Enhanced Genetic Instability and Dasatinib Sensitivity in Mammary Tumor Cells Lacking NEDD9

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Abstract

Elevated expression of the NEDD9/HEF1/Cas-L scaffolding protein promotes tumor cell invasion and metastasis in multiple cancer cell types. Conversely, generation of mammary tumors in the mouse mammary tumor virus (MMTV)-polyoma virus middle T (PyVT) genetic model is delayed by a Nedd9−/− genotype. These activities arise from the role of NEDD9 in assembling complexes and supporting activity of cancer signaling proteins, including FAK, Src, Shc, and AKT, and would support evaluation of NEDD9 expression as an unambiguous biomarker for tumor aggressiveness. However, we here show that despite the initial delay in tumor growth, cells derived from MMTV-PyVT;Nedd9−/− tumors are characteristically hyperaggressive versus MMTV-PyVT;Nedd9+/+ cells in anchorage-independent growth, in growth on three-dimensional matrix produced by tumor-associated fibroblasts, and in formation of tumors after mammary orthotopic reinjection and of lung metastases after tail vein injection. This reversal suggests the specific selection of MMTV-PyVT;Nedd9−/− cells for growth in an in vivo microenvironment. Indeed, MMTV-PyVT; Nedd9−/− cells have increased cell cycle, centrosomal, and mitotic defects, phenotypes compatible with the increased selection of these cells for aggressive growth. Intriguingly, in spite of their aggressive phenotype, MMTV-PyVT;Nedd9−/− cells persistently have low levels of Src activation and are hypersensitive to the Src kinase inhibitor dasatinib. These studies identify NEDD9 as a complex modulator of different aspects of mammary tumor growth. Cancer Res; 70(21): OF1–10. ©2010 AACR.

Introduction

The NEDD9/HEF1/Cas-L noncatalytic scaffolding protein coordinates signaling cascades that control migration and invasion (1–3), cell cycle transition through M-phase (4–6), and apoptosis (7). Upregulation of NEDD9 is associated with enhanced invasion and metastasis in human and murine tumors and cell lines including melanoma, lung, breast, and brain (reviewed in ref. 8). Reciprocally, we recently reported that a Nedd9−/− genotype increased the latency of tumors formed in the aggressive mouse mammary tumor virus (MMTV)-polyoma virus middle T antigen (PyVT) mouse mammary tumor model (9). NEDD9 is a central component of integrin-dependent signaling cascades that activate the FAK and Src kinases to promote cell migration and is also involved in lateral communication through Shc and other proteins to the Ras signaling cascades (8, 9). In MMTV-PyVT;Nedd9−/− tumors, activation of FAK, Src, AKT, and extracellular signal–regulated kinase (ERK) was typically significantly reduced, as was the migratory and invasive behavior of isolated cell lines (9). Taken in sum, these data support the straightforward interpretation that NEDD9 is a tumor-promoting factor and that high levels of NEDD9 in tumors correlate with poor prognosis and treatment resistance. As such, this would support adaptation of NEDD9 expression as a biomarker for aggressive disease, as has been proposed (10).

However, some observations argue that NEDD9 may have a more complex role in tumor formation, invasion, and metastasis. For example, whereas the majority of studies have shown a positive effect of NEDD9 on regulation of cell migration, two works have reported that NEDD9 can negatively regulate the migration of MCF10A normal breast epithelial cells, and mRNA profiling experiments suggest that Nedd9 expression negatively correlates with the metastasis of MDA-MB-231 breast cancer cells to bone (11, 12). This may reflect cell type–specific differences in NEDD9 function or, alternatively, indicate that cellular response to altered expression of NEDD9 is dynamic at different stages of tumor growth. In potential support of the latter interpretation, we have previously shown that NEDD9 directly binds and regulates the activity of the Aurora-A (AurA) kinase (5, 6, 13). Because of its role in timing mitotic progression, AurA levels and activity are highly regulated within a narrow range, with...
both overexpression and depletion causing centrosomal and mitotic defects and contributing to genomic instability (14). In cell culture experiments, we have determined that exogenous overexpression or siRNA depletion of NEDD9 results in similar cell cycle phenotypes, based on aberrant regulation of AurA (5).

These data led us to hypothesize that although the absence of NEDD9 might initially delay tumor appearance, it may also increase in vivo selection pressures affecting tumor growth, thus reconciling contradictory observations. In this study, we have analyzed early-passage cells derived from the mammary tumors of MMTV-PyVT;Nedd9−/− and MMTV-PyVT;Nedd9+/− mice to test this idea. The results of this study strongly support the idea that constitutive absence of Nedd9 in tumors induces defects in cell cycle associated with reduced function of AurA and a more aggressive tumor phenotype. Intriguingly, our data also indicate that the absence of NEDD9 causes a persistent deficiency in Src activation and influences the susceptibility of mammary cancer cell lines to growth inhibition by dasatinib, a drug targeting Src kinase. These studies in sum should significantly inform the use of NEDD9 as a biomarker for clinical response.

Materials and Methods

Cell lines and cell growth measurements

The protocol for derivation of mammary tumor cell lines from MMTV-PyVT;Nedd9−/− and MMTV-PyVT;Nedd9+/− mice was described in ref. 9. Basal proliferation rates for all cell lines were measured by seeding 1 × 10^5 cells into 0.1% gelatin–coated T25 flasks and then propagating them for up to 96 hours, after which cells were trypsinized and counted using trypsin blue exclusion analysis at the times indicated. To contrast cell proliferation on tissue culture plastic or on matrix produced by NIH3T3 cells or tumor-associated fibroblasts (TAF) to simulate an in vivo microenvironment, matrix was prepared and measurements were made using protocols described in detail in refs. 15, 16. Typically, cell lines were plated at a density of 50,000 cells per well in 12-well plates directly onto tissue culture plastic, NIH3T3-derived three-dimensional (3D) matrix, or TAF-derived 3D matrix. Cells were grown for 24, 48, 72, and 96 hours and then treated with 10% (v/v) Alamar Blue solution (Invitrogen); fluorescence was measured with a plate reader. For drug treatment experiments, cells were seeded into 48- or 96-well plates. After 24 hours, vehicle (0.1% DMSO) or drugs [dasatinib and erlotinib (obtained from the Fox Chase Cancer Center Institutional Animal Care and Use Committee and followed the NIH guidelines). For orthotopic injection, 1 × 10^6 cells in 200 μL of PBS were injected (bilateral inguinal) into the fourth mammary fat pad of severe combined immunodeficient (SCID) mice (five mice per cell line). Mice were palpated twice weekly for tumor onset. Tumors were measured by caliper beginning 6 days after injection, and volume was calculated as width × length × 0.4. The mice were euthanized by methoxy-flurane (Mefofane) inhalation when the longest dimension of the largest tumor reached 1.5 cm or, alternatively, if mice exhibited signs of illness or distress. For each mouse, the tumor and lungs were excised, divided in half, and processed either for Western blot or pathologic analysis. Xenografted tumors and both lungs were fixed in 10% phosphate-buffered formaldehyde for 24 hours, embedded in paraffin, sectioned, and stained with H&E. Three sections of each lung separated by 1 mm were evaluated for metastases. Metastases were counted by a pathologist (A.K.S.), using a Nikon Eclipse 50i microscope. The surface area of the lungs was determined with a planimetric software (Image Pro Plus, Media Cybernetics). Metastases were expressed as number of metastases per square centimeter.

For tail vein injections into SCID mice, 0.35 × 10^6 cells suspended in 200 μL of PBS were injected per mouse (five mice per cell line). The SCID mice were monitored daily for signs of developing tumor burden, such as weight loss, reduced mobility, hunched posture, and ruffled fur. All mice were sacrificed at the end of week 3, when two mice showed signs of breathing problems. For each mouse, the lungs were excised, divided in half, and processed for Western blot or pathologic analysis.

Biochemical analysis

For Western blot analysis, tumor sections histologically confirmed to contain >90% tumor tissue were harvested, homogenized, and lysed in PBS-TDS buffer (1× PBS, 1% Triton X-100, 0.1% SDS, 20% glycerol) containing complete protease and phosphatase inhibitor cocktail (Roche Diagnostic). Whole-cell lysates from the MMTV-PyVT;Nedd9−/− and MMTV-PyVT;Nedd9+/+ cell lines were prepared using CellLytic MT Mammalian Tissue Lysis/Extraction Reagent (Sigma). The primary antibodies used were targeted to NEDD9 [2G9; ref. 5; diluted 1:1,000], p130Cas (Santa Cruz Biotechnology; 1:1,000), Aurora (BD Biosciences; 1:1,000), Lyn (1:1,000; Cell Signaling), and phospho-Aurora (1:1,000), AKT (1:1,000), AKT-S 475 (1:1,000), AKT-T 308 (1:500), ERK1/2 (1:1,000), ShcA (1:1,000; Abcam), phospho-ERK1/2-T202/Y204 (1:500), AKT-S 473 (1:1,000), AKT-T 308 (1:500), ERK1/2 (1:1,000), AKT (1:1,000; Cell Signaling), phospho-Lyn-Y 507 (1:500), Lyn (1:1,000; Cell Signaling), and β-actin (1:20,000; Sigma). Secondary horseradish peroxidase–conjugated antibodies were from Pierce Biotechnology. Proteins were visualized using the West-Pico system (Pierce). Image analysis was done using NIH ImageJ (NIH), with signal intensity normalized to β-actin.

Orthotopic and tail vein injections

Care of mice and injection protocols were approved by the Fox Chase Cancer Center Institutional Animal Care and Use Committee and followed the NIH guidelines. For orthotopic injection, 1 × 10^6 cells in 200 μL of PBS were injected (bilateral inguinal) into the fourth mammary fat pad of severe combined immunodeficient (SCID) mice (five mice per cell line). Mice were palpated twice weekly for tumor onset. Tumors were measured by caliper beginning 6 days after injection, and volume was calculated as width × length × 0.4. The mice were euthanized by methoxy-flurane (Mefofane) inhalation when the longest dimension of the largest tumor reached 1.5 cm or, alternatively, if mice exhibited signs of illness or distress. For each mouse, the tumor and lungs were excised, divided in half, and processed either for Western blot or pathologic analysis. Xenografted tumors and both lungs were fixed in 10% phosphate-buffered formaldehyde for 24 hours, embedded in paraffin, sectioned, and stained with H&E. Three sections of each lung separated by 1 mm were evaluated for metastases. Metastases were counted by a pathologist (A.K.S.), using a Nikon Eclipse 50i microscope. The surface area of the lungs was determined with a planimetric software (Image Pro Plus, Media Cybernetics). Metastases were expressed as number of metastases per square centimeter.

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For in vitro kinase assays, cell lysates prepared from MMTV-PyVT;Nedd9+/− versus MMTV-PyVT;Nedd9+/− cell lines were immunoprecipitated using anti-Aurora A antibody (BD Transduction Laboratories) and incubated with γ-32P-ATP and histone H3 (Upstate) substrate, and standard methods were followed for kinase assay as described in ref. 5. Aliquots of the reaction mix were used for SDS-PAGE and Western blot analysis to confirm levels of AurA.

**Immunofluorescence**

Epifluorescence microscopy was done using an inverted Nikon TE300 microscope. All images were acquired as 12-bit images with a Spot RT1 monochrome camera (Diagnostic Instruments). Secondary antibodies labeled with Alexa-488, Alexa-568, and 4,6-diamidino-2-phenylindole (DAPI; to stain DNA) were from Molecular Probes/Invitrogen. To measure cell spreading, cells were fixed in 4% paraformaldehyde for 10 min, permeabilized in 0.2% Triton X-100 for 5 min, and blocked with 3% bovine serum albumin (BSA) in PBS. Anti-paxillin monoclonal antibodies (BD Transduction Laboratories) were used to mark focal adhesions and outline cells. Cell area measurements were made for ~100 cells per experiment using MetaMorph or MetaVue software (Molecular Devices, Universal Imaging) to score pixels within cell perimeters.

To count centrosomes and analyze mitotic spindles, cells were fixed with 4% paraformaldehyde followed by methanol; permeabilized with 1% Triton-X100 in PBS; blocked in 1x PBS, 3% BSA; and incubated with antibodies to γ-tubulin (Sigma; 1:200) and α-tubulin (Sigma; 1:800) to visualize centrosomes and mitotic spindles, respectively.

**Statistical analysis**

We used Kaplan-Meier curves for survival outcomes. For figures, we used generalized linear models with restricted cubic splines estimated by generalized estimating equations. We also used semiparametric regressions and generalized linear mixed effects models. Approaches are similar to those detailed in ref. 9. Analyses were conducted in R (R Foundation for Statistical Computing) and STATA 10 (Stata Corp.).

**Results**

**Lack of NEDD9 increases the aggressiveness of selected MMTV-PyVT mammary tumor cell lines**

We previously compared mammary tumor development in MMTV-PyVT;Nedd9+/− and MMTV-PyVT;Nedd9+/− mice (9). This work indicated that mammary tumor development was significantly delayed in mice with an MMTV-PyVT;Nedd9+/− genotype ($P = 0.0001$). Based on this observation and additional analysis indicating reduced activation of FAK, Src, AKT, and ERK1/2 in MMTV-PyVT;Nedd9+/− versus MMTV-PyVT;Nedd9+/− tumor lysates (9) and reduced cell migration by mammary tumor–derived cell lines lacking Nedd9, we concluded that NEDD9 acted positively in promoting tumor growth. However, continuing analysis of the data from the study indicated that although the initial appearance of MMTV-PyVT;Nedd9+/− tumors was delayed, once tumors had attained palpable mass, the average time until euthanasia was required because of tumor size was similar to that of MMTV-PyVT;Nedd9−/− animals (average, 129 versus 133 days; median, 128 versus 134 days; Fig. 1A).

This adaptation suggested that the absence of NEDD9 might select for compensatory changes that increase tumor aggressorion. To test this idea, we used three MMTV-PyVT;Nedd9+/− and three MMTV-PyVT;Nedd9−/−– derived cell lines for orthotopic reimplantation into mammary fat pads and again monitored the growth of tumors (Fig. 1B and C). All three MMTV-PyVT;Nedd9−/− cell lines formed palpable tumors within 14 days (median, 11 days), reaching maximal size within 40 days after appearance. In contrast, two of the three MMTV-PyVT;Nedd9+/− cell lines yielded mammary tumors that were detected within 55 days after injection (median, 33 days) and grew more slowly, whereas the third cell line failed to form any tumors over a 4-month period (Fig. 1C). In a complementary analysis, we used four cell lines of each genotype to perform tail vein injections followed by measurement of metastases in the lung after 3 weeks. Strikingly, four of four injected MMTV-PyVT;Nedd9−/− cell lines generated a large number of metastases in 20 of 20 injected animals (with an average of 73 metastases/cm²). In contrast, a single micrometastasis was detected in 1 of 20 mice injected with one of the four MMTV-PyVT;Nedd9+/− cell lines assessed, whereas 19 of 20 mice had none at the same time point (Fig. 1D). These data clearly supported the idea that the absence of NEDD9 ultimately selected for more aggressive tumor growth in vivo.

**MMTV-PyVT;Nedd9−/− cells have a consistent growth advantage on 3D matrices and in soft agar**

We had previously determined that MMTV-PyVT;Nedd9+/− cell lines were less migratory and more sensitive to detachment-induced anoikis than MMTV-PyVT;Nedd9+/− cell lines (9). Given these observations, the most plausible explanation for the increased aggressiveness of the MMTV-PyVT;Nedd9+/− cells in vivo would be if they had undergone specific selection for proliferation in a tumor microenvironment. To begin assessing this possibility, we first compared the coefficient of variation in growth rate in a larger panel of cell lines derived from the two genotypes (Fig. 2A). This analysis indicated that among a group of 12 cell lines, the range of doubling times of cells cultured on plastic was significantly greater with the MMTV-PyVT;Nedd9+/− genotype ($P < 0.0001$), with some growing very rapidly and others growing relatively slowly.

Second, the growth of cells on 3D matrices secreted either by immortalized fibroblast cell lines or by primary fibroblasts that have been “primed” to support the growth of tumor cells (i.e., TAFs) can simulate a stromal tumor microenvironment (18). We compared the proliferation rates of a set of MMTV-PyVT;Nedd9+/− versus MMTV-PyVT;Nedd9−/− cell lines cultured on tissue culture plastic,

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on 3D matrix prepared from NIH3T3 cells, and on 3D matrix generated from TAFs (Fig. 2B). Strikingly, although there was no overall statistically significant difference between the two genotypic groups cultured on plastic (as both fast-growing and slow-growing *MMTV-PyVT;Nedd9*−/− lines were assessed), all *MMTV-PyVT;Nedd9*−/− cell lines grew much faster on NIH3T3- or TAF-derived matrix (*P* < 0.001).

Third, we also compared the growth of *MMTV-PyVT;Nedd9*−/− versus *MMTV-PyVT;Nedd9*+/+ cell lines cultured in soft agar to fully assess anchorage-independent growth. In this case, 3 of 4 *MMTV-PyVT;Nedd9*−/− cell lines efficiently formed large colonies in soft agar, whereas 0 of 4 *MMTV-PyVT;Nedd9*+/+ cell lines formed large colonies (Fig. 2C). The increased growth rate of *MMTV-PyVT;Nedd9*−/− versus *MMTV-PyVT;Nedd9*+/+ cells on 3D matrices, in soft agar, and *in vivo*, coupled with the greater growth variability of *MMTV-PyVT;Nedd9*−/− cell lines on tissue culture plastic, suggested that *MMTV-PyVT;Nedd9*−/− cell lines may be more subject to selective pressures for growth in the *in vivo* microenvironment, particularly as the effects were seen in early-passage (<6) populations of cells recovered from tumors.

**MMTV-PyVT;Nedd9**−/− cells have more cell cycle, spindle, and centrosome abnormalities than *MMTV-PyVT;Nedd9*+/+ cells

To begin to establish the basis for the increased variability in the growth of *MMTV-PyVT;Nedd9*−/− cells, we directly assessed whether these cells had signs of increased genomic instability and aneuploidy. Indeed, flow cytometric analysis (Fig. 3A and data not shown) indicated a greater proportion of >4N cells and more abnormal profiles in cells with the *MMTV-PyVT;Nedd9*−/− genotype. These cells typically had a much greater number of abnormal mitoses than *MMTV-PyVT;Nedd9*+/+ lines, including both monopolar (19% versus 13%, *P* = 0.002) and multipolar (67% versus 38%, *P* = 0.001) spindles (Fig. 3B), with consistent increases observed in all cell lines of the *MMTV-PyVT;Nedd9*−/− genotype. Commensurate with this observation, a higher proportion of these cells had supernumerary centrosomes (Fig. 3C).

We have previously shown that NEDD9 binds and controls the activation of the mitotic kinase AurA (5, 6). Defects in AurA activation are often associated with...
centrosomal abnormalities and aneuploidy in human cancers. Intriguingly, we found that the levels of AurA kinase activity were sharply reduced in MMTV-PyVT;Nedd9<sup>−/−</sup> cells (Fig. 3D, top). Further, and unexpectedly, this activity drop correlated with a reduction in the overall level of AurA kinase in this genetic background, rather than a proportional reduction in AurA activity (Fig. 3D, middle). A NEDD9-dependent decrease in AurA might provide an initiating event for the genomic instability manifested by the MMTV-PyVT;Nedd9<sup>−/−</sup> cells. However, overexpression of

![Graph](https://example.com/graph.png)
neither GFP-Nedd9 nor RFP-AurA in these cell lines reverses the centrosomal abnormalities, implying that at minimum, other changes in the regulatory machinery had become critical (Fig. 3D, bottom).

The MMTV-PyVT;Nedd9−/− genotype is sensitized to dasatinib

These data in sum showed that the absence of NEDD9 resulted in a very different profile of mammary tumor cell cycle abnormalities in MMTV-PyVT;Nedd9+/+ versus MMTV-PyVT;Nedd9−/− cell lines. A, fluorescence-activated cell sorting analysis of asynchronously growing MMTV-PyVT;Nedd9−/− versus MMTV-PyVT;Nedd9+/+ cell lines indicates more cells with 4N or greater DNA content. B, mitotic profiles in asynchronously growing cells show fewer normal bipolar spindles (P ≤ 0.001) and more multipolar (P = 0.001) and monopolar (P = 0.002) spindles in MMTV-PyVT;Nedd9−/− versus MMTV-PyVT;Nedd9+/+ cell lines. More than 50 mitoses were counted per cell line in each of three independent experiments. Representative cells were visualized with DAPI (blue), α-tubulin (red), and γ-tubulin (green). Bar, 5 μm. C, increased frequency of supernumerary centrosomes in MMTV-PyVT;Nedd9−/− versus MMTV-PyVT;Nedd9+/+ cell lines (P ≤ 0.001). More than 100 cells were counted per cell line in each of three independent experiments. Images indicate representative cells with normal centrosome count (left) and supernumerary centrosomes (right), visualized with DAPI (blue) and γ-tubulin (green). Bar, 10 μm. D, IP:AurA, immunoprecipitation of total AurA from cell lines followed by in vitro kinase assay with phosphorylated histone H3 as substrate visualized by [γ-32P]ATP and with immunoprecipitated, autophosphorylated (T288) AurA, total AurA, and H3 substrates visualized by Western blot analysis. WCL, Western blot analysis of T288-phospho-AurA and total AurA in whole-cell lysates (WCL). Bottom, the change in the proportion of MMTV-PyVT;Nedd9−/− cells with >2 centrosomes following expression of GFP (G) versus GFP-Nedd9 (G-Nedd9) or RFP (R) versus RFP-AurA (R-AurA) is insignificant (P > 0.1).
growth, and signs of genomic instability were associated with loss of AurA. We had previously shown that Src and FAK had consistently reduced activity in the tumors and derivative cell lines of MMTV-PyVT;Nedd9−/− mice, whereas Shc, ERK, and AKT had statistically significant reduced activity in primary MMTV-PyVT;Nedd9−/− tumors, but greater tumor-to-tumor variability (9). We therefore assessed whether mammary tumor cells positive or negative for NEDD9 displayed different profiles for response to drugs targeting proteins with which NEDD9 functionally interacts.

Strikingly, MMTV-PyVT;Nedd9−/− cell lines were much more sensitive to dasatinib, an inhibitor of the Src kinases, than were MMTV-PyVT;Nedd9+/+ cells (Fig. 4A). This correlated not only with a significantly decreased basal ability of MMTV-PyVT;
Nedd9−/− cell lines to spread but also with enhanced inhibition of spreading following plating in low doses of dasatinib, indicating an inhibition of cytoskeletal rearrangement (Fig. 4B), as well as with the depressed activation of FAK we had previously noted in these cell lines (9). Reanalysis of FAK and Src activation in lysates from the primary tumors arising from orthotopic reinjection (Fig. 1B) indicated that activation of Src remained consistently significantly depressed in tumors of the MMTV-PyVT;Nedd9−/− genotype, although FAK activation was similar in tumors of both genotypes (Fig. 4C).

These data suggest that MMTV-PyVT;Nedd9−/− cells might be specifically vulnerable to additional dasatinib-based targeting of Src because of predisposing prior reduction in pathway activity arising from the absence of the Src partner NEDD9. If so, and given the prior data suggesting that MMTV-PyVT;Nedd9−/− cells were more adapted to extrinsic microenvironment, 3D matrix cues might be expected to rescue cell growth. Strikingly, plating of cell lines on 3D TAF matrix produced markedly different results between MMTV-PyVT;Nedd9−/− and MMTV-PyVT;Nedd9+/+ cell lines (compare Fig. 4D, top and center, with 4A). Although the MMTV-PyVT; Nedd9−/− lines remained more sensitive to dasatinib, overall IC50 values were significantly increased in 3 of 4 lines (Fig. 4D, bottom). In contrast, MMTV-PyVT;Nedd9+/+ cell lines as a group had little responsiveness to 3D TAF matrix, with 3 of 4 lines instead becoming more sensitive to dasatinib.

We also compared the responsiveness of MMTV-PyVT; Nedd9−/− and MMTV-PyVT;Nedd9+/+ cells to C1368, which targets the AurA kinase (Fig. 5A), and erlotinib, which targets epidermal growth factor receptor (EGFR)–dependent signaling (Fig. 5B). In neither case was a specific difference observed, suggesting that the sensitization we observed was specific for Src. Interestingly, although the activity of the EGFR effectors ERK, Shc, and AKT had also been depressed in primary tumors, as in the case with FAK, this was not observed in tumors arising from orthotopic reinjection (Fig. 5C), in which levels were comparable or higher than that in MMTV-PyVT;Nedd9−/− cells, and some tumors showed evidence of recovery of AurA levels (not shown).

Finally, although Src is often considered the primary physiologic target of dasatinib in solid tumors (19), this drug can affect other relevant cellular kinases including Src-family kinases such as LYN (20) as well as c-Kit (19), EGFR (19), Ephrin (21), and others. Given the close physical interactions between Nedd9 and Src kinases (1–3), one possibility was that tumors developing in the absence of NEDD9 might affect the basal expression or activity of Src-related kinases. We determined that Src and Lyn were

Figure 5. Nedd9 status does not affect the response to inhibitors of AurA or EGFR. A, experiment as described in Fig. 4A with the AurA inhibitor C1368 for two cell lines of each genotype; resulting data were averaged (IC50 1.11 versus 2.21 μmol/L; not significant). Similar results were obtained with a second AurA inhibitor, PHA 680632 (results not shown). B, experiment as described in A with the EGFR inhibitor erlotinib (IC50 1.29 versus 1.37 μmol/L; not significant) indicates no cell genotype–dependent difference in IC50 value. C, immunoblots of tumor lysates as described in Fig. 4C. D, expression and activation (reflected by levels of autophosphorylated forms) of Src and Lyn in MMTV-PyVT;Nedd9−/− versus MMTV-PyVT;Nedd9+/+ cell lines treated for 2 h with 200 nmol/L dasatinib.
similarly expressed in MMTV-PyVT;Nedd9−/− and MMTV-PyVT;Nedd9+/+ cells (Fig. 5D) and also established that the activation of these kinases was similarly inhibited by dasatinib. The expression and activity of a number of other dasatinib targets (including EGFR and c-Kit) were also unaffected by Nedd9 genotype (not shown).

Discussion

The variant proliferation rates, increased percentage of cells characterized by supernumerary centrosomes, and anomalous cell cycle compartmentalization in MMTV-PyVT;Nedd9−/− tumor-derived cell lines suggest that in each case, the Nedd9−/− genotype has provided a selection for mutations in the tumor population. The most direct explanation for this selection lies in the deficient expression and activation of AurA in Nedd9−/− cells. AurA activation is required for mitotic entry and transit, and we have shown that siRNA depletion of NEDD9 impairs AurA activation and mitotic progression, leading to the accumulation of aneuploid binucleate cells (4, 5). Our present data show that genetic loss of NEDD9 results in a similar phenotype, in which context it is remarkable that the consequences of a Nedd9−/− genotype are predominantly restricted to effects on tumorigenesis. We suggest that this may reflect a specific requirement for Nedd9 scaffolding function in sustaining the activity and expression of some of its partner proteins in the constitutive growth environment of a tumor. By contrast, the transient activation of focal adhesion–associated proteins, such as Src or FAK, in a normally growing or quiescent cellular context may not require NEDD9 interactions but may be supported by the NEDD9 paralogue BCAR1/p130Cas (discussed in ref. 22), whereas AurA activation may be supported by its other partners, such as Ajuba (23) and Tpx2 (24). The fact that very significant NEDD9 upregulation has been observed in a large subset of aggressive tumors from the lung, breast, and brain (refs. 10, 25, 26; discussed in ref. 22) and head and neck (27), whereas BCAR1, Ajuba, FAK, and Tpx2 have not been reported to be thus overexpressed, is compatible with such a model.

Identifying effective biomarkers for cancer prognosis and drug responsiveness is of great importance in improving the clinical management of cancer. Whereas earlier studies focusing on NEDD9 upregulation in tumors suggest that high NEDD9 levels are specifically associated with poor prognosis and metastasis, the work presented here and in ref. 9 indicates a more nuanced role for NEDD9 in mammary tumor biology, in which both elevated or absent levels of Nedd9 may be associated with aggressive tumor phenotypes. This argues against the straightforward consideration of high NEDD9 levels in tumors as a predictive biomarker. However, the observation that the absence of NEDD9 greatly sensitizes cells to the Src-family targeting agent dasatinib is particularly significant, given the increasing use of dasatinib in the clinic for treatment of breast (28) and other cancers. MMTV-PyVT;Nedd9−/− cells maintain persistently low levels of active Src, suggesting the “double hit” of dasatinib and that a MMTV-PyVT;Nedd9−/− genotype is particularly deleterious to cell survival signaling networks. In this context, the modulation of sensitivity by the 3D matrix produced by TAFs suggests that in vivo, specific niches of tumor cells may be protected, whereas other populations may be more vulnerable. Conversely, the subset of metastatic tumors overexpressing NEDD9 (8, 10, 25, 26, 29) may be particularly resistant to Src-targeting agents and independent of microenvironment-associated survival cues.

MMTV-PyVT;Nedd9−/− cells do not have increased sensitivity to EGFR- or AurA-targeting agents. In contrast to the situation with Src, the cells have overcome the inhibition of the EGFR effectors AKT and ERK observed in primary tumor lysates. Whereas the levels of AurA remain depressed in these tumors, much of the actual cell killing associated with Aurora-inhibiting small molecules has been proposed to be associated with inhibition of Aurora-B, rather than AurA kinase (30), compatible with our observation that reductions in AurA expression do not correlate with increased sensitivity to Aurora-targeting inhibitors. Based on these data, we suggest that NEDD9 may be an excellent candidate to examine as a modulator of response to dasatinib and potentially other Src kinase inhibitors in the ongoing clinical development of this and other Src-targeting agents.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Of interest


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