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REGγ potentiates TGF-β/Smad signal dependent epithelial-mesenchymal transition in thyroid cancer cells

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ABSTRACT

Thyroid cancer is the most common endocrine cancer with an increasing incidence and mortality. Epithelial-mesenchymal transition (EMT) is a biological process contributing to tumor progression, metastasis, and the acquisition of chemotherapy resistance. The impact of the REGγ proteasome activator on EMT in human thyroid cancer cells and the molecular mechanism is still unclear. Here, we found silencing REGγ in thyroid cancer cells inhibited cell migration and invasion, with concurrent upregulation of E-cadherin and Smurf2 expression. Mechanistically, REGγ dependent regulation of Smurf2, an E3 ligase for Smad3, contributed to alteration of Zeb1/2, Snail, Slug, and Twist. Consistently, TGF-β mediated suppression of E-cadherin was attenuated in REGγ deficient cells, coupled with changes in cell morphology, migration and invasion. Furthermore, xenograft metastasis mouse model showed a reduced E-cadherin expression at both mRNA and protein levels, and decreased cell migration. Taken together, our findings provided an important evidence for the role of REGγ in tumor suppression, thereby implicating REGγ as a potential anti-cancer strategy in thyroid cancer therapy.

1. Introduction

REGγ (also known as PA28γ or PSME3) is a member of 11S proteasome coactivator family [1]. REGγ participates in proteolysis in a ubiquitin- and ATP- independent manner [2]. Previous studies have reported that REGγ is highly expressed in human lung cancer [3], breast cancer [4], hepatocellular carcinomas [5], thyroid cancer [6], and colorectal cancer [7]. In addition, recent studies have shown that REGγ plays an important role in in protein degradation of tumor suppressor p53 [8], steroid receptor coactivator-3 (SRC-3) [2], as well as cell cycle regulator proteins including p21, p16, and p19 [9]. However, the precise role of REGγ in the regulation of thyroid cancer cell migration and invasion is still unclear.

Thyroid cancer is the most frequent endocrine cancer with a hiking incidence and mortality worldwide [10]. Anaplastic (ATC), follicular (FTC), and papillary (PTC) thyroid cancer arise from endodermal-derived follicular cells which represent the plenty of cellular population of thyroid gland [11]. PTC comprises 80% to 85% of all thyroid neoplasms, whereas FTC is the second most common thyroid cancer, accounting for approximately 10 to 15% of cases. However, ATC, responsible for only 1–2% of all the thyroid cancer cases, is considered as the most aggressive and the fastest progression type of cancer in thyroid gland [12,13]. Thyroid cancer incidence has been increased by an average of 4.5% per year, and ranks 8th most frequent cancer in China. Thyroid cancer is considered as a multi-causal disease related to environmental and genetic predisposing factors, including exposure to ionizing radiation, obesity, genetic and epigenetic alternations, yet significant uncertainty remains regarding its causes [14]. The strategy for treating these aggressive thyroid cancer patients remains an arduous task. Multi-targeted inhibitors including sorafenib and levatinib have been approved by FDA for personalized thyroid cancer therapy. Nevertheless, there is still an urgent need for developing novel therapy strategies or new drug targets against aggressive and radioactive iodine-refractory differentiated thyroid cancer (RAI-R-DTC) [10].

Metastasis is the most common cause of human cancer death and the underlying regulatory pathways remain elusive, given that epithelial-mesenchymal transition (EMT) has been recognized as a critical mechanism in cancer metastasis [16]. EMT is a complex molecular and cellular process through which epithelial cells shed specific characteristics including lack of motility, cell-cell adhesion, and planar and apical-basal polarity in order to acquire mesenchymal features such as resistance to apoptosis, motility and invasiveness [17,18]. EMT plays a key step during embryonic development and is recognized as an important contributor to the progression of primary tumor towards metastasis. The initiation of EMT can be identified by the downregulation or loss of adhesion molecule E-cadherin, whereas N-cadherin and

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Vimentin levels are increased [19]. The transcriptional factors Zeb, Snail and Twist have been described as direct repressors of E-cadherin *in-vitro* and *in-vivo* [17]. Several cytokines such as transforming growth factor-beta (TGF-β) have been reported to induce EMT, such as TGF-β. TGF-β signal is involved in the regulation of diverse cellular functions, including cell proliferation, differentiation, migration *via* specific complexes of type I (TβRI) and type II Ser/Thr kinase receptors [20]. The activated TGF-β type I receptor induces Smad2/3 phosphorylation; phosphorylated Smad2/3 forms hetero-oligomers with Smad4, which accumulate in the nucleus to regulate the expression of target genes [21]. Negative feedback loop of TGF-β signaling is composed of Smad7 and Smad ubiquitin regulatory factors 2 (Smurf2), which is an HECT type E3 ubiquitin ligase [20,22]. The Smurf2 signaling is activated through its interaction with Smad7, The Smad7-Smurf2 complex is subsequently exported from nucleus and targeted to TβRI in plasma membrane. Smurf2 delivers ubiquitin to both TβRI and Smad3 [23] for proteasomal degradation. The reduction of TβRI leads to the termination of TGF-β signaling and inhibits the excess proceeding of signal. For this reason, Smurf2 is known as a negative regulator of TGF-β signal pathway [20,22].

In the present study, REGγ was silenced with short hairpin RNA (shRNA) in ATC and FTC cell lines. We have shown that REGγ deficiency inhibited the cell migration and invasion of thyroid cancer cells (SW1736, K18, and FTC) by inducing E-cadherin *via* regulation of transcriptional repressors. Our study illustrated a new anti-cancer mechanism for REGγ inhibition and provided a promising anti-cancer target for thyroid cancer. In addition, we analyzed the invasive potential of thyroid cancer cells in animal model. We found that REGγ-deficient ATC (SW1736 and K18) and FTC (FTC) cells had significantly attenuated EMT progression.

2. Material and methods

2.1. Cell lines and cell culture

Human thyroid cancer cell lines (SW1736, K18, and FTC) and 293 T cells were purchased from the American Type Culture Collection (ATCC, Rockville, USA). SW1736 and K18 cells were grown in Roswell Park Memorial Institute-1640 (RPMI-1640) medium, FTC and 293 T cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco; Invitrogen; Life Technologies, Germany) with 10% fetal bovine serum (FBS) (Gibco) and 100 μg/mL penicillin/streptomycin. Cells were incubated at 37 °C in a humidified incubator with 5% CO_2 and 95% air atmosphere. The SW1736, K18, and FTC stable cell lines were generated by integration of retroviral shRNA vectors specific for REGγ or a control gene from OriGene (Rockville, MD).

2.2. Antibodies, reagents, and plasmids

Antibodies for E-cadherin, N-cadherin, Smurf2 were obtained from (Cell Signaling Technology) and Zeb1, Snail, Slug, Twist, Smad3, and β-actin were obtained from Santa Cruz Biotechnology. Anti-REGγ was purchased from Invitrogen. Anti-mouse and anti-rabbit fluorescent-labeled secondary antibodies were purchased from Jackson immnoResearch laboratories. Recombinant human TGF-β1, was purchased from R&D Systems (Abingdon, UK), TGF-β inhibitor (SB 431542) was purchased from Sigma-Aldrich St. Louis, Mo, USA. PCDNA3.1-HA-REGγ was previously constructed in our laboratory. The siRNA-REGγ (5′- GAAUCAAUAUGUCACUCUAUU-3′) and siRNA -Smurf2 (5′-GAUGAGAACACUCCAAUUA-3′) were purchased from Genepharma (Shanghai, China). Lipofectamine 2000 was obtained from Invitrogen.

2.3. Mice

$REG\gamma^{+/+}$ and $REG\gamma^{-/-}$ mice with C57BL/6 were kindly provided by Dr. John J. Monaco (University of Cincinnati). Mice were maintained according to the ethical and scientific standards protocol by the Animal Center at East China Normal University.

2.4. qRT-PCR analysis

To assess mRNA levels, RNA was isolated from cells using TRIzol reagent (Takara), and cDNA was synthesized using MLV reserve transcriptase from Promega. For the qRT-PCR analysis, the reverse-transcribed cDNA was subjected to RT-PCR using SYBER Green master mix (BIORAD) and the Mx3005P qRT-PCR system (Stratagen). Each experiment was performed in duplicates and repeated three times. The relative gene expressions were normalized to 18S RNA using the 2-ΔΔct method. The primer sequences are described in Supplementary Table. S1.

2.5. Western blot analysis

Western blot was performed using standard methods. Total proteins (10–20 μg) were isolated from cells or tissues. The concentration was measured by BSA protein assay kit (Pierce, USA). Proteins were isolated by SDS-PAGE and then transfer to nitrocellulose membrane (Bio-Rad). Membranes were blocked with 5% non-fat dried milk and incubated primary antibodies at 4 °C overnight. After washing with PBST 3-time, the membranes were incubated with fluorescent-labeled secondary antibodies and detected by using Odyssey LI-COR scanner.

2.6. Cell migration and invasion assays

Cell migration and invasion was measured by using transwell chamber (8-μm pore size, Corning, USA) with or without Matrigel (Becton Dickinson, New York, USA). At 24 h transfection, cells in serum-free media were placed into the upper chamber with or without 10 μg/mL Matrigel. Medium containing 10% FBS was added into the lower chamber. After 24 h of incubation, cells remaining in the upper membrane were fixed with methanol, stained with 0.1% crystal violet and counted under a microscope. Three independent experiments were carried out.

2.7. Three-dimensional (3D) and two-dimensional (2D) cell culture

Cellular growth 3D culture analysis was performed with modified version of 3D assay previously described [24,25]. First, Matrigel basement layer was prepared by pre-coated in phenol red-free Matrigel (80 μL/well) in 48-well plate, followed by incubation at 37 °C for 30 min to allow get solidification. Cells were harvested by trypsinization and pelleted by centrifugation at 115 ×*g* before re-suspension in serum free media and seeded into Matrigel-coated at final concentration of 0.2 × 105 cells/cm^2. The cells were allowed to settle and attach to Matrigel for 30 min at 37 °C, following which they were overlaid with 500 μL of fresh media containing 2% (*V*/V) Matrigel. Cell culture was maintained for upto 15 days and medium was replaced every three days with supplemented Matrigel.For 2D cell culture, SW1736 shN and SW1736 shR cells were seeded in 24-well plate at an initial density of 20,000 cells per well in 2D matrix. Cells were allowed to grow for 10 days with medium changes every 3 days.

2.8. Immunohistochemistry

All procedures performed in studies involving human participants were in accordance with the Ethics Committee of East China Normal University. Thyroid cancer tissues were collected by and specimens were fixed in 10% neutral formalin, embedded on paraffin and cut into 4 μM for immunohistochemical staining.

2.9. Luciferase assay

SW1736 shN and shR cells were transfected with pGL-3 luciferase reporter construct, harvested in lysis buffer provided with luciferase assay kit (Promega). After a brief overtaxing, whole-cell lysates were centrifuged at 12,000 rpm for 10 min at 4 °C. Then 20 μl of supernatant was added to an equal amount of luciferase assay substrate. Luminescence was measured as relative light units, twice for each lysate using LUMIstar OPTIMA (BMG Labtech) illuminometer.

2.10. Xenograft tumorigenicity analysis

SW1736 shN and shR cells with stable expression of luciferase reporter construct were delivered to 4–6 weeks old BALB/c nude mice by left ventricular injection of 2×10^6 cells in 100 μL PBS. Optical *in vivo* imaging of cancer metastasis was monitored with *in vivo* luminescence imaging system (IVIS; Xenogen Crop., Alameda, CA).

2.11. Statistical analysis

The statistical data were obtained by GraphPad Prism 5.0 software. The results were expressed as the mean ± SD. Statistical analysis was performed using two-tailed paired Student's *t*-test. *P* value if < 0.05 was considered statistically significant.

3. Results

3.1. Ablation of REGγ inhibits cell migration of thyroid cells in vitro

To determine the impact of REGγ on the migratory abilities in thyroid cancer cells, we analyzed SW1736 and K18 cells with or without stable integration of a well-established shRNA against REGγ [26] in transwell chambers. Data showed that cell migration was significantly inhibited in REGγ depleted (shR) SW1736 and K18 cells compared to the shN controls (Fig. 1A,B). To substantiate the inhibitory effect of REGγ deficiency on cell migration *in-vitro*, we performed wound healing assays with 12 h intervals in the above cells. We observed that SW1736 shR cells had a larger wound area at 48 and 72 h after scratches respectively, while SW1736 shN cells displayed faster healing (Fig. 1C,D). These results indicate a positive role of REGγ in thyroid cancer cell migration Consistent with correlation between REGγ and cell migration, the cell morphology was different before and after REGγ knockdown in thyroid cancer cell lines. The REGγ-depleted cells had enlarged cell size and adopted epithelial sheet like structure (Fig. S1A). To gain detailed EMT-like phenotype of thyroid cancer cells, we used two-dimensional (2D) cell culture approach [27]. SW1736 shR cells exhibited a tightly packed cubical epithelial-like morphology, while SW1736 shN cells retained fibroblastic spindle-shaped morphology after culture in 2D differentiation media for ten days (Fig. S1B). Next, we used three-dimensional culture method to determine the acinus formation by SW1736 shN and SW1736 shR cells. Results indicated that REGγ-depleted SW1736 cells had a highly organized acinar structure and remained in circular colonies. In contrast, the control SW1736 shN cells displayed a relatively disorganized structure in 3D cell culture (Fig. S1C). These data suggest that REGγ may affect cell adhesion molecules to potentiate cell migration.

3.2. REGγ modulates EMT effectors and regulators

Given the regulation of REGγ on cell migration and cell morphology, we sought to determine whether REGγ acts *via* the EMT pathway. In SW1736, K18 and FTC cells, REGγ knockdown (shR) significantly upregulated expression of the epithelial marker E-cadherin and repressed N-cadherin, a mesenchymal marker, compared to the shN controls (Fig. 2A,B,C). Transient depletion of REGγ by siRNA also enhanced the expression of both E-cadherin protein and mRNA in thyroid cancer cell lines (Fig. S2A & S2B). Interestingly, both E-cadherin protein and mRNA levels were significantly enhanced in *REGγ*$^{-/-}$ thyroid tissues compared with the *REGγ*$^{+/+}$ controls (Fig. S2C).Human thyroid cancer tissues having high REGγ expression showed lower E-cadherin levels compared with normal thyroid tissues (Fig. 3SA), which is in line with negative correlation between E-cadherin and REGγ in 293 T cell line (Fig. 3SB). These results suggest that REGγ negatively regulates E-cadherin expression in thyroid cancer cells and tissues.

Furthermore, we have observed a downregulation of EMT-related transcription factors including Zeb1, Snail and Slug in REGγ-silenced SW1736, K18 and FTC cells (Fig. 2A,B,C). Quantitative RT-PCR revealed that mRNA expression of both E-cadherin and ZO-1 (zonula occludens protein), a different EMT marker, was elevated, whereas N-cadherin and Vimentin levels were decreased in the REGγ deficient thyroid cancer cell lines (Fig. 2D,E,F). Correspondingly, mRNA expression of EMT transcriptional factors Zeb1, Zeb2, Snail, Slug and Twist was lower in REGγ knockdown thyroid cancer cells (Fig. 2D,E,F). These results demonstrate that REGγ regulates transcription of EMT effectors and regulators in thyroid cancer cells.

3.3. REGγ-mediated regulation of E-cadherin is Smurf2 dependent

Smurf2, one E3 ligase to mediate degradation of Smad3, has been known as a target of REGγ for ubiquitin-independent proteasomal degradation [28]. Consistent with the previous report, we found that REGγ knockdown induced Smurf2 protein expression (Fig. 3A), however, no changes in mRNA levels were observed in SW1736, K18, and FTC cells (Fig. 3B), indicating a REGγ-dependent regulation of Smurf2. To determine if REGγ regulates EMT *via* Smurf2, we silenced Smurf2 in SW1736 and K18 cells. Strikingly, ablation of Smurf2 significantly attenuated expression of E-cadherin in REGγ deficient thyroid cancer cells where E-cadherin is usually elevated (Fig. 3C). In fact, Smad3 expression was found to be significantly lower in SW1736, K18, FTC cells (Fig. 3D). Our data suggest that the REGγ regulatory effect on EMT is Smurf2 dependent and is likely *via* modulation of the TGF-β pathway.

3.4. REGγ impinges on TGF-β-mediated EMT regulation

TGF-β is a major inducer of EMT during embryogenesis, cancer progression and fibrosis. To determine if the thyroid cancer cells is responsive to TGF-β-mediated EMT, we analyzed morphological changes in SW1736 cells before and after TGF-β treatment. We observed that SW1736 cells lose their polarized epithelial cell morphology and become scattered and spindle-like resembling mesenchymal cell morphology, However, the epithelial morphological features was obtained by the stimulation of TGF-β inhibitor which is the characteristic of EMT phenotype (Fig. S4A). To clarify if REGγ mediated regulation of EMT acts through TGF-β signaling, we examined TGF-β-depentedent repression of E-cadherin in cells with or without RNA interference. REGγ depletion drastically attenuated reduction of E-cadherin following TGF-β stimulation compare to control cells (Fig. 4A). Upon the treatment with TGF-β inhibitor (SB 431542), the thyroid cancer cells restored expression of E-cadherin even in the presence of TGF-β (Fig. 4B). Accordingly, the mRNA expression of E-cadherin and N-cadherin was inversely regulated by TGF-β, whose action was potentiated in the presence of REGγ and impaired in shR thyroid cancer cells (Fig. 4C). Next, TGF-β dependent cell migration and invasion ability, hall marks of EMT and invasiveness, was investigated in transwell assays. Our results demonstrated that TGF-β stimulation increased 15–20% cell migration and invasion ability of SW1736, K18, and FTC cells, respectively (Fig. 4D & Fig. S4B). Yet TGF-β action on cell migration and invasion was still strikingly dependent upon the presence of intact REGγ. In addition, wound closure assays showed that TGF-β could increase cellular migration at the 36 h (SW1736), 60 h (K18) and 24 h (FTC) duration respectively, which was attenuated in cells with REGγ knockdown (Fig. S5A–C). These results indicate that

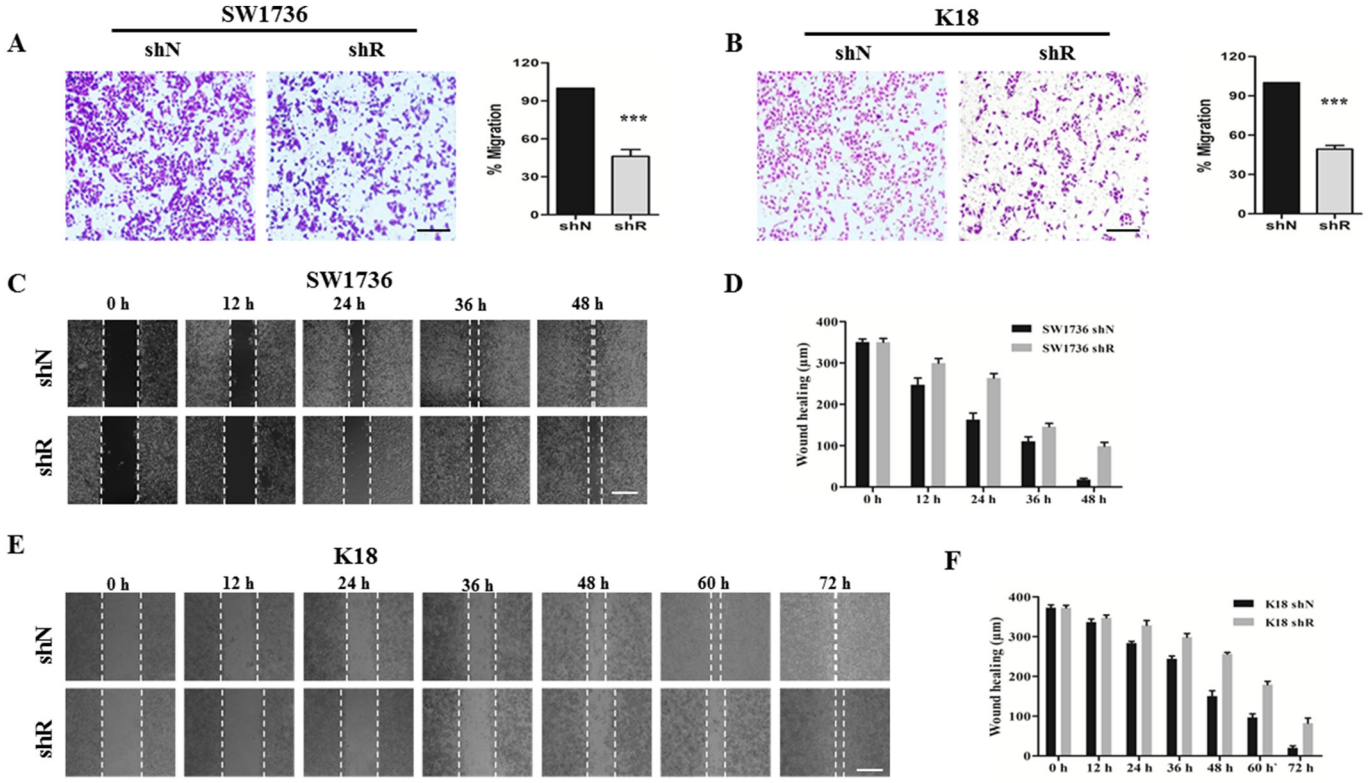

Fig. 1. REGγ knockdown inhibits cell migration of thyroid cancer cells *in-vitro*.
(A&B). Cell migration of stable knockdown REGγ cells (SW1736 and K18)were determined by using Boyden transwell chambers coating without Martigel. Left panel represents images of migration. Scale bar, 100 μm (magnification, ×10). The microscopic fields were counted for each sample. Percentage of cell migration is displayed in the right panels. (C&D). Wound was created in confluent cultures of stable knockdown expression of REGγ in SW1736 cells. Cell migration ability was recorded at 0, 12, 24, 36 and 48 h. (E&F) Scratches were created in K18 shN and shR cells. Wound healing capacity was measured at 0, 12, 24, 36, 48, 60 and 72 h. Scale bar, 50 μm (magnification, ×20).

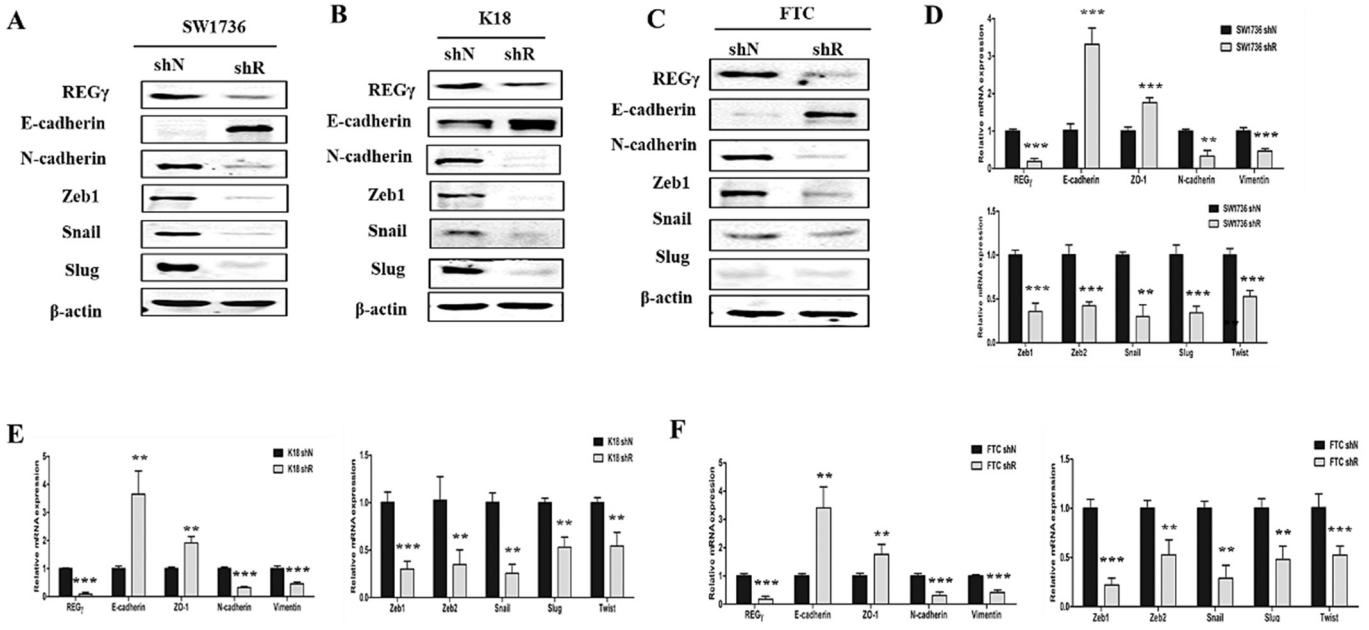

Fig. 2. REGγ deficiency inhibits EMT progression in thyroid cancer cells.
(A&B&C) The protein expression of EMT-related genes and regulators such as E-cadherin, N-cadherin, Zeb1, Snail and Slug in stable knockdown REGγ SW1736, K18 and FTC cells were determined by western blot. β-actin was used as loading control. (D&E&F) SW1736, K18, FTC REGγ shN and shR cells were subjected to total RNA isolation. The mRNA expression of EMT markers and REGγ were determined through qRT-PCR analysis. And the mRNA expression of EMT transcriptional factors was determined. Error bars represent mean ± SD from three independent experiments; $^{**}P < .01$, $^{***}P < .001$ vs control.

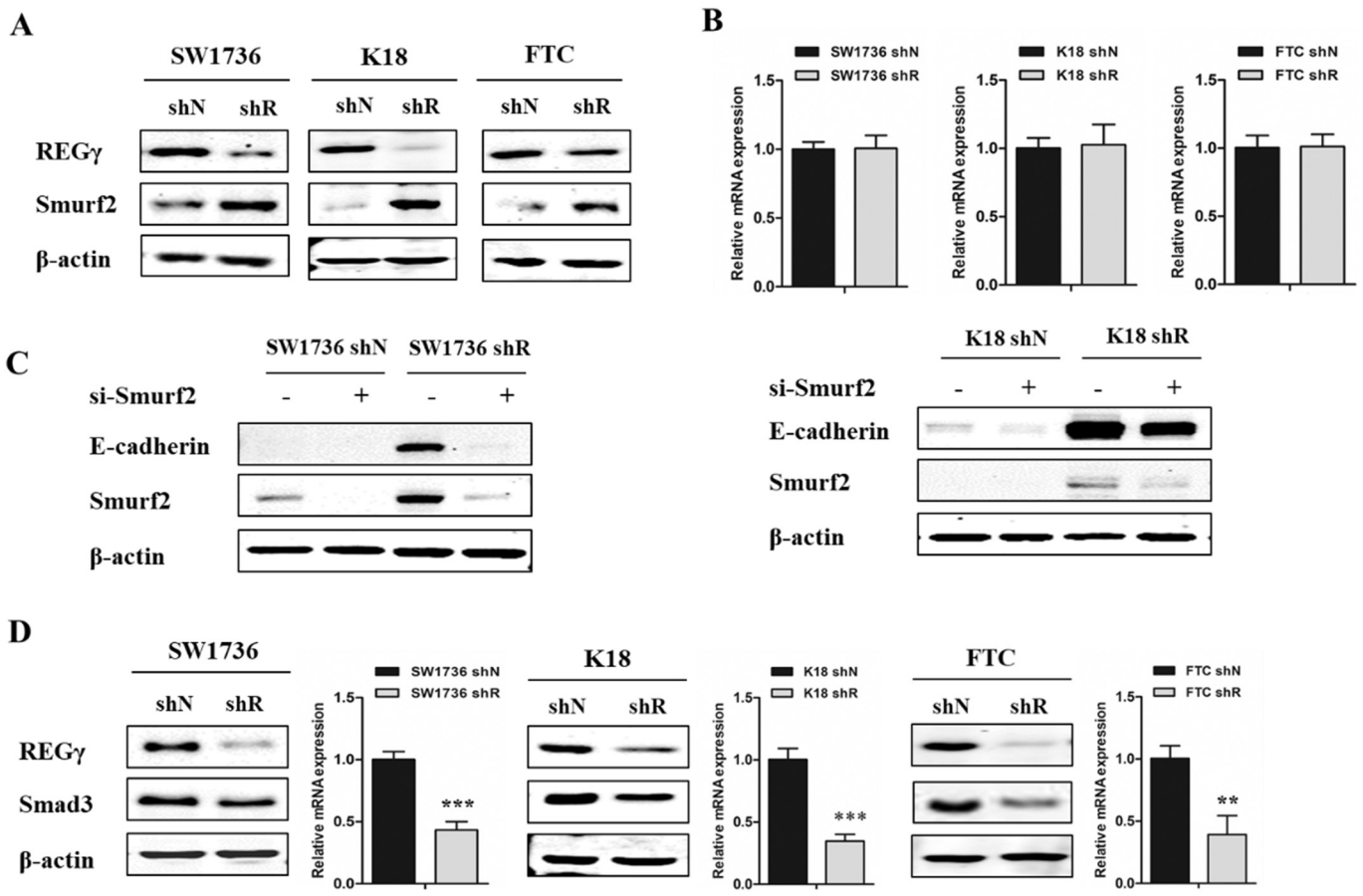

Fig. 3. REGγ-mediated regulation of E-cadherin is Smurf2 dependent in thyroid cancer cells.
(A & B) Protein and mRNA expression of Smurf2 in SW1736, K18 and FTC cells were determined by Western blot and qRT-PCR analysis. β-actin was used as loading control. Error bars represent mean ± SD from three independent experiments. (C) SW1736 and K18 cells were transfected with scrambled siRNA and Smurf2-siRNA concentration of 20 μmols for 48 h. Cells were lysed and protein were immunoblotted against Smurf2 and β-actin antibodies. (D) The protein and mRNA expression of Smad3 was determined in REGγ depleted SW1736, K18 and FTC cells. Error barrs represented mean ± SD; $^{**}P < .01$, $^{***}P < .001$, versus control cells.

REGγ modulates EMT *via* the TGF-β pathway.

3.5. REGγ promotes EMT potential of thyroid cancer cells in vivo

To determine whether REGγ may potentiate EMT in mice, SW1736 shN and shR cells were used in order to visualize *in vivo* colonization with stable expression of a luciferase reporter construct.These cells were injected intravenously *via* tail vein into nude mice to make a model of "lung metastasis". As indicated in Fig. S6A, mice injected with normal REGγ SW1736 ATC cells had higher bioluminescence signal in the lungs than those injected with REGγ-silenced cells, suggesting increased invasion and colonization *in vivo*. In order to ensure the human thyroid cancer cells implanted in nude mice still maintain EMT capability and expression profiles, cells isolated from tumors were verified in migration and invasion assays (Fig. 5A). EMT markers and transcriptional factors in these cells were found to maintain expression profiles similar to those *in vitro*, including EMT markers E-cadherin, ZO-1, N-cadherin, Vimentin and EMT regulators Zeb1, Zeb2, Snail, Slug, Twist (Fig. 5B–D). Furthermore, the protein and RNA expression of Smad3 were decreased in REGγ-silenced cells (Fig. 5E).Taken together, these data suggest that REGγ may promote tumorigenesis *in vivo*.

4. Discussion

As a member of REG family, the proteasome activator REGγ has been reported to promote degradation of numerous proteins by 20S proteasome including p21, p16, and p19 [9,29], establishing the role of REGγ in cell proliferation and cell cycle regulation. Besides, REGγ is often overexpressed in various types of cancers, illustrating roles of REGγ in cancer development [30]. Cancer promoting roles of REGγ have been demonstrated in animal models [31]. Despite that disparity studies showing increased REGγ expression during the progression of thyroid cancer [31–33], whether REGγ regulates EMT remains unclear. In the current study, we identify the impact of REGγ on cell migration, EMT and MET-related gene expression, as well as cell morphology in human thyroid cancer cell lines (SW1736, K18, and FTC cells) *via* regulation of the TGF-β pathway.

Accumulated evidence indicates that EMT is a complex phenomenon and an important driver of tumor invasion, tumor progression, and tumor metastasis. Loss of E-cadherin expression is considered as a key event during EMT induction [34]. Emerging evidence suggested that EMT is also involved in the process of tumorigenesis, whereby cancer cells tend to migrated from primary tumor site to distant organs [35]. E-cadherin plays a critical role in establishing cell polarity and cell differentiation, maintaining normal tissue morphology. Down-regulation of E-cadherin expression and acquisition of mesenchymal marker, including N-cadherin and Vimentin, result in the leads to loss of cell adhesion and facilitates cell mobility, has been considered as important characteristics of EMT [36,37]. REGγ appears to regulate EMT *via* targeting the Smurf2 E3 ligase, which regulates cellular response to TGF-β signaling and downstream effectors in these pathways, including EMT regulators, such as Twist-1, Snail-1, and Zeb-1, and EMT effectors, E-cadherin, ZO-1, N-cadherin, and Vimentin. The most critical effector E-cadherin, encoded by CDH1, is a calcium-dependent cell-cell

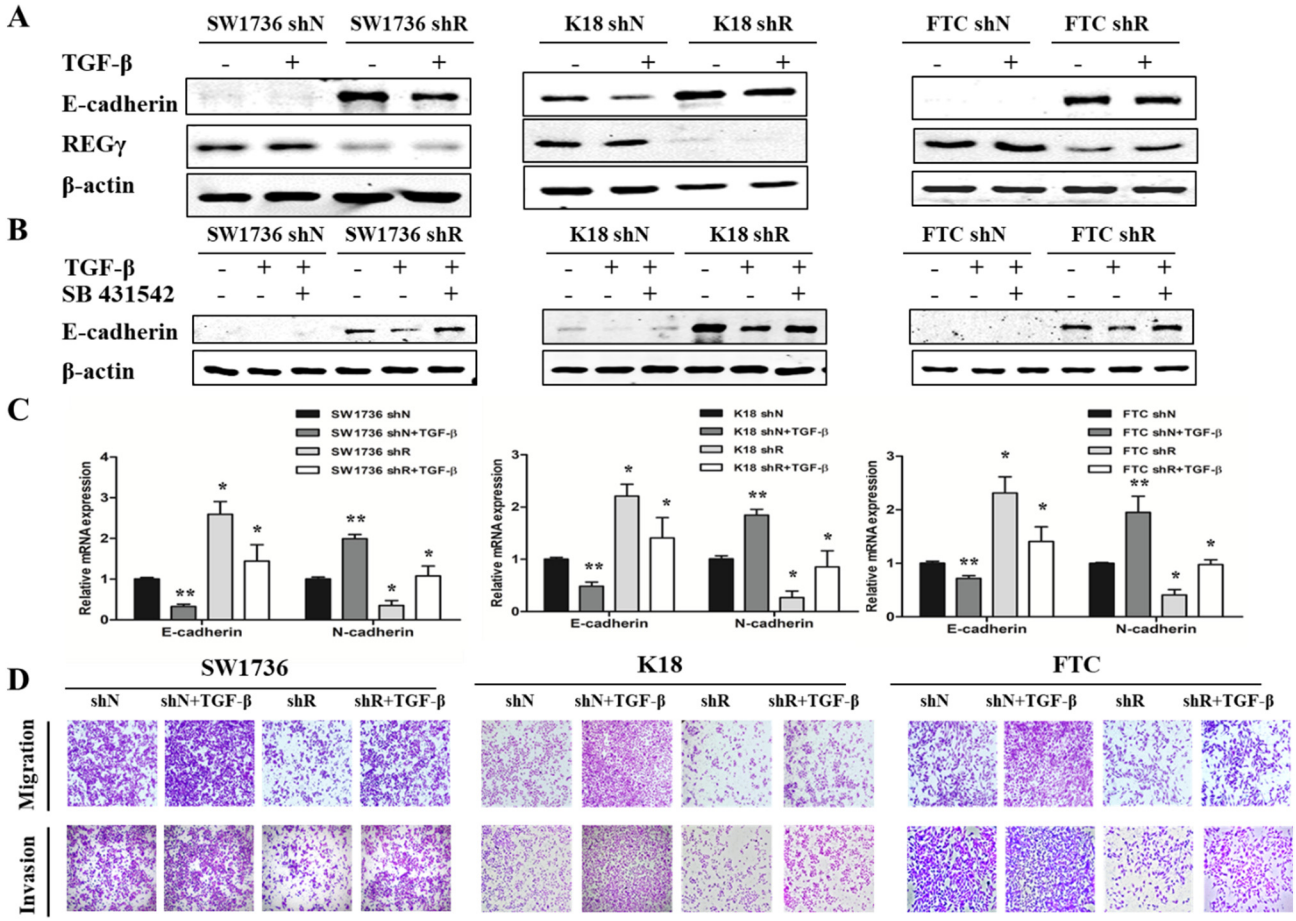

Fig. 4. Effect of REGγ deficiency on TGF-β-mediated EMT regulation.
REGγ knockdown SW1736, K18 and FTC cells treated with (A) TGF-β (5 ng/mL) (B) TGF-β (5 ng/mL) and/or SB 431542 (10 μM) for 24 h. Total protein was assessed by Western blot against E-cadherin and REGγ antibodies. β-actin was used as loading control. (C) Cells were treated with TGF-β at the concentration 5 ng/mL for 24 h. The mRNA expression of E-cadherin and N-cadherin was measured by qRT-PCR and data was normalized to 18S expression as control. Error barrs represented mean ± SD; $*P < .05$, $**P < .01$, versus control cells. (D) Cells were treated with or without TGF-β (5 ng/mL) for 24 h prior to seeding the cells to top chamber in serum free medium. Cells were allowed to migrate through 8 μM membrane towards 10% FBS medium in the chamber for 24 h. Cells were stained and photographed under microscope. Representative images of cell migration are shown in the upper panels and invasion are shown in the lower panels.

adhesion glycoprotein. Loss of E-cadherin is associated with mesenchymal transition and metastatic activity of cancer cells [38]. We noticed that nearly all the thyroid cancer cells lines had very low levels of E-cadherin, reflecting a potentially invasive phenotype of these malignant cancer cells.

In this study, we found that E-cadherin expression was induced in REGγ knockdown cells, while overexpression of REGγ represses the expression of E-cadherin. Although we mainly focused on thyroid cancer cells for EMT markers, including correlation between REGγ and E-cadherin, and behavior studies in human thyroid cancer or cancer samples, we surprisingly discovered that several *REGγ*$^{-/-}$ mouse tissues other than thyroids also had increased E-cadherin expression at both mRNA and protein levels (data not shown). Further, we also found an increased cell migration cells xenograft mouse model using luciferase reporter assay. These findings suggest that REGγ may play an important role during development and progression of cancer.Recent studies in metastatic tumor have postulated EMT as a potential mechanism by which epithelial tumor cells acquires more motile and invasive phenotype and escape from the primary tumor [39–41]. In our study, silencing of REGγ hindered cell migration and invasion as well as altered expression of proteins associated with EMT in thyroid cancer cells. During typical EMT, epithelial cells acquire mesenchymal fibroblast-like properties, reduced intercellular adhesion and increased motility. Consequently, they facilitate cancer cell invasion, migration, and metastasis [42]. Migration and invasion capacity of SW1736, K18, and FTC cells have been demonstrated in transwell Boyden chamber and scratch wound healing assays. The different methodologies and cell types used may contribute to variation in REGγ action on cell migration, as well as the interpretation of phase contrast images. Despite that REGγ has been shown to affect cell growth [30,43], we believe the impact of REGγ on cell migration/invasion is not due to its effect on cell proliferation. Most of the studies in motility or invasion have disclosed differences within 24 h, whereas cell growth such as MTT assays [44,45] need about three days before significant differences can be reached. Nevertheless, we do not exclude the possibility that growth effect might have partial contribution to cell migration.

Over the past decades, the transcriptional and posttranscriptional regulation of E-cadherin has been extensively studied. The down-regulation orE-cadherin is a key molecule in the imitation of EMT, whereas EMT regulators that suppress E-cadherin expression are recognized as potent drivers of EMT [46,47]. The major transcription factors to repress the E-cadherin expression including Snail, Zeb, and Twist. These transcription factors are able to activate EMT through direct binding to the E-cadherin promoter and repress its transcription [42,48]. Our results showed that REGγ expression was positively correlated with E-cadherin and associated transcription factors, including Zeb-1, Snail-1, Twist-1, Zeb-2, and Slug in thyroid cancer cells. Interestingly, among the known E-cadherin repressors (Snail, Slug, Twist,

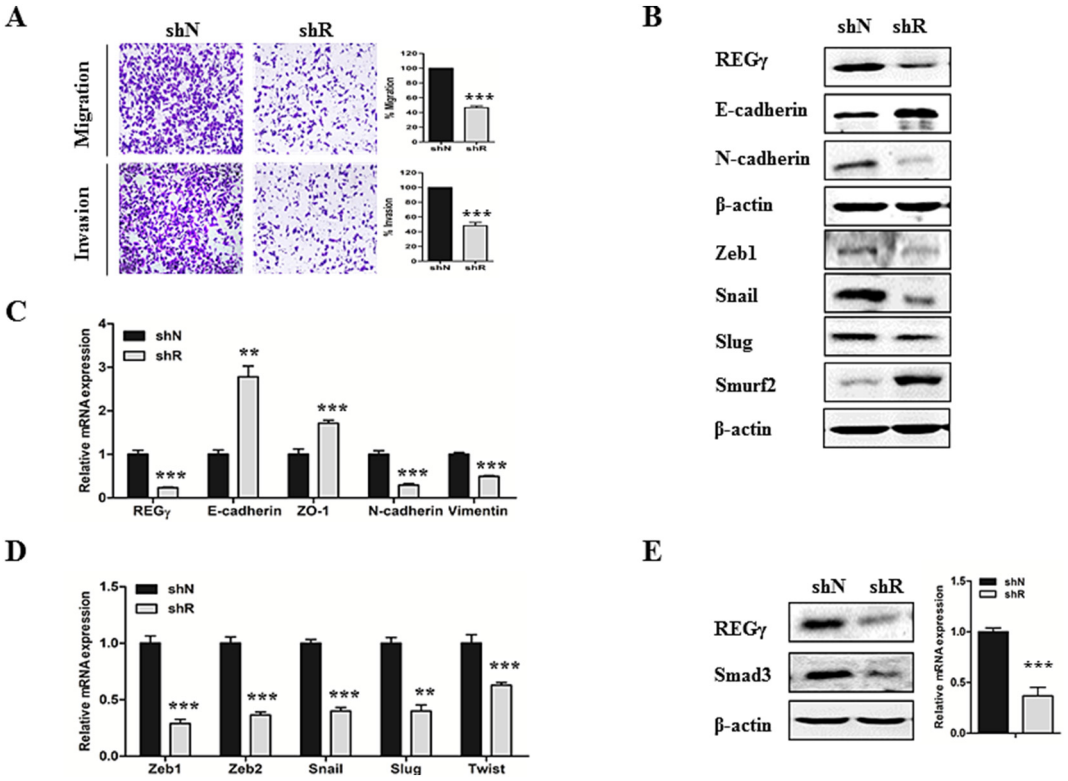

Fig. 5. Effect of REGγ expression on tumorigenicity *in-vivo*.
(A) Human thyroid cancer (SW1736 shN and shR) cells were subcutaneously injected into Balb/c nude mice for 30 days. Cell migration and invasion were determined by transwells assay. Upper panels shown migrated cells and percentage of migrated cells are shown in the right of upper panels. Representative images of the invasion cells are shown in the lower panels and percentage is represented in the right of lower panels. (B) E-cadherin, N-cadherin, Zeb1, Snail, Slug and Smurf2 expressions were determined by western blot. β-actin was used as loading control. (C-D)The mRNA expression of EMT and MET markers were determined through qRT-PCR. (E) Smad3 was determined by western blot and qRT-PCR. Results are expressed as mean ± SD of three individual experiments; significant difference $^{**}P < .01$, $^{***}P < .001$, versus control.

and Zeb1/2), Zeb-1 appears to be the most prominent regulator affected by REGγ. The importance of Zeb-1 in tumor progression is, in part, due to its overexpression in multiple cancers [49–51]. It is likely that our finding will provide better insight to understand tumor metastasis in general, not limited to thyroid cancers.

Ubiquitin-dependent protein degradation is a vital procedure in various biological progressions, in which E3 ubiquitin ligases are often regulated by frequent modifications including ubiquitination, acetylation and phosphorylation. The Nedd4-like family of E3 ubiquitin ligase members, such as Smurf1, Smurf2, WWP1, Nedd4–2, and AIP/Itch, shares a similar structure on their C-terminus of HECT domain [52]. Conversely, the REGγ proteasome promotes protein degradation independently of ubiquitination and without ATP. Previous studies demonstrate that both Smurf1/2 is targeted by REGγ proteasome system for degradation, possibly due to 83% sequence identity between Smurf2 and Smurf1 [28,53]. Similar to many REGγ substrate proteins, degradation of Smurf2 is also controlled by ubiquitin proteasome system (UPS) upon modification by ubiquitin-like protein Nedd8 [54], reflecting that important proteins need dual degradation systems to safeguard their homeostasis. However, the conditions under which ubiquitin or the REGγ-proteasome systems are activated remain unknown TGF-β signaling is widely reported to be involved in a diverse set of cellular processes, such as cell migration, adhesion, cell proliferation and differentiation. However, due to the subsequent inactivation of TGF-β signaling or the associated key target genes, malignant cells lose TGF-β tumor suppressive response [55,56]. Although TGF-β is regulated as one of the most important inducer of EMT, several non-invasive tumor cells were not able to undergo TGF-β induced EMT *in vitro* [57–59]. We have identified that knockdown of REGγ antagonizes TGF-β induced EMT by inhibiting TGF-β dependent expression changes of EMT related genes. Biologically, these poorly differentiated thyroid cancer cells often invade deeply and have a bad prognosis with a propensity for lymph node metastasis. Indeed, REGγ deletion not only inhibits lung metastasis of thyroid cancer cells in a mouse model but also induces re-differentiation of ATC cells (to be published elsewhere). Combining with the retardation of migration and invasion in these poorly differentiated thyroid cancer cells and re-differentiation after REGγ knockdown, we can conclude that REGγ plays multiple roles in malignant thyroid cancers.

In conclusion, our results are the first to indicate that REGγ is associated with cancer metastasis and EMT regulation in thyroid cancer cell lines. REGγ depletion resulted in significant induction of E-cadherin expression *in vitro* and *in vivo*. We provide strong evidence for the involvement of REGγ in the induction of EMT phenotype, cell migration and invasion. REGγ can affect TGF-β/Smad pathway by the down-regulation of Smurf2, which subsequently alters EMT markers, thus leading to inhibition of E-cadherin and elevated cell migration (Fig. 6). We therefore, conclude that REGγ promotes EMT in thyroid cancer cells by regulating TGF-β/Smad signaling pathway.

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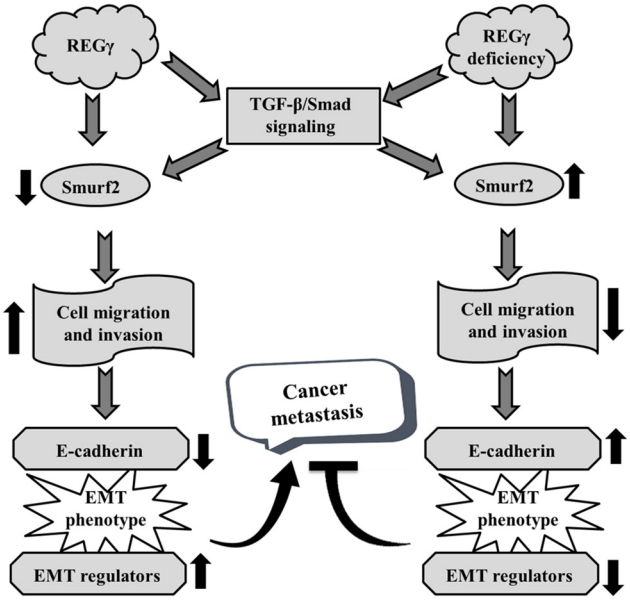

Fig. 6. Schematic diagram of REGγ regulating EMT in thyroid cancer. REGγ deficiency enhances degradation of Smurf2 protein *via* TGF-β/Smad dependent manner, its ability to inhibit EMT regulators and further elevation of E-cadherin, thus leading to inhibition of cancer metastasis. REGγ-TGF-β/Smad-EMT pathway provides a unique mechanism in the regulation of cancer metastasis.

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Appendix A. Supplementary data

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