 Slug Promotes Survival during Metastasis through Suppression of Puma-Mediated Apoptosis

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Abstract

Tumor cells must overcome apoptosis to survive throughout metastatic dissemination and distal organ colonization. Here, we show in the Polyoma Middle T mammary tumor model that N-cadherin (Cdhl2) expression causes Slug (Snai2) upregulation, which in turn promotes carcinoma cell survival. Slug was dramatically upregulated in metastases relative to primary tumors. Consistent with a role in metastasis, Slug knockdown in carcinoma cells suppressed lung colonization by decreasing cell survival at metastatic sites, but had no effect on tumor cell invasion or extravasation. In support of this idea, Slug inhibition by shRNA sensitized tumor cells to apoptosis by DNA damage, resulting in caspase-3 and PARP cleavage. The prosurvival effect of Slug was found to be caused by direct repression of the proapoptotic gene, Puma (Bbc3), by Slug. Consistent with a pivotal role for a Slug–Puma axis in metastasis, inhibition of Puma by RNA interference in Slug-knockdown cells rescued lung colonization, whereas Puma overexpression in control tumor cells suppressed lung metastasis. The survival function of the Slug–Puma axis was confirmed in human breast cancer cells, where Slug knockdown increased Puma expression and inhibited lung colonization. This study demonstrates a pivotal role for Slug in carcinoma cell survival, implying that disruption of the Slug–Puma axis may impinge on the survival of metastatic cells. Cancer Res; 74(14); 3695-706. © 2014 AACR.

Introduction

Metastasis is a well-orchestrated cascade of events involving the detachment and exit of carcinoma cells from the primary tumor, survival in the circulation, and dissemination to distant organs to build metastatic residence (1). Circulating tumor cells must overcome apoptotic stresses caused by anoikis, mechanical shearing in the bloodstream, or collisions with circulating host cells (2). Distal organ colonization is a late but rate-limiting step, which in the lungs involves sieving of tumor cells through the capillary bed, formation of intravascular emboli, extravasation, seeding, growth, and survival in the lung parenchyma (1). Although tumor cells are able to rapidly arrest in the lung capillaries, most are cleared by apoptosis, leaving less than 1% to undergo extravasation, seeding, and growth in the lungs (1, 3), underscoring the fact that metastasis is a vastly inefficient process. To overcome apoptosis, disseminated tumor cells must turn on genes to ensure survival in the circulation or in distant organs, while maintaining migratory or invasive capability. Carcinoma cells undergo epithelial to mesenchymal transition (EMT), hence facilitating invasion away from the primary tumor, entry into the vasculature, and breaching of basement membranes at distant organs (4–7). The EMT process is mediated by EMT-inducing transcription factors, involving Slug as a prominent player (4–7). Slug (Snai2) is a zinc-finger transcriptional repressor of the Slug/Snail family that promotes carcinoma cell invasion, stemness, and survival (8–10). Clinical evidence supports a role of Slug in advanced breast malignancies; high Slug levels are associated with poor prognosis, recurrence, and metastasis (11–13). Slug/Snail are known to inhibit the transcription of E-cadherin, cytokeratins, claudins, and plakoglobin, among others to attenuate the epithelial phenotype and promote malignancy (14–17). However, clinical data showed that high levels of Slug and E-cadherin were found in invasive duct carcinomas, suggesting that Slug expression does not necessarily preclude E-cadherin expression (13).

It is thought that disseminated cancer cells may revert from EMT to mesenchymal to epithelial transition (MET) at sites of metastasis to establish growth and survival of new colonies (18). In support of this notion, Slug was found to promote cell survival, and not EMT, during kidney tubulogenesis, as shown by persistent E-cadherin expression (19). Similarly, in hematopoietic progenitor cells, where EMT is not involved, Slug was found to promote survival by blocking apoptosis in response to DNA damage (20, 21). Namely, Slug knockout mice succumbed to γ-irradiation, due to apoptosis of hematopoietic progenitor
cells, thus impairing regenerative potential. In this case, Slug induced apoptosis by repressing the p53 proapoptotic target gene, Puma (21). Puma (BBC3), or p53-upregulated modulator of apoptosis, is a BH3-only member of the Bcl-2 family and a target of p53-mediated apoptosis (22, 23). It activates an apoptotic cascade by facilitating Bax activation, causing cytochrome C release from the mitochondria, caspase-3 activation, and DNA fragmentation (24, 25).

Here, we show that Slug is dramatically upregulated during metastasis in the PyMT-N-cadherin (PyMT-N-cad) mouse, which exhibits enhanced lung metastasis as compared with the PyMT mouse (26). Slug expression was increased in PyMT-N-cadherin mammary tumors and metastases relative to PyMT controls, and was further increased in distal metastases relative to primary tumors. Slug knockdown in metastatic tumor cells did not inhibit invasion, arrest, or extravasation in the lungs, but greatly reduced colonization. Consistent with FGFR potentiation by N-cadherin (N-cad; ref. 27), inhibition of FGFR suppressed Slug expression and stimulated apoptosis. Moreover, Slug knockdown sensitized cells to apoptosis, effects that were reversed bySlug reexpression. Consistent with inhibition of Puma by Slug, Slug knockdown in PyMT-N-cad cells caused increased Puma and Bax expression, whereas silencing Puma in Slug-knockdown cells inhibited apoptosis and rescued lung colonization. Conversely, overexpression of Puma in PyMT-N-cad cells suppressed metastasis. The prosurvival function of Slug–Puma was also confirmed in human breast cancer cells. Thus, our study demonstrates that Slug–Puma promotes tumor cell survival leading to distal organ colonization.

Materials and Methods

Animals

FVB female mice and athymic nude mice were obtained from Taconic. Animal protocols of this study were approved by Institute for Animal Studies at Albert Einstein College of Medicine (AECOM).

Cell lines

The PyMT and PyMT-N-cad mammary tumor cell lines were generated and characterized in 2007 as described (26). Briefly, primary mammary and metastatic tumor cell lines were derived from PyMT and PyMT-N-cad tumors or lung foci at 7 weeks after tumor onset by collagenase digestion, and plated in culture until they underwent crisis as detailed in refs. 26, 28. The MDA-MB-231 metastatic subline 3475 was obtained in 2009 from Dr. York, NY and was tested for lung colonizing activity. The BT549 cells were tested and found negative for mycoplasma.

Antibodies and reagents

The antibodies used are against N-cad, E-cadherin, plakoglobin (BD Biosciences); fibronectin, cytokeratin 18, β-actin (Sigma); Slug, vimentin, p-ERK, p-Akt, p-p53, Akt, Bcl-2, Bcl-XL, Bax, Bim, Puma, cleaved caspase-3, and PARP (Cell Signaling Technology); Erk, Bax, Noxa, and FGFR1 (Santa Cruz Biotechnology). Drugs used are PD173074 and PD0325901 (Pfizer), Iressa or ZD1839 (AstraZeneca), and MK2206 (Tocris).

Immunoblotting analysis

Cells were solubilized with RIPA lysis buffer, resolved by SDS-PAGE, and transferred to polyvinylidene difluoride membrane. Blots were probed with indicated antibodies and developed by Pierce chemiluminescence substrate (Thermo Scientific).

Slug, Puma, and N-cad shRNA and expression constructs

Two mouse Slug shRNA clones TRCN00000096227 (mature antisense: TTTTACATCAGTGGGTTGTCG), TRCN00000096228 (mature antisense: TTGTTATGACAGGTATAGGGT) and nonsilencing control shRNA in the pLKO.1 lentiviral vector (Open Biosystems) were used to knock down Slug. To generate viruses, lentiviral vectors were transfected into 293 T cells with Tat, Rev, Gag/Pol, and VSV-G vectors. Two mouse Puma shRNA clones, V3LHS_342433 (Sense: CCGATGGCGGAGACCTCA) and V3LHS_342436 (Sense: AGTACGGAGCCGAGACA) were obtained from shRNA Core Facility (AECOM). Two human Slug shRNA clones and a nonsilencing control shRNA in pLKO.1 lentiviral vectors were from Dr. Guo (AECOM). On-TARTGET plus mouse Puma shRNA (J-050032-08) and nontargeting siRNA (D-001810-01-05) were from Dharmacon. Mouse N-cad siRNA (sc-35999) and control siRNA were obtained from Santa Cruz Biotechnology. Mouse Slug cDNA was amplified by PCR and subcloned into a pLXSN retroviral vector (Clontech). For expression of Puma protein into control-sh/PyMT-N-cad cells, mouse Puma cDNA (Clone ID: 5133742) was from Thermo Scientific.

TaqMan qRT-PCR

Total RNA was isolated using the RNeasy Mini Kit and RNase-free DNase set (Qiagen), Real-time RT-PCR was carried out using the TaqMan RNA-to-Ct 1-Step Kit (Applied Biosystems) and gene-specific TaqMan probes (Applied Biosystems) in StepOnePlus Real-time PCR System. Gapdh mRNA was used for loading normalization, and a specified reference control was used for analyzing relative mRNA expression. Comparative CT (ΔΔCT) real-time analysis was performed using StepOne Software. The following are Gene Expression Assay IDs (Applied Biosystems) for each gene probe: Gapdh (Mm99999915_g1), Snail (Mm00441533_g1), Slug (Mm00441531_m1), Twist1 (Mm00442036_m1), Zeb1 (Mm00495564_m1), Sp1 (Mm00497193_m1), Foxc2 (Mm00546194_s1), Gsc (Mm00650681_g1), vimentin (Mm00449208_m1), PyMT (Mm04214513_u1), Mmp2 (Mm00439498_m1), Mmp9 (Mm00442991_m1), Mmp14 (Mm00485054_m1), and Puma (Mm00519268_m1).

Chromatin immunoprecipitation

Cells were fixed with 1% paraformaldehyde in PBS for 10 minutes, and pelleted nuclei were lysed in SDS lysis buffer supplemented with protease inhibitor cocktail (Roche) and 2 mmol/L phenylmethylsulfonylfluoride. The chromatin was sheared by sonication using a Branson 250 sonifier with a...
2-mm microtip to generate 300- to 1,000-bp fragments. Equal amounts of solubilized chromatin samples were incubated with either 1.5 μg of Rabbit anti-Slug antibody or rabbit IgG for 24 minutes in an ultrasonic bath at 4°C. Antigen–antibody complexes were collected with protein G–conjugated Dynabeads (Invitrogen) at 4°C for 45 minutes. The beads were extensively washed in low and high salt washing buffers, and the immunoprecipitated DNA samples were eluted and decross-linked by boiling the Dynabeads for 15 minutes in presence of Chelex-100 resin (Bio-Rad). Purified DNA samples were subjected to qPCR using SYBR Green in a StepOnePlus Real Time PCR system to specifically amplify Slug-binding region in intron 1 of the Puma gene and also a negative control region with no Slug-binding sequences located 9.4 kbs downstream from the Puma gene on chromosome 7. The primer sets used for the qPCR assays were: Puma intron 1, sense 5′-ACTAAGGCTGGCCAGGC-3′ and antisense 5′-GCGAGCCC-GAACCCTCTTG-3′; negative control region, sense 5′-AGC-CACTGTTTCAGACGTTAGT-3′ and antisense 5′-GCTCCAGTCTTTTCCAGCC-3′. Specific enrichment was calculated and expressed as the percent recovery of input. Data were shown as mean ± SEM. Unpaired t-test was used to determine P < 0.05.

Migration/invasion

Cell migration and invasion assays were performed in a Boyden chamber using 8-μm Transwell filters as described (26).

Extravasation

Trans-endothelial migration was performed as described (29). Fluorescently labeled tumor cells were cultured on top of a 3B-11 endothelial monolayer on Transwell filters. Bone marrow macrophages were plated on the reverse side of the Transwell. After 36 hours, transmigrated cells were counted and shown as mean ± SEM (N = 12); unpaired t-test, P < 0.05.

Lung colonization

Half million cells were injected via tail vein into 6-week-old female FVB mice, and mice were incubated for 24, 48, 96 hours, and 2 weeks or else as indicated. Lung metastases were quantified as described (26). Briefly, formalin-fixed/paraffin-embedded lungs were cut at 5-μm thickness in five sets with 50-μm intervals between each set. Sections were hematoxylin and eosin (H&E) stained, and lung foci were counted. To visualize small cluster of cancer cells in the lung tissue, sections were further immunostained with N-cad cytosolic tail antibody or pan-cytokeratin antibody. To quantify arrest of tumor cells in the lungs after intravenous injection, we applied qPCR analysis as described (30). Briefly, 1 × 10^6 PyMT-N-cad cells expressing either nontargeting sequence as control. In knockdown cell lines, Slug mRNA was increased by 7.6- and 61-fold in lung metastatic lines as compared with one of the PyMT-N-cad primary tumor cell lines (Fig. 1B). By contrast, Twist, Snai1, or vimentin mRNAs were unchanged (Fig. 1B). The apparent variation in the extent of Slug mRNA upregulation in the individual PyMT-N-cad cell lines may reflect tumor cell heterogeneity that is inherent to breast tumors (34). We further tested for potential changes in other EMT factors, including Zeb1 (Zeb1), Sip1 (Zeb2), Foxc2 (Foxc2) and Goosecoid (Gsc) in PyMT-N-cad cell lines (12). Except for Foxc2 mRNA, which was increased by 5-fold in PyMT-N-cad metastatic cells, all other factors were unchanged (Fig. 1C). Slug was localized to the nucleus, and N-cad to cell–cell junctions in PyMT-N-cad cells (Fig. 1D), whereas E-cadherin remained expressed in these cells (Fig. 1D).

We compared Slug expression in primary tumors and lung metastases from PyMT-N-cad and PyMT mice by IHC. Slug-positive cells were found in undifferentiated tumor areas and were more abundant in PyMT-N-cad than in PyMT tumors (Fig. 1E and G). Moreover, Slug expression was greater in metastases relative to primary tumors from both genotypes (Fig. 1E and F), and was further exacerbated in PyMT-N-cad relative to PyMT metastases (Fig. 1F and H).

Slug knockdown has no effect on epithelial markers or cell migration and invasion

We next sought to determine whether Slug affects EMT or invasion. We knocked down Slug in PyMT-N-cad metastatic cells using two lentiviral mRNA-targeting sequences and a nontargeting sequence as control. In knockdown cell lines, Slug protein and mRNA expression was inhibited, as shown by
immunostaining, immunoblotting (Fig. 2A and C), and qRT-PCR (Fig. 2B). Slug knockdown did not increase epithelial E-cadherin, cytokeratin-18 or plakoglobin expression, or diminish mesenchymal N-cad or fibronectin expression (Fig. 2C). The expression of FGFR or EGFR was unaffected by Slug (Fig. 2C). These data were confirmed in PyMT-N-cad cells in vivo in five individual mice (Supplementary Fig. S1). Staining for pan-cytokeratin or E-cadherin showed similar expression in Slug knockdown and control tumors, whereas vimentin was only present in stromal cells (Fig. 2D). Furthermore, Slug knockdown did not inhibit cell migration or invasion (Supplementary Fig. S2 A and S2B), and while it increased MMP-2 mRNA, it did not increase the level of active/cleaved MMP-2, as shown by zymography (Supplementary Fig. S2 C and S2D).

**Slug knockdown suppresses metastatic colonization**

To examine the role of Slug in metastasis, we used tail vein injection of carcinoma cells into syngeneic female mice, which measures lung colonization, a late but rate-limiting step in metastasis (1). Distal organ colonization is thought to measure tumor cell extravasation and survival, two hallmarks of metastatic potency (1). To test the effect of Slug on lung colonization, PyMT-N-cad cells expressing a nontargeting sequence (control), or two Slug shRNA sequences (Slug-sh) were injected into the tail vein of syngeneic female mice, and the number of metastatic foci was determined in the lungs, 2 weeks after inoculation. We found a dramatic inhibition (70%) of lung colonization as a result of Slug knockdown in two PyMT-N-cad cell lines (Fig. 2E and F). Similarly, Slug inhibition in the PyMT-N-cad cell lines was confirmed by qRT-PCR (Supplementary Fig. S2 B and S2C).
lung metastatic cell line, Met-1 (35), which expresses Slug and N-cad, also suppressed colonization (Fig. 2G and H), without however increasing the levels of E-cadherin (Fig. 2H).

**Slug knockdown disrupts metastatic seeding at an early phase of lung colonization**

These data support a critical role for Slug in metastatic colonization. However, it was unclear which stage of tumor cell dissemination was regulated by Slug. We sought to determine whether Slug protected tumor cells from apoptosis before or after extravasation in the lungs. One possibility is that Slug-knockdown cells arrived to the lungs, extravasated, and then underwent apoptosis due to low Slug levels. Alternatively, cells might have died in the circulation before reaching the lungs. Arrest in the lungs is a rapid process that occurs within 5 minutes following intravenous injection (29, 30). However, the majority of arrested cells (99%) usually undergo apoptosis within 24 hours, leaving less than 1% to extravasate, seed, and grow in the lungs (1, 3, 29, 30). To determine the effect of Slug on lung colonization, we measured the onset of metastatic foci in the lungs at 24, 48, and 96 hours after tail vein injection of $5 \times 10^5$ PyMT-N-cad cells into syngeneic female mice. We followed tumor cell seeding in the lungs by tracking carcinoma cells with pan-cytokeratin (Fig. 3A) or...
N-cad immunostaining (Fig. 3C and D). About 25 colonies of 2 to 6 cell clusters of control PyMT-N-cad cells were observed in the lungs 48 hours after injection, an effect that was sharply reduced in mice injected with Slug-knockdown cells (Fig. 3B). At 96 hours, there were 4 times more foci in control lungs as compared with Slug-sh lungs (Fig. 3B). No carcinoma cells were detected in the lungs 24 hours after injection, likely due to apoptosis and/or to that single cells are not easily detected by immunostaining (Fig. 3C; refs. 3, 29, 30). To determine the effect of Slug knockdown on tumor cell apoptosis in vivo, we costained lungs for N-cad and TUNEL at 24, 48, 96 hours, and 2 weeks after injection (Fig. 3C and D). We were unable to detect TUNEL activity in the lungs at these time points, suggesting that apoptotic cells are rapidly cleared from the lungs (Fig. 3C). However, staining of metastases at 2 weeks showed that foci derived from Slug-knockdown cells contained a higher proportion of TUNEL-positive cells than control foci, suggesting a higher rate of apoptosis caused by Slug inhibition (Fig. 3D and E).

To determine if Slug affects survival of tumor cells in the blood circulation before arrest in the lungs, we tracked PyMT-N-cad cells in the lungs 5 minutes after tail vein injection using PyMT qPCR (30). We found that Slug-shRNA did not attenuate survival of PyMT-N-cad cells in the circulation, because the real-time PCR showed a slight increase (1.5 fold) in the number of Slug-knockdown cells arrested in the lungs compared with controls (Fig. 3F).

To rule out a potential defect in tumor cell extravasation in the lungs caused by Slug depletion, we performed a transendothelial cell migration assay. This assay measures tumor cell transmigration of an endothelial cell layer in response to cues from macrophages in the bottom chamber, which release VEGFs that stimulate endothelial cell permeability, mimicking an in vivo setting in the lungs (29). Using Met-1 and PyMT-N-cad cells, we found in Met-1 no difference in transmigration between control and Slug-knockdown cells (Fig. 3G), whereas PyMT-N-cad/Slug-sh cells exhibited a 1.7-fold increase in extravasation relative to control cells (Fig. 3H), which corresponded to the extent of arrest in the lungs (Fig. 3F). These data argue that Slug knockdown does not inhibit arrest or extravasation, but inhibits the survival of carcinoma cells in the lungs after extravasation.

Figure 3. Slug knockdown inhibits tumor cell survival following arrest in the lungs. A, lung colonization of control or Slug-sh–expressing PyMT-N-cad was determined at 24, 48, and 96 hours after tail vein injection. Images of H&E (top) or pan-cytokeratin (bottom) stained control or Slug-sh lungs at 96 hours are shown. B, the number of foci in 5 animals at 24, 48, and 96 hours is shown as mean ± SEM (N = 5). Unpaired t-test; ***, P = 0.0096 (48 hours); ***, P = 0.002 (96 hours). C and D, TUNEL and N-cad staining of PyMT-N-cad control versus Slug-sh cells in the lungs at 24 and 48 hours (C), 96 hours, and 2 weeks (D) after injection is shown. E, TUNEL-positive cells at 2 weeks after injection are shown as mean ± SEM (N = 5). Unpaired t-test; ***, P < 0.0001. F, PyMT-N-cad control or Slug-sh1 cells were injected via tail vein (N = 3); 5 minutes later, lung DNA was extracted and analyzed by qPCR using PyMT primers. The relative increase in PyMT DNA in Slug-sh1 compared with control-sh is shown as mean ± SEM (N = 3); ***, P < 0.0049. G and H, fluorescently labeled control or Slug-shRNA–expressing Met-1 and PyMT-N-cad cells were cultured onto an endothelial monolayer in the Transwell. After 36 hours, extravasation of Slug-sh cells compared with control shRNA cells is shown as mean ± SEM (N = 12). Unpaired t-test; ***, P < 0.001.
The FGF receptor regulates Slug expression in PyMT-N-cad lung metastatic cells

FGFR1 amplification was found to be one out of a 66-gene signature that is strongly associated with reduced breast cancer patient survival duration (36). We showed that FGFR1 interaction with N-cad stabilizes the FGFR to promote invasiveness (27). We confirmed that PyMT-N-cad cells expressed higher levels of FGFR1 than PyMT cells (Supplementary Fig. S3A). We tested whether FGFR regulates Slug expression, using an FGFR inhibitor, PD173074 (FGFRi), or an EGFR inhibitor, Iressa (EGFRi) as control (26). Treatment of PyMT-N-cad cells with 0.5 to 2.0 μmol/L FGFRi inhibited the expression of Slug protein and mRNA (Supplementary Fig. S3B and S3C), whereas EGFRi had no significant effect (Supplementary Fig. S3B and S3D). Because ERK and Akt phosphorylation were attenuated by FGFRi, we tested whether these pathways regulate Slug expression. Inhibition of ERK by the MEK1 inhibitor PD0325901 (MEKi) or Akt by MK2206 (AKTi; Supplementary Fig. S3E) did not appreciably inhibit Slug expression. Therefore, FGFR, and not EGFR, regulates Slug, independently of ERK or Akt activation. Consistent with an effect of N-cad on FGFR leading to Slug upregulation, N-cad knockdown in PyMT-N-cad cells attenuated Slug expression, without however affecting E-cadherin levels (Supplementary Fig. S3F).

Slug inhibition primes tumor cells to apoptosis

Because lung colonization, and not extravasation, is regulated by Slug, which in turn is controlled by the FGFR, we tested whether Slug inhibition by shRNA, or by FGFR inhibition, affects cell survival. Treatment of PyMT-N-cad cells with FGFRi increased cleaved caspase-3 and PARP levels, especially at 1.0 μmol/L, which was strongly inhibitory of Slug (Fig. 4A). Consistent with these effects, treatment of Slug-sh1 cells with FGFRi enhanced caspase-3 and PARP cleavage relative to control cells (Fig. 4A). Similar effects were obtained with Slug-sh2 cells (Supplementary Fig. S4A). In line with these data, Slug shRNA induced the expression of monomeric (23 kD) and dimeric (46 kD) form of the apoptotic effector, Bax (24, 25), which was further increased by FGFRi (Fig. 4A). By contrast, the levels of the p53 target gene Noxa, the non–p53-regulated Bim gene (Supplementary Fig. S4B), or the antiapoptotic Bcl-2 and Bcl-xL proteins (37–39), were not affected by Slug shRNA (Fig. 4A). Thus, Slug protects PyMT-N-cad cells from apoptosis triggered by FGFR inhibition.

To confirm the effect of Slug on cell survival, we reexpressed mouse Slug in Slug-knockdown cells using a retroviral vector (Fig. 4B). This resulted in rescuing the effect of FGFRi by reducing levels of cleaved caspase-3 or PARP and dimeric Bax (Fig. 4B), while having no effect on Bcl-2 or Bcl-xL expression (Fig. 4B).

To further validate the effect of Slug on resistance to apoptosis, we used doxorubicin, a drug used for treatment of breast cancer. Treatment of PyMT-N-cad cells with 1 μmol/L doxorubicin caused a 4-fold increase in active caspase-3 levels in Slug-sh as compared with control PyMT-N-cad cells (Fig. 4C and D). Furthermore, a combination of doxorubicin and FGFRi enhanced the levels of apoptotic markers, especially in Slug-sh cells (Fig. 4E).
Slug promotes cell survival by repressing the cell death protein, Puma.

Because Slug attenuation sensitized cells to apoptosis, we determined whether it affected the expression of the proapoptotic gene Puma, which was shown to be repressed by Slug (21). Treatment of PyMT-N-cad cells with FGFRi, which inhibits Slug expression, caused increased Puma protein and mRNA levels (Fig. 5A and B). In vivo, mammary tumors...
from Slug-sh cells also displayed higher Puma levels (Fig. 5C and D).

Because Puma is a p53 response gene (21), we also examined whether Slug regulates Puma in response to DNA damage by γ-irradiation. Treatment of cells with 15 Gy γ-irradiation stimulated Puma mRNA and protein expression in Slug-sh cells relative to PyMT-N-cad control cells (Fig. 5E and F). As control for induction of DNA damage, p53 phosphorylation was included (Fig. 5F).

Binding of Slug protein to the first intron of mouse Puma gene and subsequent transcriptional repression of Puma by this interaction was first elaborated in mouse hematopoietic progenitor cells (21). To verify the physical interaction of Slug to a known target regulatory region of the Puma gene, we performed chromatin immunoprecipitation (ChIP) in PyMT-N-cad tumor cells with anti-Slug antibody or anti-rabbit IgG as control. Slug occupancy was significantly enriched at Puma intron 1 in control PyMT-N-cad cells as compared with Slug-knockdown cells (Fig. 5G). Enrichment of Slug at a negative control region with no Slug-binding sites, located 9.4 kbs downstream from Puma gene on chromosome 7, did not exhibit notable differences in control PyMT-N-cad cells as opposed to Slug-knockdown cells (Fig. 5G). These data demonstrate that Slug specifically binds to the first intron on the Puma gene, consequently suppressing Puma gene expression in PyMT-N-cad cells.

To verify whether Puma contributes to apoptosis in Slug-knockdown cells, Puma was silenced in PyMT-N-cad/Slug-sh cells using a pool of Puma siRNAs (Fig. 5H). Treatment with FGFRi decreased caspase-3 activation in Puma-siRNA–treated cells relative to control cells (Fig. 5H), as well as reduced the number of active caspase-3–positive cells by 60% (Fig. 5I). These findings suggest that Slug contributes to cell survival by inhibiting Puma.

To unequivocally prove the function of the Slug–Puma axis in metastatic colonization, we knocked down Puma in Slug-sh cells using two lentiviral Puma shRNA sequences as compared with a control sequence (Fig. 6A). Cells were injected intravenously into syngeneic FVB mice, and metastatic foci were monitored in the lungs 96 hours later by staining lungs for N-cad (Fig. 6C). We found a dramatic upregulation in the
number of foci in the lungs of mice injected with Slug/Puma knockdown cells relative to control lungs (Fig. 6B). Conversely, transient expression of Puma in PyMT-N-cad cells (Fig. 6D) suppressed lung colonization (Fig. 6E and F). Thus, it seems that the Slug–Puma axis is a powerful axis that drives distal organ colonization.

The contribution of SLUG–PUMA to the survival of human metastatic breast cancer cells

Slug expression was found to be inversely correlated with Puma gene expression in breast cancer genes data sets (40). To address the role of Slug in lung colonization by human breast cancer cells, we used the MDA-MB-231 metastatic 3475 subtype, which has been selected for its ability to colonize the lungs (41). We silenced Slug using two shRNA lentiviral sequences and a control nontargeting sequence (Fig. 7A). This resulted in increased Puma expression, especially in FGFRi-treated 3475/Slug-knockdown cells (Fig. 7B). Moreover, Slug knockdown in BT549 breast cancer cells also caused increased Puma levels (Fig. 7C). Consistent with our data in the PyMT-N-cad model, Slug inhibition stimulated Puma without inducing E-cadherin expression in 3475 (Fig. 7A) or BT549 cells (Fig. 7C). This was independent of N-cad, which was present in BT549 and not in 3475 cells (Fig. 7A and C). Next, we tested lung colonization of 3475 control and Slug-knockdown cells following tail vein injection in athymic nude mice. This led to an approximately 70% reduction in colonization for both Slug-knockdown cell lines compared with 3475 control cells (Fig. 7D). Thus, repression of Puma by Slug in human breast cancer cells contributes to survival during metastatic colonization.

Discussion

Slug is a transcriptional repressor, which promotes EMT, a process that accompanies cancer cell invasion and metastasis (6, 7). Slug upregulation was observed in high-grade invasive duct carcinomas, which exhibit high rate of recurrence, metastasis, and drug resistance (11, 13). Our studies showed that N-cad is expressed in aggressive breast cancers (42), and that it promotes EMT and metastasis by potentiating the FGFR (26, 27). Consistent with these findings, others have shown a strong association between N-cad (CDH2) and Slug (SNAI2) in breast cancer data sets (40). Moreover, FGFR1 amplification is part of a 66-gene signature in breast cancers that is strongly associated with reduced patient survival duration (36). Here, we show that N-cad expression in PyMT tumor cells in vivo causes striking upregulation of Slug, at the exclusion of other EMT factors. However, Slug was affecting the behavior of disseminated cancer cells, not through EMT, but by promoting cell survival at distant organs. Namely, Slug inhibition did not alter the expression of epithelial or mesenchymal markers, nor promoted motility, invasion, or extravasation, implying that the pro-metastatic function of Slug can be dissociated from its EMT-inducing effect (19). We believe that N-cad overexpression did not affect the ability of Slug to suppress E-cadherin in metastatic cells as Slug knockdown in the E-cadherin/N-cad positive BT549 or negative 3475 cell line did not increase or induce E-cadherin expression. It is believed that cancer cells undergo EMT to promote invasiveness but may also revert to MET at distant organs to resume growth and survival (18). We speculate that although Slug may be important for inducing EMT of cancer cells, especially at the primary tumor site, to facilitate detachment from the tumor mass and exit into the circulation,
it may revert from promoting EMT to activating survival at metastatic sites. Although Slug expression is found to be regulated by the FGFs, it was not due to ERK or AKT activation downstream of FGF. Interestingly, PyMT-N-cad tumor cell invasion was found to be attenuated by ERK inhibition, whereas cell migration was negatively regulated by the Akt3 isoform (26, 28). These findings suggest that Slug function in metastatic PyMT-N-cad cells is unlikely to be causing invasion or motility, and is most likely required for cell survival. Although EMT and survival are not necessarily conflicting states, it is conceivable that the EMT and survival-inducing functions of Slug may be mutually exclusive at distant sites, where survival may prevail over invasion.

Our findings demonstrate that Slug knockdown in PyMT-N-cad cells suppresses lung colonization. It was surprising that Slug, alone, would play such a dominant role. Given that lung colonization is a critical step in metastasis, which relies on tumor cell extravasation and survival in the lungs (1), we speculated that Slug promotes distal organ colonization by inhibiting apoptosis. We found that control and Slug-knockdown cells were efficiently arrested in the lungs 5 minutes after intravenous inoculation, but we were unable to detect them in the lungs before 48 hours after injection, which may be due to apoptosis of tumor cells after extravasation and/or to low sensitivity of N-cad immunodetection of small tumor clusters in the lungs. Others have described this phenomenon as "metastatic insufficiency," which is related to the poor survival of disseminated cancer cells due to apoptotic insults encountered throughout metastasis and especially at the stage following the arrest in the lungs (1, 3). In support of a role for Slug in suppressing apoptosis, Slug was found to protect tumor cells from apoptosis induced by radiation, which is reminiscent of the protective effect of Slug against DNA damage observed in hematopoietic progenitor cells (20, 21). In the latter, both Slug and Puma were shown to be targets of p53. Our results agree with these findings, showing that Slug targets Puma, and no other proapoptotic gene, including the p53-response gene, Noxa, or the non-p53 target gene Bim, to suppress apoptosis. It implies that the dual control of Slug and Puma by p53 may constitute a built-in molecular module to ensure a swift transition from cell survival to cell death in response to DNA damage. 

The involvement of Puma in apoptosis is underscored by the fact that Puma siRNA lowered the threshold for induction of apoptosis by doxorubicin. Importantly, the Slug–Puma axis was found to be operative in vivo as shown by that Puma shRNA in Slug-knockdown cells rescued lung colonization, whereas Puma overexpression in PyMT metastatic cells suppressed colonization. In sum, our study points to a pivotal function for Slug in metastasis, which allows tumor cells to overcome apoptosis, survive, and thrive throughout dissemination and colonization of distal organs (1, 2, 3). The latter was achieved by repression of the proapoptotic gene Puma. Hence, disrupting the Slug–Puma axis in cancer cells may impinge on the survival of metastatic cells.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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