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Akt and ERK Control the Proliferative Response of Mammary Epithelial cells to the Growth Factors IGF-1 and EGF through the Cell Cycle Inhibitor p57^{Kip2}*

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Abstract

Epithelial cells respond to growth factors including epidermal growth factor (EGF), insulin-like growth factor 1 (IGF-1), and insulin. Using high-content immunofluorescence microscopy, we quantitated differences in signaling networks downstream of EGF, which stimulated proliferation of mammary epithelial cells, and insulin or IGF-1, which enhanced the proliferative response to EGF but did not stimulate proliferation independently. We found that the abundance of the cyclin-dependent kinase inhibitors p21^{Cip1} and p57^{Kip2} increased in response to IGF-1 or insulin but decreased in response to EGF. Depletion of p57^{Kip2}, but not p21^{Cip1}, rendered IGF-1 or insulin sufficient to induce cellular proliferation in the absence of EGF. Signaling through the PI3K- (phosphatidylinositol 3-kinase) Akt-mTOR (mammalian target of rapamycin) pathway was necessary and sufficient for the increase in p57^{Kip2}, whereas MEK [mitogen-activated or extracellular signal-regulated protein kinase (ERK) kinase]-ERK activity suppressed this increase, forming a regulatory circuit that limits proliferation in response to unaccompanied Akt activity. Knockdown of p57^{Kip2} enhanced the proliferative phenotype induced by tumor-associated PI3K mutant variants and released mammary epithelial acini from growth arrest during morphogenesis in three-dimensional culture. These results provide a potential explanation for the context-dependent proliferative activities of insulin and IGF-1 and for the finding that the CDKN1C locus encoding p57^{Kip2} is silenced in many breast cancers, which frequently show hyperactivation of the

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PI3K pathway. The status of p57^{Kip2} may thus be an important factor to assess when considering targeted therapy against the ERK or PI3K pathways.

Introduction

In mammalian cells, extracellular growth factor-mediated signaling, which is essential for cellular proliferation, is frequently disrupted in cancer. Activation of growth factor receptors leads to the stimulation of numerous downstream pathways that modulate cellular metabolism, control gene transcription, and engage the cell cycle. Of these pathways, the Ras- Raf- ERK (extracellular signal-regulated kinase) and PI3K- (phosphatidylinositol 3-kinase) Akt pathways play a central role in driving many of the phenotypic changes induced by growth factors. The Ras-Raf-ERK pathway (hereafter the ERK pathway) is typically stimulated by recruitment of guanine nucleotide exchange factors such as SOS (son of sevenless) to the growth factor receptor, inducing the small guanosine triphosphatase Ras to bind guanosine triphosphate (GTP). Following this activation step, Ras binds to and activates the kinase Raf, which stimulates a kinase cascade culminating in the phosphorylation of ERK by MEK (mitogen-activated or extracellular signal-regulated protein kinase kinase). Phospho-activation of ERK results in its nuclear translocation and phosphorylation of numerous substrates to both promote proliferation and inhibit pro-apoptotic signals (1). The PI3K-Akt pathway (hereafter the Akt pathway) is activated by binding of the PI3K p85 regulatory subunit to tyrosine-phosphorylated sites on growth factor receptors or on associated adaptor proteins such as IRS-1 (insulin receptor substrate 1) or Gab1. This binding leads to activation of the associated p110 catalytic domain, which phosphorylates phosphatidylinositol (4,5)-bisphosphate in the plasma membrane to generate phosphatidylinositol (3,4,5)-trisphosphate (PIP3). Akt contains a pleckstrin homology (PH) domain, which binds to PIP3, enabling recruitment of Akt to the membrane, where it is phosphorylated and activated by another PH-domain containing protein kinase, PDK1 (phosphoinositide-dependent protein kinase 1), and by the TORC2 [target of rapamycin (TOR) complex 2] signaling complex. As a downstream effector of PI3K signaling, Akt promotes protein synthesis, cell proliferation, cell metabolism, and cell survival (2).

The ERK and Akt pathways work coordinately or synergistically to promote cell growth and progression through the cell cycle to enable DNA replication. Both pathways are involved in the activation of the kinase mTOR (which controls the rate of cellular growth by promoting protein translation) (3), c-Myc (a key transcription factor controlling both metabolism and cell cycle progression)(4), and cyclin D (a central regulator of the transition from G1 to S phase of the cell cycle) (5, 6). Other downstream effectors are specific to one of the two pathways; for example, the Akt pathway stimulates the uptake of glucose (7), whereas ERK activates immediate-early transcription factors including c-Fos and Ets-1. Because the ERK and Akt pathways are pleiotropic and interconnected, it is difficult to determine the distinct contribution of each to the overall proliferative response to growth factors. Nonetheless, both phospho-Akt and phospho-ERK are frequently used as readouts of proliferative or oncogenic signaling.

The ERK and Akt pathways also engage in negative crosstalk. For example, Akt suppresses ERK pathway signaling through inhibition of Raf (8–10). Moreover, ERK and its target p90RSK (p90 ribosomal S6 kinase) contribute to mTOR activation through phosphorylation of the tuberous sclerosis complex (TSC1/2), and the downstream mTOR effector S6 kinase 1 (S6K1) stimulates a negative feedback loop, thereby suppressing Akt activity (3). Thus, negative crosstalk could maintain a proper balance between the output of each pathway, preventing hyperactivation of a single pathway that might lead to uncontrolled proliferation. However, how the dynamic interplay between these pathways controls proliferation, what combinations of signals constitute pathological or deregulated states, and what mechanisms are used by the cell to detect and suppress potentially oncogenic signaling states remain to be fully elucidated.

Also central to cell cycle control are the cyclin-dependent kinase (CDK) inhibitors (CKIs), which fall into two families: INK4 (inhibitor of kinase 4) and CIP/KIP (CDK interacting protein/kinase inhibitory protein). The CIP/KIP family, consisting of CDKN1A, CDKN1B, and CDKN1C (p21^{Cip1}, p27^{Kip1}, and p57^{Kip2} respectively, hereafter referred to as p21, p27, and p57), bind to multiple cyclin-CDK complexes and can arrest the cell cycle during G1, S, or G2 phases. These proteins may also play a critical role in cell cycle progression by acting as assembly factors for the CDK4/6-cyclin D complex, promoting late G1 to S phase transition (11). Within the context of growth factor-mediated signaling, p27 is recognized as a central regulator of the balance between quiescence and proliferation (12). Akt phosphorylates p27, inducing its translocation to the cytoplasm, where it is degraded (13). p21 is best known for its role in p53-mediated cell cycle arrest in response to DNA damage or other stresses (14, 15), and has been reported to be either stimulated or suppressed by Akt (16, 17), and potentially activated by the ERK pathway (18). Although less is known about p57, it is the only member of the CIP/KIP family essential in mouse embryonic development (19). p57 has tumor suppressor functions, being silenced in many cancers and implicated in Beckwith-Wiedemann syndrome, a congenital disorder associated with tissue overgrowth and an increased risk of cancer (20). At the mRNA level, p57 is known to be stimulated by glucocorticoids (21) and suppressed by the Myc-induced miR-221/222 (22, 23).

Here, we have identified p57 as an effector of crosstalk between the ERK and Akt pathways; activation of the Akt pathway increased the abundance of p57, whereas the ERK pathway suppressed it. This cross-regulatory motif played a key role in determining the proliferative response of mammary epithelial cells to EGF and insulin-like growth factor 1 (IGF-1) or insulin. Activation of the PI3K pathway in the absence of ERK activity, either by IGF-1 or insulin or by mutant PI3K isoforms, led to an increase in the abundance of p57 and proliferative arrest. Depletion of p57 enabled IGF-1-induced proliferation in the absence of EGF, enhanced proliferation of cells harboring endogenous PI3K mutations, and abrogated proliferative arrest in 3D culture. Thus, regulation of p57 by ERK and Akt acts as a network sensor capable of detecting and limiting the proliferative response to “imbalanced” signaling states in which the Akt pathway is activated in isolation.

Results

Growth factor-mediated changes in p57 abundance control proliferation

MCF-10A mammary epithelial cells are typically cultured in growth medium containing both insulin and EGF (with insulin present at a concentration capable of stimulating both the insulin and IGF-1 receptors). To examine the contribution of these mitogens to proliferative behavior, we cultured a clonal derivative of MCF-10A cells in the presence of various concentrations of EGF and either insulin or IGF-1 and assessed proliferation by high-content immunofluorescence microscopy (HCIF). We used phosphorylated retinoblastoma protein (pRb) as an indicator of cell cycle progression (24). A small percentage of cells (5%) were pRb-positive following deprivation of both EGF and insulin for 24 hours, whereas the greatest amount of pRb staining (81%) was observed at maximal concentrations of EGF in the presence of IGF-1 or insulin (Fig. 1A). Although EGF alone was capable of stimulating cell proliferation in a dose-dependent manner, concentrations of insulin and IGF-1 capable of fully activating the Akt pathway (25) had no effect. At sub-maximal concentrations of EGF (0.5 ng/ml), co-stimulation with IGF-1 or insulin displayed a cooperative effect, increasing the pRb-positive population from 22% to 45% or 35% respectively. Thus, both EGF and IGF-1 induced proliferative signals, but those downstream of IGF-1 required additional input from the EGF-stimulated network to be manifest.

In agreement with previous work (26), EGF potently stimulated phosphorylation of ERK, but stimulated the Akt pathway relatively weakly, as indicated by a modest shift (from 1.18 to 1.14) in the nuclear/cytoplasmic ratio of FoxO3a (a more sensitive indicator of Akt activity than phospho-Akt itself in MCF-10A cells; Fig. 1B). In contrast, IGF-1 induced stronger cytoplasmic localization of FoxO3a (nuclear/cytoplasmic ratio=1.04), indicating high PI3K-Akt activity, but did not induce detectable phosphorylation of ERK.

To examine the signals contributing to proliferation downstream of EGF and IGF-1, we measured a panel of 12 proliferation-related signaling proteins by HCIF (Fig. 1C). This quantitative analysis confirmed the differences between EGF and IGF-1 in activation of ERK and Akt signaling. As expected, the abundance or phosphorylation of numerous proteins that signal downstream of both ERK and Akt, including c-Myc, c-Fos, and phospho-S6, changed in response to both EGF and IGF-1. For most of these co-regulated proteins, induction by EGF was stronger than by IGF-1, suggesting that the differences in proliferative response between EGF and IGF-1 could in part be due to stronger activation of ERK effectors, including c-Fos and c-Myc. However, abundance of the cell cycle regulators p21 and p57 increased specifically in response to IGF-1, but not EGF, suggesting that these proteins may contribute to the differential phenotypic response to the two growth factors.

A more detailed analysis of p57 dynamics revealed that saturating concentrations of IGF-1 increased the median p57 abundance by ~2-fold (Fig. 2A,B). Co-stimulation with EGF blocked this increase in a dose-dependent manner; IGF-1 upregulation of p57 was fully blocked by concentrations of EGF of 2 ng/ml or higher. EGF also suppressed the basal (non-IGF-stimulated) p57 expression. Changes in p21 abundance followed a similar trend, but were less pronounced and more prone to experimental variability (Fig. 2C and Fig. S1). Covariate analysis of single-cell data for p57 in combination with pRb revealed that p57 was

present primarily in pRb-negative cells. When the frequency of pRb-positive cells was evaluated as a function of p57 abundance using a sliding window, the percent of pRb-positive cells decreased markedly as p57 abundance increased (Fig. 2D). This relationship, which was consistent across all treatment conditions (Fig. S2), enabled us to define a threshold representing the critical amount of p57 required for its cell cycle-inhibitory effect. The frequency of cells in which p57 was above this critical threshold (p57-positive cells) increased from 27% in untreated cells to 57% in IGF-1 treated cells (Fig. 2E). Co-treatment with 20 ng/ml EGF reduced the frequency of p57-positive cells to 8%. Thus, IGF-1 and EGF induce changes in p57 abundance that are quantitatively relevant to the control of cell cycle progression.

Covariate analysis of p21 and pRb revealed a similar relationship to that seen with p57 (Fig. 2D). However, the range in percentage of p21-positive cells across all conditions was smaller than that observed with p57 (Fig. 2E; 24% to 55% for p21, compared to 8% to 57% for p57). Importantly, the abundances of p21 and p57 were not correlated at the single-cell level (Fig. 2F). This relationship supports the hypothesis that these CKIs act independently to limit proliferation. The quantitative difference in response between these CKIs argues that p57 plays a larger role in controlling the overall proliferative rate of the population in response to IGF-1 and EGF stimulation.

Accordingly, we next asked whether activation of p21 or p57 was involved in suppressing cell proliferation in response to IGF-1. We depleted p57 in MCF-10A cells using four unique shRNA constructs (Fig. 3A,C and fig S3). Relative to cells transduced with a control shRNA vector or uninfected cells, cells expressing shRNAs targeting p57 displayed a 1.5- to 2-fold increase in proliferative response to IGF-1 (Fig. 3D). This increase in proliferation rate was more subtle than that produced by saturating concentrations of EGF, but nonetheless lead to a significant ($p < 0.001$) increase in cell number, as confirmed in growth curve experiments (Fig 3E). In contrast, no such enhancement of IGF-1-stimulated proliferation was observed in p21^{-/-} MCF-10A cells (Fig. 3F) (27). Thus, p57, but not p21, appears to limit IGF-1- or insulin-induced proliferation. We therefore focused on the mechanisms involved in IGF-1- and EGF-dependent regulation of p57.

The Akt pathway increases p57 abundance

Because insulin activated the Akt pathway more strongly than did EGF, we examined whether this pathway was involved in stimulating the increase in p57 abundance. Small molecule inhibitors of PI3K activity (GDC-0941), mTOR kinase activity (Torin-1), or both (BEZ-235) attenuated IGF-1-mediated p57 upregulation (Fig. 4A,B). Of these inhibitors, Torin-1 was most effective in reducing the frequency of p57-positive cells (to 5%, relative to 62% for the DMSO control) followed by BEZ-235 (10%), and GDC-0941 (22%). Rapamycin, which specifically targets mTOR Complex 1 (mTORC1), had a modest effect, reducing the frequency of p57-positive cells to 40%. The efficacy of inhibitors capable of blocking both mTORC1 and mTORC2 (Torin-1 and BEZ-235), together with the weaker effect of rapamycin, suggest that the IGF-1-stimulated increase in p57 abundance is driven by mTORC2 or a combination of mTORC1 and mTORC2.

To determine whether Akt pathway activation alone was sufficient to increase p57 abundance, we expressed an inducible myristoylated Akt variant (myrAkt-ER) that can be selectively activated by treatment with 4'-hydroxytamoxifen (4OHT) (28) in MCF-10A cells. Cells expressing myrAkt-ER were first cultured for 48 hours in the absence of growth factors to suppress all other proliferative signals, and then stimulated with 4OHT at various time points prior to fixation. Treatment with 4OHT alone induced a marked increase in p57, whereas co-treatment with EGF attenuated the 4OHT-induced p57 response (Fig. 4C). Temporal analysis revealed that the induction of p57 began four hours after treatment with 4OHT and increased steadily through the 24 hour time period (Fig. 4D). However, no proliferative response, as assessed by pRb staining, was observed following 4OHT treatment (Fig. S4). Therefore, activation of Akt is sufficient to increase the abundance of p57, a response that can be suppressed by an EGF-stimulated pathway.

The ERK pathway suppresses p57

Because EGF is a potent activator of the ERK signaling pathway, we assessed the involvement of ERK activity in suppressing p57. Treatment of EGF-, or EGF- + IGF-1-stimulated cells with a MEK inhibitor (PD325901) led to a marked increase in the frequency of p57-positive cells (from 6% to 56% in EGF-treated cells, and from 9% to 87% in EGF + IGF-1 treated cells; Fig. 5A,B). In contrast, MEK inhibition in unstimulated or IGF-1-stimulated cells did not substantially increase the abundance of p57, in accord with the lack of detectable ERK activity under these conditions. Additionally, overexpression of H-Ras^{V12}, which constitutively stimulates ERK activity, resulted in nearly complete suppression of IGF-1-stimulated p57 induction (Fig. 5C). Treatment of H-Ras^{V12}-expressing cells with a MEK inhibitor reversed this suppression p57, confirming the requirement for ERK-mediated signaling.

To elucidate the relationship between the ERK and Akt pathways in controlling p57 abundance, we examined the effects of inhibiting both pathways simultaneously. Under conditions of EGF stimulation, the increase in p57 manifest with MEK inhibition was effectively blocked by Torin-1 or BEZ-235 (Fig. 5D), indicating that PI3K and mTOR activity induced by EGF is sufficient to upregulate p57, but is typically blocked by concomitant activation of the ERK pathway. Additionally, we plotted p57 abundance as a function of that of pERK and pAkt for all conditions shown in Figs. 2A, 4A, and 5A (including varying concentrations of EGF, insulin, IGF-1, MEK inhibitors, and PI3K-TOR inhibitors) (Fig. 5E). From this plot, it is apparent that under conditions of low ERK activity, p57 abundance is proportional to that of pAkt. However, p57 abundance is very low with increased pERK, regardless of the amount of pAkt. Thus, p57 is directly stimulated by Akt signaling, whereas ERK acts as a dominant suppressor of this activation.

Western blotting confirmed that the IGF-1-PI3K and EGF-ERK- mediated effects on p57 immunofluorescence reflected changes in total cellular p57 protein (fig S5). To assess whether these effects on p57 abundance occur at the transcriptional level, we measured p57 mRNA abundance by quantitative real-time PCR (qRT-PCR). Although insulin stimulated a modest (1.2-fold) increase in p57 mRNA abundance, treatment with the dual PI3K and mTOR inhibitor PIK-90 reduced p57 mRNA abundance by ~2-fold (Fig. 5F). In contrast,

insulin-stimulated p57 mRNA abundance was reduced 3-fold by EGF treatment, and this effect was fully blocked by the MEK inhibitor PD98059. Additionally, under direct stimulation of Akt signaling using the myrAkt-ER variant, p57 mRNA abundance was increased ~4.3-fold above the control (Fig. 5G). These changes in p57 mRNA abundance closely resemble the regulation detected by HCIF (Figs. 2, 4, and 5), suggesting that the changes in p57 observed here occur mainly at the level of mRNA abundance.

To establish the timing of changes in p57 mRNA in response to growth factor signaling, we performed time-course measurements of p57 mRNA upon EGF treatment or withdrawal. When cells growing in the presence of IGF-1 alone were co-treated with EGF, p57 mRNA abundance decreased rapidly between 3 and 6 hours (Fig. 5H). When MCF-10A cells growing in the presence of EGF and IGF-1 were shifted to IGF-1 alone, an increase in the abundance of p57 mRNA was apparent by 2 hours and reached a plateau by 12 hours (Fig. 5I). These kinetics are consistent with the time scales of transcriptional induction by growth factor signaling pathways and mRNA turnover (29, 30).

The ERK and Akt pathways control p57 in epithelial and tumor cells

To determine the range of cell types in which Akt- and ERK-dependent regulation of p57 is relevant, we assessed the function of this network in various epithelial, non-epithelial, and cancer cell lines. With MCF-12A and 184A1 cells, non-transformed mammary epithelial lines derived from different individuals, p57 responses to IGF-1 and EGF stimulation and ERK or PI3K inhibition were essentially concordant with those seen in MCF-10A (Fig. 6A, fig. S6 and S7); differences in the response of 184A1 cells could be ascribed to efficiency of pathway inhibition (Fig. S7). Similar responses were also found in an immortalized prostate epithelial line, PWR-1E (Fig. 6B and S6). In agreement with the mammary epithelial lines, PI3K-mTOR inhibition in PWR-1E caused a marked reduction in p57 abundance, whereas inhibition of the ERK pathway strongly increased p57 abundance. Non-epithelial human umbilical vein endothelial cells (HUVECs) had little basal p57, but nonetheless modestly downregulated p57 upon PI3K-mTOR inhibition (Fig. S8). Thus, stimulation of p57 by the PI3K pathway, and inhibition by the ERK pathway, appears to be a common program in non-tumor epithelial cells.

Among breast cancer cell lines examined, nuclear p57 was difficult to detect above background fluorescence, in agreement with previous observations of low p57 abundance in these lines (31, 32). In MD-MBA-468 and T47D cells, treatment with inhibitors of MEK or of DNA methylases had limited or no effect on the abundance of p57, whereas the histone deacetylase inhibitor trichostatin A (TSA) induced a 2–4 fold increase in p57 abundance (Fig. 6C,D). In the context of TSA treatment, p57 abundance was not further increased by MEK inhibition, but was markedly reduced by PI3K-mTOR inhibition. These results suggest that, in these breast cancer cells, p57 is primarily suppressed by chromatin modification and requires the PI3K-mTOR pathway for full induction. In contrast, in U2OS osteosarcoma cells, which are derived from a non-epithelial lineage, inhibition of either ERK or PI3K-mTOR increased p57 abundance by 2–3 fold (fig S9). Together, these observations indicate that the p57 network originally identified in non-tumor cells remains

partially intact in tumor cells; in some tumors p57 is suppressed directly by high ERK activity, whereas in others this network is overridden by chromatin modification.

The frequent loss of p57 in tumors and its ability to specifically limit IGF-1-driven proliferation suggests that it may suppress oncogenic signaling through the PI3K pathway. To test this hypothesis, we employed MCF-10A cells in which PIK3CA mutations associated with human tumors (E545K or H1047R) have been stably integrated at the genomic PIK3CA locus (33). As previously reported, cells harboring either mutation maintain moderate proliferative activity in the absence of EGF. Strikingly, the percentage of pRb-positive PI3K mutant cells was enhanced 2–4 fold in the absence of EGF by shRNA-mediated depletion of p57, relative to mutant cells expressing non-targeting shRNA (Fig. 6E–G). In agreement, p57 depletion also resulted in an increase in cell number (Fig. 6H).

We next assessed the role of p57 in a three-dimensional in vitro model of cellular transformation (34). In this model, mammary epithelial cells form multicellular spheroids resembling mammary acini in vivo, initially dividing but eventually reaching a state of proliferative arrest. Microarray analysis revealed that p57 mRNA abundance increased steadily over the course of morphogenesis, reaching an 18-fold increase by day 15 (Fig. 7A). This increase occurred simultaneously with proliferative arrest, as assessed by DNA content analysis. In contrast, p21 and p27 mRNAs showed a much more modest increase (~2-fold) over the same period. Cells transduced with p57-targeting shRNA formed acini ~1.5-fold larger than those formed by cells transduced with control shRNA vectors (Fig. 7B,D). This increase in size was accompanied by a marked increase in the number of cells positive for the proliferation marker Ki-67 within the acini at day 10 (Fig. 7C). p57-depleted acini frequently displayed filled lumens, which may result from an increase in proliferation exceeding the rate of cell death in the inner cells. Thus, p57 acts as a barrier to uncontrolled proliferation in 3D as well as monolayer culture.

Discussion

Here we demonstrate a mechanism by which ERK and Akt signals are integrated in the control of cellular proliferation through differential regulation of the CDK inhibitor p57. Under conditions in which Akt is activated in the absence of detectable ERK activity (for example, in MCF-10A cells treated with insulin or IGF-1 alone or induced to express activated Akt), p57 abundance increases, suppressing cell proliferation. In contrast, under conditions in which ERK is activated simultaneously with Akt (for instance, in EGF-treated cells or IGF-1-treated cells expressing activated H-Ras) this increase in p57 abundance is suppressed, permitting proliferation. Additionally, knockdown of p57 in the context of insulin or IGF-1 treatment or expression of a stably integrated oncogenic mutant variant of PI3K enhanced proliferation of mammary epithelial cells. These results indicate that p57 plays a critical role in suppressing proliferation in mammary epithelial cells, and that the abundance of p57 is sensitive to the strength of PI3K-Akt and ERK signaling.

The regulation of p57 by opposing ERK and Akt signals has not previously been described. Previous work has identified numerous integration points downstream of the ERK and Akt pathways, including cyclin D1, c-Myc, and mTOR; however, in these pathways, the

integrators respond positively to both ERK and Akt signals (4, 35–38). In contrast, we describe a mechanism whereby one growth factor-stimulated pathway (ERK) is required to negate the anti-proliferative function of another (Akt). Such a regulatory mechanism could serve to “tune” the proliferative response of a cell to the specific intensity of signaling or stimuli. For example, the proliferative response to activation of the insulin or IGF-1 receptors is highly context dependent. Treatment with either of these factors alone does not support proliferation in many cell contexts (including, notably, in MCF-10A cells grown in serum-free medium) (39–41), whereas either one alone is sufficient to induce proliferation in other contexts (42, 43). Our data indicate that changes in p57 abundance due to the differential induction of Akt and ERK determine, in part, the response to stimulation by these growth factors. These observations can also be seen as a point of cooperation or synergy between the ERK and Akt pathways: the full Akt-mediated effect on proliferation is only achieved when p57 is concomitantly suppressed by ERK. In some contexts however, control of p57 by ERK and Akt may be superseded by other means of regulation, such as chromatin remodeling or genomic imprinting at the CDKN1C locus (44–46).

The other members of the CIP/KIP family, p21 and p27, display different response profiles to ERK, Akt, and other signals (13, 16, 17). Thus, the proliferative response to specific upstream signaling pathways may be regulated by modulating the particular complement of CIP/KIP family members present in the cell. Our examination of different cell lineages suggests that p57 may be of particular importance in epithelial cells. The importance of maintaining such a balance in physiological situations is supported by our finding that downregulation of p57 causes hyperproliferative effects in 3D acini, a model for growth regulation of ductal structures in mammary tissue.

The ERK-Akt-p57 circuit could also serve as sensor for “imbalanced” or oncogenic signaling. Many cancers carry mutations in the PIK3CA isoform of PI3K or loss of PTEN. Based on our studies, such alterations would be predicted to induce activation of the Akt pathway without activating ERK, leading to induction of p57 and preventing inappropriate cell proliferation. This hypothesis would suggest that the barrier imposed by p57 would need to be overcome at some point in the progression of tumors driven by PI3K network hyperactivation. The ability of the ERK pathway to suppress p57 may create a selective pressure favoring concomitant mutations in the PI3K and ERK pathways (47). A careful bioinformatic study of the mutational status of these pathways in conjunction with p57 status will be necessary to evaluate this hypothesis.

Consistent with its tumor suppressor role, p57 is frequently inactivated through multiple mechanisms in cancer (20). The large majority of breast cancers display decreased p57 abundance at the histological level (48), and p57 expression is frequently suppressed by epigenetic silencing of the CDKN1C locus in both breast and ovarian cancers (31, 49, 50). Analysis of microarray data from a large panel of breast cancer cell lines (32) revealed that 46 out of 49 tumor-derived lines displayed low p57 mRNA abundance relative to the two non-tumor cell lines included in the panel, MCF-10A and MCF-12A. In the immortalization process of normal mammary epithelial cells, p57 is upregulated following bypass of replicative senescence, but silenced upon full immortalization (51). Together, these data suggest that the regulation of p57 by ERK and Akt plays a role in suppressing proliferation

in response to aberrant signaling during the initial stages of carcinogenesis; this regulatory function is then lost in the later stages of transformation when the CDKN1C locus is silenced.

Although our data demonstrate regulation of p57 at the mRNA level by EGF and IGF-1 through the ERK and Akt pathways, respectively, the intermediate links between these kinases and p57 mRNA regulation remain to be elucidated. The ERK pathway activates various transcriptional modifiers, including AP-1 (activating protein 1), c-Myc, Elk-1, and EZH-2 (enhancer of zeste homolog 2), whereas the Akt pathway controls Foxo3a, CREB (cAMP-response element binding protein), AP-1, c-Myc, and others. The histone methyltransferase EZH-2, which has been implicated in repression of p57 expression, is activated transcriptionally by ERK (52) and inactivated through phosphorylation by Akt (53), making it a potential mediator of p57 regulation. Another attractive mechanism for downregulation of p57 by ERK is the c-Myc-induced miRNA-221/222, which targets p57 (22, 23). However, because both IGF-1 and EGF increase c-Myc abundance in MCF-10A cells (with EGF doing so more strongly, see Fig. 1), this mechanism is insufficient to explain the difference between IGF-1 and EGF in p57 regulation, unless miR-221/222 induction is highly sensitive to different amounts of c-Myc. It is likely that a number of distinct transcriptional and post-transcriptional regulators contribute to p57 regulation by the ERK and Akt pathways.

Because insulin and Akt suppress ERK activation (10), it is possible that Akt induction of p57 could occur through decreased ERK suppression of p57 mRNA transcription. However, several results argue against this mechanism and indicate that p57 induction requires a positive input from the Akt pathway, not simply suppression of ERK signaling. First, myrAkt is able to increase the abundance of p57 mRNA and protein in cells deprived of growth factors for 48 hours, where there is no detectable pERK signal. Second, induction of p57 by MEK inhibition in the context of EGF stimulation is blocked by PI3K-mTOR inhibition.

A major challenge in signal transduction research is to understand how complex networks integrate multiple signals to reach a phenotypic cell fate decision. Computational models have been developed that focus on predicting the degree and kinetics of ERK and Akt activation in response to receptor activation or pharmacological inhibition (54, 55). However, the connections downstream of ERK and Akt that determine cell cycle control and proliferative outcome have yet to be extensively modeled. Developing a quantitative understanding of the connection between upstream signals and downstream phenotypes will be essential for predicting the efficacy of inhibitors targeted toward these pathways, and these models will depend on accurate “wiring diagrams” of the multiple integration points between ERK and Akt pathway effectors. The mechanism described here adds a new integration point critical for epithelial cell cycle regulation by these signals; additional studies will be needed to understand the relative importance of this and other integration mechanisms, which will likely be highly context-dependent.

Materials and Methods

Cell Culture

MCF-10A mammary epithelial cells and the clonal derivative 5E (56), were cultured as previously described (34) in MCF-10A growth medium (DMEM/F12 (Invitrogen) supplemented with 5% horse serum, 20 ng/ml EGF, 10 µg/ml insulin, 0.5 µg/ml hydrocortisone, 100 ng/ml cholera toxin, 50 U/ml penicillin, and 50 µg/ml streptomycin). Experimental treatments with varying concentrations of growth factors were prepared in GM-GFS medium (DMEM/F12 supplemented with 0.3% BSA, 0.5 µg/ml hydrocortisone, 100 ng/ml cholera toxin, and 50 U/ml penicillin, and 50 µg/ml streptomycin). HUVEC cells were obtained from Invitrogen, and 184A1 cells from ATCC. MCF-12A and 184A1 cell lines were cultured in the same growth medium as MCF-10A cells, PWR-1E cells in keratinocyte serum-free medium (Invitrogen), HUVEC cells in M200+LSGS (Invitrogen), U2OS cells in McCoy's 5A medium supplemented with 10% FBS, and T47D and MD-MBA-468 cells in RPMI medium supplemented with 10% FBS.

Reagents

The following primary antibodies were used: p21 [2947 Cell Signaling (CS), 556430 BD Biosciences (BD)], p27 (36865 CS, 610241BD), p57 (56341 Santa Cruz (SC), 2557 CS), phospho-ERK1/2 (4370 CS), phospho-Rb-S780 (12901 SC), phospho-Akt-S473 (4060 CS), FoxO3a (2497 CS), c-Fos (2250 CS), c-Myc (5605 CS), phospho-S6 (4838 CS), c-Jun (9165 CS), Egr-1 (4153 CS), Fra-1 (28310 SC), Ets-1 (55581 SC), Elk-1 (65986 SC), Alexa fluor 488, 555, 647 (Invitrogen). The following compounds and small molecule inhibitors were used: IGF-1 (R&D Systems), DMSO (Sigma D2650), PIK90 (Axon Medchem-1362), GDC-0941 (Axon-1377), BEZ-235 (Axon-1281), Rapamycin (Calbiochem-553210), PD325901 (Calbiochem-444966), PD98059 (Calbiochem-513000), Torin-1 (Nathanael Gray), trichostatin A (Calbiochem), 5'-aza-deoxycytidine (Calbiochem).

DNA Vectors and Generation of Cell Lines

The pMSCV-puro-ER-myrAkt retroviral vector was generated from pWZLmyrAkt-HA-ER (a gift from R. Roth, Stanford University, Stanford, CA). pBabe-puro and pBabe-puro-HRas^{V12} vectors were previously described (57). Lentiviral vectors (pLKO.1puro) encoding anti-p57 short hairpin RNAs (shRNAs) A (TRCN0000039679), B (TRCN0000039680), C (TRCN0000010486), and D (TRCN0000039678) were obtained from the RNAi Consortium. To generate cell lines, MCF-10A or MCF-10A-5E cells were infected with the retroviral or lentiviral vectors, and stable populations were selected with 2 µg/ml puromycin.

High-Content Immunofluorescence Microscopy

Cells cultured in 96-well optical bottom plates (Corning) were fixed with 2% paraformaldehyde (PFA) and permeabilized with methanol at -20°C. Odyssey Blocking Buffer (Li-Cor) was used for blocking and antibody dilution. Following incubation with primary and secondary antibodies, plates were imaged on a CellWoRx Scanner (Applied Precision). Three to four image fields were collected in each well of a 96 well plate (uniform count per experiment) and processed in MATLAB (Mathworks) using segmentation routines

derived from CellProfiler (58), with Otsu thresholding for nuclear identification and an annulus of 5 pixels radial to the nucleus for the cytoplasmic region. Routine inspection of cell segmentation typically revealed successful identification of nuclei for greater than 85–90% of cells. Single-cell fluorescence intensity values for each channel were calculated as the mean pixel value for the nuclear and cytoplasmic regions.

Growth curve analysis

Cells were seeded in optical-bottom 96 well plates and cultured under the indicated conditions, and medium was replaced every 2 days. At the indicated time points, cells were fixed in 100% methanol, stained with DAPI, and imaged with >95% well coverage. Nuclei were counted computationally following segmentation using ImageRail (59). Cell counts were normalized to the number of cells on duplicate plates fixed at the time of initial treatment (day 0).

Quantitative Real-Time PCR

Total RNA was prepared from cells using TRIzol Reagent (Invitrogen). cDNA was synthesized from 2µg RNA using the SuperScript First-Strand Synthesis System (Invitrogen). Real-time PCR was performed using an ABI Prism 7900HT Fast Real-Time PCR System with gene-specific primers (cdkn1c forward 5'-GCGGCGATCAAGAAGCTG-3', reverse 5'-CGACGACTTCTCAGGCGC-3'; RPLPO-forward 5'-ACGGGTACAAACGAGTCCTG-3', RPLPO-reverse 5'-CGACTCTTCCTTGGCTTCAA-3') and Power Sybr Green PCR Master Mix (Applied Biosystems). Relative mRNA abundances were determined and normalized to the ribosomal protein RPLP0.

3D Morphogenesis Assays

3D culture of MCF-10A cells in Matrigel (BD Biosciences) was performed as previously described (34). MCF-10A control and p57 knockdown acini were cultured in assay medium (DMEM/F12 supplemented with 2% horse serum, 5 ng/ml EGF, 10 µg/ml insulin, 0.5 µg/ml hydrocortisone, 100 ng/ml cholera toxin, 50 U/ml penicillin, 50 mg/ml streptomycin, and 2% Matrigel) and re-fed every 4 days.

Microarray and Immunofluorescence Analysis

Microarray and immunofluorescence analysis of 3D acinar structures were performed as described previously (34, 60). Indirect immunofluorescence and phase imaging were performed on a Nikon TE300 microscope equipped with a mercury lamp and CCD camera.

Statistical Analysis

For quantitative PCR experiments, averaged values represent the averages from independent replicate experiments and error bars the standard error of measurement (SEM). For HCIF experiments, values from individual wells containing 2,000–10,000 cells were represented as the median single-cell intensity value. Because of day-to-day variations in fluorescence values, quantitation of HCIF experiments is shown as the averaged median fluorescence value of triplicate samples collected on the same day, and error bars represent the standard

deviation (SD) All experiments were repeated independently at least three times to confirm that the data shown were representative in both magnitude and overall trend. *p* values were calculated by Student's two-tailed *t* test and indicated as follows: *p*<0.05, (*); *p*<0.01, (**); (***) *p*<0.001.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Summary

The cell cycle inhibitor p57^{Kip2} is induced by Akt but suppressed by ERK in non-transformed mammary epithelial cells, forming a regulatory circuit that controls the differential proliferative response to growth factors and limits oncogenic behavior.

Figure 1. Differential regulation of proliferation and the Akt and ERK pathways by EGF, insulin, and IGF-1

A. Percentage of pRb-positive cells at various EGF concentrations in the absence or presence of 10 μ g/ml insulin or 100 ng/ml IGF-1. pRb was detected by HCIF after 24 hours. Values shown indicate the mean $\pm$ SD of triplicate measurements from one representative experiment that was repeated three times.

B. Immunofluorescence images of pERK, FoxO3a, and pRb in response to 20 ng/ml EGF or 100 ng/ml IGF-1. Numbers in the lower right indicate the average ratio of nuclear to cytoplasmic staining intensity for Foxo3a. Images are representative of three independent experiments.

C. Quantitation of key signaling proteins in cells treated with 20 ng/ml EGF or 100 ng/ml IGF-1. The indicated protein signals were detected by HCIF after 24 hours. Histograms represent distributions of single-cell intensities, where the x-axis represents intensity of the signal, and the y-axis represents the frequency of cells at each intensity normalized with the mode as 1. Data are representative of two independent experiments.

Figure 2. Dynamics of p57 and p21 regulation coordinated by IGF-1 and EGF

A. HCIF images of p57 in response to varying combinations of EGF and IGF-1. MCF-10A cells were treated as in Fig. 1.

B,C. Histograms of p57 (B) or p21 (C) abundance determined by HCIF for the conditions shown in (A).

D. Covariate single-cell analysis of p21 or p57 and pRb. Top: density scatter plots of p57 or p21 vs. pRb signal for individual cells as measured by HCIF. Bottom: analysis of pRb as a function of p57 or p21 for cells treated with 0.5 ng/ml EGF and 100 ng/ml IGF-1. Individual cell measurements were binned according to p57 or p21 abundance (x-axis), and the percentage of pRb-positive (%pRb+) cells present in each bin was calculated. Curves indicate the mean, and points the individual values, of the %pRb+ for each bin from triplicate measurements. Vertical pink lines denote thresholds for p57 or p21 abundance determined by the midpoint of the decline in %pRb+.

E. Frequency of p57- or p21-positive cells under different growth factor conditions, using the thresholds defined in (D).

F. Covariate analysis of p21 and p57 abundance for cells treated with 0.5 ng/ml EGF and 100 ng/ml IGF-1.

All data shown are from individual experiments representative of at least three independent replicates.

Figure 3. Enhancement of IGF-1-stimulated proliferation in p57-depleted cells

A. HCIF analysis of p57 knockdown. MCF-10A cells stably expressing shRNA hairpins targeting p57 or a non-targeting control hairpin were grown in the presence of 100 ng/ml IGF-1 and the absence of EGF for 2 days.

B. HCIF analysis of Proliferative response in p57-depleted cells. Control and p57 shRNA-expressing MCF-10A cells were cultured in the presence of the indicated growth factors for 2 days.

C. Quantitation of p57 depletion by shRNA. Histograms represent distributions of p57 intensity for cells cultured as in (A), and knockdown is shown as the percent change in median p57 intensity.

D. Quantitation of pRb-positive p57-shRNA cells cultured in the absence of growth factors or the presence of 100 ng/ml IGF-1 or 20 ng/ml EGF for 2 days. Bars represent the average of triplicate wells from one representative experiment, $\pm$ SD.

E. Growth curve analysis of p57-depleted cells. MCF-10A cells stably expressing the indicated shRNAs were cultured in the presence of the indicated growth factors. Cell counts were determined by high-throughput imaging of DAPI-stained cells and normalized to the number of cells at the time of treatment. Error bars indicate SD of triplicate wells, and results shown are representative of three independent experiments.

F. Quantitation of pRb-positive p21 $\pm$ or $-/-$ MCF-10A cells cultured as in (D). Bars represent the average of three independent wells, $\pm$ SD.

All data shown are from individual experiments representative of at least three individual replicates.

Figure 4. Stimulation of p57 by the Akt network

A. HCIF images of MCF-10A cells cultured in the presence of IGF-1 (100 ng/ml) and 1 μ M Torin-1, 0.5 μ M BEZ-235, 0.2 μ M GDC-0941, or 20 nM rapamycin for 24 hr.

B. Quantitation of nuclear p57 by HCIF under the conditions shown in (A). Left: histograms of p57 abundance. Right: frequency of the percentage of 57-positive (%p57+) cells. The threshold for p57-positive cells was defined as in Fig. 2; bars indicate the mean, and error bars the range, of duplicate measurements.

C. HCIF quantitation of p57 stimulation by inducible myristoylated-Akt. MCF-10A cells stably expressing an inducible Akt variant were cultured in the absence of growth factors for > 48 hr and then stimulated with vehicle (EtOH) or inducer (1 μ M 4'-OHT) in the presence or absence of 20 ng/ml EGF for the indicated times.

D. Timing of p57 induction by Akt measured by HCIF. Cells were treated as in (D) for varying periods of induction prior to fixation. Curves indicate the average, and error bars SD, of four replicate measurements of median p57 intensity.

All data shown are from individual experiments representative of at least three independent replicates.

Figure 5. Opposing regulation of p57 mRNA by the Akt and ERK pathways

A. HCIF images of p57 in MCF-10A cells cultured with EGF (20 ng/ml) or IGF-1 (100 ng/ml), in the presence of vehicle (DMSO) or 1 μ M MEK inhibitor (PD0325901) for 24 hours.

B. Quantitation of p57 intensity for the conditions shown in (A). Histograms represent distributions of p57 abundance, and vertical dashed lines indicate thresholds for p57-positive cells, determined as in Fig. 2. Percentage values indicate the frequency of p57-positive cells for DMSO- (gray) or inhibitor-treated (blue) conditions.

C. HCIF analysis of p57 suppression by activated H-Ras. MCF-10A cells stably expressing vector control or H-Ras^{V12} were cultured in the presence of IGF-1 or 1 μ M MEK inhibitor (PD0325901) for 24 hours.

D. HCIF analysis of combined MEK and PI3K-mTOR inhibition. MCF-10A cells were cultured in the presence of 20 ng/ml EGF with or without 1 μ M PD0325901 plus 1 μ M Torin-1, or 0.5 μ M BEZ-235 as indicated.

E. Summary of p57 intensity as a function of pERK and pAkt abundance. For all conditions shown in Figs. 2A, 4A, and 5A, pERK, pAkt, and p57 were measured by HCIF and shown as a scatter plot; p57 abundance is represented by size and intensity of the symbols.

F. Quantitation of p57 mRNA abundance by qPCR. Cells were treated with the indicated conditions for 24 hours. Values shown are the average of four independent experiments, $\pm$ SEM.

G. Quantitation by qPCR of p57 mRNA abundance in response to Akt induction. MCF-10A cells stably expressing inducible Akt were cultured as in Fig. 4. Values are the average of three independent experiments, $\pm$ SEM.

H,I. Time-dependence of EGF- and IGF- mediated changes in p57 mRNA abundance. H. MCF-10A cells cultured in the presence of IGF-1 were shifted to medium containing IGF-1 and EGF at time 0 (red) or maintained in IGF-1 alone (gray). I. MCF-10A cells cultured in the presence of EGF and IGF-1 were shifted to medium containing only IGF-1 at time 0 (red) or maintained in EGF and IGF-1 (gray). p57 mRNA was quantitated by qPCR at the indicated times. Values and error bars represent the mean and SEM of three independent experiments.

Unless otherwise noted, all data shown are from individual experiments representative of at least three independent replicates.

Figure 6. Regulation of p57 in epithelial and cancer cell lines

A, B. Quantitation of p57 by HCIF in (A) MCF-12A mammary epithelial cells and (B) PWR-1E prostate epithelial cells treated with the indicated growth factors and inhibitors of MEK (PD) or PI3K-mTOR (BEZ) for 24 hours. The percentage of p57-positive cells was determined as in Fig. 2. Error bars represent the SD of triplicate wells.

C, D. Quantitation of p57 by HCIF in T47D (C) and MD-MBA-468 (D) cells cultured in the presence of the indicated inhibitors of MEK (PD), PI3K and mTOR (BEZ), histone deacetylases (trichostatin A TSA), or DNA methyltransferases (5'-aza-deoxycytidine - AZA) for 24 hours. Bars represent the average p57 intensity of triplicate wells $\pm$ SD. Under some conditions (marked by †) a large percentage of cells committed apoptosis; p57 fluorescence values were derived from surviving cells.

E. HCIF images of shRNA-mediated p57 depletion in PIK3CA-mutant cells. MCF-10A cells containing mutant alleles of PIK3CA (E545K or H1047R) at the endogenous locus and stably expressing non-targeting (NT) or p57-specific shRNA were cultured in the absence or presence of EGF for 2 days.

F. HCIF images of Rb phosphorylation in PIK3CA mutant cells. Cells were cultured as in (E).

G. Quantitation of pRb-positive cells for the conditions in (E). Bars represent the average of three independent wells, $\pm$ SD.

H. Growth curve analysis of p57-depleted PIK3CA mutant cells. Cells were cultured as in (E), and cell counts were determined by HCIF analysis of DAPI-stained nuclei. Error bars represent the SD of triplicate wells.

All data shown are derived from individual experiments representative of at least three independent replicates.

Figure 7. Hyperproliferation in p57-depleted cells in 3D culture

A. Microarray and proliferation analysis of CDKN1 family mRNA abundance in MCF-10A cells during 3D morphogenesis. mRNA abundance for p21, p27, and p57 are shown normalized to the amount on day 2, +/- SEM of triplicate measurements (left vertical axis). The percentage of cells with >2N DNA content (% in G2-S, gray) was assessed by flow cytometry following trypsinization and staining with propidium iodide (right vertical axis). Data shown are from one experiment representative of two independent replicates.

B. Phase-contrast images of acinar structures formed by MCF-10A cells transduced with control or p57-targeting shRNA vectors at day 10 in the 3D morphogenesis assay. Data shown are from one experiment representative of four independent replicates.

C. Immunofluorescence detection of Ki-67 in control or p57-shRNA acinar structures at day 10 of 3D culture. Data shown are from one experiment representative of two independent replicates.

D. Quantitation of acinar structure size for control and p57-shRNA acinar structures at day 17 in 3D culture. Data shown are from one experiment representative of three independent replicates.