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Wnt/β-catenin signaling inhibits death receptor-mediated apoptosis and promotes invasive growth of HNSCC

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Abstract

The Wnt/β-catenin signaling pathway plays a critical role in cell proliferation and oncogenesis. It has been found to be chronically activated in a variety of human cancers, including head and neck squamous cell carcinoma (HNSCC). Previously, we have found that the activation of the Wnt/β-catenin signaling pathway inhibits mitochondria-mediated apoptosis. In this study, we extended our studies to determine whether the Wnt/β-catenin signaling pathway inhibited death receptor-mediated apoptosis in HNSCC cells. We found that Wnt/β-catenin inhibited not only tumor necrosis factor (TNF)/c-Myc-mediated apoptosis, but also cell detachment-mediated apoptosis (anoikis) which is dependent on the death receptor signaling pathway. Interestingly, we also observed that the Wnt/β-catenin signaling pathway induced HNSCC cell scattering and promoted cell invasion in the Matrigel, both of which are hallmarks for the invasive growth of HNSCC. Consistently, the over-expression of β-catenin promoted HNSCC tumor growth in nude mice. Taken together, our results suggest that the Wnt/β-catenin signaling pathway plays dual functions in HNSCC development: promoting both cell survival and invasive growth of HNSCC cells.

Keywords: Cell growth; Squamous cell carcinoma; TNF; Apoptosis; Wnt

1. Instruction

The Wnt family genes encode a group of secretory glycoproteins [1]. The canonical Wnt signaling pathway is transduced through β-catenin which is regulated by the adenomatous polyposis coli/Axin/glycogen synthase kinase 3β complex [1–5]. Due to mutations of Wnt signaling components, the cytosolic level of β-catenin has been found to be elevated in a variety of human cancers, resulting in the constitutive activation of β-catenin/T cell factor (Tcf) transcription [1–5]. Multiple key oncogenic proteins, including c-Myc, Cyclin D1, and Cox-2, have been found to be regulated by the Wnt/β-catenin signaling pathway [3–5]. Recently, growing evidence has suggested that the abnormal activation of Wnt/β-catenin signaling pathway is associated with the development and progression of human HNSCC [6,7]. For example, Leethanakul et al. [6] found that most HNSCC cells over-expressed members of the Wnt signaling pathway by the use of laser capture microdissection and cDNA arrays. Rhee et al. reported that the levels of Wnt-1, 10b, and the Wnt receptor Frizzled-2 proteins were also markedly increased in HNSCC cells, relative to normal epithelial cells [7]. However, although it has been extensively studied in colorectal cancer and other neoplasms, there are few mechanistic studies that explore the role of Wnt/β-catenin signaling in HNSCC cells.

HNSCC is the most common cancer in the head and neck regions with low survival and high morbidity. The poor prognosis of patients with HNSCC is mainly due to the high invasive potential of these tumors, resulting in early regional lymph node and subsequent distant metastatic spread. The invasive growth has been found to play a critical role in the

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development and progression of HNSCC. The invasive growth is a complex multistage process in which cell proliferation combines with cell–cell dissociation and movement, matrix degradation and survival [8–10]. Previously, we have found that Wnt/β-catenin signaling inhibited mitochondria-dependent apoptosis in colorectal cancer cells [4]. Since the death receptor signaling pathway has been found to be required for cell detachment-mediated apoptosis (anoikis) which plays a critical role in the inhibition of the invasive growth of HNSCC [8], it is interesting to know whether the Wnt/β-catenin signaling pathway inhibited the death receptor-mediated apoptosis, thereby promoting HNSCC invasion.

It is well known that tumor necrosis factor (TNF) is one of primary inducers for the death receptor-mediated apoptosis [11–13]. TNF is a founding member of the TNF super-family proteins, which was originally identified as a product of macrophages that can kill certain types of mouse tumor cells. It is well known that TNF-induced cytotoxicity is enhanced in the presence of either a protein synthesis inhibitor or a transcription inhibitor [11–13]. The engagement of TNF receptor 1 by TNF recruits a TNF receptor-associated death domain containing protein (TRADD). Subsequently, TRADD transduces death signal by the interaction with Fas-associated death domain protein and caspase-8 to form the death-inducing signaling complex [11,12]. Cell sensitivity to TNF has been found to be regulated by a variety of stimuli and signaling pathways. TNF-mediated apoptosis is also regulated by viruses and oncogenes. For example, activation of c-Myc renders cells susceptible to TNF-induced apoptosis in fibroblasts [14–16]. The c-Myc proto-oncogene is a transcription factor that controls a variety of cellular processes including cell proliferation and growth. c-Myc on the one hand promotes proliferation and on the other hand can induce or sensitize cells to apoptosis. Under serum deprivation and growth factor withdrawal, c-Myc has been found to induce apoptosis in hematopoietic cells and fibroblasts. Over-expression or amplification of c-Myc is observed in over 30% of human cancers including human lymphoma, colorectal carcinoma, and HNSCC [17–24]. The amplification or over-expression of c-Myc often correlated with constitutive activation of Wnt/β-catenin signaling. Elegant studies by He et al. [3] have identified c-Myc as a target gene of the Wnt/β-catenin signaling pathway. During the study of the oncogenic interaction between Wnt signaling and c-Myc, we have found that Wnt/β-catenin signaling inhibited c-Myc-mediated apoptosis and promoted oncogenesis [5].

In this report, we extended our previous studies to determine whether Wnt signaling inhibited TNF/c-Myc-mediated apoptosis. Using an inducible system of c-Myc activation, we found that Wnt signaling potently inhibited c-Myc-mediated TNF sensitization which was independent of NF-κB function. Additionally, we also found Wnt/β-catenin signaling promoted HNSCC cell scattering and invasion that are typical changes for HNSCC cells during tumor progression and invasion. These results provide a mechanistic explanation for the abnormal activation of the Wnt/β-catenin signaling pathway in HNSCC, suggesting that the Wnt/β-catenin signaling pathway may be an important target for HNSCC therapy.

2. Materials and methods

2.1. Cell culture and retroviral infection

Human KB, UMSCC1 and rat intestinal epithelial cells (RIE) were maintained in DMEM supplemented with 10% fetal bovine serum (FBS). To establish cell lines stably expressing c-MycER, Wnt-1, or β-catenin, a retrovirus expression system was utilized, allowing whole populations of cells to be studied with minimal expansion in culture. Retroviruses were produced by transfecting the retroviral constructs into 293 T cells using a calcium phosphate precipitation method. Forty-eight hr after transfection, retrovirus-containing supernatants were collected and stored in −70 °C. Cells were infected with retroviruses in the presence of 6 μg/ml polybrene (Sigma). Forty-eight hours after infection, cells were selected with puromycin (1.5 μg/ml), hygromycin (600 μg/ml), or neomycin (600 μg/ml) for one week, respectively. The resistant clones were pooled and confirmed by Western blot analysis [4,5].

2.2. Cell fractionation and Western blot analysis

The cell fractionations were performed as described previously [5]. Whole cell extracts were prepared with radioimmune precipitation assay buffer containing 1% NP40, 5% sodium deoxycholate, 1 mM PMSF, 100 mM sodium orthovanadate, and 1:100 protease inhibitors cocktail (Sigma). The protein concentrations were determined by the Bradford assay. Fifty-μg aliquots of whole cell extracts were separated on a 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and transferred to a PVDF membrane (BioRad) by an electroblotting apparatus. The membrane was blocked with Tris-buffered saline containing 5% dry milk overnight and then incubated with the indicated primary antibodies. Immnuocomplexes were detected with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin (IgG) or anti-mouse IgG and visualized using a SuperSignal kit according to the manufacturer's instruction (Pierce). The following primary antibodies were used: monoclonal antibodies against β-catenin (1:2000; Transduction Laboratory), monoclonal antibodies against α-tubulin (1:7500; Sigma), monoclonal antibodies against HA epitope (1:500; Clontech) and polyclonal antibodies against c-Myc (1:500; Santa Cruz).

2.3. Cell viability and DNA fragmentation

Cells were pretreated with OHT (100 nM) or vehicle control for 5 h and then stimulated with TNF (50 ng/ml) for 24 h. The detached and attached cells were collected and

incubated with 0.1% trypan blue dye, and the viable and dead cells were counted with a microscope. To examine DNA laddering, the attached and detached cells were collected at the indicated time points following OHT and TNF treatment. Genomic DNA was isolated and separated on a 1.2% agarose gel as described previously [4,5].

2.4. Matrigel invasion assay

Invasion assays were performed in BioCoat Matrigel Invasion Chambers (Becton Dickinson) according to the manufacturer's protocol. Briefly, cells (1×10^5 per well) were seeded onto the filters which were coated with the reconstituted Matrigel of the upper compartment of each chamber and incubated with DMEM medium. The lower chamber was filled with DMEM medium supplemented with 2% FBS. The chambers were then incubated for 48 h at 37 °C. After incubation, cells on the upper side of the filters were removed and the filters were stained with Diff-Quick solution (Sigma). Cells in the lower surface of the filter were considered to have invaded through the Matrigel and were counted using light microscopy (200× magnification).

2.5. Transfection and luciferase assay

RIE cells (0.5×10^5) were plated in 6-well plates in duplicates. Cells were transfected using lipofectamine according to the manufacturer's instruction (Gibco). Briefly, plasmids were mixed with lipofectamine (1:4 ratio) in OPTI-MEM medium (Gibco) and complexes were incubated for 30 min at room temperature. For internal control, pRL-TK *Renila* luciferase reporter was co-transfected for normalizing transfection efficiency. Cells were washed with PBS and the DNA-lipofectamine mixtures were added to the cells and incubated for 8 h at 37 °C. After incubation, cells were replenished with DMEM containing 10% FBS. Twenty-four hr after transfection, cells were washed with 1 × ice-cold PBS and lysed with passive lysis buffer (Promega). Luciferase assays were performed using a dual luciferase system (Promega).

2.6. Immunostaining

Cells were plated on chamber slides overnight and fixed with 4% paraformadehyde in PBS for 30 min at room temperature Cells were permeabilized with 0.2% Triton X-100 and stained with anti-HA monoclonal antibodies (1:100) or normal IgG overnight at 4 °C. The complexes were detected with Cy2-conjugated AffiniPure Donkey anti-mouse IgG (1:100; Jackson). For staining nuclei cells were incubated with propidium iodide (Sigma).

2.7. In vivo tumor growth

Both UMSCC1/V and UMSCC1/β-Cat cells (2×10^6 cells) were inoculated subcutaneously into the back of 7–8 week old nude mice (Taconic). Tumor growth was monitored every week for 4 weeks. These procedures were approved by the University of Michigan Committee on Use and Care of Animals (Ann Arbor, MI).

3. Results

3.1. Wnt signaling inhibits TNF/c-Myc-mediated apoptosis

In order to induce apoptosis by TNF, the protein synthesis or transcription inhibitors were required [10,12]. However, these inhibitors may be physiologically irrelevant. Earlier studies found that over-expression of c-Myc sensitized cells to TNF-mediated apoptosis in fibroblasts [14–16]. Since most tumors are derived from an epithelial origin and because c-Myc is over-expressed in many human

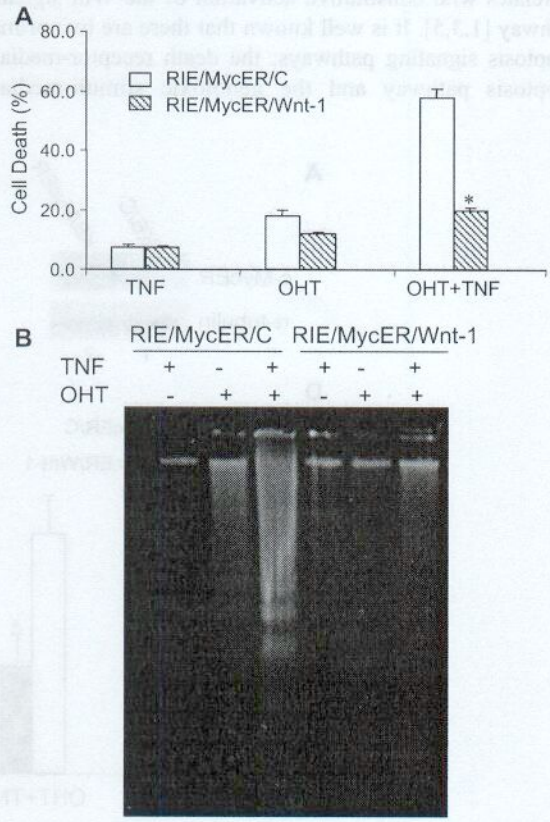

Fig. 1. Wnt-1 inhibits TNF/c-Myc-mediated apoptosis in RIE cells. (A) Wnt-1 inhibited c-Myc-mediated TNF sensitization. Both RIE/MycER/C and RIE/MycER/Wnt-1 cells were pre-treated with OHT or vehicle control (100 nM) for 5 h and then treated with TNF (50 ng/ml) for 24 h. After treatment, the attached and detached cells were collected and incubated with 0.1% trypan blue dye. The viable and dead cells were counted with a microscope. The assays were performed in duplicate, and the results represent the average values from three independent experiments. Statistical differences between each group were determined by the Student's *t* test. *, $p < 0.01$. (B) Wnt-1 inhibited TNF/c-Myc-mediated apoptosis. Cell treatment was performed as described in (A). The attached and detached cells were harvested and cells were lysed. Genomic DNAs were extracted with phenol–chloroform and separated on a 1.4% agarose–formaldehyde gel.

cancers [18–23], we were interested in determining whether c-Myc could render epithelial cells sensitive to TNF-mediated apoptosis. Previously, we established an inducible c-MycER expression model system in RIE cells (RIE/MycER). c-MycER is a fusion protein in which c-Myc is fused with a portion of an estrogen receptor and kept in the cytoplasm by chaperone proteins. c-Myc could be activated by stimulation with the synthetic steroid 4-hydroxytamoxifen (OHT) [5,25]. Therefore, we utilized this cell line to determine whether c-Myc could sensitize epithelial cells to TNF-mediated apoptosis. As shown in Fig. 1, activation of c-Myc by OHT had minimal effects on cell death under normal conditions. However, co-stimulation with OHT and TNF (TNF/c-Myc), but not TNF alone, killed over 60% of cells, indicating that c-Myc was capable of sensitizing cells to TNF in epithelial cells. In human cancers, the over-expression or amplification of the *c-myc* oncogene often correlates with constitutive activation of the Wnt signaling pathway [1,3,5]. It is well known that there are two primary apoptosis signaling pathways: the death receptor-mediated apoptosis pathway and the genotoxic stimuli-mediated apoptosis pathway [26–28]. Since Wnt signaling inhibited genotoxic drug-induced apoptosis, we were interested in determining whether Wnt-1 could inhibit TNF/c-Myc-mediated apoptosis using RIE/MycER cells expressing Wnt-1 (RIE/MycER/Wnt-1) [5]. As shown in Fig. 1A, over-expression of Wnt-1 strongly suppressed TNF/c-Myc-mediated killing compared to control cells. Apoptosis is characterized with caspase activation and DNA fragmentation [28]. To determine whether Wnt-1 inhibited TNF/c-Myc-mediated killing through the inhibition of apoptotic program, DNA fragmentation analysis was performed. As shown in Fig. 1B, the DNA ladder formation induced by TNF/c-Myc was significantly reduced by over-expression of Wnt-1, suggesting that Wnt-1 mediated survival by suppressing apoptosis (Fig. 1B).

Since Wnt family proteins have been found to be over-expressed in HNSCC [6,7] and to be associated with front growth, we determined whether Wnt-1 could suppress c-Myc-induced TNF sensitization in KB cells, a HNSCC cell line. First, we stably expressed c-Myc-ER in KB cells using retrovirus-mediated transduction as described previously.

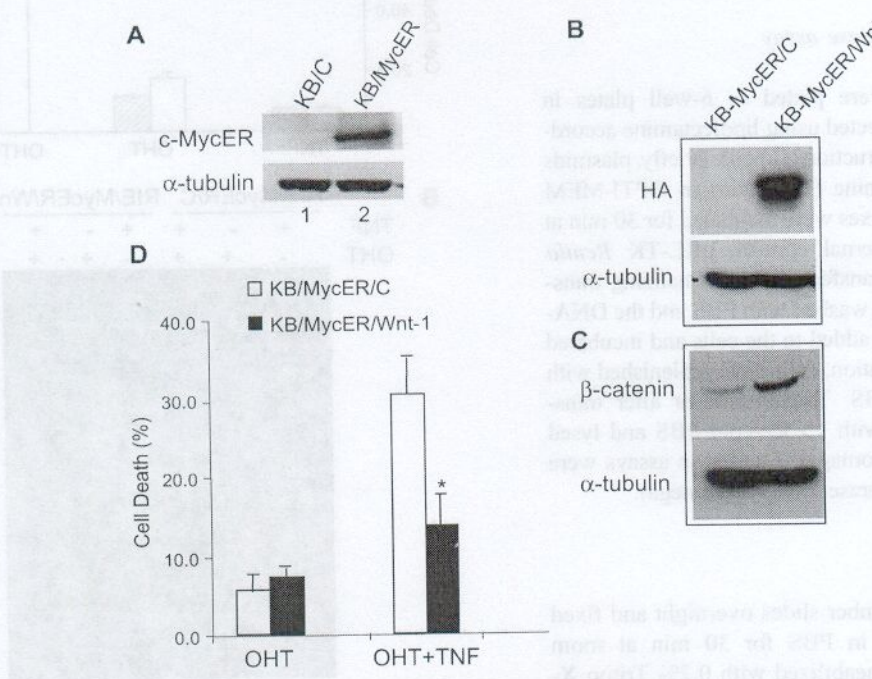

Fig. 2. Wnt-1 inhibited TNF/c-Myc-mediated apoptosis in KB cells. (A) The establishment of KB cells expressing c-MycER. KB cells were transduced with retroviruses expressing c-MycER or control vector in the presence of polybrene (6 μg/ml). Forty-eight hr after infection, cells were selected with puromycin (1 μg/ml) for one week and the surviving cells were pooled. The expression of c-MycER in KB cells was examined with Western blot analysis. For loading control, the membrane was stripped and re-probed with monoclonal antibodies against α-tubulin. (B) The establishment of KB/MycER cells expressing Wnt-1. KB/MycER cells were transduced with retroviruses expressing HA-Wnt-1 or control vector. Forty-eight hr after infection, cells were selected with hygromycin (600 μg/ml) for one week and the surviving cells were pooled. The expression of Wnt-1 in KB/MycER cells was determined with Western blot analysis. For loading control, the membrane was stripped and re-probed with monoclonal α-tubulin antibodies. (C) Wnt-1 increased the level of cytosolic β-catenin. Both KB/MycER/Wnt-1 and KB/MycER/C cells were fractionated and cytosolic extracts were probed with monoclonal antibodies against β-catenin (1 : 5000). For loading control, the membrane was stripped and re-probed with monoclonal antibodies against α-tubulin. (D) Wnt-1 inhibited TNF/c-Myc-mediated apoptosis in KB cells. Both KB/MycER/Wnt-1 and KB/MycER/C cells were pretreated with OHT for 5 h and then treated with TNF (50 ng/ml) for 24 h. After treatment, the attached and detached cells were harvested and cell viability was determined with the trypan blue exclusion assay. The assay was performed in duplicate, and the results represent average values from three independent experiments. Statistical differences between each group were determined by the Student's *t* test. *, $p<0.01$.

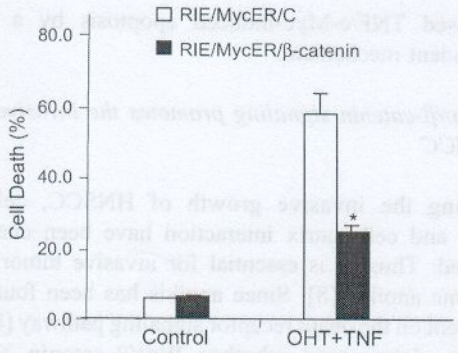

Fig. 3. Ectopic expression of β-catenin inhibits TNF/c-Myc-induced apoptosis. Both RIE/MycER/C and RIE/MycER/β-catenin cells were pretreated with OHT for 5 h and then treated with TNF for 24 h. After treatment, the attached and detached cells were harvested and cell viability was determined with the trypan blue exclusion assay. The assay was performed in duplicate, and the results represent average value from three independent experiments. Statistical differences between each group were determined by the Student's t test. *, $p<0.01$.

Briefly, KB cells were infected with retroviruses expressing Myc-ER or control vector in the presence of polybrene. Following the retroviral infection, cells were treated with puromycin (1 μg/ml) for one week and the resistant clones were pooled and subjected to Western blot analysis. As shown in Fig. 2A, KB cells expressing Myc-ER (KB/MycER) or control cells (KB/C) were obtained. Subsequently, KB/MycER cells were transduced with retroviruses expressing Wnt-1 or control vector as described previously. KB/MycER cells expressing Wnt-1 (KB/MycER/Wnt-1) or control vector (KB/MycER) were confirmed by Western blot analysis. As predicted, over-expression of Wnt-1 increased the cytosolic level of β-catenin in KB cells by cell fractionation (Fig. 2C, third panel). As shown in Fig. 2D, the combination of TNF stimulation and c-Myc activation significantly killed KB/MycER cells, whereas OHT alone only induced minimal cell death. In contrast, cell death induced by TNF and OHT treatment was significantly suppressed by Wnt-1 in KB cells. Taken together, these results demonstrate that activation of c-Myc sensitized TNF-mediated apoptosis in SCC cells and that Wnt signaling could counteract c-Myc-mediated sensitization.

The Wnt signaling pathway transduces oncogenic signals through stabilizing β-catenin [29,30]. The elevated level of β-catenin has been found in a variety of human tumors. Previously, we have established a RIE/MycER cell line expressing an oncogenic β-catenin (S37A) (RIE/MycER/β-catenin) which was found in several human cancers. Oncogenic β-catenin (S37A) has a serine to alanine point mutation at residue 37 which cannot be phosphorylated by glycogen synthase kinase 3β and subsequently degraded by the 26 S proteasome pathway [5]. Thus, we performed the experiments to determine whether ectopic expression of a mutant β-catenin (S37A) could inhibit TNF/c-Myc-induced apoptosis. As shown in Fig. 3, while 60% of control cells were killed, only 25% of RIE/MycER/β-Cat cells were killed following TNF and OHT treatment. Similarly, over-expression of β-catenin also inhibited TNF/c-Myc-mediated apoptosis in KB cells (data not shown). Our results suggest that Wnt signaling suppresses TNF/c-Myc-mediated apoptosis through β-catenin.

3.2. Inhibition of TNF/c-Myc-mediated apoptosis by Wnt/β-catenin is independent of NF-κB

Spiegelman et al. [31] reported that Wnt signaling induced the expression of β-transducing repeat-containing protein (β-TRCP), a component of the skp1-cullin-F-box ubiquitin ligase complex, to promote IκBα degradation, thereby enhancing NF-κB activation. We and others have found that the transcription factor nuclear factor-kappa B (NF-κB) activation plays a critical role in the regulation of TNF-induced apoptosis. The inhibition of NF-κB activation renders cells sensitive to TNF-induced apoptosis [32–36]. NF-κB is a transcription factor that plays an important role in inflammation, immunity and oncogenesis. The classical NF-κB is a heterodimer consisting of a transactivation subunit (RelA/p65) and a DNA-binding subunit p50. In unstimulated cells, NF-κB is retained in the cytoplasm by IκB

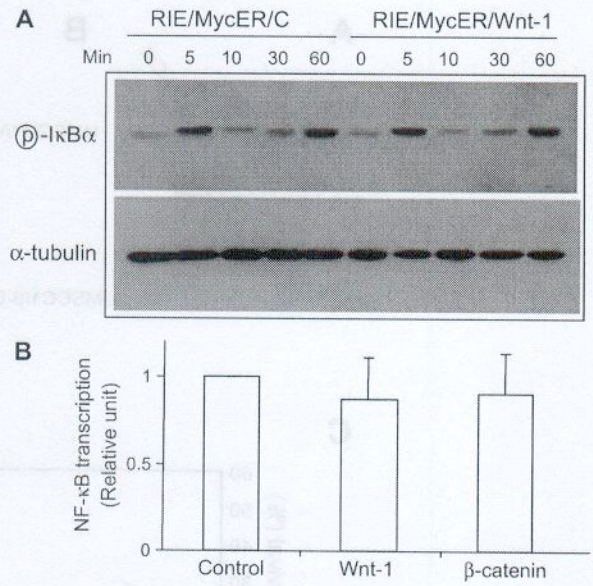

Fig. 4. Wnt-1 does not modify TNF-induced NF-κB activation. (A) Both RIE/MycER/C and RIE/MycER/Wnt-1 cells were pre-treated with OHT for 5 h and then treated with TNF (50 ng/ml) for the indicated time points. The whole cell protein extracts were prepared and probed with monoclonal antibodies against phospho-IκBα and polyclonal antibodies against IκBα. For loading control, membranes were stripped and re-probed with monoclonal antibodies against α-tubulin. (B) Wnt/β-catenin did not modify NF-κB transcription. RIE cells were co-transfected with a 2× NF-κB luciferase reporter and the expression vectors for Wnt-1 and β-catenin or the control vector using lipofectamine. Twenty-four hr after transfection, cells were harvested and lysed with the passive lysis buffer and luciferase activities were measured with a dual luciferase assay system. The assay was performed in duplicate, and the results represent average values from three independent experiments.

family proteins. Stimulation with TNF activates the IκB kinase (IKK) complex, resulting in degradation and phosphorylation of IκB and subsequently liberating NF-κB to the nucleus where it induces gene expression [36,37]. Numerous anti-apoptotic genes such as *Bcl-X_L*, *A1*, *A20*, and inhibitor of apoptosis have been found to be transcriptionally regulated by NF-κB [12,26,38–41]. Paradoxically, Deng et al. [42] have found that Wnt/β-catenin signaling inhibited TNF-mediated NF-κB activation. Since NF-κB activation plays a critical role in suppression of TNF-induced apoptosis and since c-Myc promotes TNF-mediated apoptosis by inhibiting NF-κB activation, we also determined whether Wnt-1/β-catenin modified NF-κB activation in the RIE cells. As shown in Fig. 4A, TNF induced the phosphorylation and degradation of IκB with similar kinetics in both Wnt-expressing cells and control cells. To further determine whether Wnt-1 stimulated NF-κB transcription, a 2× κB-dependent luciferase reporter assay was performed. As shown in Fig. 4B, Wnt-1 or β-catenin did not activate NF-κB transcription. Similarly, Wnt-1 also did not modify NF-κB activation in KB cells (data not shown). Taken together, these results suggest that Wnt signaling suppressed TNF/c-Myc-induced apoptosis by a NF-κB-independent mechanism.

3.3. Wnt/β-catenin signaling promotes the invasive growth of HNSCC

During the invasive growth of HNSCC, cell-to-cell contact and cell-matrix interaction have been changed or disrupted. Thus, it is essential for invasive tumor cells to overcome anoikis [8]. Since anoikis has been found to be dependent on the death receptor signaling pathway [8,10,12], we also determined whether Wnt/β-catenin signaling inhibited anoikis in HNSCC cells. Because UMSCC1 cells expressed very low levels of cytosolic β-catenin, we over-expressed oncogenic β-catenin (S37A) in UMSCC1 cells by retrovirus-mediated transduction as described above. Western blot analysis confirmed that β-catenin (S37A)-expressing UMSCC1 cells (UMSCC1/β-Cat) were generated (Fig. 5A). Our immunostaining demonstrated that oncogenic β-catenin (S37A) was also located in the nucleus, suggesting that it activated transcription (Fig. 5). To determine whether oncogenic β-catenin (S37A) inhibited anoikis, both

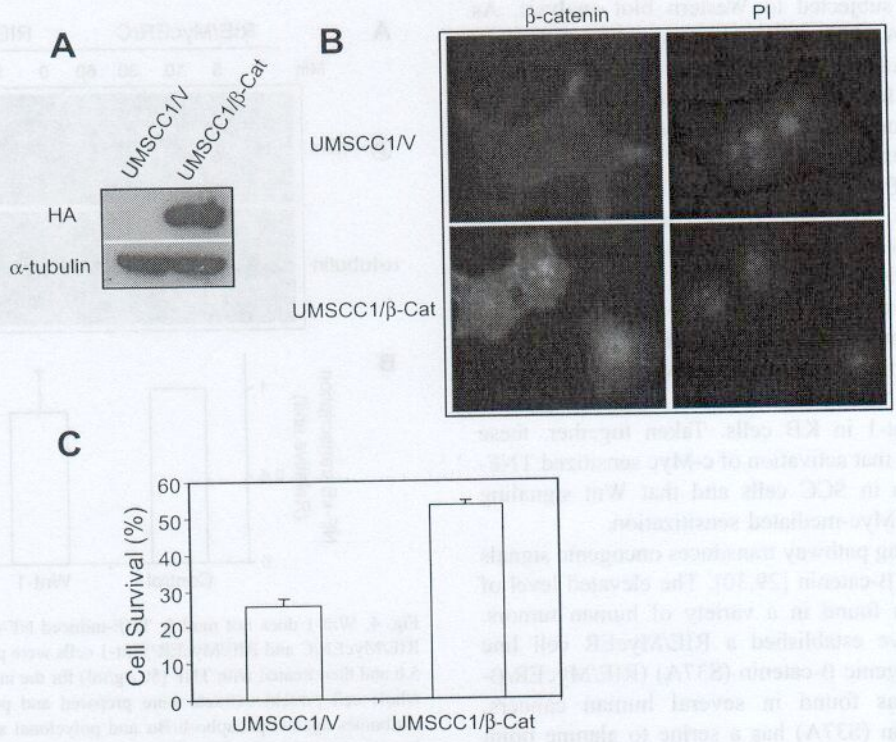

Fig. 5. β-catenin signaling inhibits anoikis in SCC cells. (A) The establishment of UMSCC1 cells expressing oncogenic β-catenin (S37A). UMSCC1 cells were infected with retroviruses expressing HA-β-catenin (S37A) or control vector and selected with neomycin (600 μg/ml) for ten days. The resistant clones were pooled and probed with monoclonal antibodies against the HA epitope. (B) The Immunolocalization of β-catenin. Both UMSCC1/V and UMSCC1/β-Cat cells were stained with monoclonal anti-HA antibodies and detected with Cy2-conjugated AffiniPure Donkey anti-mouse IgG. For staining nuclei, cells were incubated with propidium iodide (PI). (C) β-catenin inhibited anoikis. Both UMSCC1/V and UMSCC1/β-Cat cells were plated on 0.6% soft agar in the presence of growth medium, as described previously. After 48 h, cell viability was determined with the trypan blue viability assay. The assay was done in duplicate, and the results represent average values from three independent experiments. Statistical differences between each group were determined by the Student's *t* test. *, $p<0.01$.

UMSCC1/β-Cat and control UMSCC1/V cells were grown in a suspension condition. After 48 h, while ~75% of UMSCC1 cells were dead, over 50% of UMSCC1/β-Cat cells remained viable, suggesting that β-catenin also inhibited anoikis in HNSCC cells.

In addition to cell survival, we observed that β-catenin signaling promoted HNSCC cell scattering. As shown in Fig. 6A, UMSCC1/V cells formed compact cell islands in tissue culture plates, remained in tight cell–cell contacts and exhibited the typical morphology of epithelial cells. In sharp contrast, UMSCC1/β-Cat cells were scattered and exhibited the typical morphology of mesenchymal fibroblasts, suggesting that the constitutive activation of β-catenin signaling might induce epithelial–mesenchymal transition [9]. Next,

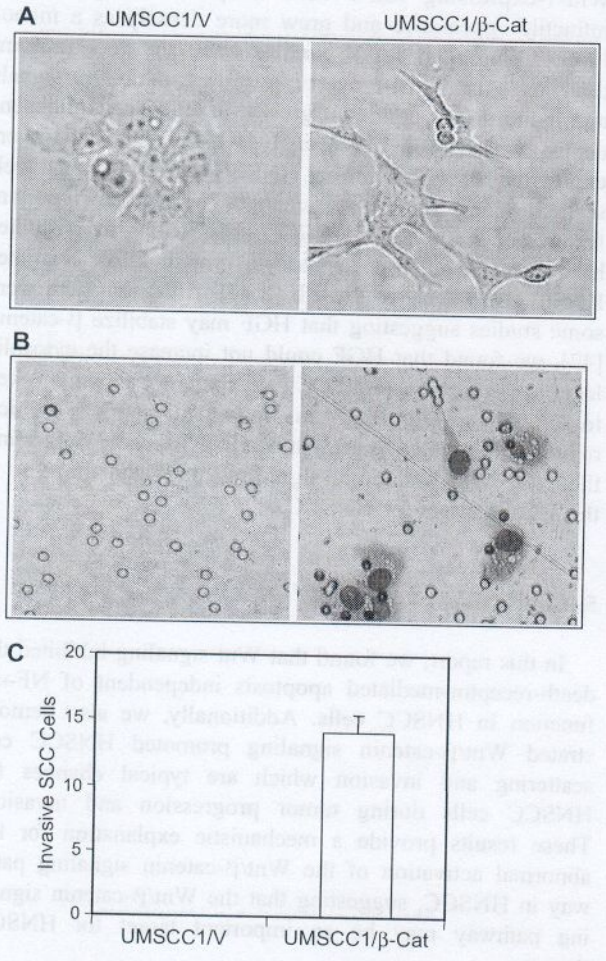

Fig. 6. β-catenin signaling promotes HNSCC cell scattering and invasion. (A) Induction of SCC cell scattering by β-catenin. Both UMSCC1/V and UMSCC/β-Cat cells were plated in low density. After 36 h, cell scattering was examined and photographed using light microscopy. (B) and (C) β-catenin induced HNSCC cell invasion. Both UMSCC1/V and UMSCC1/β-Cat cells (1×10^4) were plated on the Matrigel. After 48 h, the invasive cells were stained with Diff-Quick solution. The number of invasive cells was counted from three different fields and averaged. The results represent average values from three independent experiments. UMSCC1 cells were unable to invade through the Matrigel.

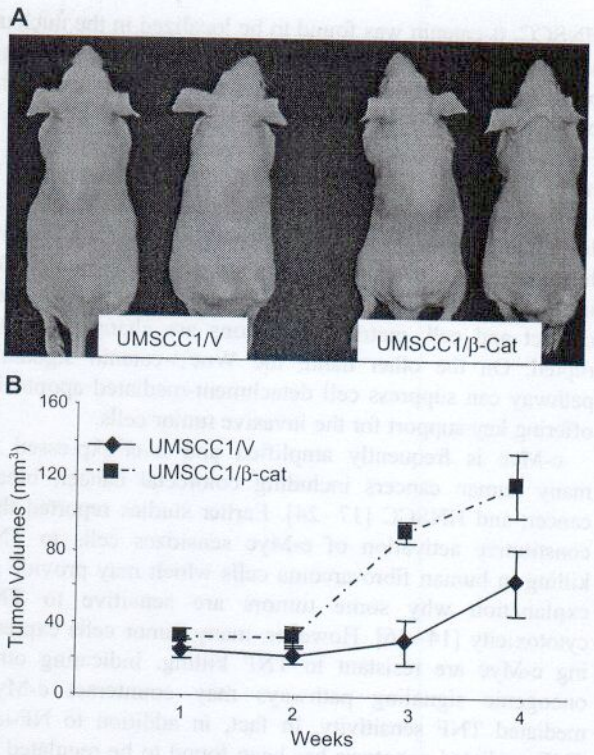

Fig. 7. β-catenin signaling promotes HNSCC tumor growth in vivo. (A) and (B) Both UMSCC1/V and UMSCC1/β-Cat cells were transplanted subcutaneously into the back of nude mice ($n = 10$). The tumor growth was measured every week for four weeks.

we examined whether the constitutive activation of β-catenin signaling stimulated cell invasion using the Matrigel chamber assays in which reconstituted basement membrane (Matrigel) has been used to mimic the naturally occurring basement membrane to assess the invasiveness of HNSCC cells. As shown in Fig. 6B and C, while UMSCC1/V cells could not migrate to the lower chamber, significant numbers of UMSCC1/β-Cat cells were found to invade through the Matrigel without stimulation.

To further determine whether oncogenic β-catenin promoted tumor growth and progression of HNSCC cells in vivo, both UMSCC1/V and UMSCC1/β-Cat cells were subcutaneously inoculated into nude mice. Although both cells formed similar numbers of tumors in nude mice, the average size of tumors from UMSCC1/β-Cat was significantly larger than that from SCC/V cells (Fig. 7). Taken together, these results suggest that the Wnt/β-catenin signaling pathway may promote the development and progression of HNSCC by promoting invasive growth.

4. Discussion

The Wnt/β-catenin signaling pathway has been found to be associated with HNSCC according to pathological studies [6,7,9]. Interestingly, in the cells at the invasive front of

HNSCC, β-catenin was found to be localized in the nucleus and cytoplasm. It suggested that the Wnt/β-catenin signaling pathway was constitutively activated in these cells and might play a critical role in the stimulation of invasive growth of HNSCC cells [9]. Our studies presented here provided a molecular explanation for these pathological observations. It is likely that the Wnt/β-catenin signaling pathway plays at least two roles in the invasive growth of HNSCC. On one hand, the Wnt/β-catenin signaling can directly promote SCC invasion. However, during the invasive growth, cell–cell contact and cell–matrix interactions are changed or disrupted. On the other hand, the Wnt/β-catenin signaling pathway can suppress cell detachment-mediated apoptosis, offering key support for the invasive tumor cells.

c-Myc is frequently amplified and over-expressed in many human cancers including colorectal cancer, breast cancer, and HNSCC [17–24]. Earlier studies reported that constitutive activation of c-Myc sensitizes cells to TNF killing in human fibrosarcoma cells which may provide an explanation why some tumors are sensitive to TNF cytotoxicity [14–16]. However, many tumor cells expressing c-Myc are resistant to TNF killing, indicating other oncogenic signaling pathways may counteract c-Myc-mediated TNF sensitivity. In fact, in addition to NF-κB, TNF-mediated apoptosis has been found to be regulated by multiple signaling pathways such as the Smad, PI3 kinase/Akt and the ERK signaling pathways [43–45]. In this report, we found that the Wnt/β-catenin signaling pathway was capable of acting against c-Myc and inhibited TNF/c-Myc-mediated apoptosis, indicating that Wnt signaling may provide a survival function in HNSCC. Our results provide new insights into the regulatory network of oncogenic signaling which controls cancer cell survival and proliferation. These results suggest that there is a delicate balance in cancer cells between different oncogenic signaling pathways which ultimately promote cell survival and proliferation.

Interaction between the Wnt signaling pathway and NF-κB is controversial. Given the fact that activation of NF-κB plays a critical role in TNF-mediated apoptosis and that c-Myc sensitized TNF-mediated apoptosis by inhibiting NF-κB [15], we have examined whether Wnt signaling cross-talked with NF-κB in our cell model system. Spiegelman et al. [31] demonstrated that β-catenin/Tcf transcription induced the expression of β-TrCP which promoted the ubiquitination and subsequent degradation of IκB. This induction resulted in enhancing NF-κB transactivation without affecting the activity of IκB kinase. On the contrary, Deng et al. [42] recently found that β-catenin physically interacted with NF-κB. Over-expression of β-catenin inhibited NF-κB DNA binding and transactivation activity and sensitized TNF-mediated apoptosis. Additionally, they found that over-expression of β-catenin did not inhibit IKK activity and IκBα degradation. In contrast to these studies, we found that the Wnt signaling pathway neither suppressed IκBα degradation nor NF-κB transcription. The Wnt signaling pathway inhibited TNF/c-Myc-mediated apoptosis independent of NF-κB activation. Furthermore, supporting our results, we found that NF-κB-regulated anti-apoptotic genes such as *TRAF-1*, *TRAF-2*, *Bcl-X_L*, *XIAP*, *c-IAP1* and *2*, and *NDED*[26,39–41] were not induced by Wnt-1 in RIE or KB cells. Currently, we cannot provide an explanation for these conflicting results. We previously found that Wntz/β-catenin induced Cox-2 and WISP-1 to inhibit mitochondria-dependent apoptosis [5]. In the future, it will be interesting to examine whether these genes are able to inhibit the death receptor-mediated apoptosis.

Previously, over-expression of Wnt-1 has been found to induce morphological transformation in Rat-1 fibroblasts and C57MG mammary epithelial cells [46]. For example, Wnt-1-expressing Rat-1 cells adopt an elongated and refractile appearance and grew more densely as a monolayer, forming cord-like bundles lined up in a uniform direction. Also, Wnt-1 overcame growth-inhibitory signals and induced post-confluent growth in Rat-1 cells independent of serum [46]. Interestingly, we found that the over-expression of β-catenin in HNSCC cells promoted cell scattering and fibroblastic changes which are important features of the invasive HNSCC cells. So far, most studies have focused on how hepatocyte growth factor regulates HNSCC cell invasive growth [8,47]. Although there were some studies suggesting that HGF may stabilize β-catenin [47], we found that HGF could not increase the cytosolic level of β-catenin in HNSCC cells (data not shown). Taken together, we identified an important pathway which regulates the invasive growth of HNSCC cells, suggesting that the Wnt/β-catenin signaling pathway may be a therapeutic target for HNSCC.

5. Conclusions

In this report, we found that Wnt signaling inhibited the death-receptor-mediated apoptosis independent of NF-κB function in HNSCC cells. Additionally, we also demonstrated Wnt/β-catenin signaling promoted HNSCC cell scattering and invasion which are typical changes for HNSCC cells during tumor progression and invasion. These results provide a mechanistic explanation for the abnormal activation of the Wnt/β-catenin signaling pathway in HNSCC, suggesting that the Wnt/β-catenin signaling pathway may be an important target for HNSCC therapy.

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