Cytoplasmic p27 promotes epithelial–mesenchymal transition and tumor metastasis via STAT3-mediated Twist1 upregulation

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INTRODUCTION

Metastasis is the leading cause of cancer-related death in most tumor types. Despite its devastating consequences, metastasis has long been recognized as an inefficient process in which cancer cells must overcome a series of challenges to spread from primary to distant sites.1 Cancer cells must acquire the ability to invade locally, intravasate, extravasate and colonize organs to form distant metastases.1 Local invasion, a critical event in which cancer cells dissociate from a primary tumor and invade surrounding stroma, involves reactivation of an embryonic developmental program referred to as the epithelial-to-mesenchymal transition or EMT. EMT is characterized by loss of E-cadherin (CDH1) expression via the upregulation of genes encoding CDH1-repressing transcription factors including TWIST1, SNAI1 and ZEB1.2 There is evidence that cancer cells recirculate after seeding to distant sites, and can reseed the primary tumor.3 Thus, the EMT may be needed not only to initiate but also to maintain the metastatic process. A greater understanding of how EMT promotes invasion and metastasis may ultimately permit the design of more effective cancer therapies.

The present study provides novel evidence that the CDK inhibitor, p27, may acquire oncogenic potential to activate an EMT and drive subsequent metastasis. p27 was initially identified as a cyclin _E–CDK2_ inhibitor that mediates the G1-phase cell cycle arrest.4–7 In keeping with its CDK inhibitory function, p27 knockout mice show increased body size, and p27 haploinsufficient mice are prone to carcinogen-mediated tumor formation, suggesting that p27 controls both tissue expansion and cell proliferation.8–11 Loss of nuclear p27 is frequent in many human cancers and correlates with worse prognosis.12 In contrast to other tumor suppressors such as p53 or p16, p27 is rarely deleted or fully lost in human cancer.12,13 This is likely because p27 deregulation can confer pro-oncogenic gain of function. Although cell cycle inhibition is a nuclear function of p27, many cancers undergo pro-oncogenic changes when p27 is mislocalized to the cytoplasm.12,13

Deregulated, cytoplasmic p27 promotes tumor progression through the activation of cell motility and invasion.14–16 In hepatocellular carcinoma cells, TAT-p27 protein transduction increased motility and cytoplasmic p27 colocalized with actin fibers to stimulate cell migration.17 p27-null mouse embryonic fibroblasts have decreased motility that is rescued by either wild-type p27 or by a p27 mutant defective for cyclin and CDK binding (p27CK−−), indicating that p27 effects on cell motility are independent of its cell cycle actions.15 Cytoplasmic p27 can bind RhoA, impair RhoGEF action and reduce RhoA–ROCK signaling, leading to dynamic cytoskeletal rearrangements that increase cell motility.15

Here, we identify EMT-promoting effects of p27 that are driven by oncogenic PI3K pathway activation. Several PI3K effectors can phosphorylate p27 in the C terminus to shift its steady-state

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localization from the nucleus to the cytoplasm. Phosphorylation of p27 at threonine 157 (T157) by AKT18-20 or SGK21 impairs its nuclear import, whereas phosphorylation at T198 by AKT22,23 or RSK24,25 stabilizes cytoplasmic p27.26,27 These C-terminal p27 phosphorylations appear to be processive and to promote binding and inhibition of RhoA-Rock to drive tumor invasion.25 This may explain, in part, how activated Ras cooperates with p27CK– to induce murine tumorigenesis.26 Indeed, our earlier work showed that a dual phosphatidylinositol 3-kinase/mammalian target of rapamycin (PI3K/mTOR) inhibitor could abrogate bone metastasis of a highly metastatic breast cancer line through restoration of nuclear p27.29

The present work reveals a novel mechanism whereby PI3K pathway activation and the accumulation of C-terminally phosphorylated p27 promote human tumor metastasis. We provide evidence that the C-terminal p27 phosphorylations at T157 and T198 confer a pro-oncogenic function to promote tumor metastasis by activating STAT3 (signal transducer and activator of transcription 3) to induce TWIST1 and drive EMT. Highly metastatic phenotypes in breast and bladder cancer models were reversed by p27 knockdown and rescued in part by constitutively activated STAT3 (STAT3CA). These data provide a novel mechanism whereby p27 deregulation by oncogenic PI3K/mTOR activates pSTAT3 to drive human cancer progression. Pharmacological inhibition of signaling pathways that drive p27-mediated EMT may ultimately prove effective in preventing or reversing cancer metastasis.

RESULTS

Overexpression of phosphomimetic p27CK-DD induces/enhances EMT in human mammary epithelial and cancer cells

Prior work showed that mutations converting T157 and T198 to aspartate in p27 are phosphomimetic.21,24,25 To test if negative charges at both sites cooperate to drive these effects, single and double phosphomimetic mutations (T157D, T198D or DD) were inserted into a p27 mutant that cannot bind either cyclins or CDKs (CK–) (Supplementary Figure S1A).30,31 To test if C-terminally phosphorylated p27 may contribute early in the process of malignant transformation, these different phosphomutant p27 vectors were transduced into the immortalized, non-transformed human mammary epithelial cell line MCF-12A (MCF-12A-p27CK-DD). While the expression of each phosphomimetic p27 mutant significantly increased cell migration, p27CK-DD enhanced MCF-12A migration most significantly and caused these cells to acquire the ability to invade matrigel (Figures 1a and b).

Similarly, in MDA-MB-231 cells, while p27CK– alone had a very modest effect, each phosphorylation site appears to contribute to cytoplasmic p27 localization and p27CK-DD caused the greatest increase in cell motility, invasion and p27 mislocalization (Supplementary Figures S1B–E). Neither p27CK– nor any of the CK– mutants bearing single or double phosphomimetic mutations affected the cell cycle (Supplementary Figure S1F). Relative levels of endogenous p27 and p27CK-DD are shown in Supplementary Figures S1G and H for the 231 model and in Supplementary Figures S2A and B for MCF-12A.

Notably, p27CK-DD-overexpressing MCF-12A cells underwent a progressive, morphological switch from a typical, cobblestone-like epithelial appearance to an elongated, spindle-like, mesenchymal shape over the next 4 weeks (Figure 1c), indicative of EMT. p27CK-expressing cells retained their cobblestone morphology, suggesting that the C-terminal phosphorylation of p27 is required for its action on the EMT (Figure 1c). p27CK-DD-overexpressing MCF-12A showed decreased levels of the epithelial marker, E-cadherin, and increased mesenchymal markers, N-cadherin and vimentin (Figures 1d, f). In MCF-12A cells, expression of vector alone or of the p27CK– vector lacking T157D and T198D mutations did not upregulate EMT drivers and mesenchymal markers (Supplementary Figure S2C). Thus, introduction of the CK– mutations and loss of cell-cycle inhibitory p27 function are not sufficient to drive p27-mediated EMT, and p27 phosphorylations at T157 and T198 appear to be required.

Similar findings were observed in the hTert-immortalized, normal human mammary epithelial HME3 line,6,26 which showed a significant increase in mesenchymal marker expression, and enhanced cell migration and invasion following stable p27CK-DD transduction (Supplementary Figures S2D–G). Thus, overexpression of a p27 protein defective for CKD inhibition and bearing phosphomimetic mutations at T157 and T198 induces an invasive EMT phenotype in non-tumorigenic human mammary epithelial cells.

The EMT program is initiated by EMT-inducing transcription factors including Snail, Slug, zinc-finger E-box-binding homeobox 1 (Zeb1) and Twist.27 To elucidate which transcription factors contribute to p27CK-DD-mediated EMT, several were screened by QPCR in MCF-12A-p27CK-DD and control cells. Although p27CK-DD modestly increased SNAI1 (encoding Snail), SNAI2 (encoding Slug) and ZEB2 expression, TWIST1 expression increased by 20-fold (Figure 1e), suggesting that TWIST1 may have a critical role during p27CK-DD-induced EMT. p27CK-DD also increased Twist1 protein and its nuclear localization was confirmed by direct immunofluorescence (Figures 1d, f). Indeed, TWIST1 knockdown significantly attenuated the EMT phenotype, causing re-expression of E-cadherin, loss of mesenchymal markers, N-cadherin and vimentin (Figure 1g), and loss of the excess motility and invasive potential of MCF-12A-p27CK-DD cells (Figures 1h and i), supporting the notion that TWIST1 induction is a major driver of the p27CK-DD-induced EMT phenotype in immortalized human mammary epithelial cells.

p27CK-DD overexpression in the luminal A, MCF-7 breast cancer line also induced a morphological change compatible with EMT, with increased expression of mesenchymal markers (N-cadherin and vimentin) and TWIST1, and an increase in MCF-7 cell migration and invasion (Supplementary Figures S3A–E). Moreover, the effects of C-terminally phosphorylated p27 on EMT are not exclusive to breast cancer, as p27CK-DD significantly enhanced mesenchymal features of a human bladder cancer cell line, UMUC3 (Supplementary Figures S4A–E). Taken together, these data support a novel, oncogenic role in which p27 promotes EMT in human mammary epithelial cells and in weakly or non-metastatic breast and bladder cancer models.

Loss of p27 decreases mesenchymal characteristics in PI3K-activated metastatic lines

To assay if C-terminal phosphorylation of endogenous p27 contributes to EMT maintenance, the weakly metastatic MDA-MB-231 (231) breast cancer line and a derivative with enhanced lung metastatic ability, MDA-MB-231-4175 (4175)34 were compared. In all, 4175 cells showed strong PI3K pathway activation: activating phosphorylations of AKT, SGK and PDK1 were increased relative to parental 231 cells (Figure 2a). When equal amounts of cellular p27 were loaded from each line, 4175 cells showed a marked increase in both p27pT157 and p27pT198 compared with 231 cells (Figure 2b, upper panel). Subcellular fractionation followed by immunoblotting showed greater cytoplasmic p27 in 4175 cells compared with 231 cells (Figure 2b, lower panel). Stable p27 knockdown in 4175 increased E-cadherin, and decreased vimentin and Twist1 (Figures 2c and d). In 4175 cells, migration and matrigel invasion were both markedly decreased following p27 knockdown (Figure 2e). Stable p27 knockdown in 4175 cells did not alter cell cycle distribution (Figure 2f). Notably, small
interfering (siRNA)-mediated p27 knockdown significantly reduced TWIST1 expression within 8 h, followed by an increase in E-cadherin protein by 48 h (Figures 2g and h).

To validate these findings in a cancer from a different organ, we compared the UMUC3 bladder cancer line with a highly metastatic sister cell line, UMUC3-LuL2 (UMUC3-L), derived following selection and expansion of lung metastases from serial tail vein injections.35 As in the breast cancer models, the highly metastatic UMUC3-L line showed significant PI3K pathway activation and increased p27 compared with control UMUC3 (Figure 2i). Similarly, p27 knockdown significantly reduced mesenchymal characteristics, decreasing vimentin, N-cadherin and Twist1 expression (Figures 2j and k) and reduced cell motility (Figure 2l). Thus, in both metastatic breast and bladder cancer models with oncogenic PI3K activation, p27 appears to maintain an EMT program with increased cell motility and invasion.

p27CK-DD overexpression activates STAT3 to induce TWIST1 and activate EMT

To elucidate the oncogenic signaling pathway(s) involved in p27CK-DD-induced EMT, we screened for signaling kinases known to contribute to EMT. Y705-phosphorylated and total STAT3 protein and STAT3 mRNA were increased in MCF-12A-p27CK-DD cells compared with control MCF-12A (Figure 3a), as was nuclear STAT3 localization (Figure 3b). Nuclear STAT3 was minimal in
control MCF-12A and in MCF-12A-p27CK-DD cells treated with an 
STAT3 inhibitor. Prolonged STAT3 inhibition over 2 weeks in 
culture reversed the mesenchymal phenotype of MCF-12A-p27CK-
DD cells, shown by morphological change from spindle-shaped 
mesenchymal cells to a cobble-stone epithelial appearance 
(Figure 3b), increased E-cadherin and decreased mesenchymal 
markers and Twist1 expression (Figure 3c). STAT3 inhibitor 
treatment reduced the intense nuclear Twist1 expression in 
MCF-12A-p27CK-DD to levels seen in MCF-12A controls 
(Figure 3d). Interestingly, p27CK-DD overexpression markedly 
increased AKT activation, and this was attenuated by STAT3 
inhibitor treatment (Figure 3e), suggesting that AKT activation in 
p27CK-DD is STAT3 dependent. Transduction of dominant-
negative STAT3 (STAT3DN) into MCF-12A-p27CK-DD cells 
pheno-copied pharmacological STAT3 inhibition, with reversion of EMT 
(Figure 3f), and marked attenuation of p27CK-DD-induced cell 

Figure 2. Loss of p27 reverts EMT in p27pT157pT198-enriched metastatic lines. (a) Western of PI3K activation and p27 in MDA-MB-231 (231) 
and MDA-MB-231-4175 (4175). (b) Lysates containing equal amounts of p27 were immunoprecipitated for p27pT157 or p27pT198 or total p27, 
and immunoblotted for p27 in 231 and 4175 cells (top). 2X indicates 231 protein lysate used was two times the amount used for 4175 cells. 
Fractionated lysates show p27 distribution in the nucleus, N, and cytoplasm, C, (bottom). (c–f) 4175 cells were transduced with shp27 (+) or sh-
scramble controls (−) and compared as follows: QPCR for mRNA encoding p27, E-cadherin and vimentin (c), Western of p27 and EMT markers 
(d), Transwell migration (left) and transwell matrigel invasion (right) (e) and cell cycle distribution by flow cytometry (f, g). The 4175 cells 
were transfected with sip27 and then assayed over the times indicated for TWIST1 expression by QPCR (g), and p27 and E-cadherin expression 
by western blot (h). (i) Western blot of total and phosphorylated PI3K effector kinases in UMUC3 and UMUC3-LuL2 (UMUC3-L). (j–l) UMUC3-L 
cells with (+) or without (−) stable p27 knockdown (shp27) were compared by: western blot for indicated proteins (j), QPCR of p27, 
N-cadherin and vimentin (k, top). QPCR for p27 and TWIST1 mRNA in UMUC3-L cells treated with siRNA against p27 (+ sip27) or control oligo 
(−) (k, bottom). UMUC3-L cells with (+) or without (−) stable p27 knockdown (shp27) were assayed for transwell motility (l). All data graphed 
represent mean of at least three repeat ± s.e.m. *P < 0.05 by Student’s t-test compared with control.
migration and invasion (Figures 3g and h). Thus, p27CK-DD-induced EMT is reversible in immortalized, non-malignant mammary epithelial cells and STAT3 signaling is critical for p27-dependent TWIST1 induction and maintenance of this EMT phenotype.

p27 binds JAK2, increases JAK2-STAT3 complexes and enhances STAT3 transcriptional activity at the TWIST1 promoter

We next assayed effects of p27 on transcriptional activity at the TWIST1 promoter. As was the case for TWIST1 expression (Figures 2d, g), TWIST1 luciferase activity was also attenuated by p27 knockdown but not by scrambled short hairpin RNA controls (Figure 4a). Overexpression of STAT3-CA restored TWIST1 promoter activity in p27 knockdown cells. STAT3 inhibition reduced TWIST1 promoter activity in 4175 cells to the same extent as p27 knockdown (Figure 4a).

Quantitative chromatin immunoprecipitation (ChIP) assays revealed that STAT3 occupancy of the TWIST1 promoter was increased in 4175 cells compared with 231 cells and attenuated by p27 knockdown, whereas p27CK-DD transduction increased STAT3 binding to the TWIST1 promoter in 231 cells (Figure 4b). p27CK-DD expressing UMUC3 showed increased STAT3 binding to the TWIST1 promoter and this was reversed by STAT3DN; and p27 knockdown in UMUC3-L decreased STAT3 occupancy of the TWIST1 promoter (Figure 4c), supporting a model in which C-terminally phosphorylated p27 promotes STAT3 activation, driving STAT3 binding to the TWIST1 promoter and TWIST1 activation. Notably, ChIP assays failed to reveal co-occupancy of p27 with STAT3 on the TWIST1 promoter.

Figure 3. p27CK-DD drives STAT3 activation to induce EMT. (a) Western blot of pSTAT3 (Y705) and total STAT3 in vector control MCF-12A, C and MCF-12A-p27CK-DD (p27CK-DD) cells (left) and QPCR for STAT3 mRNA in control MCF-12A and MCF-12A-p27CK-DD cells (right). (b) Immunofluorescence (top) and phase-contrast (bottom) images of pSTAT3 in control MCF-12A and MCF-12A-p27CK-DD cells with or without treatment for 2 weeks with STAT3 inhibitor (STAT3-I). (c–e) Control MCF-12A and MCF-12A-p27CK-DD cells treated with or without STAT3-I for 2 weeks were assayed for: Twist1 and EMT markers by western blot (c), Twist1 protein by indirect immunofluorescence (d) and total and activated STAT3 and Akt levels by western blot. (f–h) MCF-12A vector-only controls and MCF-12A-p27CK-DD cells with or without STAT3DN transduction were compared for Twist1 and EMT markers expression (f), transwell migration (g) and transwell invasion (h). All data are graphed as mean ± s.e.m. *P < 0.05 by Student’s t-test.
We next assayed if p27pT157pT198 might bind to JAK2-STAT3 to promote STAT3 activation. p27 has been shown to bind JAK2 via both its catalytic and FERM domains. Treatment of 4175 cells with a dual PI3K/mTOR inhibitor, PF-04691502 (PF), at 250 nM reduced pAKT to parental 231 levels by 48 h (Figure 4d, top panel). This drug dose, which reduces p27pT157 and p27pT198 levels, also reduced total and activated STAT3, whereas JAK2 was unchanged (Figure 4d, top). Notably, JAK2-bound STAT3 and p27 were increased in 4175 cells compared with 231 cells (Figure 4d, bottom panel). Although total and activated STAT3 were modestly increased in 4175 cells compared with 231 and PF-treated 4175 cells, densitometric
quantitation showed the increases in JAK2-bound STAT3 and p27 in 4175 cells were greater than predicted by their abundance alone (Figure 4e), suggesting that p27 binding may facilitate recruitment of STAT3 to JAK2. p27pT198 precipitation using a T198-phospho-specific antibody revealed associated STAT3 in 4175 cells (Figure 4f). Although these complexes were detected in 231, the p27pT198 and associated STAT3 levels were too low to permit accurate quantitation (not shown).

Notably, when p27CK-DD was stably expressed in the weakly metastatic UMUC3 bladder cancer line, a trimeric JAK2/p27CK-DD/STAT3 complex was detected. JAK2-bound STAT3 was increased and STAT3 was activated (Figure 4g).

STAT3 inhibition attenuates p27CK-DD-induced cancer metastasis
As both induction and maintenance of an EMT program are critical for metastatic tumor progression,4,5,37,38 we next assayed effects of p27CK-DD overexpression with and without STAT3 inhibition on invasion and metastasis in our breast and bladder cancer models. p27CK-DD transduction into 231 cells increased STAT3 levels and
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STAT3 activation, increased Twist1, decreased E-cadherin (Figure 5a) and markedly increased cell migration and invasion (Figures 5b and c) over control 231 cells. Transduction of STAT3DN into 231-p27CK-DD cells reverted the mesenchymal phenotype, causing loss of Twist1, increased E-cadherin and reversed the p27-driven increase in cell migration and invasion (Figures 5a–c).

Upon tail vein injection into nude mice, both the number and size of lung metastasis formed from p27CK-DD-expressing 231 cells were significantly increased, as was cumulative tumor bioluminescence compared with parental 231 controls (Figures 5d and f). The p27CK-DD-mediated gain of metastatic potential was STAT3 dependent: STAT3DN transduction attenuated lung tumor formation by 231-p27CK-DD cells to levels similar to those of parental 231 (Figures 5d–f).

The prometastatic effect of p27CK-DD was confirmed in the UMUC3 bladder cancer model. p27CK-DD overexpression in UMUC3 parental cells activated STAT3, modestly increased STAT3 mRNA and induced a STAT3-dependent increase in N-cadherin and Twist1, and increased cell migration and invasion (Figures 5g–i and Supplementary Figure S4F). Moreover, p27CK-DD overexpression caused widespread multiorgan tumor metastasis in vivo, detected by direct immunofluorescence of metastatic tumor that was not observed in parental UMUC3, and this was attenuated by coexpression of STAT3DN (Figure 5j). These data provide strong evidence that STAT3 activation is required for p27CK-DD-induced EMT and enhanced metastasis in these models.

p27 knockdown reduces metastasis in vivo and this is reversed by STAT3-CA

As loss of p27 reversed the EMT phenotype in the highly metastatic 4175 and UMUC3-L (Figure 2), we next assayed effects of p27 knockdown on their metastatic potential in vivo. In 4175 cells, p27 knockdown markedly reduced pSTAT3 and caused a modest decrease in STAT3 mRNA and protein levels (Figures 6a and c). This is consistent with the known action of pSTAT3 to transactivate the STAT3 gene. An increase in STAT3 mRNA expression was also observed in p27CK-DD-transduced cells (Figure 3a and Supplementary Figure S4F). Similarly, in UMUC3-L, p27 knockdown decreased pSTAT3 (Figure 6g). As observed in vitro for cell migration and invasion (Figure 2), loss of p27 also significantly decreased experimental lung metastasis formed by both 4175 (P < 0.001) and UMUC3-L cells (P < 0.001) (Figures 6d–f and h–j). The loss of metastatic ability could not be attributed to a cell-cycle effect for either cell model (Figure 2f and Supplementary Figure S5). Since STAT3 activity was decreased by p27 CK-DD knockdown, a STAT3-CA was introduced into 4175shp27 cells to test if restoration of STAT3 activity could rescue the loss of metastasis caused by p27 knockdown. In 4175shp27 cells, STAT3-CA expression partially restored loss of metastasis following p27 knockdown (Figures 6d–f), suggesting that STAT3 CA serves as a critical mediator of the prometastatic effects of p27PT157PT198 in vivo.

p27PT157 correlates with STAT3 and PI3K/mTOR activation

Proteomic analysis of 747 primary human breast cancers from The Cancer Genome Atlas (TCGA)/The Cancer Proteome Atlas (TCPA) data set showed that p27PT157 and p27PT198 are highly correlated (correlation coefficient R = 0.411, P = 0) and both are strongly associated with the activation of kinases downstream of PI3K, p70S6K, p90rsk3, S6pS23 and S6pS240 (R = 0.13–0.41, all P-values < 9E–08) and correlate negatively with PTEN (R = 0.24, P = 8.9E–11 and R = –0.14, P = 0.0002, respectively) (data shown for p27PT157 in Figure 7a). Box plots show elevated p27PT157 (Figure 7a) and pSTAT3 (Figure 7b) correlated significantly with the levels of indicated proteins above the mean. Elevated pSTAT3p117 levels correlate significantly with both p27PT157 (R = 0.102, P = 0.006) and with the activation of PI3K/mTOR effectors pAKT (pT308 and pT473), p70S6K and p90rsk3, S6pS235 and S6pS240 (R = 0.103–0.428; P-values between 0.006 and 1.3E–10) and correlated inversely with PTEN (R = –0.10, P = 0.009) (Figure 7b). Owing to the recent collection of TCGA samples, correlation with patient outcome was not possible. The correlation between pSTAT3 and PI3K/mTOR pathway activation was validated in a second data set of 712 primary breast cancers (AKTpS473, R = 0.137, P = 0.0003; mTORpS2448, R = 0.172, P = 3.9E–06). Unfortunately, p27PT157 and p27PT198 data were not available in the second breast cancer data set.

The strong correlation between activation of STAT3 and activation of multiple PI3K/mTOR pathway effector kinases was confirmed in over one thousand bladder, renal and lung cancers from TCGA data (Figure 7c). Patient follow-up time for these data sets was also too short to permit outcome analysis.

DISCUSSION

Distant metastases are the cause of most cancer patient deaths.39 Despite substantial progress in our understanding of the complex process of human tumor metastasis, the lack of knowledge of molecular mechanisms underlying metastasis has limited our ability to specifically target tumor spread.40 Cytoplasmic p27 acquires an oncogenic role, via RhoA-Rock inhibition, to increase cell migration.15,41 Present data indicate that in addition to facilitating RhoA binding,27 p27 phosphorylations at T157 and T198 link PI3K activation with an STAT3-driven metastatic cascade in vivo in human tumors. Knockdown of cellular p27 in PI3K-activated cancer models attenuates metastasis, whereas phosphomimetic p27CK-DD causes non-transformed mammary epithelial cells to acquire invasive ability in vitro and increases metastasis of cancer cells in vivo. Furthermore, we identify a mechanism in which C-terminally phosphorylated p27 drives tumor metastasis in vivo via STAT3 activation, which induces TWIST1 and EMT.

To initiate metastasis, tumor cells must transgress cell–cell junctions and acquire the ability to invade beyond surrounding basement membranes.42 Reactivation of EMT is thought to initiate this early step in the metastatic cascade.33,38 Present data demonstrate that p27CK-DD is sufficient to induce a reversible, EMT-like phenotypic switch in two immortal, non-tumorigenic mammary epithelial lines, and to upregulate this process in tumorigenic cancer cells. Conversely, in PI3K-activated, metastatic 4175 breast and UMUC3-LuL2 bladder cancer models with high endogenous p27PT157PT198, p27 knockdown reverted the EMT phenotype, reduced cell invasion and substantially reduced the formation of experimental lung metastasis. The loss of metastatic ability following p27 knockdown in these models could not be attributed to effects on cell-cycle progression or proliferation. Thus, PI3K activation is accompanied by an oncogenic gain of function in which p27PT157PT198 promotes metastasis in part by triggering EMT-like changes. Cancer cells gain an advantage subverting a CDK inhibitor to promote tumor progression. This may explain why p27 is rarely completely lost, despite the decreased nuclear p27 observed in a majority of human cancers.14,43

EMT is initiated by several transcription factors, including Twist1, Snail and Slug.44,45 Twist1 can act independently of Snail and Slug to repress E-cadherin and upregulate N-cadherin.2 As a master regulator of embryonic morphogenesis, Twist1 has been shown to promote cancer metastasis by activating EMT.45 Here, we show p27 knockdown in p27PT157PT198-enriched highly metastatic lines decreased TWIST1 expression, whereas p27CK-DD transduction led to TWIST1 overexpression in low metastatic cells. TWIST1 knockdown reversed the p27CK-DD-induced mesenchymal phenotype, indicating TWIST1 upregulation is key to p27CK-DD-induced EMT.
STAT3 is aberrantly expressed and activated in many cancers including breast cancer and has critical roles in malignant transformation and tumor progression. p27 knockdown in p27 PT157 PT198-enriched cancer cells decreased total and phosphorylated STAT3. Conversely, p27 CK-DD expression significantly increased total and phosphorylated STAT3 (Y705) in immortal mammary epithelial cells and cancer cells. STAT3 inhibition or STAT3DN reversed p27 CK-DD-mediated EMT, reverted mesenchymal to epithelial morphology, upregulated epithelial markers and attenuated the p27 CK-DD-mediated increases in cell invasion and cancer metastasis. Thus, STAT3 appears to be critical for the maintenance of p27-driven EMT and metastasis.

In cancer cells expressing high endogenous p27 PT157 PT198, we observed high TWIST1-luciferase activity and TWIST1 expression. STAT3 inhibition and p27 knockdown, in 4175 cells, both attenuated TWIST1 promoter activity and TWIST1 expression, consistent with reports that TWIST1 is induced by STAT3. In both breast and bladder cancer models, p27 CK-DD increased, whereas p27 knockdown decreased STAT3 occupancy and activation of the TWIST1 promoter. p27 can act...
and activation, and STAT3-dependent increase in AKT activation. In addition, cellular p27pT198 phosphorylation would increase p27pT157pT198 to increase STAT3 activity, further activate AKT, induce STAT3-dependent TWIST1 expression and drive EMT, contributing thereby to the acquisition and maintenance of metastatic potential. Combined inhibition of PI3K/mTOR and JAK2/STAT3 in p27pT157pT198-enriched human cancers may ultimately have therapeutic potential to limit p27-mediated EMT and cancer metastasis.

MATERIALS AND METHODS

Cell lines and reagents

The immortalized human mammary epithelial MCF-12A cell lines (ATCC Manassas, VA, USA) and HME3 cell lines were cultured as described.22 MDA-MB-231-luc and MDA-MB-4175-luc cells (from J Massague, MSKCC, New York, NY, USA)14 and MCF-7 cells were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum. Lenti-X 293T cells from Clontech (Mountain View, CA, USA) were cultured in Dulbecco’s modiﬁed Eagle’s medium with 10% fetal bovine serum. Lenti-X 293T cells from Clontech (Mountain View, CA, USA) were cultured in Dulbecco’s modiﬁed Eagle’s medium with 10% fetal bovine serum.

Table 2

**Correlation of pSTAT3 with PI3K/mTOR pathway activation**

| Kinase | Breast (n=747) | Bladder (n=127) | Kidney (n=454) | LungAD (n=237) | LungSQ (n=195) |
|--------|----------------|----------------|----------------|----------------|----------------|
| AKT pS473 | 0.238 | 0.214 | 0.216 | 0.388 | 0.500 |
| mTOR pS2448 | 0.103 | 0.33 | 0.00015 | 0.205 | 1.11E-05 |
| p70S6K | 0.428 | 0.298 | 8.74E-11 | NS | NS |
| S6K pT146 | 0.169 | 0.158 | 0.0012 | 0.33 | 5.94E-06 |
| S6K p Thr180/244 | 0.136 | 0.197 | 2.4E-05 | 0.300 | 4.34E-05 |
| PTEN | -0.098 | 0.291 | 2.53E-10 | NS | NS |

*R=correlation coefficient

Figure 7. Primary human cancers show significant associations between PI3K pathway activation, pSTAT3 and p27pT157. (a and b) RPPA analysis of 747 primary breast cancers from the TCGA/TCGA data set show significant Spearman’s correlations between enhanced p27pT157 (a) and STAT3pY705 (b), with each other and with PI3K pathway effectors shown. Box graphs depict tumors below and above 50th percentile expression for each variable as low (L) and high (H). Welch two-sample t-test was used to test significance. *P-values for all data are shown. (c) Spearman’s correlation analysis was performed between STAT3pY705 and the activated phosphorylation status of multiple PI3K/mTOR pathway effector kinases represented in the RPPA data in over 1000 cancers from the TCGA data sets, including breast, bladder, kidney and adeno (LungAD) and squamous lung (LungSQ) cancers is shown. n = number of tumors in the TCPA data set. NS, not significant; R-value, correlation coefficient.
Reagents were obtained as follows: lentivirus vector Plvx-AcGFP (from Clontech); lenti-STAT3DN (from Addgene, Cambridge, MA, USA); pBabe-STAT3-CA (from J Bromberg; MSKCC); PI3K/mTOR inhibitor PF-04691502 (used at 250 nM) (from Pfizer, New York, NY, USA); 5, 15-diphenyloxazole (used at 50 μM) to inhibit STAT3 (from Calbiochem, Billerica, MA, USA); and short hairpin RNAs of p27 and TWIST1 (from Open Biosystems, GE Dharmacon, Lafayette, CO, USA).

**p27 mutants**

Phosphomimetic p27 mutations were introduced into EGFP-p27CK–30 (from S Dowdy, UCSD, La Jolla, CA, USA) by site-directed mutagenesis and subcloned into Plvx-AcGFP to generate Plvx-AcGFP-p27CK and Plvx-AcGFP-p27CK-DD vectors for virus packaging to infect target cells.

**Lentivirus production and stable line generation**

Details of the generation of cells expressing lentivirus vectors encoding three different short hairpin RNAs to CDKN1B (p27) and TWIST1 (Open Biosystems) or Plvx-AcGFP and Plvx-AcGFP-p27CK-DD are in given in the Supplementary Information.

**Transwell migration and invasion assay**

A total of 2 × 10⁴ cells were seeded for transwell migration assays and 10⁵ cells were seeded for invasion assays into either modified Boyden chambers or automated transwell assays using the Real-Time Cell Analysis system from Xcellence (ACEA Biosciences, San Diego, CA, USA)29 (see Supplementary Information for details).

**Western blotting**

Western blots were performed as described. Antibodies used in western blots were as follows: anti-PAKT S473, AKT, pPDK1 S241, PDK1, pSTAT3 Y705 and STAT3 (from Cell Signaling); for E-cadherin, N-cadherin and p27 from Transduction Labs; and for p27/pT157 and p27/pT198 from R&D Systems. p27 immunoprecipitation used PAb C-19 from Santa Cruz Biotechnology (Dallas, TX, USA) and used as per the manufacturer’s instructions.

**Drug treatment**

MCF-12A-p27CK-DD cells were treated with or without STAT3 inhibitor 5, 15-diphenyloxazole (50 μM) for 2 weeks before fluorescence immunocytochemistry, cell invasion and western blot analysis. MDA-MB-4175 cells were treated with or without PI3K/mTOR inhibitor PF-04691502 (250 nM) for one day before p27 or JAK2 immunoprecipitations.

**Fluorescence immunocytochemistry**

Fluorescence immunocytochemistry was performed as described. Primary antibodies used were: E-cadherin (1:1000), N-cadherin (1:500), vimentin (1:500), pSTAT3 (1:1000) and Twist1 (1:500), followed by incubation with Alexa Fluor 594-conjugated secondary antibody (1:200); Nuclei were stained with 4',6-diamidino-2-phenylindole, and cells were mounted in Prolong Gold antifade reagent (Invitrogen). Photomicrographs were taken using a Zeiss Axiosview microscope (Zeiss, Jena, Germany).

**Immunoprecipitation**

MDA-MB-231 and 4175 cells treated with or without PF-04691502 (250 nM) for 24 h were lysed, and 2 mg lysate from MDA-MB-231 cells and 1 mg lysate from 4175 cells were incubated with 1 μg of p27 antibody (C-19; Santa Cruz Biotechnology), collected on protein G-agarose and washed three times with IP buffer and then analyzed by western blot for bound STAT3 and p27. Antibody-alone controls were run with all immunoprecipitations.

**Chromatin immunoprecipitation assay**

ChiP assays for STAT3 on TWIST1 promoter were as described MDA-MB-231 and UMUC3 cells expressing control vector or p27CK-DD, or p27CK-DD-STAT3DN, 4175scr, 4175shp27, UMUC3-LuL2scr and UMUC3-LuL2shp27 cells were serum deprived and then refed with media plus 20 ng/ml epidermal growth factor for 1 h followed by ChiP assays (see Supplementary Methods for details).

**Human phosphokinase array**

To determine which phosphokinases are changed by p27CK-DD expression, MCF-12A-GFP and MCF-12A-p27CK-DD cells were assayed using a human phospho-kinome array (ARY003, Proteome Profiler; R&D Systems, Minneapolis, MN, USA) as per the manufacturer’s protocol.

**Luciferase assay**

Cells were seeded at 5000 cells per well in an opaque 96-well plate. After 24 h, cells were transfected with 50 ng of TWIST1 promoter or control promoter luciferase vector (SwitchGear Genomics, Menlo Park, CA, USA) using Fugene-6 (Roche, San Francisco, CA, USA). Luciferase activity was assayed 24 h later, using LightSwitch Assay Reagent (SwitchGear Genomics) and a GloMax-Multi+ Detection System with Instinct Software (Promega, Madison, WI, USA). Relative luciferase expression was calculated as a percent maximum of the highest sample in each group.

**Bioluminescence imaging and analysis**

Mice were anesthetized and injected intraperitoneally with 1.5 mg of D-luciferin (15 mg/ml in phosphate-buffered saline) and imaged using Xenogen IVIS system (Xenogen, Caliper, Hopkinton, MA, USA) as in Wander et al. Values were normalized to those obtained immediately after xenografting (day 0) so that all mice had an arbitrary starting bioluminescence signal of 100. Average normalized photon flux is plotted over time ± s.e.m. Representative individual mice were photographed and presented along with a standardized scale.

**Reverse-phase protein array analysis of human tumors**

Revers-phase protein array analysis of human tumors was performed at the Functional Proteomics Reverse Phase Protein Array Core facility at MD Anderson to test the expression of 178 proteins and phosphoproteins in 747 breast cancers from TCGA project as described. RPPA data processing used SuperCurve (Coombes K, et al., SuperCurve SuperCurve Package, R package version 1.4.1.2011) as described. The protein expression data (RPPA) for breast, lung, kidney and bladder cancers samples were downloaded from the TCPA website (http://bioinformatics.mdanderson.org/main/TCPA). For detailed RPPA method, see Supplementary Information.

**Statistical analysis**

All graphed data are from at least three experiments and presented as means ± s.e. Two-tailed Student’s t-tests were used to test differences. P-values < 0.05 were considered statistically significant. The statistical differences between curves were calculated using ‘compareGrowthCurves’ function of the StatMod software (http://bioinf.wehi.edu.au/software/compareCurves) for IVIS metastatic tumor growth curves.

To test the correlations between pSTAT3, p27/pT157 and activation of PI3K/mTOR pathway kinases in the RPPA data, Spearman’s correlation analysis was performed between pairs of protein levels among proteins represented in the RPPA data. P-values within boxplots were determined using the Welch two-sample t-test.
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