**TUBB3/βIII-Tubulin Acts through the PTEN/AKT Signaling Axis to Promote Tumorigenesis and Anoikis Resistance in Non–Small Cell Lung Cancer**

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**Abstract**

βIII-tubulin (encoded by TUBB3) expression is associated with therapeutic resistance and aggressive disease in non–small cell lung cancer (NSCLC), but the basis for its pathogenic influence is not understood. Functional and differential proteomics revealed that βIII-tubulin regulates expression of proteins associated with malignant growth and metastases. In particular, the adhesion-associated tumor suppressor maspin was differentially regulated by βIII-tubulin. Functionally, βIII-tubulin suppression altered cell morphology, reduced tumor spheroid outgrowth, and increased sensitivity to anoikis. Mechanistically, the PTEN/AKT signaling axis was defined as a critical pathway regulated by βIII-tubulin in NSCLC cells. βIII-Tubulin blockage in vivo reduced tumor incidence and growth. Overall, our findings revealed how βIII-tubulin influences tumor growth in NSCLC, defining new biologic functions and mechanism of action of βIII-tubulin in tumorigenesis.

**Introduction**

Lung cancer is a lethal adult cancer accounting for the most cancer-related deaths worldwide (1). The most common form, non–small cell lung carcinoma (NSCLC) represents >80% of all cases (2). Over half of the patients with NSCLC have developed metastasis at the time of diagnosis and 5-year survival is approximately 14% (2). Advanced NSCLC is poorly responsive to therapy and the mechanisms responsible for its resistance and aggressive behavior are not well defined.

Alterations in expression of microtubule proteins in cancer cells are emerging as important contributors to chemotherapy resistance (3, 4). Microtubules are cytoskeletal proteins that comprise α- and β-tubulin heterodimers and are involved in many important cellular processes including maintenance of shape, intracellular transport, and mitosis (reviewed in ref. 4). These critical roles make microtubules attractive drug targets for anticancer therapies. Indeed, chemotherapy agents including the microtubule stabilizing (e.g., taxanes) and destabilizing (e.g., vinca alkaloids) drugs exert their toxic effect by binding the β-tubulin subunit of microtubules to induce a potent mitotic block (reviewed in ref. 4). β-Tubulin has seven isoforms that display tissue-specific expression. For example, β1-tubulin is constitutively expressed in many tissues, whereas β3-tubulin and βIII-tubulin are expressed in neuronal tissues, and βIV-tubulin is restricted to hematopoietic tissues (3, 4). Clinical studies show that high βIII-tubulin expression correlates with chemoresistance and poor survival in different tumor types, including breast, ovarian, gastric, and NSCLC (5, 6). Previously, we demonstrated a functional role for βIII-tubulin in regulating chemosensitivity in NSCLC using RNA interference to silence βIII-tubulin expression in NSCLC cells (7–10). Knockdown of βIII-tubulin increased sensitivity via an increase in apoptosis to chemotherapeutic agents both in vitro and in vivo (7–10). However, despite evidence to link βIII-tubulin expression to chemotherapy drug sensitivity, its roles in the tumorigenic and metastatic potential of NSCLC are unknown. Recent clinical data have suggested that high βIII-tubulin expression is correlated to aggressive and metastatic tumors (11, 12). In addition, we recently showed that silencing βIII-tubulin expression in the absence of chemotherapy delayed tumor growth in a mouse model of lung cancer (10). However, how βIII-tubulin exerts its effect on tumor growth and its role in metastases of tumor cells remain unknown.

In this study, we show for the first time that stable suppression of βIII-tubulin in NSCLC cells alters the expression of key proteins involved in regulating tumorigenic and metastatic potential, alters cell morphology, increases anoikis sensitivity, and modulates PTEN/AKT signaling. Finally, we demonstrate that knockdown of βIII-tubulin in NSCLC cells decreases the incidence and growth of lung tumors in two different preclinical mouse models.
Materials and Methods

Cell culture
H460 and A549 NSCLC cells were obtained from ATCC and grown as described (8–10). Upon receipt from ATCC, cell master stocks were prepared and cells for experiments were passaged for less than 6 months. H460 master stock cells were validated by PCR (STR profiling) in December 2012 by Cell Bank Australia (Sydney, Australia). Stable βIII-tubulin shRNA expressing clones were validated by PCR (STR profiling) in October 2014 by the Molecular Genetics Facility at the Garvan Institute (Sydney, Australia). H460 (pRS/βIIIsh144 and pRS/βIIIsh155) and A549 (pRS/βIIIsh161) cell clones stably expressing shRNA targeting βIII-tubulin or control (nonfunctional) shRNA (pRS/Ctrlsh11, pRS/Ctrlsh12, pRS/Ctrlsh12), and two H460 βIII-tubulin "rescue" cell clones (pRS/βIIIsh144/R6 and pRS/βIIIsh144/R17) were developed and characterized as described (10). All cells were routinely screened and found to be free of mycoplasma.

Two-dimensional differential gel electrophoresis
Two-dimensional differential gel electrophoresis (2D-DIGE) is a fluorescent-based proteomics approach that uses charge and size-matched CyDye fluorophores that covalently attach to lysine residues for protein labeling before fractionation. A key strength of this assay is the ability to directly compare protein samples using a multiplex approach, while incorporating a standardized internal control (13, 14). To identify differentially expressed proteins in NSCLC cells with stable suppression of βIII-tubulin, we performed 2D-DIGE on H460 βIII-tubulin shRNA (pRS/βIIIsh144 and pRS/βIIIsh155) or control shRNA (pRS/Ctrlsh11 and pRS/Ctrlsh12) cells (Supplementary Fig. S1). 2D-DIGE was performed as we previously described with modifications (15). Refer to Supplementary Materials and Methods for detailed methodology. For the analysis of the effects of βIII-tubulin knockdown, H460 pRS/βIIIsh14 and pRS/βIIIsh155 cells were grouped and compared with controls, H460 pRS/Ctrlsh11 and pRS/Ctrlsh12 cells. These comparisons were performed for both nuclear and cytoplasmic fractions at two pH ranges, pH 4.5–5.5 (narrow) or pH 4–7 (broad). Analysis of the fluorescent gel images was performed using the Batch Processor and Biological Variation Analysis (BVA) Module of the DeCyder software version 5.0. The BVA module was used to compare each group and data are expressed as average expression ratios and Student t tests of individual protein spots.

Matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry
Protein spots were excised from SYPRO Ruby–stained gels and matrix-assisted laser desorption/ionization–time-of-flight (MALDI-TOF) mass spectrometry performed as described previously (15). Peptide masses were searched against SwissProt/ Trembl protein databases using the Peptidtent tool on ExPASY (http://www.expasy.org/tools/peptident.html) for protein identification.

Western blotting
Western blot analysis was performed using the following antibodies: βIII-tubulin (clone TUJ1, Chemicon), maspin (BD Biosciences), profilin 2 (AbCam), peroxiredoxin 4 (AbCam), total β-tubulin (AbCam), lamin B1 (AbCam), p-AKT (S473), pAKT (T308), total AKT, PTEN (Cell Signaling Technology), and GAPDH (Abcam) as described (10, 16).

Immunofluorescence microscopy
The actin cytoskeleton of NSCLC cells was visualized by phalloidin staining and imaged using a Zeiss immunofluorescence microscope (Zeiss).

Real-time quantitative PCR analysis
The expression of βIII-tubulin and maspin mRNA in NSCLC cells was examined by quantitative real-time PCR (qPCR) as described (10, 16). All data were normalized to the housekeeping gene β2-microglobulin.

Spheroid outgrowth assay
Tumor multicellular spheroids were generated in 96-well round bottom plates as described (17). After formation, spheroids of uniform size were transferred to 6-well tissue culture plates and left to attach and grow for 24 hours. Images were captured on a Zeiss Axiovert 200M inverted microscope and cell outgrowth quantified using the AxioVision software package.

Anoikis assay
To measure anchorage-independent cell death, βIII-tubulin shRNA expressing (pRS/βIIIsh144 and pRS/βIIIsh155), control (pRS/Ctrlsh11 and pRS/Ctrlsh12), or βIII-tubulin rescue (pRS/βIIIsh144/R17) cells were seeded onto poly-(2-hydroxyethyl methacrylate)-coated tissue culture plates. Forty-eight hours after seeding cells, cell death was measured as described (10, 18).

In vivo mouse models

Experimental metastasis model. All animal experiments were approved by the Animal Ethics committee, UNSW (ACEC no. 12/41A). Balb/c nude mice received a tail vein injection of 5 × 105 NSCLC cells expressing βIII-tubulin (pRS/βIIIsh144 and pRS/βIIIsh155), control (pRS/Ctrlsh11 and pRS/Ctrlsh12), or βIII-tubulin rescue (pRS/βIIIsh144/R17) cells were seeded onto poly-(2-hydroxyethyl methacrylate)-coated tissue culture plates. Forty-eight hours after seeding cells, cell death was measured as described (10, 18).

Tumorigenicity model. BALB/c nude mice received subcutaneous inoculations into the flank with 1 × 105 NSCLC cells expressing βIII-tubulin (pRS/βIIIsh144 and pRS/βIIIsh155) or control (pRS/Ctrlsh11 and pRS/Ctrlsh12) shRNA. The detection of tumors within the lung was monitored by micro-CT imaging.

Micro-CT tumor analysis
The presence of lung tumors in the mice was monitored by micro-CT scanning. The micro-CT data were acquired using an Inveon system (Siemens) at 71.88 pixel size, 220 projections, 250 ms integration time, 50 keV photon energy, and 450 μA current. Image data were evaluated using the Inveon software package (Siemens). Tumor margins were identified by contrast thresholding, which allowed the tumor margins (soft tissue density) to be defined against the surrounding lung (air density).
**Statistical analysis**

Data are expressed as the mean ± SE and analyzed using ANOVA or Student t test followed by the nonparametric Dunnett test using the GraphPad Prism program. A P value of <0.05 was considered statistically significant.

**Results**

**Suppression of βIII-tubulin alters the expression of proteins involved in regulating tumorigenesis and metastases**

Previously, we established NSCLC cells that have stable and potent suppression of βIII-tubulin (10). These cells displayed increased chemosensitivity and decreased tumor incidence (10). However, despite clinical reports correlating increased levels of βIII-tubulin in NSCLC with aggressive disease, studies describing its role in tumorigenesis and metastases are limited. To investigate the role of βIII-tubulin, 2D-DIGE proteomics was performed on both the cytoplasmic and nuclear fractions of two independent TUBB3/βIII-tubulin shRNA–expressing H460 NSCLC cell clones (pRS/βIIIhs64 and pRS/βIIIhs159) and two independent control shRNA H460 NSCLC cell clones (pRS/CtrlSh1 and pRS/CtrlSh2 (Supplementary Figs. S1 and S2). Differences in protein expression between the βIII-tubulin shRNA–expressing and control cells were quantitatively determined using the DeCyder image analysis software, which provided statistical information on each individual protein identified on the 2D-DIGE gel. Protein spots of interest were excised and identified by mass spectrometry. Eleven out of a total of 963 proteins [1.1%; isoelectric point (pI) 4-7, broad range] and 56 (21 upregulated and 35 downregulated) out of a total of 1,331 proteins (1.1%; pI 4-7, narrow range) were significantly altered in the cytoplasmic fractions of H460 pRS/βIIIhs64 and Sh59 cells compared with controls (pRS/CtrlSh1 and Sh12). Moreover, 33 out of a total of 1,331 proteins (2.5%; pI 4-7, broad range) and 42 (9 upregulated and 33 downregulated) out of a total of 502 proteins (8.4%; pI 4-5.5, narrow range) were significantly altered in the nuclear fractions of pRS/βIIIhs64 and Sh59 cells compared with controls. A number of proteins identified as being differentially expressed in the βIII-tubulin knockdown cells, are involved in modulating tumor biology (Supplementary Tables S1 and S2). To confirm changes in protein expression and mass spectrometry analysis, a select number of proteins were validated by Western blotting as being differentially expressed in both individual βIII-tubulin shRNA cell clones (pRS/βIIIhs64 and Sh59) compared with controls (pRS/CtrlSh1 and Sh12; Supplementary Fig. S4).

**βIII-Tubulin levels influence the expression of the tumor suppressor maspin in NSCLC cells**

Maspin also known as Serpin B5 is a member of the serine protease inhibitor/noninhibitor superfamily (reviewed in ref. 20) and is classified as a tumor suppressor in different cancer types (20). Maspin was identified by 2D-DIGE as being upregulated (2.29-fold, P < 0.05) in the βIII-tubulin shRNA–expressing cells (Supplementary Table S1). Western blot analysis confirmed the increase in maspin protein levels in pRS/βIIIhs64 and Sh59 cells when compared with controls (Fig. 1A and B). Recently, studies have described the localization of maspin in the cytoplasm or the nucleus as being an important determinant in its tumor suppressor activity (21, 22). To examine whether there was any difference in the cellular distribution of maspin in βIII-tubulin shRNA–expressing cells, cytoplasmic and nuclear extracts were collected and maspin expression measured by Western blot analysis. Control shRNA–expressing cells had higher maspin expression in their cytoplasm when compared with the nucleus (Fig. 1C). βIII-Tubulin shRNA–expressing cells also had increased maspin expression in their cytoplasmic fraction compared with the nuclear fraction (Fig. 1C). However, the expression of maspin was increased in both the cytoplasmic and nuclear fractions of the βIII-tubulin shRNA cells compared with control cells (Fig. 1C). To determine whether this difference was reflected at the gene level, maspin gene expression was measured by qPCR. Maspin mRNA levels were increased in both βIII-tubulin shRNA–expressing cell clones (pRS/βIIIhs64 and Sh59) compared with controls (pRS/CtrlSh1 and Sh12; Fig. 1D). The increase in maspin expression was also confirmed in another H460 NSCLC βIII-tubulin shRNA–independent cell clone (pRS/βIIIhs60), which also displayed potent knockdown of βIII-tubulin (Supplementary Fig. S5). Notably, this cell clone was not used in the 2D-DIGE experiments, thus adding further support to the correlation between βIII-tubulin knockdown and increased maspin expression.

To determine whether βIII-tubulin expression can directly influence maspin expression, βIII-tubulin was restored back into the βIII-tubulin shRNA–expressing cells as described (10) and maspin expression measured. Restoration of βIII-tubulin expression in two individual βIII-tubulin rescue clones (pRS/βIIIhs64/R6 and pRS/βIIIhs159/R17) completely abolished the increased expression of maspin (Fig. 2A and B). In contrast, the empty pREPvector clone (pRS/VE) that maintained suppressed βIII-tubulin expression (Fig. 2A) did not alter maspin expression (Fig. 2A and B). Collectively, these results show for the first time that βIII-tubulin levels directly influence the expression of a tumor suppressor protein.

**βIII-Tubulin suppression induces cell morphology changes in NSCLC cells**

Malignant transformation of tumor cells is associated with changes in organization of the cell cytoskeleton, decreased cell adhesion, increased cell migration and resistance to anchorage-independent apoptosis (23). Given that suppression of βIII-tubulin led to alterations in the expression of proteins involved in regulating tumor growth and metastases, we assessed whether suppression of βIII-tubulin led to changes in cell morphology of NSCLC cells. βIII-Tubulin knockdown produced marked changes in cellular morphology when compared with controls. The control cells had a rounded morphology and poorly defined actin stress fibers (Fig. 3A and B). In contrast, the βIII-tubulin shRNA cells were flatter and displayed prominent actin stress fibers as evidenced by phalloidin staining (Fig. 3C and D). To establish whether the reduced expression of βIII-tubulin was responsible for the change in cell shape, we also examined the cellular morphology of two individual H460 NSCLC βIII-tubulin rescue clones. Strikingly, the actin stress fibers were markedly reduced in these cells and their shape appeared to be very similar to the controls (Fig. 3E and F).

**βIII-Tubulin suppression inhibits tumor spheroid outgrowth, cell migration, and sensitizes NSCLC cells to anoikis**

To determine the functional significance of the altered cell morphology in βIII-tubulin knockdown NSCLC cells, we first measured their ability to grow out from tumor spheroids in 3D culture. In this system, spheroids are formed by the self-assembly of clusters of cell colonies under anchorage-independent conditions where cell–cell interactions dominate (24). Notably, after
transfer of the spheroids to a standard tissue culture plate, H460 βIII-tubulin shRNA–expressing cells failed to attach and grow out from the spheroids (Fig. 4A, panel II). In contrast, control shRNA cells readily attached and grew out from the spheroids (Fig. 4A, panel I). The importance of high βIII-tubulin levels in regulating cell attachment and spheroid outgrowth was further highlighted in another independent NSCLC cell line (A549) that stably expresses TUBB3/βIII-tubulin shRNA. These cells displayed potent and stable knockdown of βIII-tubulin (Supplementary Fig. S7). In agreement with H460 βIII-tubulin shRNA cells, A549 βIII-tubulin knockdown cells (pRS/βIIIsh61) also showed a significant delay in attachment and spheroid outgrowth when compared with controls (Fig. 4B, panels I and II). The ability of βIII-tubulin knockdown cells to adhere to an extracellular matrix (ECM) protein fibronectin (a major ECM protein expressed in the lung and tumor stroma; ref. 25) was also measured. Detachment of tumor cells from the ECM is a critical step in the formation of metastases. We showed that suppression of βIII-tubulin in NSCLC cells induced a modest but significant increase in adhesion to fibronectin (Supplementary Fig. S6A).

Next, we examined whether suppression of βIII-tubulin would influence cell migration. Briefly, A549 NSCLC cells stably expressing βIII-tubulin shRNA (pRS/βIIIsh61) or control shRNA (A549 pRS/Ctrlsh27) were seeded into the wells of modified Boyden chambers, and cell migration was assessed 24 hours later (Supplementary Methods). Stable suppression of βIII-tubulin expression markedly reduced the migratory capacity of NSCLC cells (pRS/βIIIsh61 migration index of 3.95 ± 0.70) when compared with controls (A549 pRS/Ctrlsh27; migration index of 30.93 ± 4.81; Supplementary Fig. S6B). Collectively, these results provide strong evidence that βIII-tubulin plays an important role in regulating cell–cell and cell–matrix interactions as well as the migration capacity of NSCLC cells.

Typically, detachment of normal epithelial cells from ECM results in programmed cell death "anoikis." Tumor cells with high metastatic potential are resistant to anoikis, which allows them to survive under nonadherent conditions and travel in the blood or lymph to form metastases. To determine whether...
Suppression of βIII-tubulin modulated anoikis sensitivity. βIII-tubulin shRNA NSCLC cells were plated in poly-HEMA-coated culture plates to prevent cell adhesion. Cell death was then measured by Annexin V staining. Suppression of βIII-tubulin did not induce cell death in adherent conditions (Fig. 4C). In contrast, when kept in nonadherent conditions, βIII-tubulin knockdown significantly increased anoikis sensitivity in both the pRS/βIII-SH1 and pRS/βIII-SH27 cells when compared with controls (Fig. 4C). To confirm a direct correlation between βIII-tubulin expression and increased anoikis sensitivity, the same experiment was performed using the βIII-tubulin rescue (pRS/βIII-SH27/R17) cells. Reexpression of βIII-tubulin abolished the increased sensitivity to anoikis (Fig. 4D).

βIII-Tubulin modulates AKT activity and PTEN expression in NSCLC cells

To investigate the molecular mechanisms that mediate increased sensitivity to anoikis in βIII-tubulin knockdown cells, we examined the activation of the PI3K downstream substrate AKT kinase. AKT is commonly dysregulated in tumor cells and is important in promoting cell survival and resistance to anoikis (26). To examine whether there was any difference in AKT activity in βIII-tubulin shRNA–expressing cells, we measured AKT phosphorylation (p-AKT) at residues S473 and T308 (phosphorylation at both sites is required for full AKT activation) between control (pRS/CtrlSH1 and SH27) and βIII-tubulin knockdown (pRS/βIII-SH1 and SH27) H460 and A549 NSCLC cells. Both βIII-tubulin shRNA–expressing NSCLC cell lines displayed a clear reduction in p-AKT expression at both S473 and T308 residues when compared with controls (Fig. 5A). Remarkably, restoration of βIII-tubulin back into the H460 βIII-tubulin shRNA cells completely restored p-AKT levels at both phosphorylation sites (Fig. 5B). Next, the expression of the upstream tumor suppressor PTEN, which is a key regulator of AKT activation, was measured. Loss of PTEN is observed in many types of cancer including NSCLC, which often leads to increased p-AKT (27). Control cells had little PTEN expression (Fig. 5C). Strikingly, NSCLC cells with stable βIII-tubulin knockdown displayed markedly increased PTEN expression (Fig. 5C). Moreover, restoration of βIII-tubulin expression abolished the increase in PTEN expression, thus confirming that the low levels of βIII-tubulin were responsible for the differential expression of PTEN (Fig. 5C). Finally, to determine whether the decrease in p-AKT activity in the βIII-tubulin knockdown cells, was in part mediated via increased PTEN expression, we transfected βIII-tubulin shRNA cells with PTEN siRNA (Fig. 6D). Cells transfected with nonfunctional siRNA served as controls. Notably, the decrease in p-AKT expression levels was restored in the βIII-tubulin knockdown cells to the level of control cells (Fig. 5D). These results suggest that high levels of βIII-tubulin in NSCLC cells influence p-AKT activity via PTEN.

To examine whether AKT activity is altered when NSCLC cells are exposed to nonadherent conditions, p-AKT levels were compared between the βIII-tubulin shRNA (pRS/βIII-SH14 and S164) and control shRNA (pRS/CtrlSH12 and S127) H460 and A549 cells at differing time points when in suspension. In H460 cells, a progressive time-dependent decrease in p-AKT expression was observed in both control and βIII-tubulin shRNA cells (Fig. 6A). However, p-AKT levels in βIII-tubulin shRNA (pRS/βIII-S14) cells

Figure 2.

Rescue of βIII-tubulin reverses the increase in maspin expression in βIII-tubulin shRNA NSCLC-expressing cells. A, representative Western blot and densitometry graph showing that in two individual βIII-tubulin rescue cell clones (H460 pRS/βIII-R6 and pRS/βIII-R17), maspin protein expression is restored to control (pRS/CtrlSH1 and SH2) levels when compared with βIII-tubulin shRNA (H460 pRS/βIII-pshRNA) or empty vector control (H460 pRS/EV); n = 6 independent experiments. All samples were normalized to the housekeeping protein GAPDH (*, P < 0.001, H460 pRS/βIII-shRNA and pRS/EV vs. control clones H460 pRS/CtrlSH1 and SH2; #, P < 0.001, H460 pRS/βIII R6 and R17 vs. pRS/C3 or pRS/EV). B, a graph showing that maspin mRNA expression levels are restored to control (H460 pRS/CtrlSH2 and SH27) or empty vector control (H460 pRS/EV) in two individual βIII-tubulin rescue cell clones (H460 pRS/βIII R6 and pRS/βIII R17); n = 4 independent experiments. All samples were normalized to the housekeeping gene β2-microglobulin (*, P < 0.001, H460 pRS/βIII-pshRNA and pRS/EV vs. control clones pRS/CtrlSH1 and SH2; #, P < 0.001, pRS/βIII R6 and R17 vs. pRS/C3 or pRS/EV).
became undetectable as early as 10 minutes after exposure to nonadherent conditions, p-AKT activation in control (pRS/ CtrlSH2) cells was still evident after 20 minutes in suspension (Fig. 6A). In A549 cells, a marked time-dependent increase in p-AKT activation was observed in control cells (pRS/CtrlSH2), whereas little to no p-AKT expression was observed in the βIII-tubulin shRNA cells (pRS/βIIIsh4 and sh5; Fig. 6A). To determine whether the increased levels of PTEN in the βIII-tubulin knockdown cells was responsible for the suppression of AKT activation when the cells were in suspension, PTEN was silenced in βIII-tubulin knockdown cells (pRS/βIIIsh4) using siRNA. Notably, knockdown of PTEN fully restored AKT activation at all of the time points assessed (5, 10, and 20 minutes) in the βIII-tubulin knockdown cells (pRS/βIIIsh4) when compared with cells treated with control siRNA (Fig. 6B). Together, these results clearly show that the activity of a prosurvival signaling protein is suppressed in NSCLC cells with low βIII-tubulin expression when exposed to nonadherent conditions, and that PTEN appears to mediate the suppression of AKT activation.

**βIII-Tubulin suppression increases anoikis sensitivity in vivo**

To expand our findings into a clinically relevant context, and to investigate whether increased sensitivity to anoikis and reduced p-AKT activity induced by βIII-tubulin knockdown would affect the tumorigenic and/or metastatic potential of NSCLC cells, we injected control shRNA or βIII-tubulin shRNA cells into the tail vein of mice and monitored the formation of metastatic lung tumors by micro-CT imaging. After 50 days, 3 of 5 (60%) mice injected with control shRNA cells (pRS/CtrlSH2) developed lung tumors, whereas only 1 of 5 (20%) mice injected with βIII-tubulin shRNA cells (pRS/βIIIsh4) developed lung tumors (Fig. 6C). The presence of tumors within the lungs of mice was confirmed by micro-CT and histology (Fig. 6D and E). This finding suggests that NSCLC cells with low βIII-tubulin expression have reduced tumorigenic and metastatic potential when exposed to the circulatory system.

Finally, to reinforce that βIII-tubulin knockdown cells have a reduced capacity to form tumors in the absence of adhesion to ECM, we assessed tumor growth in mice following subcutaneous implantation of control shRNA or βIII-tubulin shRNA cells suspended in PBS to mimic an ECM-free environment, or in Matrigel to mimic attachment to ECM. After 21 days, tumors derived from control shRNA (pRS/CtrlSH2) cells that were administered in PBS were markedly bigger as compared with tumors derived from βIII- tubulin shRNA (pRS/βIIIsh4) cells in PBS (Fig. 7A). In contrast, when βIII-tubulin shRNA cells were suspended in ECM we observed no significant difference in the size of the tumors generated by control (pRS/CtrlSH2) or βIII-tubulin shRNA cells (pRS/βIIIsh4; Fig. 7B). Similar results were observed when comparing another individual βIII-tubulin shRNA cell clone (pRS/βIIIsh15) and its control (pRS/CtrlSH; data not shown). Collectively, the data provide strong evidence in two independent mouse models of NSCLC that βIII-tubulin levels influence tumor growth via dependence of βIII-tubulin–depleted cells on cell adhesion.

**Discussion**

Overexpression of TUBB3/βIII-tubulin in tumor cells is often associated with resistance to chemotherapeutic drugs. Recently, attention has turned to its clinical correlation with an aggressive tumor phenotype. However, despite reports highlighting βIII-tubulin as a potential biomarker for tumor aggressiveness, studies describing its functional role in tumorigenesis and metastases are limited. Herein, we report for the first time novel roles for βIII- tubulin in (i) altering the expression of proteins involved in promoting tumor growth and metastases; (ii) anoikis sensitivity; (iii) modulating PTEN/AKT signaling; and (iv) promoting tumor incidence and growth in vivo.

Cancer cells with high tumorigenic and metastatic potential differentially express a host of proteins including tumor suppressors, oncogenes, and regulators of the cell cytoskeleton, which enable them to escape apoptosis and achieve rapid growth and motility. To gain an understanding as to whether NSCLC cells with high βIII-tubulin levels have an altered proteomic profile, we performed 2D-DIGE proteomic analysis on isolated cytoplasmic and nuclear fractions from NSCLC cells with stable and potent βIII-tubulin knockdown. Our data demonstrated that βIII-tubulin can alter the expression of key proteins involved in modulating tumor growth and metastases. We demonstrated for the first time that the levels of βIII-tubulin in NSCLC cells are critical in modulating the expression of tumor suppressor proteins. For instance, maspin a member of the serpin family of serine protease inhibitors was originally identified as a tumor suppressor that is
expressed in normal mammary epithelial cells, but is reduced or absent in breast tumor cells (22). Recent studies have explored the potential of maspin as a prognostic marker in different tumor types. Several reports have shown high maspin expression to be a favorable predictor for different tumors, including NSCLC (20). However, for other tumor types such as pancreatic, gallbladder, colorectal, and thyroid, high maspin expression has been associated with a poor prognosis (20). These differences are thought to be attributed to its differential expression within the cell (i.e., cytoplasmic vs. nuclear; ref. 20). Nevertheless, in NSCLC there is an increasing body of evidence to suggest that tumors with high maspin expression have a favorable prognosis (28–30). These studies have been supported using in vitro and in vivo models of lung cancer. Beltran and colleagues (31) demonstrated that restoration of maspin expression in NSCLC cells using artificial transcription factors combined with chromatin modifier compounds, reduced NSCLC metastatic behavior (31). Maspin was also shown to be involved in regulating the survival of lung cancer cells to chemotherapy drugs (32). Our studies showed that suppression of ßIII-tubulin led to an increase in maspin expression, and that rescue of ßIII-tubulin expression back into these cells was sufficient to bring maspin gene and protein expression back to control levels. This result confirms that the levels of ßIII-tubulin are directly responsible for the differential expression of maspin in NSCLC cells. The regulation of maspin in tumor cells is thought to involve a number of processes including control by transcription factors such as p53, ATF-2, PTEN, and Snail (33–35). Interestingly, reports have demonstrated that the carboxy-terminal tail of ßIII-tubulin is subject to posttranslational modifications, which can allow it to form protein–protein complexes with
Suppression of βIII-tubulin modulates AKT activity and PTEN expression in NSCLC cells. A, representative Western blots demonstrating that βIII-tubulin shRNA-expressing cells (H460 pRS/βIII-tubulin shRNA) have significantly decreased phosphorylation of AKT at S473 and T308 residues versus control (H460 pRS/CtrlSH2). Total AKT levels were unchanged. B, representative Western blot analysis showing that reexpression of βIII-tubulin (H460 pRS/βIII R17) back into βIII-tubulin shRNA (H460 pRS/βIII-tubulin shRNA) cells restores p-AKT activity at both S473 and T308 residues back to control (H460 pRS/CtrlSH2). C, representative Western blot analysis demonstrating increased PTEN expression in βIII-tubulin shRNA (H460 pRS/βIII-tubulin shRNA) cells when compared with control (H460 pRS/CtrlSH2). Restoration of βIII-tubulin expression (H460 pRS/βIII R17) returns PTEN expression back to control (H460 pRS/CtrlSH2). Total AKT levels were unchanged. D, representative Western blot analysis and densitometry graph showing restoration of p-AKT S473 expression in βIII-tubulin shRNA (H460 pRS/βIII-tubulin shRNA) cells 72 hours after transfection with PTEN siRNA. Cells transfected with nonfunctional siRNA served as controls. Total AKT levels were unchanged.

*, P < 0.05. GAPDH was used a protein loading control for all Western blots. All data are representative of three independent experiments.

signaling proteins such as small GTPases and/or PKC in cancer cells (36, 37). Furthermore, binding to these proteins initiated signaling cascades, which promoted cellular survival under stress conditions. Therefore, it is possible that high levels of βIII-tubulin in NSCLC cells enhance protein–protein interactions, which in turn activate signaling cascades that control the expression of maspin. Further studies aimed at understanding the molecular link between βIII-tubulin and maspin in NSCLC cells are required.

Our findings showing that the levels of βIII-tubulin in NSCLC cells affects the expression of proteins involved in modulating tumorigenic and metastatic potential prompted us to explore the functional significance of βIII-tubulin in these processes. We showed that suppression of βIII-tubulin had a profound effect on the cell morphology of NSCLC cells as evidenced by their flattened appearance and prominent actin stress fibers. The altered morphology of the βIII-tubulin shRNA cells was not associated with any significant change in the expression of total actin or tubulin (results not shown). Reorganization of the cell cytoskeleton due to loss or gain in the expression of proteins associated with the cytoskeleton has been reported in tumor cells. For instance, Bharadwaj and colleagues (38) demonstrated that breast cancer cells with high tumorigenic and metastatic potential had reduced amounts of the actin-binding protein tropomyosin 1 (TM1; ref. 38). Reintroduction of TM1 into these cells produced a striking alteration in the cytoskeleton with a flattened phenotype and prominent actin stress fibers. This correlated with a reduction in their tumorigenic potential by increased sensitivity to anoikis (38). Notably, our proteomic analysis identified a significant increase in TM1 expression in the βIII-tubulin shRNA cells (Supplementary Table S1). In addition, studies have also reported that maspin can modulate changes in the expression of proteins associated with the cytoskeleton that correlate to increased cell adhesion and a reduced migratory phenotype (39). We cannot exclude the possibility that maspin may be acting in concert with other proteins and it is possible that both TM1 and maspin may in part be involved in the reorganization of the cell cytoskeleton in NSCLC cells with suppressed βIII-tubulin.

Cancer cells must detach from an ECM matrix, survive under anchorage-independent conditions to travel in the blood or lymphatic system, and adhere and proliferate at new organ sites (40). Therefore, tumor cells with metastatic potential have acquired altered mechanisms of cellular adhesion as well as resistance to anoikis (40, 41). We demonstrated that suppression of βIII-tubulin prevented/delayed the ability of NSCLC cells to adhere and grow out from multicellular tumor spheroids. In addition, we showed that βIII-tubulin knockdown increased cell adhesion to fibronectin. These results support the notion that βIII-tubulin plays an important role in regulating cell–cell and cell–matrix interactions. We also demonstrated that suppression of βIII-tubulin sensitized NSCLC cells to anoikis. Importantly, anoikis was reversed by reexpression of βIII-tubulin, indicating a direct
correlation between βIII-tubulin levels and anoikis sensitivity. To gain an understanding into the mechanisms that link βIII-tubulin knockdown and increased anoikis, we examined the activation of the prosurvival signaling protein AKT in βIII-tubulin knockdown cells. There are numerous reports that highlight the importance of the AKT signaling pathway in tumor cells as a mechanism that promotes resistance to various forms of apoptosis (40). Interestingly, we showed that suppression of βIII-tubulin correlated with a decrease in AKT phosphorylation at both phosphorylation sites (S473 and T308). Strikingly, AKT phosphorylation was fully restored following reexpression of βIII-tubulin. One of the key regulators of AKT activity is the tumor suppressor PTEN. The principal catalytic function of this phosphatase is to dephosphorylate phosphatidylinositol-triphosphate, which is a potent activator of AKT (42). Loss of PTEN function leads to AKT signaling hyperactivation and is a common feature in a wide range of tumors (43). In agreement with our finding that inhibition of βIII-tubulin expression correlates with decreased AKT phosphorylation, we showed that expression of PTEN is markedly increased in βIII-tubulin shRNA–expressing cells. Furthermore, knockdown of PTEN using siRNA in the βIII-tubulin shRNA cells restored p-AKT activity back to controls. Finally, we showed that when kept in nonadherent conditions, which is a surrogate marker for tumorigenicity and is an environment in which cells are susceptible to anoikis, AKT activation in βIII-tubulin shRNA cells became undetectable much earlier compared with control cells.

Figure 6. Suppression of βIII-tubulin reduces tumor incidence in vivo. A, representative Western blots from βIII-tubulin shRNA (H460 pRS/βIIIsh4 and A549 pRS/βIIIsh61) NSCLC cells showing a decrease in p-AKT (S473) expression levels at different time (5, 10, 20 minutes) points when exposed to nonadherent conditions. Total AKT levels were unchanged. GAPDH was used as a protein loading control; n = 3 independent experiments. B, representative Western blot analysis showing that knockdown of PTEN in βIII-tubulin shRNA H460 (pRS/βIIIsh4) cells using siRNA completely reverses the decrease in p-AKT (S473) expression over time (5, 10, 20 minutes) when exposed to nonadherent condition. Cells (pRS/βIIIsh4) treated with nonfunctional siRNA served as controls; n = 3 independent experiments. C, graphic representation of tumor incidence for control (H460 pRS/Ctrlsh2) and βIII-tubulin shRNA (H460 pRS/βIIIsh4) cells; n = 5 animals per group. D, representative micro-CT images of lung tumors (arrowheads) on the axial, coronal, and sagittal planes 50 days after tail vein injection of control (H460 pRS/Ctrlsh4) or βIII-tubulin shRNA (H460 pRS/βIIIsh4) NSCLC cells. E, representative hematoxylin and eosin stain showing the presence of tumors within the lung of mice injected systemically with control shRNA (H460 pRS/Ctrlsh2) or βIII-tubulin shRNA (H460 pRS/βIIIsh4) NSCLC cells. T, tumor; NL, normal lung; and H, heart.
Suppression of βIII-tubulin reduces tumor growth in an anchorage-independent environment. A, graph showing tumor volumes (mm³) following subcutaneous injection of control shRNA (pRS/CtrlSH2) or βIII-tubulin shRNA NSCLC cells (pRS/βIIIISH4) when administered in an anchorage-independent environment (PBS). B, graph demonstrating tumor volumes (mm³) following subcutaneous injection of control shRNA (pRS/CtrlSH2) or βIII-tubulin shRNA NSCLC cells (pRS/βIIIISH4) when administered in an anchorage-dependent environment (Matrigel). n = 3–5 mice for βIII-tubulin knockdown and control cells in PBS; n = 6–7 animals for βIII-tubulin knockdown and control cells in Matrigel; values presented as mean ± SEM.

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Disclosure of Potential Conflicts of Interest

M. Kavallaris received an industry research grant from Benitec Biopharma to develop a lung cancer therapeutic. This grant did not fund this research. M. Kavallaris has ownership interest in a patent on methods for detecting and modulating the sensitivity of tumor cells to antimitotic agents. No potential conflicts of interest were disclosed by the other authors.
References

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