ORIGINAL RESEARCH COMMUNICATION

NADPH Oxidase NOX4 Is a Critical Mediator of BRAF^{V600E}-Induced Downregulation of the Sodium/Iodide Symporter in Papillary Thyroid Carcinomas

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Abstract

Aims: The BRAF^{V600E} oncogene, reported in 40%–60% of papillary thyroid cancer (PTC), has an important role in the pathogenesis of PTC. It is associated with the loss of thyroid iodide-metabolizing genes, such as sodium/iodide symporter (NIS), and therefore with radioiodine refractoriness. Inhibition of mitogen-activated protein kinase (MAPK) pathway, constitutively activated by BRAF^{V600E}, is not always efficient in resistant tumors suggesting that other compensatory mechanisms contribute to a BRAF^{V600E} adaptive resistance. Recent studies pointed to a key role of transforming growth factor β (TGF- β) in BRAF^{V600E}-induced effects. The reactive oxygen species (ROS)-generating NADPH oxidase NOX4, which is increased in PTC, has been identified as a new key effector of TGF- β in cancer, suggestive of a potential role in BRAF^{V600E}-induced thyroid tumors.

Results: Here, using two human BRAF^{V600E}-mutated thyroid cell lines and a rat thyroid cell line expressing BRAF^{V600E} in a conditional manner, we show that NOX4 upregulation is controlled at the transcriptional level by the oncogene *via* the TGF- β /Smad3 signaling pathway. Importantly, treatment of cells with NOX4-targeted siRNA downregulates BRAF^{V600E}-induced NIS repression.

Innovation and Conclusion: Our results establish a link between BRAF^{V600E} and NOX4, which is confirmed by a comparative analysis of NOX4 expression in human (TCGA) and mouse thyroid cancers. Remarkably, analysis of human and murine BRAF^{V600E}-mutated thyroid tumors highlights that the level of NOX4 expression is inversely correlated to thyroid differentiation suggesting that other genes involved in thyroid differentiation in addition to NIS might be silenced by a mechanism controlled by NOX4-derived ROS. This study opens a new opportunity to optimize thyroid cancer therapy. *Antioxid. Redox Signal.* 26, 864–877.

Keywords: NADPH oxidase, ROS, NOX4, BRAFV600E, papillary thyroid cancer, NIS

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Innovation

Our results establish a link between BRAF^{V600E} and NOX4, which is confirmed by a comparative analysis of NOX4 expression in human (TCGA) and mouse thyroid cancers. Remarkably, analysis of human and murine BRAF^{V600E}-mutated thyroid tumors highlights that the level of NOX4 expression is inversely correlated to thyroid differentiation, suggesting that other genes involved in thyroid differentiation in addition to NIS might be silenced by a mechanism controlled by NOX4-derived ROS.

Introduction

PAPILLARY THYROID CANCER (PTC) is the most frequent histologic type of human thyroid carcinoma (26). BRAF mutations are found in 40%–60% of the cases (7) and the most frequent is the BRAF^{T1799A} point mutation that encodes the BRAF^{V600E} oncogenic constitutively active protein kinase (50). This mutation is associated with more extensive disease, a higher rate of recurrence, and decreased survival (51). It is also associated with thyroid cell dedifferentiation with a decreased expression of thyroid functional genes (7).

The property of thyroid cells to accumulate iodide is mediated by the sodium/iodide symporter (NIS) (13). This is clinically highly relevant because it enables treatment of thyroid cancers with radioiodine. However, BRAF^{V600E}-positive tumors are often associated with a significant decrease or a complete loss of NIS expression.

BRAF mediates the signal transduction of the mitogen-activated protein kinase (MEK)-extracellular-signal regulated kinase (ERK) pathway and the presence of a BRAF mutation predicts for a high sensitivity to MEK inhibitors in human cancers (28). Thus, BRAF^{V600E}-positive thyroid cancer cell lines are sensitive to the growth suppressive effects of mitogen-activated protein kinase (MAPK) pathway inhibitors (28, 38) and small-molecule BRAF or MEK inhibitors restore radioiodine incorporation in the thyroid tumors of transgenic mice with doxycycline-inducible expression of BRAF^{V600E} (8). However, it has been also observed that MEK inhibition did not rescue BRAF^{V600E}-induced functional NIS repression in a rat thyroid cell line (41) and in some patients harboring thyroid cancer with BRAF mutation indicating that the MAPK pathway inhibition did not always fully revert cancer progression and BRAF^{V600E}-induced effects (19). A possible explanation is that MAPK signaling is incompletely inhibited in some BRAF-mutant tumors because of relief of negative feedback effects on upstream components of the pathway (30, 35). The strong activation of the MAPK pathway also activates additional pathway(s) contributing to dedifferentiation and epithelial-mesenchymal transition (EMT) in tumors harboring oncogenic BRAF.

Several studies have shown an important role of transforming growth factor β 1 (TGF- β) in this process. TGF- β is overexpressed in human tumors (40), including thyroid malignancies (31, 47), and is a potent pro-oncogenic and prometastatic factor. Its role in cancer biology and in cell signaling is complex, and the cellular context appears to be a crucial determinant of the ultimate outcome of TGF- β signaling in normal and tumor cells. TGF- β was first shown to

play a major role as a local modulator of thyroid by inhibiting both growth and differentiation in several species (17, 44, 46). It can be a potent repressor of the expression of thyroid-specific functional genes, such as the NIS, thyroglobulin (Tg), and thyroperoxydase (TPO) (11, 24, 37). BRAF^{V600E} expression induces the production of functional TGF- β 1, which leads to a TGF- β -driven autocrine loop that mediates, at least in part, the effects of the BRAF^{V600E} oncoprotein, in particular the decreased expression of NIS (41) and the promotion of cell migration, invasiveness, and EMT (25). However, the molecular basis of the BRAF^{V600E}-induced TGF- β 1-loop in the development of thyroid cancers remains unclear.

Recent observations suggest that reactive oxygen species (ROS) play an important role in the TGF- β 1-induced EMT and cell mobility of many cell types. The NADPH oxidases (NOXs and DUOXs) generate ROS in a wide variety of tissues (4). Cancer cells produce high levels of ROS, and in some cases, the source of these ROS has been linked to NOX/DUOX deregulation as reported for prostate cancer (4), melanoma (52), and glioblastoma (20). Among the NADPH oxidases, NOX4 is prominently expressed in a number of tumors such as papillary thyroid cancers (48) and recent studies underscored NOX4 as a new key effector of TGF- β in cancers (5). This led us to consider NOX4 as a potential candidate for an ROS producing system regulated by the BRAF^{V600E}-induced TGF- β -loop.

In this report, we show that NADPH oxidase NOX4 is regulated by BRAF^{V600E} in a TGF- β -Smad3-dependent pathway in thyroid cancer cells, and that NOX4-dependent ROS generation has a critical role in NIS repression induced by mutated BRAF. We confirmed our findings in both patient tissue samples from thyroid cancers harboring the BRAF^{V600E} mutation and in mouse BRAF^{V600E}-induced thyroid tumors by showing a positive correlation with enhanced NOX4 expression. Strikingly, BRAF-mutated tumors have a thyroid differentiation score (TDS) inversely correlated to NOX4 expression level supporting a model where NOX4-derived ROS silence the transcription of genes involved in thyroid differentiation during tumorigenesis.

Results

Activated BRAF oncogene promotes ROS generation via the NADPH oxidase NOX4

p22^{phox} forms a functional heterodimeric complex with NOX4, and accordingly, we evaluated by real-time quantitative polymerase chain reaction (QPCR) the time course changes in the levels of both mRNAs in two human thyroid cancer cell lines harboring the BRAF^{V600E} mutation (BCPAP and 8505C). Figure 1A and Supplementary Figure S1A (Supplementary Data are available online at www.liebertpub.com/ars) show that the expression of NOX4 and p22^{phox} mRNAs increased with time in culture in the two cell lines. This was associated with an increased expression of both NOX4 and p22^{phox} proteins in both cell lines (Fig. 1B and Supplementary Fig. S1B). Thyroid cells also express two other NADPH oxidases generating H₂O₂ at the apical plasma membrane: DUOX2, which is involved in thyroid hormone biosynthesis, and DUOX1, whose physiologic role in this tissue is still unknown. The mRNA levels of two DUOXs were not increased in these experimental conditions (Supplementary Fig. S1C). Knocking down NOX4 or p22^{phox} with

FIG. 1. Expression of NADPH oxidase NOX4 and its functional partner p22^{phox} under the control of BRAF^{V600E} oncogene. Time course expression of NOX4 and p22^{phox} in BCPAP. (A) NOX4 and p22^{phox} mRNA were quantified by real-time quantitative reverse transcription PCR (qRT-PCR). (B) NOX4 and p22^{phox} proteins were analyzed by Western blot as described in the Materials and Methods section. Densitometric quantification of Nox4 and p22^{phox} levels normalized to actin levels and presented as fold change compared with the basal level (24 h). (C) Intracellular ROS detection in BCPAP cells by DCF fluorescence using flow cytometry 48 h after knocking down of NOX4 or p22^{phox} with specific siRNA (10 nM). The relative fluorescence units (RFU) are normalized to 100 in the control. (D) Immunoblot detection of NOX4 and p22^{phox} in BCPAP cells after pharmacological inhibition of BRAF^{V600E} oncogene by PLX4032 (10 μ M). Densitometric quantification of Nox4 and p22^{phox} levels normalized to actin levels and presented as fold change compared with vehicle-treated cells for 24 h. Student *t*-test is realized by comparing PLX versus DMSO for each corresponding time of the kinetic. Values are mean $\pm$ SE. **p* < 0.05, ***p* < 0.01, and ****p* < 0.001. ROS, reactive oxygen species.

specific siRNA reduced the intracellular level of ROS in both cell lines (Fig. 1C and Supplementary Fig. S1D, E). Treatment of cells for 72 h with the BRAF^{V600E} kinase inhibitor PLX4032 led to downregulation of NOX4 and p22^{phox} protein levels, indicating that mutated BRAF regulated both protein expressions in these two tumor cell lines (Fig. 1D and Supplementary Fig. S2A).

BRAF^{V600E} induced TGF- β secretion

We analyzed at different time points the cell culture supernatants for the presence of TGF- β 1 (Fig. 2A). BCPAP cells produced TGF- β 1 and this was associated with an increase in both total and phosphorylated form of Smad3, consistent with activation of the TGF- β 1/Smad signaling pathway (Fig. 2B). Treatment of cells with a TGF- β receptor inhibitor (SB431542) strongly decreased mRNA expression of NOX4 indicating that TGF- β 1 regulates the protein at the transcriptional level (Supplementary Fig. S2B). Treatment of BCPAP cells with PLX4032 inhibited TGF- β 1 secretion, total and phosphorylation of Smad3 (Supplementary Fig. S2C, D). Therefore, we conclude that mutated BRAF controls TGF- β secretion. Downregulation of BRAF by RNA interference confirmed the results (Supplementary Fig. S3A).

In 8505C cells, phospho-Smad3 level, which transiently increased during the 72-h incubation, and total Smad3 protein level were also inhibited by PLX4032 (Supplementary Fig. S2E).

TGF- β regulates NOX4 via Smad3 activation

To determine the role of phospho-Smad3 on NOX4, we treated BCPAP and 8505C cells with SIS3, a specific inhibitor of phospho-Smad3 (21). Immunoblot analysis showed that SIS3 decreased both total and phosphorylated form of Smad3 (Fig. 2C and Supplementary Fig. S3B) and down-regulated NOX4 mRNA and protein levels in both cell lines (Fig. 2C; Supplementary Figs. S2B, and S3B, C). As expected, the level of ROS was also reduced by treatment with both compounds that inhibited TGF- β 1 signaling (Supplementary Fig. S3D). Taken together, these results show that the TGF- β 1 signaling pathway upregulates NOX4 in BRAF^{V600E}-mutated thyroid cells at the transcriptional level in a Smad3-dependent manner. As shown in Figure 2D and Supplementary Figure S4A, the MEK inhibitor U0126 impaired both phosphorylation and expression of Smad3 as expressions of NOX4 and p22^{phox} in two BRAF-mutated thyroid cancer cell lines. This was associated with a

FIG. 2. BRAF^{V600E} oncogene upregulates NOX4 via TGF- β 1 signaling pathway in papillary thyroid cancer cell line. (A) Quantitative detection of secreted and accumulated TGF- β 1 in the extracellular medium of BCPAP cells using ELISA test. (B) Western blot analysis of total and phospho Smad3 proteins in BCPAP cells at indicated time of culture. Densitometric quantification of phospho Smad3 levels normalized to actin levels and presented as fold change compared with the basal level (24 h). (C) Immunoblot detection of Nox4, Smad3, and phospho-Smad3 proteins in BCPAP cells treated with SIS3 (10 μ M). Densitometric quantification of Nox4 and phospho-Smad3 levels normalized to actin levels and presented as fold change compared with vehicle-treated cells or inhibitor-treated cells for 24 h. Student *t*-test is realized by comparing SIS3 *versus* DMSO for each corresponding time of the kinetic. (D) Inhibition of NOX4, p22^{phox}, Smad3, and phospho-Smad3 protein expressions by the specific inhibitor of MAPK pathway U0126 (10 μ M). Proteins were visualized by Western blot in BCPAP cells treated with U0126 for 48 h. Densitometric quantification of Nox4, p22^{phox}, and phospho-Smad3 levels normalized to actin levels and presented as fold change compared with vehicle-treated cells for 48 h. Values are mean $\pm$ SE. **p* < 0.05, ***p* < 0.01, and ****p* < 0.001. TGF- β 1, transforming growth factor β 1.

decreased intracellular level of ROS (Supplementary Fig. S4B). Therefore, Smad3 activation and BRAF^{V600E}-induced NADPH oxidase expression were both MEK dependent.

NOX4-dependent ROS generation downregulates NIS expression in BRAF-mutated cell lines

Based on these results, we postulated that NOX4-dependent ROS generation might play a role in regulating NIS expression in BRAF-mutated thyroid cells. We first analyzed the effect of the ROS scavenger N-acetyl cysteine (NAC) on NIS expression in BCPAP cells. As shown in

Figure 3A, the ROS scavenger increased the NIS at both mRNA and protein levels indicating that ROS are involved in NIS repression at the transcriptional level. DPI, an inhibitor of flavoproteins such as NADPH oxidases, also induced NIS mRNA expression (Fig. 3B). As expected, knocking down NOX4 with specific siRNA reinduced NIS mRNA and protein levels (Fig. 3C). The same results were obtained in 8505C cells (Supplementary Fig. S4C). We next analyzed the cell surface expression of NIS, as membrane localization of the symporter is essential to its function. Cell surface biotinylation experiments were carried out with a nonpermeable crosslinker on both cell lines. Sulfo-NHS-biotin treatment revealed that a fraction of NIS protein, detected as two bands

FIG. 3. ROS generating NOX4 downregulates the NIS in both BRAF^{V600E}-mutated thyroid cell lines. (A) Expression of NIS (mRNA and protein) 24 h after treatment of BCPAP cells with 1 mM of N-acetyl-cysteine (NAC). Densitometric quantification of NIS protein level normalized to vinculin level and presented as fold change compared with vehicle-treated cells for 24 h. (B) Expression of NIS (mRNA) 24 h after treatment of BCPAP cells with 2 μ M of diphenyleneiodonium (DPI). (C) Expression of NIS (mRNA and protein) 48 h after knocking down of NOX4 in BCPAP cells. Densitometric quantification of Nox4 and NIS levels normalized to actin levels and presented as fold change compared with siRNA control-treated cells for 48 h (D) Western blot analysis of cell surface NIS expression in BCPAP cells, 48 h after knocking down of NOX4 or p22^{phox} by RNA interference. Red Ponceau staining at a corresponding molecular weight area of NIS protein was used as loading control. Densitometric quantification of NIS (hyperglycosylated form of 100 kDa) levels normalized to loading control levels and presented as fold change compared with siRNA control-treated cells for 48 h. Values are mean $\pm$ SE. * p < 0.05 and ** p < 0.01. NIS, sodium/iodide symporter.

corresponding to the hypo- and hyperglycosylated forms (29), was increased at the cell surface after NOX4 and p22^{phox} inactivation, respectively (Fig. 3D and Supplementary Fig. S4D). Collectively, these data show for the first time that a NOX4-dependent ROS generation is involved in BRAF^{V600E}-induced NIS repression via the TGF- β /Smad3 signaling pathway.

BRAF^{V600E} induced NOX4 expression through Smad signaling in thyroid PCCL3 cells

We next investigated the effect of acute BRAF^{V600E} expression on NOX4 expression in thyroid cells. To this end, we used the doxycycline-inducible BRAF^{V600E} thyroid PCCL3 cells where mutated BRAF was previously shown to decrease NIS expression through the TGF- β /Smad signaling pathway (41). Importantly, induction of BRAF^{V600E} by doxycycline, which led to activation of Smad3 phosphorylation (Supplementary Fig. S5A), upregulated NOX4 protein and mRNA levels (Fig. 4A, C). Treatment with the Smad3-specific inhibitor (SIS3) abolished the BRAF induction of NOX4 mRNA induction in these cells, which counteracted repression of NIS mRNA level by doxycycline (Fig. 4B). This

underscored that acute BRAF^{V600E} activation upregulated NOX4 at the transcriptional level in a Smad3-dependent manner. To determine the role of NOX4 in BRAF^{V600E}-induced repression of NIS, we performed interference RNA experiments. As shown in Figure 4C, NOX4 knockdown dampened the doxycycline-induced repression of thyroid stimulating hormone (TSH)-induced NIS expression. Of note, in the absence of doxycycline, NOX4-inactivated PCCL3 cells displayed a significant increase of NIS mRNA expression after TSH stimulation, indicating that NOX4 may also participate in the regulation of NIS mRNA level expression in normal thyroid cells. Expression of DUOX1 and DUOX2 mRNA levels was not affected by doxycycline treatment (Supplementary Fig. S5B).

TGF- β downregulates Na⁺/I⁻ symporter via NOX4 in human thyrocytes

To investigate whether NOX4 and p22^{phox} are regulated by TGF- β 1 in normal human primary thyroid follicular epithelial cells, we analyzed the time course effect of TGF- β 1 on both NOX4 and p22^{phox} mRNA by real-time (RT)-qPCR. Figure 5A shows that TGF- β 1 increased NOX4 mRNA in a time-dependent manner. This was associated with increased

FIG. 4. NOX4 induced by BRAF^{V600E} via Smad3 signaling pathway downregulates NIS in thyroid PCCL3 cells. (A) Western blot analysis of BRAF^{V600E} and NOX4 protein expressions in PC-BRAF^{V600E} cells treated for 48 h with or without doxycycline (1 μ g/ml). Densitometric quantification of BRAF^{V600E} levels normalized to actin levels and presented as fold change compared with vehicle-treated cells for 48 h. Densitometric quantification of Nox4 levels normalized to actin levels and presented as fold change compared with siRNA control-treated cells for 48 h in the absence of doxycycline. **(B)** QRT-PCR analysis of NIS and NOX4 mRNA levels in PC-BRAF^{V600E} cells treated for 48 h with or without doxycycline (1 μ g/ml) and SIS3 (10 μ M). **(C)** qRT-PCR analysis of NIS and NOX4 mRNA levels in PC-BRAF^{V600E} cells treated for 48 h in the absence or presence of TSH (1 mU/l, doxycycline [1 μ g/ml] and specific siRNAs [10 nM]). Values are mean $\pm$ SE. * p < 0.05, ** p < 0.01, and *** p < 0.001. TSH, thyroid stimulating hormone.

phospho-Smad3 and NOX4 protein, and inhibited by the Smad3-specific inhibitor (SIS3) (Fig. 5B). NIS mRNA was downregulated by TGF- β 1 (Fig. 5C) and SIS3 counteracted this effect (Supplementary Fig. S6A, B). Of note, TGF- β 1 did not increase DUOX1 and DUOX2 mRNA levels in these cells (Supplementary Fig. S6C). These data indicate that TGF- β 1 regulates NOX4 expression via a Smad3-dependent pathway in human thyrocytes. We next analyzed the effect of NOX4 inactivation on NIS mRNA level in both control and TGF- β 1-treated thyrocytes. Figure 5D shows that NOX4 inactivation counteracted the inhibitory effect of TGF- β 1 on NIS mRNA in the presence of TSH. This was related to a significant decrease in intracellular ROS level (Supplementary Fig. S6D). Taken together, these data show that NOX4 plays a key role in TGF- β 1-induced downregulation of NIS in normal human thyrocytes.

NOX4 is increased in BRAF-mutated papillary thyroid carcinomas

From The Cancer Genome Atlas (TCGA), it is now possible to perform combined analysis of gene expression as a function of different driver mutation groups that lead to different pathologies with distinct signaling and differentiation characteristics (7). BRAF^{V600E} status has been characterized in 390 of the 496 PTCs, and we compared NOX4 mRNA expression levels in BRAF^{WT}-PTCs (n = 170) and BRAF^{MUT}-PTCs (n = 220). NOX4 was significantly increased in PTCs harboring BRAF mutation (Fig. 6A) and this was associated with a significant decrease of NIS mRNA expression level (Fig. 6B). In BRAF-mutated PTCs, a high NOX4 mRNA level mostly correlated with a low NIS mRNA level (Fig. 6C). In addition, an inverse and highly significant correlation was

FIG. 5. NOX4 is involved in TGF- β -induced downregulation of NIS in human thyrocytes. (A) Time course expression of NOX4 and p22^{phox} mRNAs (qRT-PCR) in human thyrocytes treated with human recombinant TGF- β (10 ng/ml). (B) Western blot analysis of NOX4, Smad3, and phospho-Smad3 in human thyrocytes treated for 12 h in the absence or presence of human recombinant TGF- β (10 ng/ml) and SIS3 (10 μ M). Densitometric quantification of Nox4 and phospho-Smad3 levels normalized to actin levels and presented as fold change compared with untreated cells. Student *t*-test is realized by comparing TGF- β with Ctrl and TGF- β +SIS3 with TGF- β . (C) Time course analysis of expression of NIS mRNA (qRT-PCR) in human thyrocytes treated with human recombinant TGF- β (10 ng/ml). (D) qRT-PCR analysis of NIS mRNA expression in human thyrocytes treated or not by human recombinant TGF- β (10 ng/ml for 12 h) in the presence of specific siRNAs (10 nM for 48 h). Values are mean $\pm$ SE. **p* < 0.05, ***p* < 0.01, and ****p* < 0.001.

found between NOX4 expression and the TDS (7) (Fig. 6D). Importantly, both TGF- β 1 and Smad3 mRNA were found to be significantly increased in BRAF-mutated PTCs (Fig. 6E, F). A BRAF^{V600E}-RAS score (BRS) has been also previously developed to quantify the extent to which the gene expression profile of a given tumor resembles either the BRAF^{V600E} or RAS mutant profiles (7). We found that a higher level of NOX4 expression was associated with a negative score that characterizes the BRAF^{V600E}-like samples (Supplementary Fig. S6E). In addition, the ERK activation level, which is highly correlated to BRS (7), was associated with an increased expression of NOX4 gene (Supplementary Fig. S6F). Overall, these data support a role of NOX4 in thyroid tumors driven by BRAF^{V600E}, in particular, a role in mechanisms leading to thyroid dedifferentiation. A schematic model summarizing all the steps involved in NOX4 regulation is presented in Figure 7A.

Since dedifferentiation during tumorigenesis might follow the inverse gene expression pattern observed during normal differentiation, we wondered whether ontogenic changes of

mRNA expression of thyroid differentiation markers, such as NIS and TPO, could be associated with a modulation of the expression level of NOX4 gene. To this end, we analyzed by quantitative PCR, mRNA expression for NOX4, TPO, and NIS genes in thyroid tissues during human development between 8 and 12 gestational weeks (GW). While a significant increase in NIS and TPO mRNA levels was detected at 10 GW in accordance with the onset of thyroid function (43), no modulation of the expression of NOX4 mRNA level was observed in these conditions (Fig. 7B), meaning that only an increase of NOX4 may impact expression.

NOX4 mRNA level is inversely correlated to the thyroid differentiation score in genetically engineered mouse models of thyroid cancer

The profiles of gene expression have been previously established for different mouse PTC models induced by knockin mutations of drivers such as BRAF^{V600E} (14) and H-RAS^{G12V} in the context of NF2, PTEN, or p53 loss (15).

FIG. 6. High level of NOX4 mRNA is correlated with low level of NIS mRNA and low TDS in papillary thyroid cancer (PTC) harboring BRAF^{V600E} mutation. A homogeneous cohort of 390 PTCs (170 BRAF^{WT} PTCs and 220 BRAF^{V600E} PTCs) were included in this analysis. Correlative analysis between NOX4 mRNA (A) or NIS mRNA levels (B) and BRAF^{V600E} mutation in 390 PTCs. Correlation of NOX4 mRNA level and NIS mRNA level in 220 PTCs harboring BRAF^{V600E} mutation (C). Correlative analysis between BRAF^{V600E} mutation and TDS in 390 PTCs (D). Correlative analysis between TGF- β 1 mRNA (E) or Smad3 mRNA levels (F) and BRAF^{V600E} mutation in 390 PTCs. TDS, Thyroid Differentiation Score. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

These tumors present phenotypic differences ranging from differentiated (PTC) to poorly differentiated thyroid cancers (PDTC) and undifferentiated (anaplastic carcinoma ATC). Analysis of the gene expression data clearly shows that the NOX4 mRNA level was increased mostly in BRAF-mutated tumors (Fig. 8A), which significantly correlated with increased expression of TGF- β 1 mRNA and with activation of TGF- β /Smad3 signaling pathway (Fig. 8B and Supplementary Fig. S7A, B, C). Strikingly, BRAF-mutated tumors had a very low TDS, which, as observed in their human counterparts, was strongly inversely associated with NOX4 mRNA levels (Fig. 8C, D).

Discussion

NOX4 as a new effector of BRAF^{V600E} and TGF- β 1 oncogenic signaling in PTC

The BRAF^{T1799A} mutation, which encodes BRAF^{V600E}, is the most common genetic event in human papillary thyroid cancers (7). BRAF is a potent activator of the MAPK/ERK pathway and, as such, a critical regulator of normal growth, differentiation, and oncogenic transformation. TGF- β pathway activation is strongly associated with thyroid cancer progression in mice (25). Overexpression of TGF- β in human

thyroid tumors is consistent with this role and led us to explore downstream mechanisms that may account for these effects in thyroid malignancies. ROS are known to be important effectors of the TGF- β pathway at the molecular level and to induce EMT and cell mobility in many cell types (36, 53). NOX4-dependent ROS production mediates TGF- β -induced effects such as fibrosis (3) and EMT (6). We have previously shown that NOX4 is overexpressed in human papillary thyroid cancers, but its functional consequences have not been previously explored (48).

The present study shows that NOX4 expression is significantly higher in human and mouse papillary thyroid cancers harboring the BRAF^{V600E} mutation (Figs. 6 and 8). Using two human BRAF^{V600E} thyroid cancer cell lines and a rat thyroid cell line expressing BRAF^{V600E} in a conditional manner, we show that NOX4 upregulation is controlled at the transcriptional level by the BRAF^{V600E} oncogenic protein via the TGF- β /Smad3 signaling pathway. Inhibitors targeting specifically this pathway impaired NOX4 expression and consequently the intracellular ROS production in all thyroid cells tested, including normal human thyrocytes. Smad3 is a transcription factor known as an effector of TGF- β 1. Recent data describe the identification of a Smad-binding element involved in TGF- β 1 regulation of the NOX4 gene promoter

FIG. 7. Oncogenic BRAF-dependent regulation of NOX4. (A) A schematic model of all the steps involved in the oncogenic BRAF-dependent regulation of NOX4. Our data show that BRAF^{V600E} upregulates NOX4 expression *via* the TGF- β 1/Smad3 signaling pathway in thyroid cancer cells. The NOX4-dependent ROS production plays a key role in SLC5A5 gene repression. Consequently, it might be involved in the absence of radioiodide uptake by BRAF-mutated cancer cells. The targets of different inhibitors used in this study are indicated. (B) Ontogenic changes of mRNA expression of NIS, TPO, and NOX4. qRT-PCR analysis of NOX4, TPO, and NIS mRNA expression in 6 human thyroids at 8, 10, or 12 weeks of development. GW, gestational week; TPO, thyroperoxidase.

(6). Thus, NOX4 transcriptional regulation by TGF- β 1 in thyroid cells is likely due to an increase of NOX4 promoter activity by the binding of Smad3. NOX4 heterodimerization with p22^{phox} is sufficient to enhance its ROS generating activity and does not require cytosolic subunits that are essential for other NOX isoforms (4). NOX4 is the only NOX with a constitutive ROS generating activity that depends directly on its gene expression. The protein p22^{phox} tightly interacts with NOX4 (1). Except for a few cases and what was observed in BCPAP and 8505C cells, p22^{phox} is not consistently regulated by TGF- β 1 at the transcriptional level. NOX4 has the capacity to stabilize p22^{phox} and therefore to increase its

intracellular protein level (1). Thus, unlike NOX4, p22^{phox} mRNA expression is not the limiting factor for both p22^{phox} protein expression and formation of the active NADPH oxidase.

The MAPK pathway contributes to the progression of BRAF^{V600E}-induced thyroid cancer. Constitutive activation of the MAPK pathway is required for optimal TGF- β signaling in mouse thyroid cancer cells induced by BRAF^{V600E} (25). However, in the rat thyroid cell line expressing conditionally BRAF^{V600E}, the involvement of the MEK-ERK pathway in the mutant BRAF-induced TGF- β effects appeared to be selective. Indeed, unlike BRAF^{V600E}-induced EMT, mutant BRAF-induced NIS repression was an MEK-ERK independent process (41). In the present study, MEK inhibition impaired Smad3 activation and NADPH oxidase expression in both BRAF^{V600E} thyroid cancer cell lines indicating that BRAF^{V600E}-induced NOX4 expression that relied on the autocrine TGF- β loop is MEK-ERK dependent.

Regulation of NIS mRNA transcription

Since BRAF^{V600E}-induced repression of NIS was previously shown to be dependent of TGF- β , we wondered whether NOX4-dependent ROS generation, regulated by TGF- β 1, could be involved in this mechanism. Using antioxidant (NAC) and RNA interference-mediated NOX4 knock-down, we show for the first time that NIS gene expression could be controlled at the transcriptional level by a potential redox-sensitive mechanism in thyroid cancer cells. Our results highlight that BRAF-induced TGF- β 1 represses NIS gene expression through a NOX4-dependent pathway downstream of Smad3. An alternative mechanism to explain NIS repression by TGF- β 1 has been previously proposed. This involved a functional interaction between the transcription factor PAX8, a critical mediator of NIS gene transcription, and Smad3, resulting in inhibition of PAX8 transcriptional activity (11). However, in light of these new data, Smad3 might also contribute to the inhibition of the action of PAX8 by an oxidation process through an upregulation of NOX4-derived ROS. Moreover, a redox regulation of PAX8, involved in its DNA-binding activity, has been previously described in rat thyroid FRTL-5 cells (23). Furthermore, analysis of results obtained with human and murine tumor tissues underscore that the level of NOX4 expression is inversely correlated to thyroid differentiation, suggesting that, beyond NIS, other genes involved in thyroid differentiation might be silenced by a general mechanism finely controlled by NOX4-derived ROS. This mechanism could involve redox-sensitive epigenetic modifications, such as DNA methylation and histone acetylation (18). Hypermethylation of the NIS promoter is observed in BRAF^{V600E} harboring PTC (10, 42), and the use of demethylating agents has been shown to restore decreased iodine uptake to cells with methylated promoter regions *in vivo*. ROS have been shown to provide an oxidative stress-mediated epigenetic imbalance, which perturbed genomic integrity in numerous oxygen radical-induced injuries, disturbing the DNA demethylation mechanisms (33). It was, in particular, observed that oxidative damage could contribute to the relocalization of a silencing complex containing DNA methyl transferases (DNMTs) (39). The molecular mechanisms of a potential redox-sensitive control by NOX4-derived ROS of genes involved in thyroid differentiation

FIG. 8. High level of NOX4 mRNA is correlated with low thyroid differentiation score in genetically engineered mouse models of thyroid cancer. Gene expression profiling of GEM models of thyroid hyperplasia or the indicated thyroid cancer phenotypes. (A) Fold change in NOX4 expression compared to normal thyroid controls calculated from Affymetrix expression array data. (B) Association of NOX4 expression levels with the TGF- β transcriptional output score. Pearson correlation was used to calculate r - and p -values. (C) Thyroid differentiation score for each mouse model. (D) Correlation between thyroid differentiation score and NOX4 expression. Pearson correlation was used to calculate r - and p -values.

have to be further investigated. Interestingly, the increases in NIS and TPO mRNA expressions during human thyroid differentiation were not associated with a modulation of NOX4 mRNA, suggesting that the onset of human fetal thyroid function is not dependent on the level of NOX4.

NOX4 as a potential therapeutic target?

A clinical study using the MAPK inhibitor selumetinib to reverse refractoriness to radioiodine in patients with radioiodine refractory metastatic thyroid cancer partially restored radioactive iodine (RAI) incorporation in patients with BRAF-mutated tumors, but yielded much better results in patients with RAS-mutated tumors (10). Analysis of human PTC (TCGA) shows that the NOX4 expression level is correlated to ERK activation. Interestingly, we have recently shown that overexpression of oncogenic H-RAS^{V12} upregulates NOX4 in thyroid cells (49). This underscores that NOX4 expression in thyroid tumors may depend on the degree of MAPK activation. It was recently shown that BRAF^{V600E} PTCs are a heterogeneous group of tumors with variable degrees of thyroid differentiation (7). Since the level of NOX4 appears to be correlated with thyroid dedifferentiation in BRAF^{V600E} PTC, it would be of importance to evaluate the potential role of NOX4 as an RAI therapy response predictive

marker in patients with metastatic thyroid cancers. Moreover, this study identifies NOX4 as a potential therapeutic target through its role in NIS repression. Specific inhibitors might be used in association with MAPK inhibitors to potentiate their effect on RAI therapy.

Materials and Methods

Cell culture and reagents

Human thyroid cancer cell lines harboring the BRAF^{V600E} mutation (BCPAP and 8505C cells) were cultured in Dulbecco's modified Eagle's medium (DMEM) (4.5 g/l glucose) (Life Technologies) supplemented with 10% (vol/vol) FCS (Life Technologies) and penicillin/streptomycin (100 mg/ml; Life Technologies). Primary human thyroid cells were cultured as previously described (2).

PC-BRAF^{V600E} cells were derived from the rat thyroid cell line PCCL3 to obtain doxycycline-inducible expression of BRAF^{V600E} (34). PC-BRAF^{V600E} cells were cultured in Coon's modified Ham's F-12 medium supplemented with 5% Tet-free FCS (Life Technologies) and a six-hormone mixture, including TSH and insulin. Inhibitor of TGF- β 1 pathway (SB431542; Calbiochem), inhibitor of Smad3 phosphorylation (SIS3; Sigma-Aldrich), inhibitor of MAPK pathway (U0126; Calbiochem), inhibitor of BRAF^{V600E}

(PLX4032; Selleckchem); recombinant human TGF- β 1 (Peprotech), N-acetyl-cysteine (Sigma-Aldrich), and doxycycline (Sigma-Aldrich) were used in this study.

Transfection of small interfering RNAs

Cells were transfected at 50%–60% confluence with specific human siRNA against NOX4 (stealth RNAi duplex; Invitrogen), scrambled siRNA control (Invitrogen), or siRNA against p22^{phox}, siRNA against BRAF, and rat siRNA against NOX4 (smart pool from Dharmacon) using the INTERFERIN transfection reagent (Polyplus-Transfection) according to the manufacturer's protocol (Supplementary Table S2).

Measurement of intracellular ROS

The peroxide-sensitive fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA; Molecular Probes, Life Technologies) was used to assess the generation of intracellular ROS.

Western blot analysis and ELISA test

Cells were solubilized in the RIPA lysis buffer (Sigma-Aldrich) supplemented with a mixture of phosphatase and protease inhibitors (Roche Applied Science). Immunodetection was performed as described previously (48) using as primary antibody anti-NOX4 (OriGene); anti-pSmad3 (ser 423) (Abcam); anti-Smad3 (Abcam); anti-p22^{phox} (Santa Cruz Biotechnology, Inc.); anti-NIS (Thermo Scientific); anti-BRAF^{V600E} (Spring); anti- β -actin (Sigma-Aldrich), and anti-Vinculin (Abcam). The proteins were visualized by enhanced chemiluminescence (Amersham) using secondary antibodies conjugated to HRP: goat anti-rabbit IgG-HRP antibody (Southern Biotech) and goat anti-mouse IgG-HRP antibody (Santa Cruz Biotechnology, Inc). Secreted TGF- β was measured by the ELISA test according to the manufacturer's protocol (Promega).

Cell surface biotinylation

Membrane-associated proteins were prepared using the Cell Surface Protein Isolation Kit (Pierce, Life Technologies) and avidin agarose (Sigma-Aldrich) according to the manufacturer's protocol.

Real-time qRT-PCR

Total RNA was extracted from cells using the TRIzol reagent (Life Technologies) according to the manufacturer's instructions. The quality of RNA preparation, based on the 28S/18S rRNAs ratio, was assessed using the RNA 6000 Nano Lab-On-chip (Agilent Technologies). Total RNA (1 μ g) was reverse-transcribed using Maxima Reverse Transcriptase (Thermo Fisher Scientific) and random hexamer primers in a total reaction volume of 20 μ l of PCR buffer according to the manufacturer's protocol for 60 min at 60°C. Real-time qRT-PCR was performed as described previously (48). Sequences of primer pairs and TaqMan probes were as follows: Human DUOX1 primer: sense, 5'-CCAGCAATCATCTATGGGGGC-3' and antisense, 5'-TGGGGCCGCTGGAACC-3'; DUOX2 primer: sense, 5'-CCGGCAATCATC TATGGAGGT-3' and antisense, 5'-CCTTGGGGCCTCTGGAATT-3'; the same TaqMan fluorescent probe

for DUOX1 and DUOX2: 5' FAM-AGCAGCGTGGTGAAGGCGGAGC-3' TAMRA; NOX4 primer: sense, 5'-TGTCTTCTACATGCTGCTGACGTT-3' and antisense, 5'-CTGAGAGCTGGTTTCGGTTAAGACT-3'; TaqMan fluorescent probe: 5'-FAM-CAAACTAATTAGATACCCACCCTCCGGC-TAMRA; Human p22^{phox} primer: sense, 5'-GCGCTTCACCCAGTGGTACTT-3' and antisense, 5'-GGTGGAGCCCTTCTTCTCTT-3'; TaqMan fluorescent probe: 5'-FAM-TTGTGGCGGGCGTGTGTTGTGTG-TAMRA; Human NIS primer: sense, 5'-CCATCCTGGATGACAACTTGG-3' and antisense, 5'-AAAAACAGACGATCCTCATTG-3'; TaqMan fluorescent probe: 5'-FAM-AGAAGTCCCACTGGAAACAAGAAGCCC-TAMRA; Human TBBP primer: sense, 5'-GAATATAATCCCAAGCGTTTGC-3' and antisense, 5'-CTTAGCTGGAAAACCCAACCTTCTG-3'; TaqMan fluorescent probe: 5'-FAM-AGGATAAGAGAGCCACGAACCACGGC-TAMRA; Housekeeping gene TBBP primer: sense, 5'-GAATATAATCCCAAGCGTTTGC-3' and antisense, 5'-CTTAGCTGGAAAACCCAACCTTCTG-3'; TaqMan fluorescent probe: 5'-FAM-AGGATAAGAGAGCCACGAACCACGGC-TAMRA. Oligonucleotides were purchased from Eurofins Genomics.

For PC-BRAF^{V600E} cells, real-time qRT-PCR was performed with FastStart Universal SYBR Green Master (Roche). The sequence of primer pairs were as follows: rat DUOX1: sense, 5'-GATACCCAAAGCTGTACCTCG-3' and antisense, 5'-GTCCTTGTCACCCAGATGAAG-3'; rat DUOX2: sense, 5'-TGCTCTCAACCCCAAGTG-3' and antisense, 5'-TCTCAAACAGTAGCGATCAC-3'; rat NIS: sense, 5'-GCTCATCCTGAACCAAGTGA-3' and antisense, 5'-ACGAGCATTACCACAACCTG-3'; rat NOX4: sense, 5'-TCCATCAAGCCAAGATTCTGAG-3' and antisense, 5'-GGTTTCCAGTCATCCAGTAGAG-3'; rat GAPDH: sense, 5'-TGATTCTACCCACGGCAAGT-3' and antisense, 5'-AGCATCACCCCATTTGATGT-3'.

Expression arrays

Samples were processed and hybridized on Affymetrix MOE 430A 2.0 arrays by the MSKCC Genomic core facility. Gene expression data of all samples were log₂-transformed and quantile-normalized with Partek GS software. Fold change was calculated compared to the average expression of normal thyroid tissues (TPO-Cre negative or wild-type animals). We determined the TDS and TGF- β transcriptional output scores from thyroid specimens of different mouse models by measuring expression of genes involved in thyroid hormone biosynthesis or that are transcriptionally regulated by the TGF- β pathway (Supplementary Table S1). Score = Average of all genes (Invlog10), where each gene = log₂(gene)/log₂(controls). Genes used for the TDS were the mouse homologues of the human gene list used to develop the TDS in the TCGA article on papillary thyroid carcinoma (7). The TGF- β score was based on genes upregulated by TGF- β in mouse primary hepatocytes (12) that were concomitantly upregulated in LSL-Braf/TPO-Cre PTCs relative to normal thyroid tissue. Of note, we confirmed that these murine Braf-induced PTCs have increased activation of the TGF- β pathway (Supplementary Fig. S7A, B). For genes with multiple probes in the expression array, we used the probe with the highest standard deviation between samples of different characteristics to calculate scores. Correlations between

Nox4 expression and TGF- β output and TDS were calculated in GraphPad using Pearson correlation.

Human embryonic tissue samples

Human embryonic and fetal thyroid was extracted from tissue fragments that were obtained immediately after elective termination of pregnancy between 7 and 12 weeks of gestation in compliance with the French bioethics legislation. Approval was obtained from Agence de Biomedecine, the French competent authority, along with maternal written consent. The number of sampled thyroid tissues per GW was as follows: 2 at 8 GW; 2 at 10 GW; and 2 at 12 GW. Tissue samples were snap-frozen and stored at -80°C before RNA analysis. Total RNA was purified from frozen thyroid tissues using RNeasy extraction, according to the manufacturer's instruction (QIAGEN).

Mouse genetic models

TPO-Cre mice express Cre recombinase under the control of the thyroid peroxidase gene promoter, which is active only in thyroid follicular cells beginning at E14.5 (27). *FR-Hras^{G12V}* mice conditionally express a latent *Hras^{G12V}* allele under the regulatory control of its endogenous gene promoter (9). To generate triple transgenic mice, *TPO-Cre/FR-Hras* mice were bred with *Nf2^{fllox2}* (16), *Trp53^{fllox2}* (22), or *Pten^{fllox2}* mice (45). Mice with homozygous loss of *Nf2* develop thyroid hyperplasia, whereas *Hras^{G12V}/Nf2*-null and *Hras^{G12V}/Pten*-null develop PDTC. *Hras^{G12V}/p53* null develop either PDTC or anaplastic thyroid cancer (15). *TPO-Cre* mice crossed with *LSL-Braf^{V600E}* (32), which harbor conditional knockin allele of *Braf^{V600E}*, result in development of PTC with short latency (14). Both male and female mice were used. Animal care and all procedures were approved by the MSKCC Institutional Animal Care and Use Committee.

Statistical analysis

Statistical analyses were performed using GraphPad Instat software for analysis of variance and the Student *t*-test, with the level of significance set at $p < 0.05$.

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Author Disclosure Statement

No competing financial interests exist.

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Abbreviations Used

BSA = bovine serum albumin
DUOX = dual oxidase
DUOXA = DUOX activator
EMT = epithelial–mesenchymal transition
ERK = extracellular signal-regulated kinase
MAPK = mitogen-activated protein kinase
MEK = mitogen-activated protein kinase kinase
NAC = N-acetyl-cysteine
NADPH = reduced nicotinamide adenine dinucleotide phosphate
NIS = sodium/iodide symporter
NOX = NADPH oxidase
PTC = papillary thyroid cancer
ROS = reactive oxygen species
RT = reverse transcription
siRNA = small interfering RNA