**Article**

Thromboxane A2 receptor α promotes tumor growth through an autoregulatory feedback pathway

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Tobacco smoking can cause a number of cancers. The role of thromboxane synthase (TxAS) in smoking-related cancers is largely unknown. In this study, 37 pairs of tumor and non-tumor lung tissues of non-small-cell lung cancer, 5 lung cancer cell lines, and a mouse tumor model were used to study TxAS and its related molecules. A mouse model of smoking carcinogen 4-methylnitrosamino-1-3-pyridyl-1-butane (NNK)-induced lung tumor showed an increase in TxAS. Thromboxane A2 receptor (TP) was aberrant in lung cancer tissues of smokers. TxAS and TP were increased in lung tissues of NNK-treated mice. The in vitro studies showed that TPα rather than TPβ promoted tumor growth, and NNK increased TPα. NNK-induced TxAS, which depended on activation of cyclooxygenase-2 (COX-2), ERK and NF-κB, could be inhibited by miR-34b/c. TPα played a positive role in NNK-induced COX-2/ERK/NF-κB activation, leading to the upregulation of TxAS expression and thromboxane A2 (TxA2) synthesis. The newly synthesized TxA2 could further activate TPα, forming an autoregulatory feedback loop for TPα activation. Collectively, NNK promotes lung tumor growth via inducing TxAS and TPα, which constitutes an auto-positive feedback loop to exaggerate the growth. This study suggests that TPα and TxAS are the ideal targets against smoking-related lung cancer.

**Keywords:** thromboxane synthase, thromboxane A2 receptor, autoregulation, cancer, smoking, lung

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**Introduction**

Smoking causes a number of cancers worldwide (Jha, 2009). Among the smoking carcinogens, NNK is the most tumorigenic (Huang and Chen, 2011; Huang et al., 2011). The contribution of NNK to cancer initiation and progression has been observed in many types of cancers, especially lung cancer (Huang and Chen, 2011). Tobacco smoke and its carcinogen NNK can affect the production of cyclooxygenase (COX) and its downstream products in cancer cells (Huang et al., 2011). COXs, including COX-1 and COX-2, catalyze the conversion of arachidonic acid into prostaglandin H₂, which is further converted into various bioactive prostanooids via different downstream isomerases (Cathcart et al., 2010; Huang and Chen, 2011). Lung cancer tissues obtained from smokers show the elevated expression of COX-2 with concomitant increase in TxAS and TxA2 when compared with that from non-smokers (McLemore et al., 1988; Ernert et al., 2003; Chen et al., 2006). Therefore, COX-2 and its derived TxA2 may have a role in the progression of the smoking-related lung cancer. The inhibition of COX-2 is well known as an effective approach to prevent and treat certain cancers including lung cancer. In the past decade, the idea that targeting the downstream molecules in the COX-2 pathway has gained increasing attention as it can be more effective and also avoid or reduce the side-effects associated with COX-2 inhibitors such as myocardial infarction and stroke (Bäck et al., 2012). This strategy has been evidently supported by the studies showing that the inhibition of TxAS and/or its receptors can effectively suppress the growth and progression of bladder, prostate, lung, and glioma cancers (Moussa et al., 2008; Nie et al., 2008; Cathcart et al., 2011; Ekambaram et al., 2011; Huang et al., 2011). For example, TxAS is over-expressed in non-small-cell lung cancer (NSCLC) and inhibition of this enzyme suppresses the tumor cell proliferation via inducing apoptosis (Cathcart et al., 2011).

TxA2 acts through its signature receptors, thromboxane A2 receptor (TP) (Ekambaram et al., 2011; Huang and Chen, 2011). Two alternatively spliced human TP isoforms, TPα and TPβ, were identified. We previously demonstrated that NNK increased the expression of COX-2 and TxAS and stimulated TxA2 production in human lung cancer cells (Huang and Chen, 2011; Huang et al., 2011). Blockade of TxA2 synthesis or its action could significantly reduce NNK-promoted lung cancer cell survival and proliferation.
In both cell lines treated with NNK, TP expression by NNK is similar to the NNK-mediated change in transcriptional mechanisms, respectively. It is noted that two TP isoforms exist in human beings, while mice have only single TP isoform, whose nuclear acid sequence is significantly affected by NNK, whereas TxAS mRNA was upregulated by 1.8 folds at 34 weeks and 6.0 folds at 38 weeks compared with the respective control (Figure 1A). At the protein level, both TxAS and TP were increased in NNK-treated mice in a time-dependent manner (Figure 1B). We also confirmed that NNK upregulated COX-2 but not COX-1 expression in mouse lung tissues (Supplementary Figure S2).

NNK upregulates TP and TxAS in lung tissues of A/J mice

In a mouse model, NNK-induced lung hyperplasia occurs at 26 weeks after NNK treatment and the lung tumor is formed at 30 weeks (Li et al., 2010b). The lung tissues were thus collected at various points accordingly for detection of TP and TxAS expression. It is noted that two TP isoforms exist in human beings, while mice have only single TP isoform, whose nuclear acid sequence is significantly affected by NNK. Whereas TxAS mRNA was upregulated by 1.8 folds at 34 weeks and 6.0 folds at 38 weeks compared with the respective control (Figure 1A). At the protein level, both TxAS and TP were increased in NNK-treated mice in a time-dependent manner (Figure 1B). We also confirmed that NNK upregulated COX-2 but not COX-1 expression in mouse lung tissues (Supplementary Figure S2).

NNK induces TPα and TxAS rather than TPβ

Both NCI-H23 and A549 are lung adenocarcinoma cell lines. NCI-H460 is a large cell lung carcinoma cell line. CRL-2066 and NCI-H69 are small-cell lung cancer cells. TPα protein was expressed in all these five human lung cancer cell lines that represent different subtypes of lung cancer (Figure 2A). However, only A549, NCI-H23, and CRL-2066 expressed TPβ protein. NCI-H23 and CRL-2066 cells, which express both TPα and TPβ, were selected as models for the following study. TPα constituted 73.87% and about 50.71% of total TP proteins in NCI-H23 and CRL-2066 cells, respectively (Figure 2B). In both cell lines treated with NNK, TPα protein level was increased in a time-dependent manner, whereas the level of TPβ protein was not significantly changed (Figure 2C). It appears that the change in TPα expression by NNK is similar to the NNK-mediated change in TxAS (Huang et al., 2011). However, at mRNA level, TxAS was time-dependently increased by NNK, while the expression of either TPα or TPβ was not significantly changed (Figure 2D and E). These findings are in agreement with those observed in lung tissues from A/J mice treated with NNK (Figure 1). Thus the data strongly suggest that NNK may induce TxAS and TPα via transcriptional and post-transcriptional mechanisms, respectively.

Results

TP expression is much higher in human lung cancer tissues of smokers than non-smokers

The expression of TP was immunohistochemically evaluated. The lung cancer tissues, in particular those obtained from smokers, presented strong cytoplasmic staining for TP, whereas the adjacent non-cancer tissues showed weak or vacant cytoplasmic staining for TP (Supplementary Figure S1). A higher TP staining score was showed in 30 cases of cancer tissues than their paired corresponding non-cancer tissues (81.1%, 30 of 37 cases, P < 0.001) (Supplementary Table S1). Importantly, all tissues (cancer and non-cancer) from smokers presented positive-staining for TP. When the score 1 (≥20%) of TP-positive staining was used as the cut-off value, the Fisher’s exact test showed that in cancer tissues, TP expression was much higher in smokers than that in non-smokers (93.1%, 27 of 29 cases vs. 37.5%, 3 of 8 cases, P = 0.002).

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The TP antagonist SQ29548 can abrogate NNK-mediated ERK and Akt activation as well as cell proliferation in lung cancer cells (Huang et al., 2011). However, it is unknown which isoform of TP is involved. We therefore transfected TPα or TPβ DNA into cells and found that the levels of p-ERK1/2 and p-Akt were increased but PTEN was markedly decreased in TPα-transfected cells (Figure 2F). However, these proteins were not changed in TPβ-transfected cells. The reduction of PTEN is unlikely due to the mutation as the complete mRNA coding sequence of PTEN in the tested cells was completely identical to the published data (Genebank ID: NG_007466) (data not shown). To further explore the PTEN pathway, we examined miR-21, as PTEN is a well-known target of miR-21 (Salmena et al., 2008; Hatley et al., 2010) and smoking may affect the level of miR-21 (Maccani et al., 2010). We found that NNK significantly enhanced miR-21 (Figure 2G), suggesting that NNK may decrease PTEN via enhancing miR-21. Collectively, these results have clearly demonstrated that NNK increases TPα rather than TPβ in lung cancer cells.

The positive roles of NF-κB, ERK, and COX-2 in NNK-induced TPα and TxAS expression

NF-κB is constitutively activated in lung cancer (Saitoh et al., 2010) and NNK is able to activate it (Li et al., 2010a; Huang et al,
We therefore tested whether NF-κB was responsible for the NNK-induced TPα and TxAS expression in lung cancer cells. Western blot analysis showed that the NF-κB inhibitor SN50 completely suppressed NNK-induced TxAS expression but had no effect on TPα (Figure 3A), indicating that NF-κB signaling is required for NNK-induced TxAS expression. In our previous report, PI3K/Akt and ERK were found to be activated by NNK (Li et al., 2010a, b; Huang et al., 2011). We thus asked whether Akt or ERK signaling could affect NF-κB activation. As shown in Figure 3B, wortmannin, a specific PI3K/Akt inhibitor, did not affect p-IκB level, whereas the ERK inhibitor U0126 abrogated p-IκB expression induced by NNK in both tested cell lines. These results demonstrate that NF-κB
The role of NF-κB, ERK, and COX-2 in NNK-induced TxA2 and TPα expression. (A) Cells were serum-starved for 24 h and subsequently untreated or treated with the NF-κB inhibitor SN50 (50 μg/ml) for another 24 h following 15 min treatment of 10 μM NNK. TxA2 and TPα were measured by western blotting. (B) Cells were serum-starved for 24 h and subsequently untreated or treated with the MEK/ERK inhibitor U0126 (10 μM) or PI3K/Akt inhibitor wortmannin (2 μM) for another 24 h following 15 min treatment of 10 μM NNK. p-IκB and T-κB were evaluated by western blotting. (C) Cells were serum-starved for 24 h and subsequently untreated or treated with graded levels of the TP antagonist SQ29548 following 15 min treatment of 10 μM NNK. COX-2 was measured by western blotting. (D) The biosynthesis of TxA2 was evaluated by measuring the level of TxB2 in the culture medium. The results were presented as percentages of the control. Data are expressed as mean ± SEM of three independent experiments in triplicates. *P < 0.05 and **P < 0.01. Actin was used as a loading control and the cells without NNK treatment were employed as the additional control in all western blots. Figures are representative results from three independent experiments.

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Activation is necessary for NNK to induce TxA2 but not TPα, and that ERK but not PI3K/Akt functions upstream of NF-κB to participate in NNK-mediated TxA2.

TPα is able to induce COX-2 expression in lung cancer (Tai et al., 2007), but it is unknown whether TP activation can affect TxA2 synthesis via inducing COX-2. Our result showed that the TP antagonist SQ29548 dose-dependently inhibited NNK-induced expression of COX-2 protein in both tested cell lines (Figure 3C). Importantly, the formation of TxA2 by NNK is largely dependent on COX-2 activity in lung cancer cells, as demonstrated by the data that the COX-2 inhibitor NS398 (25 μM) reduced NNK-induced TxA2 production by ~55.86% in NCI-H23 cells and ~59.54% in CRL-2066 cells, both of which were below their control levels (Figure 3D). This is consistent with the finding that the expression of COX-2, TxA2, and TxA2 is increased in lung cancer tissues obtained from smokers compared with those from non-smokers (McLemore et al., 1988; Ermert et al., 2003; Chen et al., 2006).

Attenuation of NNK-mediated cell growth by COX-2 inhibition is rescued by TP agonist

The TP agonist U46619 can promote the growth of lung cancer cells (Tai et al., 2007; Huang et al., 2011). COX-2 plays a positive role in lung cancer growth (Tai et al., 2007; Huang and Chen, 2011). We thus assessed the role of TP in COX-2-induced cell growth. To this end, siRNA was used to knockdown COX-2 (Figure 4A). It was found that NNK remarkably increased the level of p-ERK, and the effect was alleviated by the COX-2 inhibitor NS398 or COX-2 siRNA (Figure 4B and C). Furthermore, COX-2 siRNA significantly prevented NNK-induced cell growth (Figure 4D). Importantly, the TP agonist U46619 almost restored NNK-induced cell growth and ERK activation in the presence of COX-2 siRNA. These data strongly suggest that TP functions as a key mediator in ERK/COX-2-mediated growth of NNK-treated cells.

Mechanisms used by NNK to induce TPα and TxA2

To explore the possible post-transcriptional mechanism responsible for NNK-mediated TPα upregulation, we determined TPα mRNA level in cells treated with inhibitors of several key transcriptional factors. As shown in Supplementary Figure S3, the level of TPα mRNA and protein in both tested cell lines was not significantly changed by the PPARγ ligand PGZ, NF-κB inhibitor BAY-117082, SP1 siRNA, or CREB siRNA in the presence of 10 μM NNK, indicating that the transcription does not account for the upregulation of TPα by NNK. Moreover, actinomycin D (AcD) and cycloheximide (CHX), the general transcriptional and translational inhibitors, respectively (Zhang et al., 2008), were used. NNK-induced TPα expression was not affected by AcD, but completely abrogated by CHX (Figure 5A), strongly suggesting that the translational rather than the transcriptional mechanism is the major cause of NNK-induced TxA2 expression.

Unlike TPα, the expression of TxA2 is positively associated with the NF-κB activity (Figure 3A), supporting a transcriptional regulation mechanism in NNK-induced TxA2 expression. In addition, post-transcriptional mechanism was also examined. It has been
reported that miR-34c is downregulated in the lung exposed to cigarette smoke (Izzotti et al., 2009). Moreover, according to our miRNA data mining using several public programs, we found that there are binding sites for miR-34b and miR-34c in the 3′-UTR of TxAS (Figure 5B). We thus asked whether NNK could affect the potential TxAS regulator miR-34b/c to alter the activity of 3′-UTR of TxAS. The result showed that NNK markedly reduced the levels of both miR-34b and miR-34c (Figure 5C and D). Importantly, in parallel with the reduction of both miRNAs, NNK significantly induced 3′-UTR activity of TxAS (Figure 5E). These findings indicate that miR-34b/c function as suppressors of TxAS and that NNK upregulates TxAS via inhibiting these two suppressors, suggesting a translational regulation mechanism in NNK-induced TxAS expression in lung cancer cells. In conclusion, NNK induces TxAS expression by both transcriptional and translational regulation mechanisms.

**TPα auto-activation pathway in lung cancer cells stimulated with NNK**

ERK signaling is the key mediator for TPα function in lung tumor (Li and Tai, 2009; Wei et al., 2010; Huang et al., 2011), and NF-κB functions at the downstream of ERK signaling to regulate TxAS in cells treated with NNK (Figure 3). These observations together with the fact that TxA2 is the natural and specific ligand of TPα raise the possibility that, in lung cancer cells stimulated with NNK, TPα activation may induce TxAS expression and subsequent TxA2 synthesis via the COX-2/ERK/NF-κB pathway, thereby constituting a positive feedback loop to further activate TPα in
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![Diagram](image)

**Figure 5** Mechanisms used by NNK to upregulate Tpα and TxA2. (A) Cells were serum-starved for 24 h and subsequently untreated or treated with the general transcriptional inhibitor AcD (5 μg/ml) or translational inhibitor CHX (50 μM) for 24 h following the stimulation of 10 μM NNK for 15 min. The level of Tpα was measured by western blotting. Actin was used as a loading control and the cells without NNK treatment were employed as the basic control. Representative results from three independent experiments are shown. (B) Diagram of TxA2-3'-UTR-containing reporter construct. (C and D) miR-34b/c was evaluated by real-time PCR at different time-points of NNK (10 μM) treatment as described in Materials and methods. (E) Cells were transiently transfected with 500 ng of the reporter construct. After the transfection, cells were treated with 10 μM NNK for different periods. Reporter assay was performed subsequently. The cells without NNK treatment were used as the control. Data are expressed as mean ± SEM of three independent experiments in triplicates. *P < 0.05 and **P < 0.01, compared with the control.

NNK-stimulated lung cancer cells (Figure 6A). To test this possibility, we first examined whether p-ικB could be affected by Tpα activation. Tpα-transfected cells had a higher basal level of p-ικB and NNK treatment further increased it (Figure 6B). The TP antagonist SQ29548 abolished the NNK-mediated expression of p-ικB. However, in TPβ-transfected cells, the level of p-ικB was similar to that in control cells, and neither NNK nor SQ29548 had any additional effect on p-ικB expression when compared with control cells. These findings indicate that the NNK-initiated NF-κB signaling is, at least in part, dependent on Tpα but not TPβ. Furthermore, NNK-induced upregulation of TxA2/COX-2/TxA2 and NNK-induced downregulation of PTEN were aggravated in Tpα-transfected cells, when compared with the control vector- or TPβ-transfected cells, and such effects were significantly reversed by the TP antagonist SQ29548 (Figure 6B and C). However, similar changes were not observed in TPβ-transfected cells. Since TxA2 production can be abolished by TxA2 inhibitor (Huang and Chen, 2011; Huang et al., 2011) and TP is concomitantly activated during the NNK-mediated TxA2 synthesis (Huang et al., 2011), the findings presented here strongly support the auto-activation of Tpα in NNK-treated lung cancer cells (Figure 6A).

To verify the role of Tpα auto-activation in NNK-induced growth, MTT assays were performed in cells treated with BM567 that is a dual TxA2 modulator (combined TxA2 inhibitor and TP antagonist). NNK obviously induced the cell growth (Figure 6D), which was significantly abrogated by BM567 to the level much below the control. The result was further confirmed by the apoptotic assay, which showed that in the cells treated with NNK plus another dual TxA2 modulator pinane thromboxane A2 (pTxA2), the percentage of cells in early and late apoptosis increased about 6 folds and 14 folds, respectively, when compared with the NNK-treated cells (Figure 6E). Collectively, these findings demonstrate that the inhibition of Tpα can significantly attenuate the NNK-mediated cell growth.

**Discussion**

Our initial screening of lung tissues showed that the expression of both TxA2 and TP was increased in human lung cancer tissues compared with the paired non-cancer tissues. Moreover, smokers had significant higher expression of TxA2 and TP in tumor tissues than non-smokers. In agreement with the data observed in human lung tissues, TxA2 and TP proteins were also increased in lung tissues obtained from NNK-treated A/J mice. These findings suggest that NNK may be responsible for the smoking effects on TxA2 and TP expression in lung cancer progression. Interestingly, the expression of TP mRNA in lung tissues obtained from NNK-treated A/J mice was not significantly altered. Because only single TP isoform exists in mouse (Nakahata, 2008), the subsequent mechanistic studies focused on both TP isoforms were performed on *in vitro* models. In human, we found that Tpα, but not TPβ, was widely expressed and enhanced in several lung cancer cell lines. Because TP plays a positive role in NNK-induced lung cancer cell proliferation via PI3K/Akt and ERK pathways (Huang et al., 2011), we examined which isoform of TP was responsible for this positive role. The upregulation of p-Akt and p-ERK1/2 and the downregulation of PTEN, a well-known tumor suppressor, were found in lung cancer cells with Tpα but not TPβ overexpression. The role of Tpα on PTEN downregulation is verified by the inhibitory experiments, in which the TP antagonist SQ29548 fully recovers the loss of PTEN. These findings are in line with the clinical observation of PTEN loss in NSCLC (Tang et al., 2006). Interestingly, PTEN is downregulated in a progressive smoking-dependent...
manner in smokers with chronic obstructive pulmonary disease, one of major risks for lung cancer (Shaykhiev et al., 2011). The downregulation of PTEN in smokers is further supported by the current study in that NNK can significantly decrease the level of PTEN. Furthermore, our finding suggests that the decreased PTEN is likely caused by NNK-mediated upregulation of miR-21, a known negative regulator of PTEN (Salmena et al., 2008; Hatley et al., 2010). Accompanied by the NNK-reduced PTEN, NNK increased TPα at protein level but not at mRNA level. These results confirm the positive role of TPα in lung tumor growth and also suggest that a post-transcriptional mechanism may be involved in the effect of NNK on TPα expression.

The proposed post-transcriptional mechanism of NNK-mediated TPα expression is further investigated using two strategies. The first is to exclude the role of possible transcriptional factors including CREB, NF-κB, PPARγ, and SP1 (Supplementary Figure S3) (Sugawara et al., 2002; Salmena et al., 2008; Li et al., 2010b; Yuan et al., 2010). The second strategy is to use general translation inhibitor CHX and general transcriptional inhibitor AcD. It was found that CHX but not AcD had an inhibitory effect on NNK-induced TPα protein expression. Therefore, NNK mediates TPα expression via a translational rather than a transcriptional mechanism, which is in line with a previous study showing that translational mechanism is the major cause of the upregulation of TP by lipid soluble smoking particles in organ culture of the arterial segments (Zhang et al., 2008). In support of our conclusion, TP protein elevation without significant mRNA change was also observed in bladder tumor tissues (Moussa et al., 2005).

Both mRNA and protein expression of TXAS could be induced by NNK, suggesting that, in contrast to TP, TXAS can be also controlled by transcriptional regulation, which is confirmed by the data showing that NF-κB inhibition significantly blocked the NNK-induced TXAS. Furthermore, we demonstrated the involvement of miRNAs in the NNK-mediated TXAS. We have identified the binding sites for miR-34b and miR-34c in the 3′-UTR of TXAS. NNK markedly reduced the levels of both miR-34b and miR-34c. In parallel with NNK-mediated reduction of both miRNAs, NNK significantly induced 3′-UTR activity of TXAS. These findings indicate that NF-κB upregulates TXAS, while miR-34b/c work to suppress it. These results have clearly indicated that the NNK-mediated TXAS expression is positively regulated by NF-κB at the transcriptional level and negatively regulated by miR-34b/c at the translational level.

Although NNK-mediated upregulation of TPα is independent of NF-κB, interestingly, NNK-mediated activation of NF-κB is, at least in part, TPα-dependent. Since TXAS expression depends on the NF-κB activity in NNK-stimulated cells, and TXA2, the product of TXAS, is the natural TPα ligand, we thus speculate that there is a positive feedback loop of TPα activation in NNK-stimulated cells. Such a speculation is verified by data that increased TXAS and TXA2 levels and reduced PTEN expression in response to NNK could be aggravated by TPα overexpression, and that the effect of TPα was almost completely inhibited by the TP antagonist SQ29548. These findings are further supported by observations that the production of COX-2 and TXA2 in endothelial cells is inhibited by SQ29548, the TXAS inhibitor carboxyheptyl imidazole, and COXs inhibitor aspirin, while it was promoted by the TXA2 analog, carboxyclic TXA2 (Caughey et al., 2001). Moreover, in TPα-transfected cells, COX-2 expression and its downstream metabolites PGE2 and TXA2 could be induced by the TP agonist I-BOP and inhibited by the TP antagonists SQ29548 or BMS67 (Tai et al., 2007). The effects of PGE2 on tumor development are, to some extent, controversial since there are several PGE2 binding receptors that produce the contrary effects (Huang and Chen, 2011). Therefore, the role of PGE2 in NNK-mediated lung cell growth needs further investigations. Collectively, our data suggest a TPα auto-activation mechanism (Figure 6A), which is further confirmed by the data showing that COX-2, a key upstream enzyme in the formation of TXA2 (Cathcart et al., 2010; Ekambaram et al., 2011; Huang et al., 2011), could be significantly increased by TPα overexpression, but decreased by SQ29548. In the autoregulatory feedback loop of TPα activation, both TPα and TXAS play a central role. Therefore, the dual suppression of TXAS and TPα may have dramatical inhibitory effects on NNK-mediated tumor growth, which is confirmed by the finding showing that BM567, a combined TXAS inhibitor and TP antagonist, could completely suppress lung cancer cell growth induced by NNK. This was further verified by another dual blocker pTXA2.

In summary, NNK-mediated activation of TPα stimulates the production of TXA2 via a COX-2/ERK/NF-κB/TXAS pathway. Importantly, the newly synthesized TXA2 can further activate TPα, forming an autoregulatory feedback loop for TPα activation and thus exaggerate the tumor growth (Figure 6A). Obviously, the identification of this novel pathway has made TPα and TXAS ideal therapeutic targets against smoking-related lung cancer. However, there are still some important areas remained to be explored, for example, the detailed translational mechanism responsible for NNK-mediated TPα upregulation and a lack of a suitable in vivo model targeting TPα and TXAS.

Materials and methods

Human lung tissues and immunohistochemical analysis

Thirty-seven pairs of tumor and non-tumor lung tissues were obtained from patients who underwent surgery for lung cancer at our hospital. Of these patients, 29 were tobacco smokers with an average smoking history of over 28 years and the other 8 patients were non-smokers. Tumor tissue samples were taken from the central part of the tumors. The tissue samples were stored in a liquid nitrogen tank until the experiments were performed. The samples included 11 pairs of squamous cell carcinoma tissues, 11 pairs of adenocarcinoma tissues, 5 pairs of large cell carcinoma tissues, and 10 pairs of poorly differentiated carcinoma tissues. All tissue specimens were confirmed by histological examination. The tissues were sectioned and immunohistochemical staining was performed as described previously (Chen et al., 2001). The sections were examined by two independent observers using the Zeiss Spot imaging system (Carl Zeiss) and graded according to the published procedure (Chen et al., 2001). This study was approved by local human research ethical committee and informed consent was obtained from all patients.
Figure 6 Identification of TPα auto-activation pathway. (A) The proposed model of TPα auto-activation mechanism. TPα is increased by NNK, leading to TxAS expression and subsequent TxA2 synthesis via the COX-2/ERK/NF-κB pathway. Survival pathways of PI3K/Akt and ERK are initiated to activate NF-κB. When the newly synthesized TxA2 binds to its receptor TPα, a positive feedback loop for TPα activation is formed, and tumor growth is exaggerated. (B and C) Cells were transiently transfected with the control vector, TPα, or TPβ in the serum-free medium for 24 h. The TP antagonist SQ29548 was subsequently added for 24 h following cells untreated or treated with NNK for 15 min. (B) Total protein was extracted and subjected to western blotting. Actin was used as a loading control. Representative results and the densitometry for blots from three independent experiments are shown. *P < 0.05 and **P < 0.01, when compared with the corresponding empty pcDNA3. ^P < 0.01, NNK vs. NNK + SQ29548. *P < 0.05 and **P < 0.01, the control vs. NNK in TPβ-transfected cells. (C) TxA2 was evaluated by measuring TxB2 in the culture medium. Results were presented as percentages of the empty pcDNA3 control. Data are expressed as mean ± SEM of three independent experiments in triplicates. *P < 0.05 and **P < 0.01, compared with the corresponding empty pcDNA3. ^P < 0.01, NNK vs. NNK + SQ29548. *P < 0.01, the control vs. NNK in TPβ-transfected cells. (D and E) Cells were untreated or treated with BM567 or pTxA2 for 24 h following 15 min stimulation with NNK. The cells without NNK treatment were used as the control. (D) Cell growth was evaluated by MTT assay. Results were presented as percentages of the control. Data are expressed as mean ± SEM of three independent experiments in triplicates. **P < 0.001. (E) Cells were double-stained with annexin V-FITC and propidium iodide. Flow cytometric analysis was performed to measure apoptosis. The percentages of cells in early and late apoptosis are provided in the lower right and upper right quadrants, respectively.
Mouse model

Lung tumor A/J mouse model was established according to our previous publication (Li et al., 2010b; Yuan et al., 2010). Briefly, the mice at 6 weeks old were i.p. injected with a single dose of NNK (100 mg/kg) to induce lung tumor and the growth of the tumor was monitored up to 16 weeks. The animals injected with PBS were used as controls. The use of mice was approved by local animal research ethical committee and in accord with our institute’s guidelines.

Cell culture and chemicals

Human lung cancer cell lines NCI-H23, A549, NCI-H460, CRL-2066, and NCI-H69 were obtained from American Type Culture Collection. NCI-H23, A549, and NCI-H460 are NSCLC cell lines. NCI-H23 was cultured in Dulbecco’s modified Eagle’s medium and both A549 and NCI-H460 were cultured in RPMI 1640 medium. Both CRL-2066 and NCI-H69 cell lines were derived from small-cell lung carcinoma and respectively cultured in Waymouth’s MB 752/1 medium and RPMI 1640 medium.

NNK was obtained from Toronto Research Chemicals, Inc. PPAγ ligand PGZ was kindly provided by Takeda. NF-κB inhibitors SN50 and BAY-117082 were purchased from Calbiochem and Cayman Chemical, respectively. The combined TxA5 inhibitor and TP antagonist BM567, pTxA2, specific TP antagonist SQ 29548, COX-2 inhibitor NS398, and specific TP agonist U66619 were purchased from Santa Cruz. MEK/ERK inhibitor U0126 was purchased from A.G. Scientific. The PI3K-specific inhibitor wortmannin was purchased from Sigma Chemical. General transcriptional inhibitor AcD and translational inhibitor CHX were purchased from Santa Cruz Biotechnology. The concentrations of all chemicals used were based on the product datasheets and previous publications (Tang et al., 2006; Salmena et al., 2008; Li et al., 2010a; Maccani et al., 2010; Wei et al., 2010; Yuan et al., 2010; Huang et al., 2011), and were proven to be optimal in the present study.

Transient transfection

Lung cancer cells were seeded at the same density into a 6-well culture plate, and then incubated overnight to allow cells to attach to the plate. One microgram control small interference RNA (siRNA), SP1 siRNA, CREB siRNA, or COX-2 siRNA was transfected into the cells according to the manufacturer’s instruction (Santa Cruz). The transfection of 2 μg control vector (pcDNA3) or pcDNA3 encoding TPα or TPβ was also performed in 6-well plates using the FuGENE® HD Transfection reagent (Roche).

TxB2 measurement

Lung cancer cells were seeded at the same density into a 6-well culture plate, and then incubated overnight to allow cells to attach to the plate. After proper treatments, the culture supernatant was collected and centrifuged. TxB2 was detected by peroxidase-labeled TxB2 conjugates using an enzyme immunoassay kit (Cayman Chemical).

Cell growth detection and analysis of apoptosis

Cells were seeded into 6-well plates at the same density and incubated overnight. After proper treatments, cell growth was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) assay. Cell apoptosis was analyzed by flow cytometry. Cell pellets were collected and washed twice with ice-cold PBS. After resuspension in 400 μl Annexin-binding buffer (10 mM HEPES/NaOH, 140 mM NaCl, 2.5 mM CaCl2, PH7.4), cells were stained with Annexin V fluorescent dye and propidium iodide (Molecular Probe) in dark at room temperature for 15 min. The cells were kept on ice and analyzed with Becton Dickinson FACScan (BD Biosciences) within 1 h after staining.

Construction of 3′-UTR-luciferase plasmid and reporter assays

The full-length 3′-UTR of TxAS (224 bp) was amplified using cDNA from A549 cells (Primers: Forward 5′-AATCTGAGACAA GAGGCTGCGCGGT-3′, and Reverse 5′-GACTCTACATTAGAG AGAGCAGCGGT-3′, subcloned into the XbaI site of pGL3 (Promega). For the reporter assay, NCI-H23 and CRL-2066 cells were transiently transfected with the reporter plasmid using lipofectamine 2000 (Invitrogen). Reporter assays were performed 36 h post-transfection using the Dual-luciferase-assay-system (Promega), normalized for transfection efficiency by cotransfected Renilla-luciferase.

Real-time PCR

Total RNA was extracted using Trizol reagent (Invitrogen) according to the manufacturer’s protocol. cDNA was synthesized from 2 μg total RNA using a high capacity cDNA reverse transcription kit (Promega). Aliquots of cDNA were used as template for real-time PCR with gene-specific primers and SYBR Green qPCR SuperMix (Invitrogen). Real-time PCR was performed using the ABI Prism 7900 detection system (Applied Biosystems). The expression of target genes in the treatment and control groups was normalized using the house-keeping gene β-actin or U6-snRNA, and the fold change in the expression of each target gene was calculated by the 2-ΔΔCT method.

The following primer sequences were used: β-actin, (forward) 5′-GGAATATGCGTGACAGC-3′ and (reverse) 5′-CAGCCAGCTCGTATCTTT-3′; mouse COX-1, (forward) 5′-GTCGGTGCCAACCTTAT-3′ and (reverse) 5′-GGATGATACTCCCTCTCA-3′; mouse COX-2, (forward) 5′-GATGACCTGCCAACCTCCC-3′ and (reverse) 5′-AAACCA GTTGCTCGCTTA-3′; mouse TxAS, (forward) 5′-ATCCAGAGGAGCCTC TAAA-3′ and (reverse) 5′-CAGTTTACCTGCTGTTTAT-3′; mouse TP, (forward) 5′-TTTCGCGGGTGAACATC-3′ and (reverse) 5′-GGCT CGCCAGTCCAAACA-3′; human TXAS, (forward) 5′-AAAGAAGG CAGACCAACT-3′ and (reverse) 5′-GGCTTACCCCTAGAGG-3′; mouse TP (forward) 5′-GGCGAGAGGCTGCTTACT-3′, human TPx (reverse) 5′-CCCCGCGTGAATCCTCA-3′, and human TPβ (reverse) 5′-CAAAAGGAGCACTGTACC-3′; miR-21, (forward) 5′-GCCCGACTGCTTAACAGCTGTG-3′; miR-34c, (forward) 5′-AGGC AGTGTATGCTGTTGAC-3′; miR-34b, (forward) 5′-GAACTACTAA ACCTCACTGCCAT-3′; U6, (forward) 5′-GCCGCGTGGAACCGCTTC-3′. Expression of mature miRNAs was determined by the NCodeTM EXPRESS SYBR® GreenERTM miRNA qRT–PCR Kit Universal (Invitrogen).

Western blot analysis

Cells were washed with ice-cold PBS, collected, and homogenized with RIPA lysis buffer containing 1× PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitors. Total protein was prepared and measured by the Bio-Rad protein assay (Bio-Rad laboratories). Equal amounts of protein (20 μg) were subjected to western blot analysis and the following antibodies were used. Rabbit polyclonal antibody against TP detecting both α and β isoforms (1 : 200), rabbit polyclonal
antibodies against TxAS (1:200), PTEN (1:500), and phosphorylated ERK1/2 (1:1000), and mouse monoclonal antibodies against total ERK1/2 (1:1000) and COX-2 (1:1000) were purchased from Cayman chemical. Goat polyclonal antibody against β-actin (1:1000), rabbit polyclonal antibodies against phosphorylated Akt (ser473) (1:1000), and total Akt (1:1000) were obtained from Santa Cruz. Rabbit polyclonal antibodies against phosphorylated IκBα (1:500) and total IκBα (1:500) were purchased from Cell Signaling Technology. To ensure equal protein loading, membranes were stripped and then probed with anti-total ERK1/2, anti-total Akt, anti-total IκBα, or anti-β-actin antibody.

### Statistical analysis

The Wilcoxon signed-ranks test was used to compare the difference of TxAS expression between the paired tumor and non-tumor samples. Student’s t-test was employed to compare the difference of TxAS expression in tumor tissues between smokers and non-smokers. One-way ANOVA followed by Dunnett’s test was employed for the comparison between two groups, while One-way ANOVA was used to analyze the difference among three or more groups. Data are presented as the mean ± SEM for all statistical tests. A two-side P-value of <0.05 was used to reject the null hypothesis.

### Supplementary material

Supplementary material is available at Journal of Molecular Cell Biology online.

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### Conflict of interest:

None declared.

### References


