SKI-606, an Src Inhibitor, Reduces Tumor Growth, Invasion, and Distant Metastasis in a Mouse Model of Thyroid Cancer

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Abstract

**Purpose:** Src is overexpressed or hyperactivated in a variety of human cancers, including thyroid carcinoma. Src is a central mediator in multiple signaling pathways that are important in oncogenesis and cancer progression. In this study, we evaluated the effects of an Src inhibitor, SKI-606 (bosutinib), in a spontaneous metastatic thyroid cancer model with constitutively activated Src (ThrbPV/PVPten+/− mice).

**Experimental Design:** ThrbPV/PVPten+/− mice were treated with SKI-606 or vehicle controls, beginning at 6 weeks of age until the mice succumbed to thyroid cancer. We assessed the effects of SKI-606 on thyroid cancer progression and analyzed the impact of SKI-606 on aberrant Src-mediated signaling.

**Results:** SKI-606 effectively inhibited aberrant activation of Src and its downstream targets to markedly inhibit the growth of thyroid tumor, thereby prolonging the survival of treated mice. While Src inhibition did not induce cell apoptosis, it decreased cell proliferation by affecting the expression of key regulators of cell-cycle progression. Importantly, SKI-606 dramatically prevented dedifferentiation, vascular invasion, and lung metastasis of thyroid cancer cells. These responses were mediated by downregulation of mitogen-activated protein kinase pathways and inhibition of the epithelial–mesenchymal transition.

**Conclusions:** Our findings suggest that Src is critical in the progression of thyroid cancer, making oral SKI-606 a promising treatment strategy for refractory thyroid cancer. *Clin Cancer Res; 18(5); 1281–90.*

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Our studies showed that SKI-606 treatment inhibited not only thyroid tumor growth but also distant metastasis of thyroid cancer. SKI-606 reduced tumor growth by inhibiting cell proliferation and preventing dedifferentiation of tumor cells. These responses were accompanied by down-regulation of MAPK pathways and inhibition of epithelial–mesenchymal transition (EMT). These findings from this preclinical study indicate that the Src inhibitors are potentially effective strategies for the treatment of refractory thyroid cancer.

Materials and Methods

Animals and treatment

The National Cancer Institute Animal Care and Use Committee approved the protocols for animal care and handling in the present study. Mice harboring the ThrbPV gene (ThrbPV+/PV mice) were prepared via homologous recombination, and genotyping was carried out using the PCR method, as previously described (20). Pten−/− mice were kindly provided by Dr. Ramon Parsons (Columbia University, New York, NY). ThrbPV+/PV Pten−/− mice were obtained by crossing Pten−/− mice with ThrbPV+/PV mice, followed by crossing ThrbPV+/PV Pten−/− with ThrbPV+/PV Pten−/− mice. SKI-606 (LC Laboratories) was dissolved in 0.5% Methocel/0.4% Tween-80 (Sigma-Aldrich) in water and administered by oral gavage 5 times a week at a dose of 150 mg/kg body weight (14, 18) starting at the age of 6 weeks. Mice were monitored until they became moribund with rapid weight loss, hunched posture, and labored breathing. The thyroids and lungs were dissected after mice were euthanized for weighing, histologic analysis, and biochemical studies.

Hormone assay

Serum levels of total T4 were determined by using a GammaCoat T4 assay RIA kit (DiaSorin) as previously described (21). Serum TSH levels were measured as previously described (25).

Histopathologic analysis

Thyroid glands and lungs were dissected and fixed in 10% neutral-buffered formalin (Sigma-Aldrich) and subsequently embedded in paraffin. Five-micrometer thick sections were prepared and stained with hematoxylin and eosin. For each animal, single random sections of thyroid and lung were examined. For thyroids, morphologic evidence of hyperplasia, capsular invasion, and vascular invasion, and eventually metastasis (20). We have recently shown that the Src kinase pathway is activated to promote thyroid carcinogenesis of ThrbPV+/PV mice (22). The ThrbPV+/PV Pten−/− mouse model was created by introducing haploid deficiency of the silencing of the tumor suppressor gene Pten (phosphatase and tensin homolog deleted from chromosome 10) into ThrbPV+/PV mice. Pten deficiency further exacerbates the overactivated PI3K/AKT signaling, leading to more aggressive cancer phenotype with decreased survival and increased distant metastasis, making it useful for preclinical studies (22, 23).

The aim of the present study was to evaluate the effect of inhibiting Src activity by SKI-606 in the ThrbPV+/PV Pten−/− mouse model. This spontaneous metastatic thyroid cancer model is ideal for assessing the effect of SKI-606 on cancer progression from early capsular invasion to late pulmonary metastasis. The effect of Src inhibition could be assessed in a whole-animal context because many functions of the Src family kinases in multiple signaling pathways are integrated into an intact immune system and microenvironment (7, 8, 24).

Translational Relevance

About 30% of recurrent or metastatic differentiated thyroid cancers show dedifferentiation of malignant cells and subsequently become resistant to radioiodine therapy with poor prognosis. Although some kinase inhibitors have been in trials for this subgroup of patients, no effective therapy was approved for radio-refractory differentiated thyroid cancer. Src is frequently overactivated in human thyroid cancer and is a central mediator in multiple signaling pathways critical in carcinogenesis. Our studies showed that inhibition of Src by SKI-606 decreased tumor growth, inhibited dedifferentiation, and blocked distant metastasis. The present preclinical studies indicate that the Src inhibitors are potentially effective strategies for single-drug therapy or in combination with radioiodine therapy to treat refractory thyroid cancer.
with the peroxidase substrate diaminobenzidine followed by counterstaining with Gill’s hematoxylin.

Western blot analysis
Preparation of whole-cell lysates from thyroid glands has been described previously (27). The protein sample (20–30 μg) was loaded and separated by SDS-PAGE. After electrophoresis, the protein was electrotransferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore Corp.). The antibodies p-Src (1:1,000 dilution), total Src (1:1,000 dilution), total FAK (1:500 dilution), glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:1,000 dilution), p-Rb (1:500 dilution), total Rb (1:250 dilution), CDK4 (1:1,000 dilution), CDK6 (1:1,000 dilution), Bad (1:500 dilution), cleaved caspase-3 (1:500 dilution), cleaved PARP (1:500 dilution), p-ERK (1:1,000 dilution), total ERK (1:1,000 dilution), p-p38 MAPK (1:1,000 dilution), total p38 MAPK (1:1,000 dilution), p-INK (1:1,000 dilution), total INK (1:1,000 dilution), vimentin (1:1,000 dilution), and Slug (1:1,000 dilution) were purchased from Cell Signaling Technology. Antibodies for cyclin B1 (1:500 dilution), cyclin D1 (1:300 dilution), cyclin E (1:500 dilution), E2F1 (1:200 dilution), Bax (1:200 dilution), MMP-2 (1:1,200 dilution), MMP-9 (1:200 dilution), and E-cadherin (1:250 dilution) were purchased from Santa Cruz Biotechnology. Antibody for p-FAK (1:500 dilution) was from BioSource (Invitrogen). Antibody for Bcl-XL (1:1,000 dilution), MMP-9 (F2319): 5'-AGC TGA -ACA-3', cyclin D1 (1:200 dilution), MMP-2 (1:1,200 dilution), MMP-9 (1:200 dilution), and E-cadherin (1:250 dilution) were purchased from Santa Cruz Biotechnology. Antibody for p-FAK (1:500 dilution) was from BioSource (Invitrogen). Antibody for Bcl-XL (1:1,000 dilution) was purchased from NeoMarker (Thermo Scientific). Antibody for cyclin D1 (1:200 dilution), MMP-9 (1:200 dilution), E2F1 (1:200 dilution), Bax (1:200 dilution), MMP-2 (1:200 dilution), MMP-9 (1:200 dilution), and E-cadherin (1:250 dilution) were purchased from Santa Cruz Biotechnology.

Quantitative real-time reverse transcriptase PCR
Total RNA from thyroid tumors was extracted by TRizol (Invitrogen), followed by RNase-free DNase treatment (Qiagen) and was purified as described (RNeasy Mini Kit; Qiagen). One-step real-time reverse transcriptase PCR (RT-PCR) reactions were carried out with 200 ng of total RNA using a QuantiTect SYBR green RT-PCR kit (Qiagen) in a Roche LightCycler PCR instrument (Roche) following the manufacturer’s instructions. Specific primers were as follows: MMP-2 (F1910): 5'-CAG GGA ATG AGT ACT GGG TCT ATT-3'; MMP-2 (R2030): 5'-ACT CCA GTT AAA GGC AGC ATC TAC-3'; MMP-9 (F2319): 5'-AAT CTC TTC TAG AGA CTG GGA AGG AG-3'; MMP-9 (R2445): 5'-AGC TGA TGT ACT AAA GTA GCT GGA-3'; GAPDH (F681): 5'-ACA TCA TCC CTG CAT CCA CT-3'; and GAPDH (R904): 5'-GTC CTC AGT GTC GGC CAA G-3'. The reaction conditions were 50°C for 20 minutes; 95°C for 15 minutes; 40 cycles of 94°C for 15 seconds, 60°C for 25 seconds, 72°C for 25 seconds; and 63°C to 95°C with a heating rate of 0.1°C/s and a cooling step to 40°C.

Statistical analysis
Data are presented as median/range or mean ± SD, accordingly. Mann--Whitney U test or Student t tests were used to compare continuous variables accordingly. The Kaplan–Meier method with log-rank test was used to compare survival in each treatment group. P values were 2-sided throughout, and P < 0.05 was considered statistically significant. Data were analyzed using SPSS statistics 19.0 (SPSS Inc.). GraphPad Prism 4.0a (GraphPad Software) was used to draw graphs.

Results
Src inhibitor, SKI-606, significantly increases survival of ThrbPV/PVPten+/− mice by inhibition of thyroid tumor growth
Previously, we reported that the Src kinase pathway is activated in the thyroid of ThrbPV/PVPten mice (26). To evaluate whether Src kinase is also activated during thyroid carcinogenesis of ThrbPV/PVPten+/− mice, we compared the protein levels of phosphorylated Src (p-Src), total Src, phosphorylated FAK (p-FAK), and total FAK in wild-type and ThrbPV/PVPten+/− mice (Fig. 1A). Src was highly activated as evidenced by markedly elevated phosphorylation of Src in the thyroids of ThrbPV/PVPten+/− mice as compared with wild-type thyroids [Fig. 1A (i), compare lanes 4–6 with lanes 1–3] without affecting total Src protein levels [Fig. 1A (ii)]. FAK is a tyrosine kinase which is a critical downstream effector of Src signaling, affecting cell-cycle progression and survival as well as adhesion and migration (8). Activated FAK was also evident by the increased phosphorylation of FAK in ThrbPV/PVPten+/− mice as compared with wild-type thyroids [Fig. 1A (ii), compare lanes 4–6 with 1–3]. There were no apparent changes in total FAK protein levels in ThrbPV/PVPten+/− mice as compared with wild-type thyroids [Fig. 1A (iv), compare lanes 4–6 with 1–3]. These findings suggested that the Src/FAK pathway is a potential target for treatment of thyroid cancer. Therefore, we evaluated the effect of the Src inhibitor, SKI-606, on thyroid carcinogenesis of ThrbPV/PVPten+/− mice.

To evaluate the extent of Src inhibition by SKI-606, p-Src (Y416) and total Src levels were analyzed by Western blotting. As shown in Fig. 1B (i), SKI-606 treatment inhibited the activity of Src by decreased p-Src (Y416) as compared with vehicle-only controls. Moreover, the activity of its downstream effector, FAK, was also inhibited by decreased phosphorylation of FAK in ThrbPV/PVPten+/− mice as compared with wild-type thyroids [Fig. 1A (ii), compare lanes 4–6 with 1–3]. There were no apparent changes in total FAK protein levels in ThrbPV/PVPten+/− mice as compared with wild-type thyroids [Fig. 1A (iv), compare lanes 4–6 with 1–3]. These findings suggested that the Src/FAK pathway is a potential target for treatment of thyroid cancer. Therefore, we evaluated the effect of the Src inhibitor, SKI-606, on thyroid carcinogenesis of ThrbPV/PVPten+/− mice.
As shown in Fig. 1D, thyroid weights of SKI-606–treated mice (n = 11) were significantly lower than that of vehicle-treated mice (n = 15, P = 0.02). There was no significant difference in total body weight between the mice treated with or without SKI-606 at the time of sacrifice (data not shown). These data suggested that the increased survival in SKI-606–treated mice was associated with a substantial decrease in thyroid weight as compared with vehicle-treated mice, indicating the effectiveness of SKI-606 in reducing tumor growth, leading to increased survival.

**SKI-606 inhibits vascular invasion and distant metastasis of thyroid cancer in Thrb\(^{PV/PV}\)Pten\(^{+/−}\) mice**

We further investigated whether SKI-606 treatment could prevent invasiveness and distant metastasis of thyroid cancer in Thrb\(^{PV/PV}\)Pten\(^{+/−}\) mice. As shown in Fig. 1E, vascular invasion of thyroid cancer was significantly decreased in SKI-606–treated Thrb\(^{PV/PV}\)Pten\(^{+/−}\) mice as compared with vehicle-treated mice (P < 0.001). The development of lung metastasis was also significantly reduced in the SKI-606–treated cohorts as compared with the vehicle-treated group (Fig. 1F, P = 0.001). These results suggested that SKI-606 could significantly delay the progression of thyroid cancer and prevent lung metastasis in Thrb\(^{PV/PV}\)Pten\(^{+/−}\) mice.

**TSH does not mediate inhibition of thyroid tumor growth by SKI-606 in Thrb\(^{PV/PV}\)Pten\(^{+/−}\) mice**

Recent studies show that multitargeted tyrosine kinase inhibitors, such as sunitinb and sorafenib, could influence thyroid functions (28, 29). It is known that TSH does not mediate inhibition of thyroid tumor growth by SKI-606 in Thrb\(^{PV/PV}\)Pten\(^{+/−}\) mice.

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**Figure 1.** The effects of SKI-606 on survival, tumor growth, vascular invasion, and lung metastasis of thyroid cancer in Thrb\(^{PV/PV}\)Pten\(^{+/−}\) mice. A, Src signaling pathway is activated in the thyroids of Thrb\(^{PV/PV}\)Pten\(^{+/−}\) mice as compared with wild-type mice (n = 3 for each group). Western blot analysis of phospho-Src (p-Src), total Src, p-FAK, total FAK, and GAPDH as a loading control. WT, wild-type. B, SKI-606 effectively inhibited activation of Src and FAK in Thrb\(^{PV/PV}\)Pten\(^{+/−}\) mice (n = 4) as compared with wild-type mice (n = 4). Western blot analysis of p-Src, total Src, p-FAK, total FAK, and GAPDH as a loading control. C, survival curves for Thrb\(^{PV/PV}\)Pten\(^{+/−}\) mice treated with SKI-606 (n = 11; 150 mg/kg body weight) or vehicle (n = 15) orally 5 times a week from 6 weeks of age until they had to be euthanized because of sickness. Data are presented by Kaplan–Meier methods and analyzed by log-rank test. D, thyroids of vehicle-treated or SKI-606–treated Thrb\(^{PV/PV}\)Pten\(^{+/−}\) mice were dissected from moribund mice at the age of 2.8 to 5 months and 3.4 to 6.6 months, respectively. The data are presented as ratios of thyroid weight to total body weight. The data are presented by median and analyzed by Mann–Whitney U test. E, the development of vascular invasion in Thrb\(^{PV/PV}\)Pten\(^{+/−}\) mice after treatment with SKI-606 (n = 11) or vehicle only (n = 15). The data are presented by Kaplan–Meier methods and compared by log-rank test. F, the development of lung metastasis in Thrb\(^{PV/PV}\)Pten\(^{+/−}\) mice after treatment with SKI-606 (n = 11) or vehicle only (n = 15). The data are presented by Kaplan–Meier methods and compared by log-rank test.
stimulator of thyrocyte proliferation. To evaluate whether the decreased size of thyroid tumors after treatment with SKI-606 could possibly be due to decreased TSH levels, we compared serum TSH and total T4 levels (Fig. 2). We found a small but significant increase in the levels of serum TSH by SKI-606 treatment as compared with vehicle treatment (Fig. 2A). A very small increase was also detected for total T4 (Fig. 2B). These data suggested that the decreased thyroid tumor size in SKI-606–treated mice was not due to the effects of TSH.

SKI-606 delays tumor progression by preventing dedifferentiation and inhibiting cell proliferation in thyroids of ThrbPV/PVPten+/− mice

To understand how SKI-606 inhibited thyroid tumor growth, we evaluated its effect on cell differentiation and proliferation. Remarkably, the follicular structure of the thyroid gland and its cellular polarity were largely maintained, similar to that of normal thyroid in the SKI-606–treated group as compared with vehicle-treated mice (Fig. 3A). The cell population in thyroid was clearly decreased, and the quantification of cell density showed a 38% decrease after SKI-606 treatment as compared with vehicle treatment (Fig. 3C, P = 0.003). The antiproliferative effect of SKI-606 was clearly evident by the reduced staining of Ki-67 in the nucleus of thyrocytes of ThrbPV/PVPten+/− mice (Fig. 3B, right) as compared with the control (Fig. 3B, left). Cells positively stained with Ki-67 were scored among total cells examined. Figure 3D shows about 50% reduction in the positively Ki-67–stained cells, indicating that treatment with SKI-606 led to inhibition of cell proliferation.

We further studied the protein abundance and activity of key regulators involved in cell-cycle progression in thyroid tumors affected by SKI-606 treatment. Western blot analysis shows that the protein levels of cyclin B1, cyclin E, and cyclin D1 were reduced by 20% to 50% after SKI-606 treatment [Fig. 4A (i–iii)]. The levels of cyclin-dependent kinases (CDK4 and CDK6) were also lower in the SKI-606–treated group than the vehicle-treated group [Fig. 4A (iv and v)]. In addition, SKI-606 treatment decreased the phosphorylation of the retinoblastoma [Rb; S807/811 and S780; Fig. 4B (i and ii)]. In its unphosphorylated state, Rb acts as a negative regulator of cell-cycle progression by binding to and inhibiting critical regulatory proteins, including members of the E2F family of transcription factors (Fig. 4B). The intensities of the bands in Fig. 4A and B were determined, and the quantitative comparison is shown in Fig. 4C. These results suggest that inhibition of Src signaling pathway by SKI-606

Figure 3. SKI-606 reduces tumor growth by prevention of dedifferentiation and inhibition of cell proliferation and in ThrbPV/PVPten+/− mice. A, representative microphotographs of hematoxylin and eosin staining on thyroid sections of SKI-606 (n = 11) or vehicle-treated ThrbPV/PVPten+/− mice (n = 15). i, ii, and v, vehicle-treated group; ii, iv, and vi, SKI-606–treated group. B, representative microphotographs of Ki-67 immunohistochemistry on thyroid sections of SKI-606–treated mice (n = 3) or vehicle-treated ThrbPV/PVPten+/− mice (n = 3). Left, vehicle-treated group; right, SKI-606–treated group. C, comparison of relative cell density determined by cell counting in the 2 groups. D, thyroid cell proliferative index, determined by Ki-67 immunohistochemistry in the 2 groups.
reduced thyroid tumor growth by inhibiting cell proliferation and cell-cycle progression.

We also evaluated the effect of SKI-606 on apoptotic pathways. That inhibition of Src activity by SKI-606 did not alter cell apoptosis in Thrb<sup>PV/PPten<sup>+/−/C0 thyroids was indicated by the absence of changes in the protein levels of the prosapoptotic factors Bad, Bax, and Bcl-XL (Fig. 4D). No changes of cleaved caspase-3 or cleaved PARP were detected by Western blot analysis after treatment with SKI-606 (data not shown).

**Treatment with SKI-606 inhibits the activation of MAPK pathways**

MAPKs including extracellular signal–regulated kinases (ERK1/2), c-Jun N-terminal kinases (JNK), and p38 MAPK isoforms are serine/threonine-specific kinases that respond to extracellular stimuli and regulate various cellular activities such as gene expression, mitosis, differentiation, proliferation, and cell survival (30). Src has an important role in the receptor tyrosine kinase–mediated proliferative responses through the activation of the Ras/Raf/MEK/MAPK signaling pathways (31). We therefore evaluated the levels of phosphorylated extracellular signal-regulated kinase 1/2 (p-ERK1/2) and phosphorylated p38 isoforms (p-p38) by Western blotting. Figure 5A shows that after treatment of SKI-606, the levels of p-ERK1/2 [Fig. 5A (i)] and p-p38 MAPK [Fig. 5A (iii)] were significantly lower in SKI-606–treated than in vehicle-treated thyroids of Thrb<sup>PV/PPten<sup>+/−/C0 mice. The phosphorylated JNK (p-JNK) level, which plays an important role in inflammatory response and cancer microenvironment, was also lower in SKI-606–treated mice than in control groups (Fig. 5B). The intensities of the bands in Fig. 5A and B were determined, and the quantitative comparison is shown in Fig. 5C. The ratios of p-ERK/total ERK, p-p38/total p38, and p-JNK/total JNK were decreased by 95%, 80%, and 65%, respectively, indicative of marked attenuation of MAPK/ERK signaling in mice treated with SKI-606. These data indicate that inhibition of Src by SKI-606 led to blocking of downstream signaling via inhibition of phosphorylation cascades to decrease thyroid tumor growth and increase survival.

**Src inhibition by SKI-606 reduces the EMT in thyroid carcinogenesis**

The migration of tumor cells through matrix barriers, tissue compartments, vessels, and organ boundaries is essential in the development of distant metastasis (8). Previously, Src was shown to promote the expression of matrix-degrading proteases such as matrix metalloproteinases (MMP) by diverse mechanisms. Through the ERK signaling pathway, the expression of MMP-9 is elevated, and through the CAS/Rac/JNK and paxillin/Rac/JNK pathways, the expression of MMP-2 and MMP-9 is upregulated (29, 32, 33). We therefore evaluated the expression levels of MMP-9 and MMP-2 after treatment of Thrb<sup>PV/PPten<sup>+/−/C0 mice with SKI-606. Consistent with the decreased activation of ERK and JNK signaling pathways (see Fig. 5B), the protein abundance of MMP-9 was decreased by 90% [Fig. 6A (i) and bars 1 and 2 of Fig. 6B]. Figure 6A (ii) shows that whereas...
ThrbPV/PVPten decreased by 50% in SKI-606–treated mice (Fig. 6C, $P = 0.04$ and $P = 0.009$, respectively). The reduction of these proteases led to reduction in tumor cell migration.

We also examined the markers of EMT after treatment of ThrbPV/PVPten +/− mice with SKI-606. E-cadherin is involved in cell–cell adhesion, and activation of Src is known to decrease E-cadherin expression. As shown in Fig. 6D (i), treatment with SKI-606 led to 2.2-fold increase of E-cadherin protein abundance as compared with vehicle-treated thyroids (see also the quantification in Fig. 6E). Other EMT markers, vimentin and Slug (Snail-2), involved in invasion and tumor progression in the mesenchymal phenotype were decreased after treatment with SKI-606 [Fig. 6D (ii and iii), respectively]. Taken together, our data indicate that reduction of Src signaling by SKI-606 blocks cancer progression and lung metastasis at least in part via inhibition of EMT in thyroid cancer.

**Discussion**

Genetic mutations of Src are very rare. But aberrantly activated Src and its downstream effector, FAK, are frequent in human cancers. Overactivation of Src increases cell growth and survival, as well as promoting the reorganization of the cytoskeleton and decreasing cell–cell and cell–matrix adhesion. These activities of Src ultimately facilitate cell motility and invasion (8). Thus, inhibition of Src is a promising target for controlling primary tumor growth, invasion, and metastasis. In thyroid cancer, the increased expression of Src and FAK has been reported, and overexpressed FAK has been proposed to be a marker of invasive potential in thyroid cancer (10–12). These findings suggested that Src could be a potential molecular target for treatment of thyroid cancer. The availability of ThrbPV/PVPten +/− mouse model that exhibits aggressive tumor progression and metastasis (17) provided us with an opportunity to test this possibility in vivo. In the present study, we showed that Src/FAK signaling was overactivated (see Fig. 1A and B) and therefore suitable for the evaluation of the effects of inhibiting this pathway on thyroid carcinogenesis. We showed that when the p-Src was inhibited by SKI-606, the survival of ThrbPV/PVPten +/− was prolonged, the thyroid growth was markedly decreased, the occurrence of tumor invasion and metastasis was delayed, and the degree was less severe. These findings clearly show that activation of Src/FAK signaling is critical in thyroid carcinogenesis. More importantly, the present study has identified an important potential molecular target for treatment of thyroid cancer.

By the use of SKI-606 in inhibiting the Src/FAK signaling, the present studies have uncovered downstream effectors critical in thyroid carcinogenesis of ThrbPV/PVPten +/− mice. The key cell-cycle regulators, such as cyclins (D1, B1 and E), CDKs (CDK4 and 6), Rb, and E2F1, were downstream effectors in mediating the proliferation of thyroid tumor cells of ThrbPV/PVPten +/− mice. Moreover, in addition to p38 MAPK, p-ERK1/2, total ERK1/2, p-p38 MAPK, total p38 MAPK, and p-JNK, total JNK, and GAPDH as a loading control after treatment with vehicle ($n = 6$) or SKI-606 ($n = 4$). B, Western blot analysis of p-JNK, total JNK, and GAPDH as a loading control after treatment with vehicle ($n = 3$) or SKI-606 ($n = 3$). C, quantification of relative expression of phosphorylated proteins associated with Src/FAK and MAPK pathways in thyroid cancer after using GAPDH as loading control.

Active MMP-2 was decreased by 50% without significant change in pro-MMP-2 protein levels [Fig. 6A (ii) and B]. Quantitative real-time PCR data also indicated that mRNA expression of both MMP-9 and MMP-2 were also decreased by SKI-606 (Fig. 6C, $P = 0.04$ and $P = 0.009$, respectively). The reduction of these proteases led to reduction in tumor cell migration.
MAPK, shown previously a critical downstream effector of Src/FAK signaling in the carcinogenesis of ThrbPV/PV mice (22), ERK and JNK pathways were also found to participate in the carcinogenesis of ThrbPV/PVPten+/−/C0 mice. These findings suggest that Pten haplold deficiency not only exaspe-rate the PI3K/AKT signaling but also expands the pathways downstream of Src/FAK signaling to contribute to thyroid carcinogenesis of ThrbPV/PVPten+/−/C0 mice. The identification of these downstream effectors of Src/FAK signaling raises the possibility that intervention of phosphorylation cascades by kinase inhibitors in the ERK and JNK pathways would offer additional benefits in the treatment of thyroid cancer. Currently, MEK inhibitors, AZD6244 (NCT00970359) and GSK1120212 (NCT01438554), leading to blocking the activities of immediate downstream effector, ERK, are in clinical trials for refractory thyroid cancer. JNK inhibitors, such as SP600125 and AS601245, are in the preclinical stage. Thus, the present preclinical findings would be useful in the consideration of SKI-606 as a single-agent upstream inhibitor of Src/FAK/ERK/JNK signaling or in combination with the inhibitors of ERK and JNK for effective treatment of patients with thyroid cancer.

It is known that TSH is a major stimulator of thyrocyte proliferation. However, it is important to note that the reduced thyroid growth observed in mice treated with SKI-606 did not involve TSH. A small but significant increase in the TSH and T4 levels was detected in ThrbPV/PVPten+/−/C0 mice treated with SKI-606, arguing against the role of TSH in the reduction of thyroid growth by SKI-606. These observations suggest that activation of Src/FAK signaling is most likely largely responsible for propelling proliferation of thyroid tumor cells of ThrbPV/PVPten+/−/C0 mice.

It is currently unclear whether the effect of SKI-606 on thyroid carcinogenesis of ThrbPV/PVPten+/−/C0 mice can also act via the inhibition of Abl kinase. Although it was reported that in chronic myeloid leukemia, SKI-606 could inhibit the aberrantly activated Abl kinase activity in Abl-Bcr fusion protein (34), it is unknown whether Abl could be activated via Abl-Bcr rearrangements in thyroid cancer and thyroid cancer cell lines. This question awaits further investigation.

The present studies also revealed that Src inhibition by SKI-606 reduced the extent of dedifferentiation of thyroid tumor cells during tumor progression as evidenced by regaining more normal thyroid follicular structures. Microenvironmental changes in cell–cell and cell–matrix adhesion and the activation of EMT play an important role in cancer cell invasion and metastasis. We found that the expression of E-cadherin, essential for establishing cellular polarity and maintaining epithelial integrity and cellular differentiation (35), was increased after treatment with SKI-606. Consistent with our observations, decreased expression of E-cadherin was reported to be associated with dedifferentiation, increasing frequencies of distant metastasis, and poor prognosis of follicular thyroid cancer (36, 37). Moreover, we also found that markers of the mesenchymal invasive phenotype, vimentin and Slug, were downregulated by Src inhibition. Although detailed molecular pathways...
leading to decreased dedifferentiation of tumor cells by SKI-606 treatment are not clear, it is reasonable to postulate that such events could be mediated by the changes in MAPK signaling observed in the present study. This notion is consistent with the previous reports in which inhibition of the MAPK and TGF-β signaling pathways was shown to decrease EMT and thyroid cancer cell dedifferentiation (38). Treatment with SKI-606 could lead to inhibition of dedifferentiation of thyroid tumor cells and could offer additional benefits to patients with thyroid cancer with the possibility of increasing efficacy of radioactive iodine therapy and more positive prognosis. In summary, the present study clearly shows that overactivation of Src/FAK signaling promotes thyroid carcinogenesis and that Src and its several other downstream effectors are potential molecular targets for treatment of thyroid cancer.

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

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