support the improved efficacy of combined RTK and mTOR inhibitor therapies (18, 21, 22), it remains unclear whether mTOR-mediated signaling feedback may be actually deleterious, modulating critical cell behaviors such as invasion or motility. In large part, this may be because nearly all previous studies have focused on effects in 2D monolayer cell cultures. Accordingly, a recent study illustrates how the cellular consequences of upregulated RTK signaling following PI3K/mTOR suppression are best modeled using 3D organoid budding was quantified in 5 independent experiments as the proportion of organoids with more than 2 buds (100–300 organoids/condition). Mean proportions were normalized to wild-type for each replicate, as the percentage of branched wild-type organoids varied (33%–88%) across experiments. The number of buds per organoid was visually counted for 30 to 300 organoids in one representative experiment. Luminal filling was defined as more than 50% cellular filling of internal luminal space on histologic sectioning and was assessed 3 independent experiments (24–51 organoids/condition).

Cell culture
Isogenic MCF10A parental cells and two independent clones with homozygous targeted recombination at the PTEN locus (PTEN/−− #1, PTEN/−− #2) were a gift of K. Bachman (University of Maryland, Baltimore, MD; ref. 6). Conditions for 2D monolayer culture (6), 3D acinar culture in Matrigel (30), and 3D acinar culture in collagen–Matrigel mixtures (31) have been described. MCF10A-ER-SRC cells (32) were a gift of K. Struhl (Harvard University, Cambridge, MA) and were maintained as described.

Quantification of morphogenesis in MCF10A acini
Acinar size was quantified at day 18 of culture in Matrigel by measuring diameter (Zeiss Axiosvision). Invasive protrusions were quantified at day 3 of culture in collagen–Matrigel as the proportion of acini containing at least one invasive cytoplasmic protrusion under phase-contrast imaging in 3 independent experiments (100–300 acini/condition).
Proliferation and apoptosis assays

Organoids were pulsed with 10 μmol/L BrdUrd (BD Biosciences) at day 4 or 7 of culture followed by fixation, processing, histologic sectioning, and immunohistochemistry to detect BrdUrd. BrdUrd immunohistochemistry was scored manually by counting the proportion of positively stained nuclei for at least 10 organoids per condition in 3 independent experiments that were analyzed. Cleaved caspase-3 (CC3) immunohistochemistry was quantified at day 4 or 7 of culture as the proportion of positively stained nuclei for 3 independent experiments (10 organoids/condition). For MCF10A acini, immunofluorescence for CC3 was carried out at day 18 of culture and imaged by confocal microscopy as described below. The percentage CC3-positive cells was assessed for 7 to 8 acini/genotype (50–500/cells each).

Inhibitors

Pharmacologic inhibitors were dissolved in dimethyl sulfoxide and added to culture media at day 0 upon plating at concentrations spanning the IC50 values: rapamycin (200 nmol/L; LC Laboratories), AZD0855 (500 nmol/L; LC Laboratories); torin1 (500 nmol/L; a gift of D.M. Sabatini, Harvard; ref. 33), GDC0491 (1 nmol/L; LC Laboratories), BEZ235 (1 nmol/L; LC Laboratories), erlotinib (250 nmol/L; LC Laboratories), dasatinib (100 nmol/L; LC Laboratories), LY294002 (25 μmol/L; Sigma); marimistat (5 μmol/L; Sigma), and TAPI-1 (10 μmol/L; Millipore).

Immunoblotting

Organoids, acini, and cell monolayers were immunoblotted by SDS-PAGE as described (27) using primary antibodies from Cell Signaling Technologies: p-EGFR (Y1068), p-AKT(T308), p-AKT(S473), p70S6K, p-s6, p-SRC, EGFR, pan-AKT, p-EGFR (Y1068), p-AKT(T308), p70S6K, pan-AKT, p-TSC1, p-TSC2, p-mTOR, p-pan-AKT, p-p70S6K, p-p-SRC, p-pEGFR, and/or smooth muscle actin (Sigma), or E-cadherin (Cell Signaling). Directly conjugated phalloidin (Invitrogen) was used to detect filamentous actin (F-actin). mT/mG fluorescence was directly detected in cryosections. Immunofluorescence of MCF10A acinar cultures for laminin (Millipore) and GM130 (BD Biosciences), was conducted as described (30) and confocally imaged in serial 5 μm Z-sections (Zeiss LSM 510).

Immunohistochemistry/immunofluorescence quantification

CK14 expression was quantified as proportion of CK14-expressing luminal cells/organoid on histologic sections in 3 independent experiments (6–14 organoids/condition). Mean proportions were normalized to wild-type as the average percentage CK14-positive luminal cells in the wild-type organoids varied between replicates (3.8% to 15.8%). ER expression was quantified using H-score. The H-score is calculated as 1’ (percentage 1+ intensity cells) + 2’ (percentage 2+ cells) + 3’ (percentage 3+ cells). Seven to 15 organoids were assessed/condition/experiment (30–200 cells/organoid), mT/mG immunofluorescence was quantified as the percentage of enhanced green fluorescence protein (EGFP)-expressing cells/organoid on cryosections assessing 20 organoids/timepoint from one representative experiment.

RTK array

Phospho-RTK–signaling arrays (Cell Signaling Technologies) were conducted according to the manufacturer’s instructions using cell lysates from 2D monolayers exposed to specified drug for 18 hours. Arrays were immediately captured via digital chemiluminescent imaging (Alpha Innotech).

Statistical analysis

Statistical analysis was conducted throughout using Student unpaired t test.

Results

Murine mammary organoids undergo invasive budding in response to FGF or HER ligands. In the murine mammary ex vivo culture system, ductal fragments are isolated and embedded in laminin-rich ECM where they recapitulate normal bilayered ductal organization. Although the organoids are initially cystic, 4 days following the addition of exogenous growth factor, the luminal cells proliferate to fill the interior space. By day 7, the luminal cells have invaded beyond the surrounding of myoepithelial cell layer into the ECM, recapitulating pubertal ductal morphogenesis and the early stages of invasive breast cancer (29; Supplementary Fig. S1). Although both FGF and HER ligands can independently induce invasive budding in this system (29), we found that FGF2 is more effective than TGFβ or EGF and a small but consistent synergistic response was seen with both ligands together (Supplementary Fig. S1).

As ADAM17 (a metalloproteinase that cleaves and activates membranous HER ligands) is required for ductal invasion during puberty (33), we tested whether autocrine and/or paracrine HER signaling may regulate full response to FGF or HER ligands during puberty (33), we tested whether autocrine and/or paracrine HER signaling may regulate full response to FGF or HER ligands. In the murine mammary ex vivo culture system, ductal fragments are isolated and embedded in laminin-rich ECM where they recapitulate normal bilayered ductal organization. Although the organoids are initially cystic, 4 days following the addition of exogenous growth factor, the luminal cells proliferate to fill the interior space. By day 7, the luminal cells have invaded beyond the surrounding of myoepithelial cell layer into the ECM, recapitulating pubertal ductal morphogenesis and the early stages of invasive breast cancer (29; Supplementary Fig. S1). Although both FGF and HER ligands can independently induce invasive budding in this system (29), we found that FGF2 is more effective than TGFβ or EGF and a small but consistent synergistic response was seen with both ligands together (Supplementary Fig. S1).

As ADAM17 (a metalloproteinase that cleaves and activates membranous HER ligands) is required for ductal invasion during puberty (33), we tested whether autocrine and/or paracrine HER signaling may regulate full response to FGF ligands in vitro, as documented in other epithelial systems (34). We found that treatment of organoids with marimistat or TAPI-1 (metalloprotease inhibitors) markedly inhibited budding in response to FGF2 alone in a manner that could be rescued with exogenous TGFβ (Supplementary Fig. S1, data not shown), suggesting that autocrine/paracrine HER signaling is required downstream of FGF2 addition for in vitro epithelial invasion in this system.

Short-term Pten loss is efficiently induced in primary mammary organoid culture. To query the short-term effects of Pten loss-of-function on cellular differentiation and morphogenesis in this system, we isolated mammary organoids.
from R26CreER;PTEN<sup>loxp/loxp</sup> mice. Using the mTmG reporter, we found that an 18-hour incubation with 4-OHT at day 0 of culture (followed by washout) was sufficient to induce Cre activity in 60% of cells by day 3 of culture with minimal effects on morphogenesis (Supplementary Fig. S1). Accordingly, by day 4 of culture, we observed efficient PTEN protein loss in R26CreER;PTEN<sup>loxp/loxp</sup> organoids (referred to as PTEN<sup>−/−</sup>- after 4-OHT) compared with littermate PTEN<sup>loxp/loxp</sup> controls (referred to as PTEN<sup>+/+</sup>- after 4-OHT), with concomitant increases in downstream-activated AKT (Fig. 1A).
mTORC1 signaling promotes basal-like differentiation and is necessary and sufficient to inhibit ER expression downstream of short-term PTEN loss. It remains unclear whether PTEN loss is directly responsible for basal-like differentiation in human PTEN-null tumors (4), and if so, which downstream signal transduction pathways are required. To address this question, we assessed keratin and ER expression in our benign isogenic system with short-term PTEN loss. At day 4 of culture, PTEN+/+ organoids were solid bilayered structures, with a single layer of CK14-myoepithelial cells surrounding CK8-positive luminal epithelial cells (29; Fig. 1B and C). In contrast, PTEN−/− organoids contained a mixture of CK8- and CK14-expressing cells in their lumens, with nearly a 50% increase in luminal CK14-positive cells. The increase in luminal CK14-positive cells could be due to increased proliferation or aberrant differentiation. Unlike CK14-positive myoepithelial cells in wild-type organoids, these CK14-positive cells in PTEN−/− organoids did not uniformly coexpress smooth muscle actin, suggesting that they have undergone partial but incomplete myoepithelial differentiation (Fig. 1E). In addition, the location of these cells within the luminal compartment (as opposed to the periphery) suggested that they arose from aberrant differentiation of luminally located cells, rather than due to increased relative proliferation of the CK14-positive myoepithelial compartment in the PTEN+/+ organoids. Finally, quantification of proliferation as measured by BrdUrd incorporation revealed no significant difference between the ratio of percentage proliferating CK14-positive cells and percentage proliferating non-CK14-positive cells in the PTEN+/+ and PTEN−/− organoids (1.46±0.50 vs. 1.20±0.30. P = NS), despite accumulation of CK14-positive cells specifically in the PTEN−/− organoids.

To discern which signaling pathways activated downstream of PTEN loss might mediate this basal-like differentiation, we treated PTEN−/− organoids with mTOR inhibitors. We found that rapamycin (a potent mTORC1 inhibitor, which suppresses p70S6K and p-S6 levels, Fig. 3B) substantially decreased the nuclear ER expression levels in murine organoids. Rapamycin treatment of murine organoids restored nuclear ER levels, whereas rapamycin treatment of human organoids restored nuclear ER levels to wild-type organoid levels (Fig. 1B and C). Interestingly, treatment of PTEN−/− organoids with GDC0941, which in contrast to rapamycin inhibits PI3K and mTORC2 activity in addition to mTORC1...
(see Fig. 7), resulted only in a very modest and statistically insignificant rescue of nuclear ER levels (26% increase in average H-score, \( P = 0.14 \), data not shown). Thus, in the context of constitutively suppressed PI3K/mTORC2 activity, mTORC1 inhibition is insufficient to rescue ER expression in \( \text{PTEN}^{-/-} \) cells.

To query whether mTORC1 activation independent of \( \text{PTEN} \) loss is sufficient to suppress nuclear ER expression, we generated \( \text{R26}ERCre \text{TSC}1^{lox/lox} \) organoids (hereafter referred to as \( \text{TSC}1^{-/-} \)). \( \text{TSC}1^{-/-} \) organoids have markedly increased mTORC1 activity as evidenced by increased p-S6, and this can be suppressed with rapamycin treatment (Fig. 3D). By day 7 of culture after growth factor addition, \( \text{TSC}1^{-/-} \) organoids showed reduced nuclear ER expression compared with their wild-type counterparts, an effect reversible by rapamycin (Fig. 1F and G). In contrast to \( \text{PTEN}^{-/-} \) organoids, the number of luminal CK14-positive cells was not significantly increased in \( \text{TSC}1^{-/-} \) organoids, suggesting that the changes in CK14 and ER expression are independent and that mTORC1 activity is not sufficient to increase basal-like keratin expression in this system (Fig. 1F). Thus, downstream of short-term \( \text{PTEN} \) loss, mTORC1 activity is necessary to promote basal-like differentiation and suppress ER expression in mammary organoid cultures. Furthermore, mTORC1 activation independent of \( \text{PTEN} \) loss is sufficient to inhibit nuclear ER expression, but not to promote basal-like differentiation.
Because *PTEN* loss promotes independence from HER signaling in human tumors and cell lines (6–8), we also examined whether mammary organoids with short-term *PTEN* loss could survive in the absence of EGF/HER2 signaling. Consistent with our finding that autocrine/paracrine HER signaling occurs in the organoid system in the presence of FGF2, we found that treatment with lapatinib (a combined EGF/HER2 inhibitor) decreased viability of wild-type mammary organoids cultured in FGF2 (Supplementary Fig. S2). These data suggest that in addition to modulating epithelial invasion in this system, at least minimal autocrine/paracrine EGFR/HER2 signaling is required for cell survival in wild-type organoids. Although mamristat blocks autocrine release of HER ligands, it did not result in decreased organoid viability (Supplementary Fig. S1B), likely because it results in a less complete block of this autocrine signaling compared with lapatinib. In contrast to wild-type organoids, *PTEN*−/− organoids showed no decrement in viability in the presence of lapatinib. This suggests that cell survival is no longer dependent on HER signaling in the context of *PTEN* loss, likely due to constitutive activation of downstream intracellular survival signaling pathways. Importantly, rapamycin treatment did not restore sensitivity to lapatinib (data not shown), suggesting that constitutive mTORC1 activation is not critical for cell survival in *PTEN*−/− organoids.

Short-term *PTEN* loss promotes cell and tissue growth and inhibits epithelial cell invasion of ECM. By day 7 of culture with growth factor, *PTEN*−/− mammary organoids lacked *PTEN* protein expression and showed increased AKT phosphorylation (Fig. 2A). As expected, they were also larger than their wild-type counterparts (Fig. 2A and B), due in part to the increased size of individual luminal epithelial cells (Fig. 2C), an effect reversible with rapamycin (data not shown) and independent of growth factor (Fig. 2D). In contrast to wild-type organoids, which were predominantly cystic at this timepoint, the majority of *PTEN*−/− organoids showed persistent filling of the luminal space by CK8- and CK14-expressing cells (Fig. 2D and E). Most striking, fewer than half as many *PTEN*−/− organoids responded to growth factor by developing invasive CK8-positive epithelial buds extending into the surrounding ECM (Fig. 2D and F). Taken together, *PTEN* loss drives increased tissue size and luminal filling within days in mammary organoids. Surprisingly, however, *PTEN* loss restraints growth factor-induced epithelial budding in the context of the organoid system.

mTORC1 signaling downstream of *PTEN* loss is necessary to inhibit mammary epithelial cell invasion in organoid cultures. Next, we examined which signal transduction pathways downstream of *PTEN* loss might suppress growth factor-induced mammary epithelial cell invasion. Both PI3K-mTORC1/2 inhibitors (GDC0941, Fig. 3A and LY294003, data not shown) and specific mTOR kinase inhibitors that block mTORC1/2 signaling (AZD8055 or torin1; Fig. 3A and data not shown), all potently inhibited epithelial budding in both genotypes. Thus, PI3K activation and mTOR kinase function are required for budding in this system. Surprisingly, we observed that rapamycin did not suppress epithelial invasion in wild-type mammary organoids, indicating that while mTORC1 function is not required, PI3K- and mTORC2-dependent invasion occurs in this system. In fact, rapamycin treatment rescued epithelial budding in *PTEN*−/− mammary organoids, increasing the percentage of branched organoids and the number of buds/organoid even beyond wild-type controls (Fig. 3A and C). In contrast, there were no consistent effects of rapamycin on budding in *PTEN*+/− organoids. mTORC1 inhibition was associated with an increase in PI3K activity [as measured by p-AKT (T308)], even in *PTEN*−/− organoids (Fig. 3B). Thus, these data indicate that in *PTEN*-null mammary organoids, mTORC1 activity simultaneously restrains full PI3K activation and suppresses epithelial invasion.

Because luminal cell proliferation is required for epithelial invasion in the organoid system (29), we next asked whether the increased budding in rapamycin-treated *PTEN*−/− organoids (and decreased budding in GDC0941- and AZD8055-treated organoids) could be due to changes occurring in net proliferation. Notably, both GDC0941 and AZD8055 dramatically decreased proliferation as measured by BrdUrd incorporation on day 4 (Supplementary Fig. S3), making it difficult to discern whether decreased cellular proliferation or impaired epithelial migration/invasion underlie the effects of these inhibitors on budding. Despite the fact that it increased epithelial branching in *PTEN*−/− organoids, we found that rapamycin actually suppressed proliferation as measured by BrdUrd incorporation on day 4 and increased apoptosis as measured by CC3 expression on day 7 (Supplementary Fig. S3). Thus, rapamycin likely increases budding by modulating epithelial migration and invasion.

mTORC1 activation is sufficient to restrain growth factor-induced mammary epithelial invasion. To query whether mTORC1 activation was sufficient to inhibit epithelial invasion, we studied *TSC1*−/− organoids. Compared with their wild-type counterparts, *TSC1*−/− organoids showed a depressed level of PI3K activation measured by p-AKT (T308; Fig. 3D). This correlated with a dramatic decrease in invasive buds in response to growth factor (Fig. 3E and F). In contrast, rapamycin-treated *TSC1*−/− organoids showed full rescue of epithelial invasion, with numerous CK8-positive buds beyond the myoepithelial cell layer (Fig. 3E and F), whereas rapamycin-treated wild-type organoids showed no significant effects (data not shown). Thus, mTORC1 activation is both sufficient to inhibit growth factor-induced epithelial invasion in the absence of *PTEN* loss and necessary to restrain invasion in the context of *PTEN* loss.

*PTEN* loss in MCF10A cells promoues luminal filling in 3D culture but does not disrupt apical-basal polarity specification of matrix-attached cells. To confirm our findings in a human system with long-term *PTEN* loss, we took advantage of established isogenic MCF10A cell lines with and without *PTEN* deletion (6). The wild-type parental cells formed well-developed acinar structures lined by a single layer of epithelial cells when cultured in laminin-rich ECM (Matrigel) for 18 days (Fig. 4A and C; ref. 30). In contrast, two independent *PTEN*−/− clones (dPTEN−/−-1 and dPTEN−/−-2) formed predominately solid structures that were nearly twice as large as the parental acini (Fig. 4). Despite reports of a requirement for *PTEN* in apicobasal polarity specification (35), matrix-attached cells...
from PTEN−/− clones were appropriately polarized similar to the parental clones (Fig. 4C). In contrast, internal, non–matrix-attached cells were randomly polarized in both parental and PTEN−/− clones. As PI3K activity has recently been shown to prevent apoptosis of internal, non–matrix-attached cells (23), we immunostained for CC3 and noted a decrease in apoptosis in these cells in PTEN−/− clones, although this did not reach statistical significance (Fig. 4E and F).

mTORC1 signaling restraints epithelial cell invasion and inhibits full PI3K activation in PTEN-null MCF10A acini. We observed that the PTEN-null cells developed transient cytoplasmic protrusions into the Matrigel ECM at a rate far higher than that seen in the wild-type parental clone (Supplementary Fig. S4). In collagen–Matrigel mixtures, similar protrusions were formed specifically by the PTEN−/− acini at day 3 of culture. These protrusions were F-actin–rich and invaded into the surrounding ECM, beyond the basally deposited laminin layer (Fig. S4A and B). In addition, smaller actin-rich projections were evident around the periphery of the forming PTEN−/− acini, and these were not apparent in the parental clone (Fig. S4A). However, even in collagen–Matrigel mixtures, invasive cytoplasmic protrusions were relatively rare (occurring in less than 10% of PTEN−/− acini, Fig. 5C) and frequently unstable, regressing, and reforming rapidly when viewed by time-lapse DIC imaging (Fig. 5D, Supplementary Movie S2B).

Because PI3K inhibitors suppressed epithelial invasion in murine organotypic cultures, we next asked whether PI3K activity was necessary for invasive protrusion in PTEN−/− MCF10A acini. Treatment of cells with GDC0941 (PI3K inhibitor), markedly reduced protrusions in PTEN−/− acini, suggesting that PI3K activity is also required to induce invasion in this system (Fig. 5B and C). Remarkably, treatment with either rapamycin (mTORC1 inhibitor), AZD8055, or Torin1 (mTORC1/mTORC2 inhibitor) dramatically increased acinar invasion specifically in the PTEN−/− clones, with only a minimal effect on the parental wild-type cells (Fig. 5B and C, data not shown). Time-lapse DIC imaging revealed that rapamycin stabilized the otherwise transient invasive cytoplasmic protrusions and promoted their growth into cords and branches of cells, ramifying into an interconnected ductal network that was never seen in parental wild-type cells treated with the same drug (Fig. 5D, Supplementary Movies S2A and S2C).

At the signal transduction level, rapamycin or AZD8055 treatment markedly increased upstream PI3K signaling [as indicated by p-AKT(T308) levels], more dramatically in the
PTEN<sup>−/−</sup> clones than in the wild-type cells (Fig. 5E and F). Interestingly, basal p-AKT (T308) levels appeared constitutively suppressed in the vehicle-treated PTEN<sup>−/−</sup> clones such that differences in p-AKT (T308) were not readily apparent between the PTEN<sup>−/−</sup> and wild-type clones until rapamycin or AZD8055 was added. This contrasted with p-AKT (S473) levels that were more consistently elevated in vehicle-treated PTEN<sup>−/−</sup> clones and which was increased even further by treatment with rapamycin, but suppressed as expected by AZD8055 (Fig. 5E). To examine the effects of a combined PI3K/mTOR inhibitor, we also tested BEZ235 in the MCF10A system. Interestingly, we found BEZ235 to be indistinguishable from pure mTOR kinase inhibitors, as it suppressed p-AKT (S473) levels but increased p-AKT (T308) levels and induced epithelial invasion, particularly in PTEN<sup>−/−</sup> clones (Supplementary Fig. S5). Thus, as in murine organotypic cultures, PI3K activity is required for epithelial invasion, whereas mTORC1 activity is dispensable.
In both systems, mTORC1 inhibition relieves constitutive suppression of PI3K activity in PTEN⁻/⁻ cells and the resultant increase in PI3K activity correlates with a striking increase in epithelial invasion in this setting.

mTORC1 signaling downstream of PTEN loss feeds back to inhibit upstream c-SRC and EGFR kinase activation. To test whether HER signaling might be required for rapamycin-induced mammary epithelial invasion in the setting of PTEN⁻/⁻ cells, we omitted EGF from the basal MCF10A cell culture media and examined the effects on PTEN⁻/⁻ clones, which are EGF independent (6; Fig. 6A). Strikingly, epithelial invasion in rapamycin-treated PTEN⁻/⁻ cells was entirely dependent on EGF stimulation. This suggested the possibility that mTORC1 inhibition in PTEN⁻/⁻ cells may boost PI3K activity via relief of negative feedback to upstream HER kinase activity. Accordingly, we took advantage of RTK-signaling arrays to screen for changes in tyrosine phosphorylation after treatment with mTOR inhibitors (Fig. 6B). Treatment with either rapamycin or AZD8055 resulted in increased tyrosine phosphorylation of c-SRC in PTEN⁻/⁻ clones, with smaller increases in wild-type parental clones. Treatment with AZD8055 also led to markedly increased phosphorylation of STAT3 in PTEN⁻/⁻ clones, consistent with reports that c-SRC potently activates STAT3 signaling (36). In addition, increased phosphorylation of EGFR and IGF-IR was present, though subtle, in PTEN⁻/⁻ cells treated with rapamycin or AZD8055 when visualized on the RTK arrays.

To confirm these findings, we conducted immunoblotting of cell lysates from 2D monolayer cultures after rapamycin (C) and AZD8055 (D) treatment. Representative immunoblots are shown from 3 to 5 independent experiments for each drug.
mildly suppressed in PTEN−/− cells compared with parental wild-type cells, (Fig. 6C and D) and treatment with either mTOR inhibitor markedly upregulated SRC Y416 phosphorylation consistently in the context of PTEN loss (Fig. 6C and D). In addition, treatment with either inhibitor increased EGFR autophosphorylation. Total levels of EGFR were inconsistently increased following rapamycin treatment as well. Again, this activation was more prominent in PTEN−/− cells, although in contrast to SRC, basal EGFR activation was not appreciably decreased with PTEN loss. No consistent changes were seen in phospho- or total HER2 or HER3 levels in response to mTOR inhibitor treatment (data not shown). Taken together, these data indicate that mTOR inhibition in rapamycin-treated PTEN−/− cells activates upstream c-SRC and EGFR signaling and is associated with increased PI3K activity and acinar invasion.

Simultaneous inhibition of PI3K, SRC, or EGFR/HER2 signaling is sufficient to repress epithelial invasion in rapamycin-treated PTEN-null acini. We next asked whether feedback activation of EGFR, PI3K, and SRC following mTORC1 inhibition was required for invasive cell behavior. Simultaneous treatment with GDC0941 (a PI3K inhibitor) and rapamycin eliminated PI3K activation and acinar invasion in PTEN−/− cells (Fig. 7) confirming the requirement for PI3K signaling. Similarly, treatment with lapatinib (a dual EGFR/HER2 inhibitor; Fig. 7A and B) or erlotinib (an EGFR inhibitor, data not shown) resulted in a marked reduction in epithelial invasion in rapamycin-treated PTEN−/− clones. Lapatinib markedly reduced p-EGFR levels and p-SRC levels, in addition to eliminating the increase in p-AKT seen with rapamycin treatment alone (Fig. 7C), suggesting that the SRC activation in rapamycin-treated PTEN−/− cells occurs downstream of HER signaling. Finally, concurrent treatment with dasatinib (a SRC family kinase inhibitor) also suppressed epithelial invasion in rapamycin-treated PTEN−/− acini (Fig. 7A and B). In contrast to lapatinib, dasatinib did not abrogate the rapamycin-induced increase in PI3K activity as reflected by p-AKT (T308; Fig. 7D). Interestingly, we found that constitutive SRC activation in wild-type MCF10A cells was sufficient to induce both PI3K activation and epithelial invasion (Supplementary Fig. S6). Taken together, these data suggest mTORC1 inhibition releases negative feedback to EGFR signaling, triggering a marked increase in downstream PI3K and SRC activity in PTEN−/− cells, both of which are required to induce mammary epithelial invasion.

Discussion

RTK-signaling pathways are subject to tight feedback regulation. With the advent of potent and specific mTOR inhibitors, there has been an increasing interest in the role of mTORC1 signaling in the downregulation of upstream PI3K activation and its relevance for tumor drug resistance (15).
Solid tumors have been largely resistant to rapalog monotherapy, and many show increased PI3K and MAPK activity following mTORC1 inhibition (12, 37). Early studies conducted predominantly in MEFs pinpointed S6K activity downstream of mTORC1 activation as responsible for this negative feedback via destabilization of IRS-docking proteins and inhibition of IR/IGFR signaling (9, 10). In addition to this early work in mesenchymal cells, recent work in epithelial cancer cell lines has also highlighted an important role for mTORC1-mediated inhibition of HER signaling. Studies of combined PI3K/mTOR inhibitors (BEZ235), PI3K inhibitors (XL147), AKT inhibitors (AKTIs), and mTOR kinase inhibitors (AZD8055) in epithelial cancer cell lines have all shown resistance that coincides with increased PI3K and MAPK activity and a corresponding increase in HER levels and activity, although the mechanisms involved have not been entirely worked out (18, 21–23, 38).

A common theme emerging from these data is that signaling feedback allows cells to maintain a remarkably highly regulated signaling network, even in the presence of apparently dysregulating mutations (16). Accordingly, targeted therapeutics aimed at suppressing a specific oncogenic signaling event may have the paradoxical effect of unmasking upstream RTK activity. For example, oncogenic RAS desensitizes upstream HER signaling and relief of this negative feedback via depletion of oncogenic RAS has the paradoxical effect of hyperactivating EGFR and wild-type RAS (39). Similarly, BRAF(V600E)-activating mutations suppress EGFR signaling (40, 41) and tumor types that respond to this signaling (colon cancers) are resistant to a BRAF-mutant–specific inhibitor in contrast to melanomas, which express less EGFR (42). Thus understanding the role of signaling feedback in a specific genetic and cellular context is critical to the success of targeted therapeutics.

Our study is the first to show that the effects of mTOR-mediated feedback may be specifically modulated by PTEN status in the mammary epithelium. In fact, because PTEN loss results in net increased basal PI3K activity, the assumption has been that this genetic alteration may obviate any effects of signaling feedback (43). Here, quite to the contrary, we have shown that mTORC1 activity plays a critical role in suppressing maximal PI3K signaling specifically in the setting of PTEN loss. Indeed, in PTEN-null MCF10A cells, p-AKT (T308) levels were not markedly elevated compared with wild-type cells until rapamycin was added, in large part because mTORC1 inhibition more dramatically increased p-AKT (T308) in the PTEN+/− cells (Fig. 5F). These data suggest that even in the context of long-term PTEN loss, mammary cells engage mTORC1-dependent homeostatic mechanisms to partially normalize PI3K activity.

One such potential mechanism is mTORC1-mediated feedback regulation of upstream RTK activity. In this model, mTORC1 inhibition boosts upstream RTK signaling, leading to increased PI3K recruitment, but in the wild-type setting, downstream PI3K and AKT activation are largely held in check by intact PTEN activity. In PTEN+/− cells treated with mTORC1 inhibitors, the combination of elevated RTK activity and PTEN loss leads to unflattered PI3K activity, culminating in marked downstream AKT activation. Accordingly, in the MCF10A system, treatment of PTEN+/− cells with rapamycin or an mTOR kinase inhibitor resulted in marked SRC and EGFR phosphorylation, and correlated tightly with increased downstream effector activity, including p-AKT and p-STAT3. Furthermore, pharmacologic blockade of EGFR/HER2 signaling restrained this rapamycin-induced EGFR/SRC/p-AKT activity.

However, in the context of PTEN loss, as in prior studies of HER2-expressing cancer cells, the precise molecular mechanism by which mTORC1 inhibition augments upstream EGFR signaling remains to be discovered. AKT inhibition has been reported to increase basal HER levels in cancer cells (via increased FOXO transcription; 21–23). However, FOXO-mediated transcription is likely decreased after mTORC1 inhibitor treatment due to signaling feedback, which boosts AKT activity. Though we did observe a small (but not entirely consistent) increase in total EGFR in MCF10A cells following rapamycin treatment (Fig. 6), the increase in p-EGFR was much more robust. In addition, HER2 and HER3 total levels and phosphorylation were not affected by rapamycin treatment in the MCF10A system (Fig. 6, data not shown). We are the first to report an increase in SRC activity with mTORC1 inhibitor treatment and intriguingly SRC phosphorylates and transactivates EGFR (44), raising the question of whether SRC activation may itself provide a potential mechanism for increased EGFR phosphorylation in the setting of mTORC1 inhibition. Indeed, at least one prior study has shown that rapamycin treatment increases SRC phosphorylation and results in EGFR transactivation in the absence of growth factor (45). However, it seems that SRC is activated downstream of EGFR in the MCF10A system, as treatment with HER inhibitor lapatinib was sufficient to completely abolish SRC and PI3K activation in rapamycin-treated PTEN+/− cells. Similarly, although SRC is sufficient to activate PI3K signaling (as observed with inducible v-SRC expression, Supplementary Fig. S6), SRC inhibition with dasatinib did not abolish feedback activation of EGFR or PI3K (Fig. 7D, data not shown). Thus, in our model, mTORC1 inhibition releases negative feedback to upstream EGFR phosphorylation, resulting independently in increased downstream SRC activation and PI3K activation.

Perhaps the most novel finding in the current study is that mTORC1-mediated signaling feedback modulates cell behaviors, specifically in the context of PTEN loss. Until now, studies of negative feedback regulation of oncogenic signaling pathways have focused on the implications for signal transduction and proliferation in 2D cell line monolayers or xenografts (18, 21, 22). Here, in two independent 3D culture systems, we found that mTORC1 feedback is critical for restraining growth factor-induced mammary epithelial cell invasion in the setting of PTEN loss. It is important to note that the two benign model systems we used herein are quite divergent in terms of species derivation, differentiation, and mode of cellular invasion used. It does seem that the murine organoid system requires auto/paracrine HER signaling for invasive budding downstream of FGF2 stimulation, providing an intriguing link between the HER-mediated suppression of invasion seen in the MCF10A system and the FGF2-dependent organoid system. However, many distinctions between the two systems do likely underlie some of the important differences we observed, for example, PTEN loss by itself in the organoid system.
suppresses invasion, whereas a modest increase in invasive potential is seen in the MCF10A system. Despite these limitations, the similar effect of mTORC1-mediated feedback on PTEN−/− cell invasion in each is compelling. In the bilayered murine organotypic culture system, mTORC1 signaling was both necessary and sufficient to suppress nonproliferative, collective luminal epithelial invasion in response to FGF2. Similarly, in the MCF10A system with PTEN loss, mTORC1 inhibition potently induced protrusive, single cell epithelial invasion into the surrounding ECM in response to EGF stimulation. Ultimately, the similar findings in each system are suggestive of the potentially highly conserved role of mTORC1-mediated signaling feedback in modulating cell behavior.

Just as mTORC1-mediated feedback to upstream HER signaling regulates epithelial invasion in mammary epithelial cells, we found that mTORC1 also interfaces with hormone receptor signaling pathways. Somewhat surprisingly, we found that even short-term PTEN loss was sufficient to dramatically decrease nuclear ER levels and increase basal-type keratin expression in murine organotypic cultures, potentially interrelated differentiation events dependent on mTORC1 activation. In addition, we found that in the absence of PTEN loss, mTORC1 signaling upregulation via TSC1 loss was necessary and sufficient to repress nuclear ER levels. These findings are reminiscent of recent reports that PI3K/mTOR signaling can repress AR levels and signaling in PTEN-null murine and human prostate tissues (19, 20). Given our findings in the MCF10A system, it is of particular interest that PI3K/mTOR may mediate this feedback to AR in the prostate via downregulation of HER kinase signaling (19).

Ultimately, additional studies of the role of mTORC1 suppression in modulating hormone and growth factor receptor responsiveness in the specific setting of PTEN-null human tumors will be necessary to substantiate our findings. Although rapalogs have long been known to be ineffective as monotherapies, the similar effect of mTORC1-mediated feedback on upstream tyrosine kinase inhibitors both relieve feedback to upstream tyrosine kinase activity resulting in downstream AKT activation, specifically in the setting of PTEN loss. These signaling effects are consistent with a recent cancer cell line study (18) and combined with our finding that this signaling correlates with increased epithelial invasion, suggest the novel possibility that mTOR kinase monotherapies may not only be ineffective, but potentially even detrimental in tumors with aberrantly activated PI3K signaling. Clearly, additional studies to directly address this hypothesis in malignant systems with PTEN loss are necessary and may further elucidate the importance of this critical signaling feedback pathway.

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11. Lotan T, Lotan T.L. Lotan. Invasive mammary epithelial cell invasion in each is compelling: In the bilayered murine organotypic culture system, mTORC1 signaling was both necessary and sufficient to suppress nonproliferative, collective luminal epithelial invasion in response to FGF2. Similarly, in the MCF10A system with PTEN loss, mTORC1 inhibition potently induced protrusive, single cell epithelial invasion into the surrounding ECM in response to EGF stimulation. Ultimately, the similar findings in each system are suggestive of the potentially highly conserved role of mTORC1-mediated signaling feedback in modulating cell behavior. Just as mTORC1-mediated feedback to upstream HER signaling regulates epithelial invasion in mammary epithelial cells, we found that mTORC1 also interfaces with hormone receptor signaling pathways. Somewhat surprisingly, we found that even short-term PTEN loss was sufficient to dramatically decrease nuclear ER levels and increase basal-type keratin expression in murine organotypic cultures, potentially interrelated differentiation events dependent on mTORC1 activation. In addition, we found that in the absence of PTEN loss, mTORC1 signaling upregulation via TSC1 loss was necessary and sufficient to repress nuclear ER levels. These findings are reminiscent of recent reports that PI3K/mTOR signaling can repress AR levels and signaling in PTEN-null murine and human prostate tissues (19, 20). Given our findings in the MCF10A system, it is of particular interest that PI3K/mTOR may mediate this feedback to AR in the prostate via downregulation of HER kinase signaling (19).

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Disclosure of Potential Conflicts of Interest
B.H. Park is a consultant/advisory board member in Horizon Discovery and GlaxoSmithKline. No potential conflicts of interest were disclosed by the other authors.

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mTOR Inhibits Mammary Invasion Downstream of PTEN Loss


mTOR Signaling Feedback Modulates Mammary Epithelial Differentiation and Restrains Invasion Downstream of PTEN Loss

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