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Inhibition of TWIST1 Leads to Activation of Oncogene-Induced Senescence in Oncogene Driven Non-Small Cell Lung Cancer

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Abstract

A large fraction of non-small cell lung cancers (NSCLC) are dependent on defined oncogenic driver mutations. Although targeted agents exist for *EGFR*- and *EML4-ALK*-driven NSCLC, no therapies target the most frequently found driver mutation, *KRAS*. Furthermore, acquired resistance to the currently targetable driver mutations is nearly universally observed. Clearly a novel therapeutic approach is needed to target oncogene driven NSCLC. We recently demonstrated that the basic helix-loop-helix transcription factor *Twist1* cooperates with mutant *Kras* to induce lung adenocarcinoma in transgenic mouse models and that inhibition of Twist1 in these models led to *Kras*-induced senescence. In the current study, we examine the role of *TWIST1* in oncogene driven human NSCLC. Silencing of *TWIST1* in *KRAS* mutant human NSCLC cell lines resulted in dramatic growth inhibition and either activation of a latent oncogene-induced senescence program or in some cases, apoptosis. Similar effects were observed in *EGFR* mutation driven and *c-Met* amplified NSCLC cell lines. Growth inhibition by silencing of *TWIST1* was independent of p53 or p16 mutational status and did not require previously defined mediators of senescence, p21 and p27, nor could this phenotype be rescued by overexpression of *SKP2*. In xenograft models, silencing of *TWIST1* resulted in significant growth inhibition of *KRAS* mutant, *EGFR* mutant and *c-Met* amplified NSCLC. Remarkably, inducible silencing of *TWIST1* resulted in significant growth inhibition of established *KRAS* mutant tumors. Together these findings suggest that silencing of *TWIST1* in oncogene driver dependent NSCLC represents a novel and promising therapeutic strategy.

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Conflict Of Interest

The authors declare no conflict of interest.

Keywords

TWIST1; OIS; KRAS; NSCLC; EGFR

Introduction

Lung cancer is the leading cause of cancer death in the United States and worldwide. The five year survival for all lung cancer patients is a dismal 15% (1). The recent advances in the treatment of NSCLC have come from recognition that NSCLC that is not a single disease entity but rather a collection of distinct molecularly driven neoplasms. This paradigm is typified by the recent progress made in the treatment of patients with *EGFR* mutant and *EML4-ALK* translocation driven adenocarcinomas of the lung with tyrosine kinase inhibitors targeting these oncogenes (2). Unfortunately, acquired resistance to the currently targetable driver mutations arises in nearly all cases, typically within the first two years of therapy (3, 4). Furthermore, little if any progress has been made in the treatment of patients with the most frequently observed driver oncogene, mutant *KRAS*. It has been over two decades since the first mutation in the *KRAS* oncogene was identified in non-small cell lung cancer (NSCLC). *KRAS* is mutated in one third of all malignancies and approximately 20% of all NSCLC (5). *KRAS* mutation, which results in a constitutively active oncoprotein, has been shown to predict lack of benefit from chemotherapy in the adjuvant setting, to portend worse prognosis in metastatic NSCLC, and possibly to increase risk of recurrence in early stage disease (5). Although testing for *KRAS* mutation status is routinely done, there are no currently approved therapies targeting this critical pathway in NSCLC.

The principle of “oncogene addiction”, the absolute dependency on an oncogene for a tumor's survival, has been well supported by several preclinical models and in multiple tumor types (6). An emerging concept of “non-oncogene addiction”, the dependency of tumor cells on factors which are not inherently oncogenic, but which are required for survival due to the oncogenic state of the tumor, is now gaining acceptance. To that end, several investigators have performed large scale synthetic lethal screens looking for genes which are essential for survival of *KRAS* mutant tumor cells and have suggested therapeutic strategies that may specifically target *KRAS* mutant tumors (7-11). To date it is unclear whether the inhibition of candidate genes identified in these screens can reproducibly produce synthetic lethality in *KRAS* mutant cancer specifically or are more generally required for tumor proliferation (12).

Another potential therapeutic strategy in *KRAS* mutant tumors is activation of latent oncogene-induced senescence (OIS). OIS is an irreversible cell cycle arrest that is characterized by cells displaying an enlarged, flattened cytoplasm, increased senescence associated beta-galactosidase (SA- β -Gal) activity, increased chromatin condensation and changes in gene expression associated with DNA damage and cell cycle checkpoint pathways. OIS is thought to be triggered early during tumorigenesis after oncogene activation and serves as a checkpoint to prevent pre-malignant lesions from progressing to malignancy (13).

The emerging concept of using prosenescence therapies for cancer has generated considerable interest (14, 15). Interestingly, several studies have demonstrated that bypass of senescence in mouse models of mutant *Kras*-mediated adenocarcinoma of the lung and pancreas is essential for tumorigenesis (16-18). Therefore, activation of oncogene-induced senescence (OIS) in *KRAS* mutant NSCLC may be an effective therapeutic strategy. Disruption of the p53-ARF and p16-Rb pathways was previously thought to be required for bypass of RAS mediated senescence (18). However, recent data suggests that the

requirement of these pathways is context dependent, as inactivation of Skp2 can lead to activation of OIS independent of the p53-ARF pathway through Atf4, p27 and p21 (19). Activation of suppressed senescence pathways in *KRAS* mutant NSCLC through inhibition of key downstream mediators of oncogene-induced senescence would be a valuable treatment in NSCLC.

Previous murine studies have suggested a role for Twist1 as a factor in preventing oncogene-induced senescence through inhibition of the Rb and p53 pathways (20, 21). TWIST1 is a basic helix-loop-helix (bHLH) transcription factor which plays critical roles throughout development influencing mesoderm formation, neurogenesis, myogenesis and neural crest cell migration and differentiation (22). These roles are highlighted by the findings that *Twist1* deletion is embryonic lethal in mice and that germline haploinsufficiency of *TWIST1* in humans leads to Saethre-Chotzen Syndrome, which is characterized by craniosynostosis and limb abnormalities (23, 24). A number of reports have implicated TWIST1 in oncogenesis through its ability to inhibit DNA damage-induced apoptosis, prevent OIS and promote metastasis through induction of the epithelial-mesenchymal transition (EMT) (25). Increased *TWIST1* expression is correlated with increased risk of metastasis and poor prognosis in a number of solid tumor types including breast, prostate, ovarian and lung (25). We have recently shown that *Twist1* cooperates with mutant *Kras* to induce adenocarcinoma of the lung in transgenic mouse models and inhibition of *Twist1* in these models led to activation of *Kras*-induced senescence (26). However, significant differences exist between transgenic mouse models of cancer and human disease. In the current study, we explore whether *TWIST1* plays a critical role in human NSCLC characterized by multiple defined oncogenic drivers.

Materials and Methods

Cell Culture

All human non-small cell lung cancer cell lines, (A549, H460, H358, H727, H23, Calu-1, Calu-6, H1975, H3255, H1993) and embryonic kidney cell line HEK 293T were obtained from the American Type Culture Collection and grown in media as recommended.

SA- β -gal staining

At 12 $\pm$ 2 days after infection with shRNA lentiviruses, the cells were washed twice with phosphate-buffered saline (PBS) and then fixed with PBS containing 2% formaldehyde and 0.25% glutaraldehyde for 5 min. The cells were then incubated at 37°C for 20 hr with staining solution (40 mM citric acid sodium phosphate, pH 6.0, 1 mg/ml 5-bromo-4-chloro-3-isolyl-b-D-galactoside [X-gal, Fisher, Pittsburgh, PA], 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 150 mM NaCl, 2 mM MgCl₂). After incubation, cells were washed twice with PBS and viewed with bright-field microscopy.

Immunoblot analysis

Cells were lysed on ice for 60 min in radioimmunoprecipitation assay buffer supplemented with protease and phosphatase inhibitors (Sigma-Aldrich) and clarified by centrifugation. Protein concentrations were determined by Bradford protein assay (Bio-Rad Laboratories). Equal protein concentrations of each sample were run on NuPAGE bis-Tris gels (Invitrogen) and electrophoretically transferred to polyvinylidene difluoride membranes. After being blocked with 5% dried milk in TBS containing 0.2% Tween 20, the filters were incubated with primary antibodies. The following primary antibodies were used: goat anti-Actin (C-11, Santa Cruz), rabbit anti-GAPDH (FL-335, Santa Cruz), anti-p21 (Ab-1, Calbiochem), mouse monoclonal anti-p27 (F-8, Santa Cruz), rabbit anti-Parp-1/2 (H-250, Santa Cruz) rabbit anti-cleaved Parp (Cell Signaling), anti-p53 (DO-1, Santa Cruz). After washing and incubation

with horseradish peroxidase (HRP)-conjugated anti-Goat or anti-mouse IgG (Amersham, Piscataway, NJ), the antigen-antibody complexes were visualized by chemiluminescence (ECL detection system; Perkin Elmer, Boston, MA or SuperSignal West Femto Maximum Sensitivity Substrate; Thermo Fisher Scientific, Rockford, IL).

Colony formation assay

On day 4 or 6 after infection with the indicated shRNA lentiviruses, cells were plated in 12-well plates at a density of 5,000-10,000 cells/well. On day 12, the cells were stained with crystal violet (0.5% in 95% ethanol).

Cell Cycle Analysis

Cells were harvested at indicated times, fixed with 70% cold ethanol and stained with stained with propidium iodide and analyzed by flow cytometry as previously described (27).

Lentiviral shRNA and cDNA Overexpression Experiments

293T cells were seeded (2.5×10^6 cells) in T25 flasks. Lentiviral particles were generated using a three-plasmid system and infected as per the TRC Library Production and Performance Protocols, RNAi Consortium, Broad Institute (28) shRNA constructs were obtained from the Broad RNAi Consortium and clone IDs as are follows: TWIST1: TRCN0000020539 (shTWIST1-1) and TRCN0000020543 (shTWIST1-2). P53: TRCN000003756 (shp53-1) and TRCN0000010814 (sh53-2). The pLKO.1-shRNA scramble vector was obtained from Dr. David M. Sabatini through Addgene (Addgene plasmid 1864) as previously described (29). The Tet-pLKO-puro vector was from Dmitri Wiederschain through Addgene (Addgene plasmid 21915) as previously described (30). The pLKO.1 – Neo vector was obtained from Sheila Stewart through Addgene (Addgene plasmid 13425) and oligonucleotides for TWIST1-1, TWIST1-2 and scrambled control were cloned into this vector and used to for experiments with shRNAs targeting p53. All constructs were sequence verified. Twenty-four hours after infection, cells were treated with 1 μ g/ml puromycin or 500 μ g/ml G418 and passaged once 80% confluent.

Xenograft Experiments

5×10^5 viable cells were resuspended in equal volumes of PBS and Matrigel and injected subcutaneously in adult homozygous NOD/SCID mice (Harlan Laboratories). For doxycycline inducible cell line experiments, once the tumors reached 150 mm³ (average, 14 d), the mice were given 5% dextrose either with or without 2 mg/ml doxycycline in the drinking water. Drinking water was replenished at least once a week. Statistical analyses were performed for each xenograft separately. Initial tumor volume measurements are summarized using means, SDs, and ranges. Growth patterns were summarized graphically by plotting the mean and SD for each treatment group over time. A conditional *t* test is used to compare shTWIST1 tumors with the control (shScram). A *P* value of <0.05 is considered statistically significant.

Results

Silencing of *TWIST1* activates latent oncogene-induced senescence in human non-small cell lung cancer cells

To explore the role of *TWIST1* in human NSCLC, we employed a shRNA strategy to silence *TWIST1* in NSCLC cell lines with distinct mutational backgrounds (*KRAS* mutant, *EGFR* mutant and *c-Met* amplified) (Supplemental Table 1). In our previous study and current study, we have demonstrated that the shRNAs used in this report (shTWIST1-1 and shTWIST-2) are able to significantly suppress TWIST1 levels on both the transcript and

protein level ((26) and Supplemental Fig. 1A, B). Upon silencing of *TWIST1* with two separate shRNAs compared to a control scrambled shRNA, we observed dramatic growth inhibition in colony formation assays for all ten NSCLC cell lines examined (Fig. 1). To confirm that these effects were not due to off-target effects of the shRNAs used in this study, we stably overexpressed a mouse *Twist1* cDNA in A549 cells and demonstrated this overexpression rescued the A549 cells from the growth inhibitory effects of silencing human *TWIST1* ((26) and Supplemental Fig. 2). In addition to the nine lines shown in Fig. 1, an additional *KRAS* mutant NSCLC cell line, Calu-6 demonstrated extensive apoptosis by day 4 after silencing and could not be plated for this assay. In all, we observed significant growth inhibition in seven *KRAS* mutant NSCLC cell lines, two cell lines which harbor an activating mutation in *EGFR* as well as in a cell line with *c-Met* amplification. Taken together these data suggest that *TWIST1* may play a critical role in lung cancer tumorigenesis in the context of multiple driver mutations.

We observed a dramatic activation of latent OIS as demonstrated by positive SA- β -Gal staining and characteristic enlarged, flattened cytoplasm morphology (Fig. 2) in three *KRAS* mutant NSCLC cell lines (H460, A549, and H358). These findings were further supported by profound cell cycle arrest in these cell lines (Fig. 3A) as well as by induction of the senescence markers, p21 and/or p27 in all cell lines examined (Fig. 3B). Furthermore, a gene expression analysis after silencing of *TWIST1* in three *KRAS* mutant cell lines (H460, H727, and H358) revealed several highly significant gene sets involved with growth arrest (Supplemental Figure 7 and 8 and data not shown). Collectively, these series of experiments support a role for *TWIST1* in the suppression of oncogene-induced senescence in *KRAS* mutant lung cancer cell lines. Interestingly, in a subset of cell lines (Calu-1, Calu-6), we also observed evidence of apoptosis including Parp cleavage. These findings are consistent with previously known anti-apoptotic role of *TWIST1*, and suggest complementary mechanisms by which inhibition of *TWIST1* may block tumor growth (31).

Growth inhibition by silencing *TWIST1* is not dependent on the p53-p21 pathway or p27-SKP2 pathway in NSCLC

A previous study had demonstrated a requirement for p16 in mediating OIS in pancreatic ductal epithelial cells after silencing of *Twist1* (21). Furthermore, prior work had suggested a possible role for the p53-p21 pathway in mediating OIS after suppression of *TWIST1* in human cancer cell lines (20). In the present study, we examined whether these pathways played a key role in mediating the growth inhibition observed after *TWIST1* silencing in human lung cancer cell lines. Since the p16 pathway was disrupted either through deletion, methylation or mutation in every cell line examined (Supplemental Table 1), it appeared that p16 was not required for the growth inhibition observed. In addition, only two (H460, A549) of the ten lines had wild type p53 which suggested that inhibition of p53 could not be the only mediator of growth inhibition after silencing of *TWIST1*. To test whether p53 was required for growth inhibition in p53 wildtype cell lines, we silenced p53 using a shRNA strategy and confirmed knockdown of p53 at baseline and under conditions that resulted in p53 stabilization (*TWIST1* knockdown and DNA damage) (Fig. 4A, Supplemental Fig. 3 and data not shown). After knockdown of *TWIST1*, stabilization of p53 was observed in both the shScrambled control H460 and A549 cells and was accompanied by induction of p21. However, after silencing of p53, p53 expression and p21 induction was lost (Fig. 4A and Supplemental Fig. 3A). Despite the loss of p53 in these cell lines, growth inhibition induced by suppression of *TWIST1* was unaltered (Fig. 4B and Supplemental Fig. 3B). It is possible that through disabling a critical member of the OIS pathway such as p53, the cellular response after suppression of *TWIST1* may shift from a senescence program to an apoptotic program. To assess whether a shift to an apoptotic program is occurring with depletion of p53, we have checked for evidence of PARP cleavage which is a well validated

marker of apoptosis. In H460 and A549 cells, we observed only a modest amount of apoptosis in our control shScram line after silencing of *TWIST1*. Interestingly, after depletion of p53, we observed a decrease in apoptosis compared to the control cells after silencing *TWIST1* (Figure 4). This would argue against apoptosis as significant mechanism for the observed growth inhibition since the modest decrease in apoptosis after p53 depletion did not result in even a partial rescue of the observed decrease in clonogenic survival following silencing of *TWIST1*.

Since we had observed p53-independent p21 induction in several of our p53 disrupted cell lines (Fig. 3B), we examined whether p21 was required for the growth inhibition observed. We silenced p21 using shRNAs in 3 cell lines (H460, A549, and H727) and observed a significant reduction in p21 expression but not in the cyclin dependent kinase inhibitor, p27 (Supplemental Fig. 4A). Silencing of p21 did not evidently affect the inhibition of colony formation induced by knockdown of *TWIST1* (Supplemental Fig. 4B).

Recent studies had suggested that the SKP2-p27 pathway was important for the induction of senescence (19). In addition, after silencing of *TWIST1* we observed induction of p27 in several of these cell lines (Fig. 3B). We therefore attempted to knock down p27 in H460 cells. Despite screening eight shRNAs targeting p27, we were only able to derive a partial knockdown of this gene; complete knockdown prevented the establishment of a puromycin resistant cell line even at early time points (Supplemental Fig. 5 and data not shown). It is possible that these cells lines require cytoplasmic p27, which has been demonstrated in several studies to be oncogenic and can cooperate with *KRAS* in lung tumorigenesis ((32) and reviewed in (33)). Partial knockdown of p27 markedly suppressed p27 induction after loss of *TWIST1*, however, growth inhibition was unperturbed (Supplemental Fig. 5B). To further evaluate the requirement of the p27 pathway in mediating growth suppression after loss of *TWIST1*, we overexpressed the key regulator of p27 stability, *SKP2* (34) in the A549 and H460 cell lines. Overexpression of *SKP2* largely blocked p27 induction after silencing of *TWIST1* but did not rescue loss of *TWIST1* (Supplemental Fig. 6). Interestingly, loss of *TWIST1* expression in the setting of *SKP2* overexpression did result in modest increase in apoptosis as demonstrated by the appearance of cleaved PARP.

Microarray analysis of NSCLC cell lines after silencing of *TWIST1* revealed a striking cell cycle arrest gene signature

To further elucidate possible mechanisms of *TWIST1* dependency in NSCLC, we performed microarray analysis on three *KRAS* mutant NSCLC lung cancer cells (H460, H358, and H727) after silencing of *TWIST1*. A profound cell cycle arrest signature was observed associated with silencing of *TWIST1* (Supplemental Fig. 7). Genes encoding general regulators of cell cycle progression (Cyclin D, E1, A2, B1, CDK2) and specific regulators of progression through S phase (PCNA, *SKP2*) and mitosis (*BUB1*, *CDC20*, *MAD2*, *AURKA*, *FOXM1*) were significantly decreased after silencing of *TWIST1* (Supplemental Fig. 7). Gene set enrichment analysis (GSEA) (35), comparing global gene expression patterns after *TWIST1* silencing with patterns from control (shScram) cell lines revealed a highly significant similarity to multiple gene sets for cell cycle arrest (Supplemental Fig. 8A). This dataset also demonstrated highly significant similarity to a gene set predicting resistance to doxorubicin (Supplemental Fig. 8B). This is consistent with previous studies demonstrated that *TWIST1* overexpression could confer chemoresistance in other tumor types (reviewed in (25)). Two gene sets involving the RAS signaling pathway were also highly significantly similar, including a gene signature of *NRAS* induced genes (decreased in sh*TWIST1* compared to control), and a gene signature following exposure of cells to the RAS inhibitor, salirasib (Supplemental Fig. 8C). This is consistent with our findings that inhibition of *TWIST1* is critical for *KRAS* mutant NSCLC survival. Interestingly, a gene set for downregulation of the target genes of YB-1, a known *TWIST1* target gene (36), was also

highly similar and suggests a possible role for YB-1 in mediating TWIST1 function (Supplemental Fig. 8D).

Silencing of *TWIST1* results in a significant growth inhibition in oncogene driver dependent NSCLC xenograft models

To further evaluate TWIST1 as a potential therapeutic target for NSCLC with a defined oncogenic driver, we examined the effect of TWIST1 targeting on both tumor initiation and maintenance in a xenograft hind flank model. *KRAS* mutant (A549, H358, and H727), *EGFR* mutant (H1975) and *c-Met* amplified (H1993) NSCLC cell lines were infected with either scrambled shRNA or TWIST1-1 shRNA and on day four after infection were implanted in the hind flank of NOD/SCID animals. Knockdown of *TWIST1* significantly inhibited tumor growth in each of these oncogene driven NSCLC xenograft models (Fig. 5). Remarkably, silencing of *TWIST1* in the *c-Met* amplified and dependent cell line H1993 completely prevented tumor formation, suggesting a potentially critical role for the TWIST1 pathway in this context.

To examine the role of TWIST1 in tumor maintenance of fully established tumors, we constructed syngenic A549 cells containing either shRNA targeting *TWIST1* or a scrambled control under the control of a doxycycline responsive promoter (30). In absence of doxycycline, these cell lines had similar growth characteristics. However, upon addition of doxycycline, *TWIST1* was silenced in the A549 Tet-shTWIST1 cell line, p21 was induced and growth inhibition was observed (Fig. 6A, B). To determine the requirement of *TWIST1* in established tumors, the A549 Tet-shScram and A549 Tet-shTWIST1 cell lines were implanted in the hind flank of NOD/SCID animals. After the formation of palpable tumors, doxycycline was added to the drinking water of half the animals for each tumor type. Although the administration of doxycycline to animals with A549 Tet-shScram xenograft tumors did not affect the growth kinetics, silencing of *TWIST1* by doxycycline administration to animals with the A549 Tet-shTWIST1 xenografts resulted in significant growth inhibition (Fig. 6C). Interestingly, this growth inhibition was accompanied by induction of p21 specifically in the tumors in which TWIST1 was silenced. This finding supports the role of growth arrest and OIS in mediating the observed growth inhibition *in vivo*. In summary, these data suggests that inhibition of *TWIST1* in established tumors may be an effective therapeutic strategy.

Discussion

We have demonstrated that TWIST1 is a promising novel therapeutic target for oncogene driven NSCLC, through activation of oncogene-induced senescence (OIS). We found that silencing of *TWIST1* resulted in dramatic growth inhibition and activation of latent (OIS) and/or apoptosis. This marked growth inhibition appeared to be independent of the p53-p21, p16 and SKP2-p27 pathways. Similar effects were seen in *KRAS* mutant, *EGFR* mutant and *c-MET* amplified cell lines, suggesting that inhibition of TWIST1 may be a potential therapeutic target in a large percentage of oncogene driven lung cancer patients.

Although it has been over two decades since the first mutation in the *KRAS* oncogene was identified in NSCLC, no current therapies target this critical oncogene. In this study, we have demonstrated that TWIST1 is required for proliferative growth of oncogene-driven NSCLC including multiple *KRAS* mutant lines, and that silencing of *TWIST1* leads to activation of OIS. Previous studies have defined the bypass of senescence as a requirement for mutant *KRAS* mediated carcinogenesis in the lung (16-18). In this current study, we elucidated a possible synthetic interaction between mutant *KRAS* and *TWIST1* that in most lines results not in lethality but in the activation of latent OIS. Our findings are consistent with a recent study of *KRAS* transformation of primary pancreatic duct epithelial cells that

demonstrated activated mutant *KRAS* led to increased expression of TWIST1 by a posttranscriptional mechanism and that TWIST1 expression was essential to bypassing OIS in this model (21). We have recently shown that *Twist1* overexpression cooperates with mutant *Kras* to induce adenocarcinoma of the lung in mouse models and inhibition of *Twist1* in these models led to activation of *Kras*-induced senescence (26). One possible caveat to this mouse model study was that simultaneous expression of *Kras*^{G12D} and *Twist1* during tumorigenesis could have resulted in co-dependent tumors and therefore the phenotype of tumor stasis and activation of OIS observed with removal of *Twist1* overexpression was due to this induced co-dependency. However, our current work demonstrates the *TWIST1* dependency of oncogene driven NSCLC generally, and *KRAS* mutant NSCLC in particular using both *in vitro* and xenograft approaches.

Previous work had suggested that bypass of OIS by TWIST1 required p16 and possibly a functional p53/p21 pathway (20, 21). In our multiple lung cancer lines, we observed activation of OIS in both p53 wild type and mutant backgrounds and in the absence of a functional p16 pathway. Furthermore our knockdown experiments confirmed that the observed OIS and growth inhibition was p53-p21 independent. A recent study has demonstrated a critical role of TWIST1 in sarcomagenesis through its ability to lead to p53 degradation (37) and previous studies have demonstrated the ability of TWIST1 to interact with and influence the transcriptional function of p53 (38, 39). Furthermore, p53 is required for Ras induced OIS in mouse embryonic fibroblasts (18). Although the dispensability of p53 for OIS after silencing of TWIST1 was unexpected, it is consistent with the previous work that first established the role of TWIST1 in suppressing OIS (20). Ansieau et al. observed induction of p21 and p16 after silencing of TWIST1 and subsequently proposed a role for the p53 and Rb pathways in mediating OIS after suppression of TWIST1. However, in the same study, they clearly demonstrated the induction of OIS after silencing of TWIST1 or TWIST2 in two p53 mutant cell lines (T47D and RPMI 7951) (20). The aforementioned study regarding the role of TWIST1 in sarcoma, demonstrated a requirement for p53 in mediating serum starvation senescence after silencing of TWIST1 in a single sarcoma cell line (37). It is not clear whether the requirement for p53 is a sarcoma specific effect or is required under conditions of serum starvation. Our current study suggests that both the p53 and p16 pathways are dispensable for oncogene-induced senescence and growth inhibition observed after suppression of TWIST1.

In addition, utilizing both knockdown of p27 and overexpression of SKP2 we have also demonstrated that activation of OIS is likely independent of the SKP2-p27 pathway. An important caveat is that in neither experiment were we able to completely deplete p27 from the cell and it remains possible that this is a p27-dependent process requiring only a low threshold expression of p27. Alternatively, p21 and p27 may be redundant and knockdown of both CDK inhibitors is required for abrogation of OIS induced by silencing of *TWIST1*. Furthermore, in our experiments using SKP2 overexpression cell lines, we did observe an increase in apoptosis in the SKP2 overexpressing lines compared to vector control. Therefore, we cannot rule out the possibility that the ability of SKP2 overexpression to rescue the OIS phenotype was masked in part by the increase in apoptosis that was observed. However, for all experiments, in which we have silenced TWIST1, we have assessed the cells for senescence associated beta galactosidase staining and have not seen a rescue of OIS after depletion or overexpression of any genes in this study.

Despite these caveats, these data suggest that alternative p53-p21 and SKP2-p27 independent mechanisms may exist for the activation of OIS. A recent study suggested that the endoplasmic reticulum (ER) stress protein and transcription factor, ATF4 was important for p53-independent OIS induced by Skp2 inhibition (19). In addition, there are some data suggesting that unopposed expression of oncogenes such as β -catenin can result in DNA

damage resulting in senescence and apoptosis independent of p53 (40). To better characterize the mediators of TWIST dependency, we are performing overexpression and loss of function genetic screens to elucidate the key mediators of OIS upon inhibition of TWIST1.

The requirement of TWIST1 for bypassing OIS appears to extend across multiple driver oncogenes. Interestingly, a previous study had correlated increase *TWIST1* expression with the presence of *EGFR* activating mutations (41). A follow up study found that TWIST1 may play a role in migration and EGF mediated EMT in a limited subset of *EGFR* mutant cell lines in short term experiments (42). In our present study, we demonstrated a critical requirement for TWIST1 in *EGFR* mutant NSCLC in long term growth experiments *in vitro* as well as *in vivo*.

Little is known about the requirement for *TWIST1* in *c-Met* dependent NSCLC. Interestingly, a recent report, has suggested that either pharmacological inhibition of *c-Met* or silencing of *TWIST1* could inhibit metastasis in an *in vivo* breast cancer model by preventing EMT (43). We report for the first time, that TWIST1 expression is essential for *c-Met* dependent NSCLC. The *c-Met* oncogene is amplified, mutated, and/or overexpressed in significant number of patients with NSCLC. Furthermore, there is evidence of significant crosstalk and interdependence between the *EGFR* and *c-Met* signaling pathways (44). *c-Met* amplification is a recognized acquired resistance mechanism in patients treated with *EGFR* targeted TKI (45). Multiple antibody based and TKI based therapies for targeting the *c-Met* pathway are currently in clinical trials for NSCLC (44). Therefore, targeting *TWIST1* in *c-Met* dependent NSCLC represents a novel and promising therapeutic strategy.

In summary, our data suggests that TWIST1 represents an attractive therapeutic target for oncogene driven NSCLC. Inhibition of TWIST1 resulted in OIS and growth inhibition in diverse genetic backgrounds and was effective for three major classes of driver mutations in adenocarcinoma of the lung (*KRAS* mutant, *EGFR* mutant and *c-Met* amplified). While prenatal *TWIST1* haplo-insufficiency leads to Saethre-Chotzen Syndrome (23, 24), the expression of *TWIST1* is quite restricted postnatally and some studies have even suggested that postnatal deletion of this loci may be well tolerated (46, 47). To this end, we are currently developing a cell based assay to screen for small molecules that may inhibit TWIST1 transcriptional activity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

CDK	Cyclin Dependent Kinase
GSEA	gene set enrichment analysis
EMT	epithelial-to-mesenchymal transition
NSCLC	Non-Small Cell Lung Cancer
OIS	Oncogene-Induced Senescence
SA-β-gal	senescence-associated beta-galactosidase
X-gal	5-bromo-4-chloro-3-isolyl-b-D-galactoside

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Figure 1. *TWIST1* knockdown results in growth inhibition in human non-small cell lung cancer cells

Silencing of *TWIST1* with two different shRNAs (shTWIST1-1 and shTWIST1-2) resulted in marked growth inhibition of *KRAS* mutant (H460, A549, H358, H727, H23 and Calu-1), *EGFR* mutant (H1975 and H3255) and a *c-Met* amplified (H1993) NSCLC cell lines, as shown by representative triplicates of crystal violet staining.

Figure 2. *TWIST1* knockdown activates latent oncogene induced senescence in human non-small cell lung cancer cells

A. Representative photomicrographs (20×) of increased SA-β-gal staining in 3 representative NSCLC cell lines following shRNA mediated *TWIST1* knockdown (above).

B. Quantification of SA-β-gal stained cells following shRNA mediated *TWIST1* knockdown.

Figure 3. Inhibition of TWIST1 leads to a profound cell cycle arrest and apoptosis in NSCLC cell lines

A. Cell cycle analysis with propidium iodide staining in H460 cells and A549 on day 8 and day 6 respectively after lentiviral shRNA infection. Bottom panel: mitotic trapping of cells with nocodazole (100 ng/ml) for 24 hrs prior to harvesting. **B.** *Twist1* knockdown in NSCLC cell lines results in the upregulation of markers of senescence, p21 and p27 as well as a marker of apoptosis, cleaved PARP, as shown by Western blotting on day 4 after infection.

Figure 4. Growth inhibition by silencing *TP53* in NSCLC is not p53-dependent

A. *TP53* knockdown in H460 and A549 cells prevents induction of p53 and its target gene, p21 after *TP53* knockdown as shown by Western blotting on day 12 and day 6 respectively after the shRNA infection. **B.** Representative triplicates of crystal violet staining of H460 or A549 NSCLC cells demonstrate *TP53* knockdown with 2 separate shRNAs decreases cellular proliferation despite the absence of p53.

Figure 5. Silencing of *TWIST1* results in a significant growth inhibition in oncogene dependent NSCLC xenograft models

Representative studies in which *KRAS* mutant (A549, H358 and H727), *EGFR* mutant (H1975) and *c-Met* amplified (H1993) NSCLC cell lines were infected with shRNA targeting *TWIST1* (blue filled squares) or scrambled control (shScram) (red closed circles) respectively and on day 4 after infection equal numbers of viable cells were injected in the hind flank of NOD-SCID animals and allowed to establish palpable tumors. Once established tumors formed, tumor volumes were measured every 3 days for the duration of the experiment (p value < 0.05 for all models).

Figure 6. Inducible silencing of *TWIST1* results in a significant growth inhibition of established NSCLC tumors

A. Induction of the doxycycline inducible shRNA targeting *TWIST1* after addition of doxycycline (250 ng/ml) to the media results in p21 induction as shown by Western blotting on day 8 after treatment. **B.** Representative triplicates of crystal violet staining in A549 cells carrying doxycycline inducible shRNAs targeting scrambled control or *TWIST1* respectively. **C.** A549 cells carrying a doxycycline inducible shRNA targeting scrambled control (Tet-shScram) or *TWIST1* (Tet-shTWIST1) respectively were injected in the hind flank of NOD-SCID animals and allowed to establish palpable tumors. Once established tumors formed, at least six animals in each group were given 5% dextrose with (blue filled squares) or without (red closed circles) 2 mg/ml doxycycline in the drinking water and tumor volumes were measured every 3 days for the duration of the experiment (p value < 0.05 for Tet-shTWIST1; NS for shScram). **D.** Induction of p21 in xenograft tumors containing A549 Tet-shTWIST1 in the presence of doxycycline. Tumors were harvested at 21 days after the initiation of 5% dextrose with or without 2 mg/ml doxycycline in the drinking water. Western blot analysis was performed for p21 and beta-actin.