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Activation of Snail and EMT-Like Signaling *via* the IKKαβ/NF-κB Pathway in Apicidin-Resistant HA22T Hepatocellular Carcinoma Cells

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Abstract

The molecular and phenotypic associations between chemo- or radio-resistance and the acquisition of epithelial-mesenchymal transition (EMT)-like phenotype are tightly related in cancer cells. Wnt/β-catenin and NF-κB signaling pathways play crucial roles in EMT induction. Apicidin-resistant (Apicidin-R) HA22T cells are known to activate the Wnt/β-catenin signaling pathway and MMP-2 expression *via* the IGF-IR/PI3K/Akt signaling pathway to enhance metastatic effects of cancer cells. In this study, we further investigated if Apicidin-R HA22T cells actually underwent EMT. In Apicidin-R HA22T cells, E-cadherin protein level was reduced but Vimentin, Snail and Twist were significantly activated. Activation of p-IKKαβ and p-IκBα was also observed in Apicidin-R HA22T cells. Apicidin-R HA22T cells displayed even higher NF-κB nuclear accumulation. Snail was enhanced but GSK3-β was reduced. However, unphosphorylated GSK3-β protein level was totally reversed when the Snail-specific siRNA was applied in a knockdown experiment. Taken together, Apicidin-R HA22T cells could potentiate aggressive metastasis behavior due to up-regulation of Snail expression and promoted EMT effects *via* the IKKαβ/NF-κB pathway. In addition, Snail might decrease the GSK3-β level resulting in extraordinarily activation of Wnt/β-catenin signaling pathway.

Key Words: apicidin, EMT, HA22T, IKKαβ, NF-κB, Snail

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Introduction

Epithelial-mesenchymal transition (EMT) has been recognized for several years as critical for embryogenesis (24); the process has recently been shown to be also relevant to cancer progression (25). Cells that undergo EMT during tumor invasion are characterized by the loss of cell-cell adhesion such as E-cadherin and polarity accompanied by cytoskeleton rearrangements and increased cell motility (1, 24, 25). During EMT of *in situ* cancer cells, mesenchymal markers such as vimentin, fibronectin, N-cadherin and the metalloproteinases MMP-2 and MMP-9 can be acquired, resulting in enhanced ability for cell migration and invasion (20).

In addition, mesenchymal cells have a spindle-shaped, fibroblast-like morphology, whereas epithelial cells grow as clusters of cells that maintain complete cell-cell adhesion with their neighbors. E-cadherin is most abundantly expressed in epithelial phenotype. E-cadherin levels become limiting, due to chemoresistance, which results in the loss of E-cadherin-dependent intercellular epithelial junction complex and the abolishment of E-cadherin-mediated sequestering of β-catenin in the cytoplasm (43).

Previous studies have suggested that there are molecular and phenotypic associations between chemo- or radio- resistance (37, 41) and the acquisition of EMT-like phenotype of cancer cells (12, 13, 19, 49). The zinc-finger Snail homologues, Snail1, Snail2/ Slug and Snail (28, 43), and several basic helix-loop-helix (bHLH) factors such as Twist, ZEB1, ZEB2/ SIP1 and TCF3/ E47/E12 are factors that transcriptionally repress E-cadherin (36). The Snail family of transcriptional repressors not only regulates various aspects of EMT during embryonic development but also participate in tumor progression (30). In mammalian cells, Snail has been reported to be a direct repressor of transcription of the E-cadherin gene and Snail expression induces full EMT and increases migration/invasion in different physiological and pathological situations (2, 4, 35). The bHLH transcription factor Twist represses the E-cadherin promoter and gene transcription (43). Activation of Twist expression has been positively correlated with an aggressive cancer phenotype and poor patient survival (14, 25, 50). The vimentin (VIM) gene encodes a cytoskeletal protein that is a part of the large intermediate filament (IF) gene family, which is abundant in mesenchymal cells. Vimentin expression has often been described as the end-stage progression in EMT, representing the completely dedifferentiated state in tumor cells that are highly proliferative and invasive (1, 25, 26). In addition, using tissue microarray analysis, vimentin was found to be expressed in 21 out of 272 breast cancer cases and correlated positively with tumor grade (24).

NF-κB is a structurally conserved family of dimeric transcription factors distinguished by the presence of an N-terminal 300-amino acid region, termed the Rel homology domain (RHD), which contains sequences mediating dimerization, DNA binding, nuclear localization and interaction with the inhibitory IκB proteins (8). In most cells, inactive NF-κB protein is sequestered in the cytoplasm in a complex with an inhibitor protein, termed IκB. Activation of NF-κB typically involves the phosphorylation of IκB by the IκB kinase (IKK) complex, which results in IκB degradation. This releases NF-κB and promotes it to translocate freely to the nucleus (10). The genes regulated by NF-κB include those involved in cell death, apoptosis, proliferation, inflammation, the innate- and adaptive-immune responses, the cellular-stress response and tissue remodeling (3, 7, 10, 32, 34, 51).

The activation of NF-κB is known to play critical roles in the processes of EMT, tumor cell invasion and metastasis (28). Inhibition of NF-κB activities reduces tumor cells invasion (40). NF-κB is critical for promoting and maintaining a mesenchymal phenotype in the transcription of mesenchymal genes encoding vimentin, MMP-2 and MMP-9 (28). GSK3 inhibition stimulates transcription of the human Snail gene which is mediated through NF-κB signaling (1). NF-κB has been identified as the upstream regulator of Snail expression during EMT of human breast cancer cells *via* overexpressing a constitutively active Type I insulin-like growth factor receptor (IGF-IR) (21). However, inhibition of NF-κB signaling can reverse the induction of Snail transcription during EMT. Thus, NF-κB plays a crucial role in the regulation of the Snail gene transcription. Twist is a direct transcriptional target of NF-κB (16, 33, 44). Overexpression of NF-κB in breast cancer cells induces vimentin expression and a more mesenchymal phenotype (46). Moreover, NF-κB is responsible for the activation of MMP-9 transcription (11). Therefore, NF-κB is a key mediator that promotes an invasive phenotype.

In hepatocellular carcinoma (HCC), multiple molecular alterations ensure the progressive growth of tumor cells. Rapid tumor growth is closely linked to chemotherapy resistance (42). Chemoresistance is the major problem affecting HCC therapy; there is no effective chemotherapy for HCC because the tumor cells develop resistance to cytotoxic drugs. Apicidin is a novel histone deacetylase (HDAC) inhibitor derived from a fungal metabolite (23, 27, 45). Apicidin has been reported to have a potent broad spectrum of antiproliferative activity against various cancer cell lines (9, 22, 47). The combination of apicidin and doxorubicin enhances the antitumor effects of doxorubicin on caspase activation and tumor growth in HCC (26).

However, the growth-inhibitory concentrations of apicidin in HCC were higher than in other cancer cell lines (10). Therefore, the induction of side effects and chemoresistance by apicidin could be expected in HCC treatment. Here, we aimed to firstly investigate if apicidin-resistant (Apicidin-R) HA22T hepatocellular carcinoma cells could potentiate aggressive metastasis behavior due to the up-regulation of expression of Snail family proteins and promote EMT effects *via* the IKKαβ/NF-κB pathway. Secondly, we investigated whether Snail decreased the GSK3-β to result in the activation of the Wnt/β-catenin signaling pathway.

Materials and Methods

Cell Culture

HA22T cells were maintained in Dulbecco's minimum essential medium (D5523, Sigma, MO, USA) containing 10% charcoal treated FBS (Characterized Fetal Bovine Serum, HyClone, Thermo Scientific, UT, USA) and 1% penicillin (Invitrogen Corp., CA, USA).

Establishment of Apicidin-R HA22T Hepatocarcinoma Cell Line

To establish stable liver cancer cell lines chronically resistant to apicidin, HA22T cells were exposed to increasing concentrations of apicidin. HA22T cells were first exposed to 5 μM apicidin, which resulted in greater than 95% cell death. Once surviving cells reached 80% confluence, they were passaged twice in this same concentration of apicidin, after which the process was repeated at gradational doses of apicidin until a cell population was selected that demonstrated at least a 3-fold greater IC_{50} to apicidin than the parental HA22T cell lines.

Whole Cell Extract

The cells were extracted in a cell lysis buffer (50 mM Tris-base, 0.5 M NaCl, 1.0 mM EDTA, 1% NP40, 1% Glycerol, 1 mM β-mercaptoethanol, Proteinase K inhibitor). The extracts were clarified by centrifugation.

Lowry Protein Assay

After obtaining the whole cell extracts, Lowry assay (6) is used to determine protein concentrations in these protein samples.

Western Blotting

Cultured cells were lysed with lysis buffer (250 mM sucrose, 50 mM Tris-HCl, 5 mM imidazole, 2.5 mM EDTA, 2.5 mM DTT, 0.1% Tritons X-100, pH 7.40) and protein concentration was measured using the Lowry protein assay. An aliquot of each sample equivalent to 30 μg protein was boiled after addition of the appropriate amount of 5× sample buffer (5 mM EDTA, 162 mM DTT, 5% SDS, 50% glycerol, 0.5 l bromophenol blue, 188 mM Tris, pH 8.8). The samples were separated on 10% SDS-polyacrylamide gels (SDS-PAGE) and electrophoretically transferred onto nitrocellulose filters using the Bio-Rad electrotransfer system (Bio-Rad Laboratories, Munich, Germany). Equal transfer was verified by Ponceau S staining of the Apicidin-R HA22T cells activate membranes. Antigen-antibody complexes were visualized with HRP-coupled secondary antibodies (goat anti-mouse and goat anti-rabbit, Santa Cruz Biotechnology, CA, USA) and a custom-made ECL detection system (2.5 mM luminol, 0.4 mM para-coumaric acid, 10 mM Tris base, 0.15 l H_2O_2, pH 8.5). We used the following antibodies against β-actin (C4), E-cadherin, GSK-3β (H-76), HDAC1 (C-19), IKKα/β (H-470), p-IKKα/β (Ser176), NF-κB p65 (A), Snail, twist (Twist2C1a), α-Tubulin(B-7), Vimentin (RV202), purchased from Santa Cruz Biotechnology. Antibodies against p-IκB-α (Ser32) (14D4) and p-NF-κB p65 (Ser536) were purchased from Cell Signaling Technology (Beverly, MA, USA).

Cytoplasmic and Nuclear Fractionations

Cell cytoplasmic and nuclear fractions were obtained with the Extraction Reagent, lysis buffer A (50 mM Tris-base, 0.5 M NaCl , 1.0 mM EDTA, 1% NP40, 1% Glycerol, 1 mM β-mercaptoethanol, Proteinase K inhibitor and lysis buffer B (50 mM Tris-base, 0.5 M NaCl, 1.0 mM EDTA, 1% Glycerol, Proteinase K inhibitor). In brief, 5×10^6 cells were trypsinized (0.05% trypsin/0.53 mM EDTA) and resuspended in 100 μl lysis buffer B. After 10-minute ice-cold incubation, each sample was centrifuged at 3000 g 10 min to pellet the nuclear proteins. After centrifugation, the supernatant was stored for use as the cytoplasmic Apicidin-R HA22T cells activate Snail *via* NF-κB fraction, and the nuclear fraction was lysed with 100 μl lysis buffer A.

Small Interfering RNA (siRNA) Transfection

Transient transfections were carried out by the proprietary cationic polymer reagent TurboFect™ *in vitro* Transfection Reagent (Fermentas, Thermo Scientific, UT, USA) following the manufacturer's instructions. Double-stranded siRNA sequences targeting Snail mRNA were obtained from Santa Cruz Biotechnology. The non-specific (scramble) siRNA consisted of non-targeting sequences. Cells were cultured in 60-

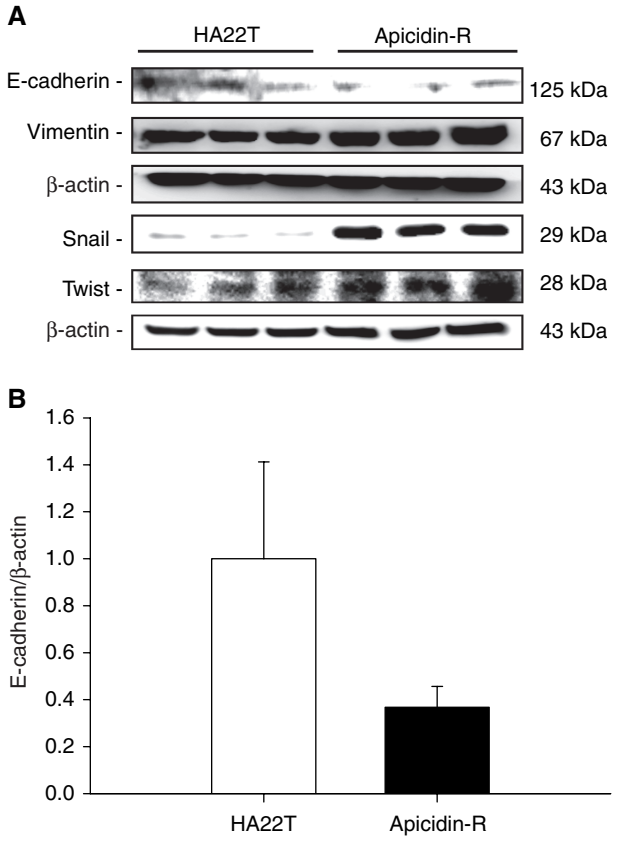

Fig. 1. Activation of the expression of mesenchymal markers in Apicidin-R cells. (A) Western blot analysis showing the expression of markers of the epithelial and mesenchymal phenotypes. (B) For E-cadherin, values were quantified as fold of Apicidin-R values relative to the parental HA22T cells levels. $P = 0.06$, n = 3.

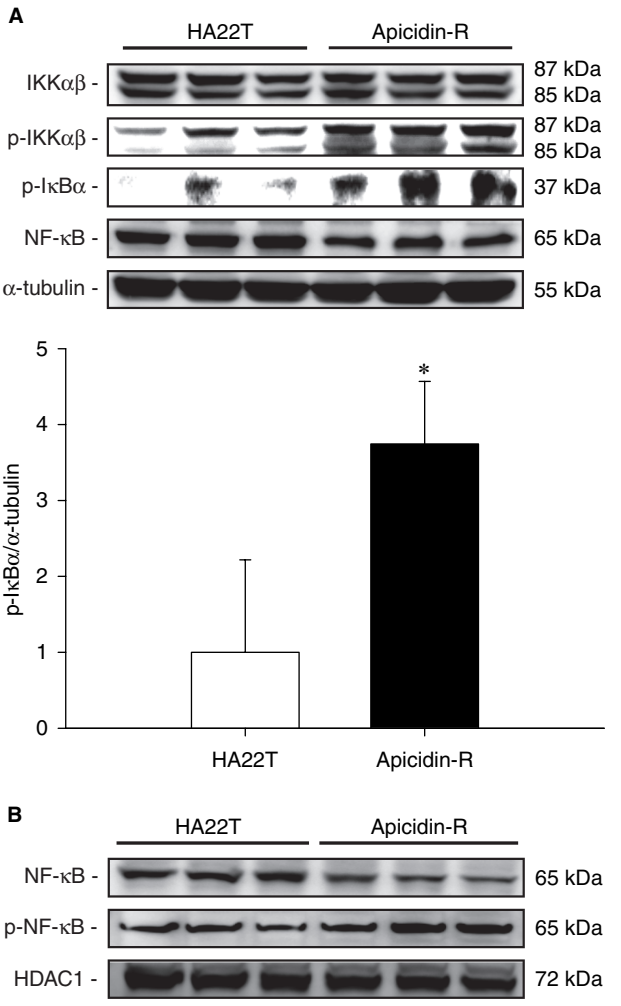

Fig. 2. Activation of the NF-κB pathway is involved in EMT in Apicidin-R cells. (A) Western blot analysis of total cellular lysates using the IKKαβ], p-IKKαβ], p-IκBα, NF-κB and α-tubulin antibodies. Results were presented as mean ± SE. $*P < 0.05$, compared with the parental HA22T cells (n = 3). (B) Cytoplasmic and nuclear fractions of Apicidin-R and parental HA22T liver cancer cells were subjected to SDS-PAGE and Snail activation by NF-κB in Apicidin-R HA22T followed by immunoblotting with anti-β-catenin and -HDAC1 antibodies. HDAC1 was used as a nuclear protein loading control.

mm dish plates in appropriate medium. Transfection of siRNA was carried out with TurboFect™ transfection reagent. Specific silencing was confirmed by immunoblotting with cellular extracts after transfection.

Statistical Analysis

Each sample was analyzed based on results that were repeated at least three times and the SigmaPlot 10.0 software and standard *t*-test were used to analyze each numeric data. In all cases, differences at $P < 0.05$ were regarded as statistically significant; values at $P < 0.01$ or $P < 0.001$ were considered highly statistical significances.

Results

Mesenchymal Markers Were Significantly Activated in Apicidin-R HA22T Cells

To confirm whether Apicidin-R HA22T cells underwent EMT, we determined the expression of markers of epithelial and mesenchymal phenotypes. In Apicidin-R HA22T cells, E-cadherin protein level was not only reduced but those of Vimentin, Snail and Twist were significantly activated when compared to the parental HA22T cells (Fig. 1).

The IKKαβ/NF-κB Pathway Was Significantly Activated in Apicidin-R HA22T Cells

In order to investigate whether the IKKαβ/NF-κB pathway was affected in Apicidin-R HA22T cells, p-IKKαβ and p-IκBα were examined. Indeed, activation

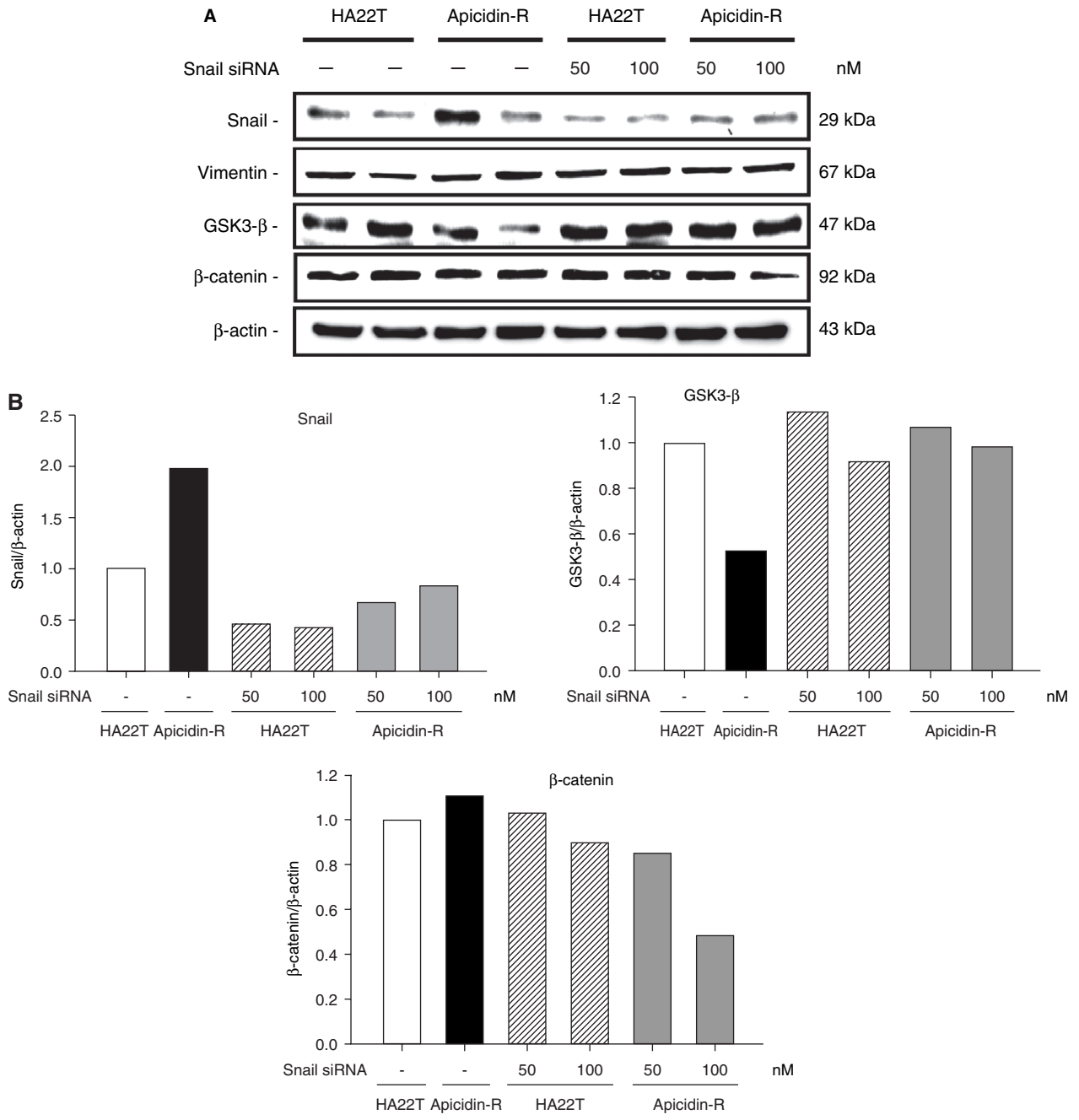

Fig. 3. Down-regulation of Snail induced reversal of the suppression of GSK3-β in Apicidin-R cells. (A) Western blotting showing the Snail, Vimentin, GSK3-β, β-catenin and β-actin expression levels of Apicidin-R cells after transfection with Snail siRNA (50 and 100 nM). (B) The expression levels were qualified by normalizing to the expression level of β-actin used as the internal control relative to the parental HA22T cells. n = 1.

of p-IKKαβ and p-IκBα was observed in Apicidin-R HA22T cells (Fig. 2A). Apicidin-R HA22T cells also displayed greater extents of NF-κB nuclear accumulation when relative to the HA22T cells (Fig. 2B).

Extraordinarily Activated β-Catenin Was Mediated Through the Snail Protein of the EMT Marker in Apicidin-R HA22T Cells

To confirm a direct mechanistic role of β-catenin in Apicidin-R HA22T cells showing EMT characteristics, expression of Snail proteins was knocked-down in Apicidin-R HA22T cells using specific siRNA. Not only the Snail protein level was significantly enhanced but GSK3-β was significantly reduced when relative to Apicidin-R and parental HA22T treated with the same siRNA. The activated GSK3-β protein level was

totally reversed when the Snail specific siRNA was used (Fig. 3). The total β-catenin protein level was greatly decreased after high doses of Snail siRNA were applied (Fig. 3). However, the level of Vimentin was not significantly changed in Apicidin-R HA22T cells with or without Snail siRNA treatment (Fig. 3).

Discussion

The change from a non-invasive to an invasive and malignant phenotype is a critical step in tumor progression and metastasis (17, 18). Wnt/β-catenin and NF-κB signaling pathways are well known for EMT induction (28, 48). Our previous data suggested that Apicidin-R HA22T cells activated the Wnt/β-catenin signaling pathway and MMP-2 expression *via* the IGF-IR/PI3K/Akt signaling pathway to enhance tumor metastatic effects (15). Therefore, we further investigated if Apicidin-R HA22T cells underwent EMT. We first determined the protein level of epithelial and mesenchymal phenotype markers. In Apicidin-R HA22T cells, there was not only reduced E-cadherin protein level but also significantly activated Vimentin, Snail and Twist when compared to the parental HA22T cells (Fig. 1). This result suggested that Apicidin-R HA22T cells might actually undergo EMT phenomenon.

Multiple important transcription factors, such as Snail and Twist, have been shown to suppress epithelial gene expression resulting in EMT induction (5, 37). Moreover, these factors are regulated either directly or indirectly by NF-κB. Therefore, we investigated whether the IKKαβ/NF-κB pathway was affected in Apicidin-R HA22T cells. Indeed, activation of p-IKKαβ and p-IκBα was observed in Apicidin-R HA22T cells (Fig. 2A). Apicidin-R HA22T cells displayed even higher NF-κB nuclear accumulation when relative to the HA22T cells (Fig. 2B). These results strongly suggest that activation of IKKαβ/NF-κB pathway is closely linked to the induction of EMT phenomenon in Apicidin-R HA22T cells.

Wnt/β-catenin signaling is associated with EMT-mediated metastasis and is highly correlated to prognostic values in cancer (31, 39). To confirm a direct mechanistic role of β-catenin in Apicidin-R HA22T cells with EMT characteristics, we knocked down the expression of Snail using specific siRNA. Under such treatment, Apicidin-R HA22T cells not only abundantly enhanced Snail protein levels but also greatly reduced GSK3-β when compared to the parental HA22T cells. However, the activated GSK3-β protein level was totally reversed when on Snail-specific siRNA knockdown (Fig. 3). As expected, the total β-catenin protein level was greatly decreased when high doses of Snail-specific siRNA were applied (Fig. 3). All these results indicate that Snail may play a negative regulatory role on GSK3-β and enhances the Wnt/β-catenin signaling pathway.

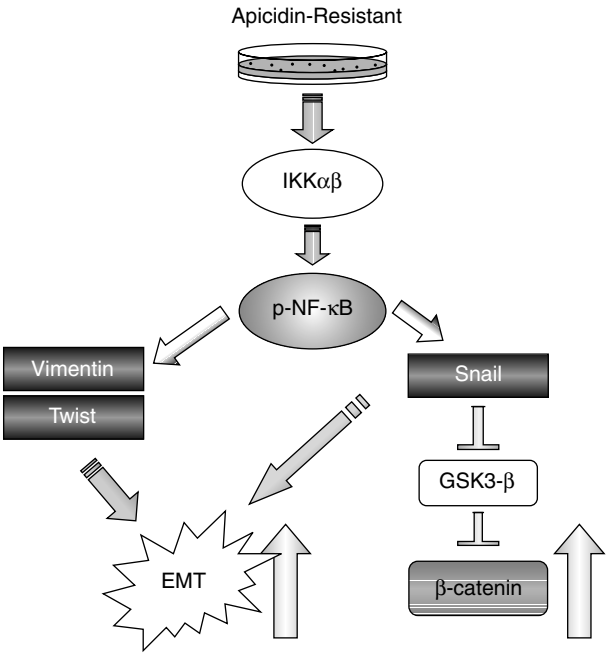

Fig. 4. A model of activation of the IKKαβ/NF-κB pathway that triggers Vimentin, Twist and Snail proteins to promote EMT-like singling of Apicidin-R HA22T cells. Snail overexpression may also play a negative regulatory role of GSK3-β and over-expresses the Wnt/β-catenin signaling pathway in Apicidin-R cells.

In summary, our results strongly suggest that Apicidin-R HA22T cells could potentiate aggressive behavior due to the up-regulation of Snail expression and the promoted EMT effects *via* the IKKαβ/NF-κB pathway. In addition, Snail may decrease the GSK3-β levels which results in the activation of the Wnt/β-catenin signaling pathway (Fig. 4).

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