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Wnt signaling promotes oncogenic transformation by inhibiting c-Myc–induced apoptosis

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Aberrant activation of the Wnt/β-catenin signaling pathway is associated with numerous human cancers and often correlates with the overexpression or amplification of the *c-myc* oncogene. Paradoxical to the cellular transformation potential of c-Myc is its ability to also induce apoptosis. Using an inducible c-MycER expression system, we found that Wnt/β-catenin signaling suppressed apoptosis by inhibiting c-Myc–induced release of cytochrome c and caspase activation. Both cyclooxygenase 2 and WISP-1 were identified as effectors of the Wnt-mediated antiapoptotic signal. Soft agar assays showed that neither c-Myc nor Wnt-1 alone was sufficient to induce cellular transformation, but that Wnt and c-Myc coordinated in inducing transformation. Furthermore, coexpression of Wnt-1 and c-Myc induced high-frequency and rapid tumor growth in nude mice. Extensive apoptotic bodies were characteristic of c-Myc–induced tumors, but not tumors induced by coactivation of c-Myc and Wnt-1, indicating that the antiapoptotic function of Wnt-1 plays a critical role in the synergetic action between c-Myc and Wnt-1. These results elucidate the molecular mechanisms by which Wnt/β-catenin inhibits apoptosis and provide new insight into Wnt signaling-mediated oncogenesis.

Introduction

The Wnt family genes encode a group of secretory glycoproteins that play important roles in embryogenesis, cell proliferation, and specification of cell fate (Nusse and Varmus, 1992; Cadigan and Nusse, 1997; Miller et al., 1999; Peifer and Polakis, 2000; Hartmann and Tabin, 2001; Huelsken et al., 2001; Kawakami et al., 2001). Wnt signaling is transduced through β-catenin which is regulated by the adenomatous polyposis coli (APC)*/Axin/glycogen synthase kinase (GSK)3β complex (Behrens et al., 1998; Bienz and Clevers, 2000; Polakis, 2000; Woodgett, 2001). In the absence of Wnt stimulation, GSK-3β constitutively phosphorylates β-catenin at both serine and threonine residues of the NH_2-terminal region (known as GSK-3β consensus sites), which is well conserved within the catenin family of proteins (Yost et al., 1996, 1998; Ikeda et al., 1998; Polakis, 2000). The phosphorylated β-catenin is ubiquitinated and degraded through the proteasome pathway (Aberle et al., 1997; Kitagawa et al., 1999; Matsuzawa and Reed, 2001; Sadot et al., 2001). In the presence of Wnt stimulation, the Frizzled receptors and low-density lipoprotein receptor-related proteins 5 and 6 synergistically stabilize β-catenin by multiple mechanisms, resulting in the accumulation of free cytosolic β-catenin (He et al., 1997; Pinson et al., 2000; Tamai, et al., 2000; Wehrli

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*Abbreviations used in this paper: APC, adenomatous polyposis coli; ARF, alternative reading frame; Cox, cyclooxygenase; GSK, glycogen synthase kinase; OHT, 4-hydroxytamoxifen; RIE, rat intestinal epithelial cells; RT-PCR, reverse transcription–PCR; Tcf, T cell factor; TUNEL, deoxynucleotidyl transferase-mediated dUTP nick end labeling.

Key words: apoptosis; β-catenin; c-Myc; oncogenesis; Wnt signaling

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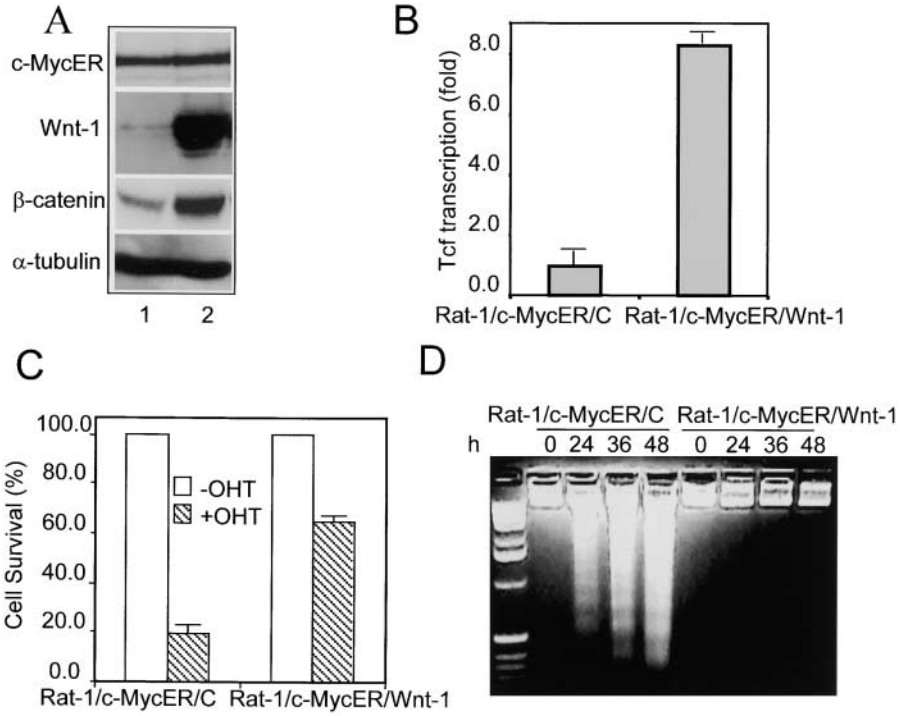

Figure 1. **Wnt-1 inhibits c-Myc–induced apoptosis in Rat-1 cells.** (A) Establishment of Rat-1/c-MycER/Wnt-1 cells stably expressing Wnt-1 and control cells (Rat-1/c-MycER/C). Rat-1/c-MycER cells were transduced with retroviruses encoding the Wnt-1 expression vector or a control vector. Cells were selected with neomycin (600 μg/ml) for 1 wk and the resistant clones were pooled. The expression of c-MycER in both Rat-1/c-MycER/Wnt-1 and Rat-1/c-MycER/C cells was confirmed with monoclonal antibodies against c-Myc by the Western blot analysis (top). HA-Wnt-1 was detected with monoclonal antibodies against HA (second panel, lane 2). The cell fractionations were performed as described previously (Shimizu et al., 1997) and cytosolic extracts were probed with monoclonal antibodies against β-catenin (third panel). For internal controls, the blots were stripped and reprobed with monoclonal antibodies against α-tubulin. (B) The activation of β-catenin/Tcf-mediated transcription by Wnt-1. Both Rat-1/c-MycER/C cell and Rat-1/c-MycER/Wnt-1 cells were transfected with either pTopflash or pFopflash luciferase reporter plasmid. The pRL-TK *Renilla* luciferase reporter was cotransfected to normalize transfection efficiency. The fold activation was determined by comparing pTopflash luciferase activity with pFopflash luciferase activity. The activation values represent triplicate samples that were counted and averaged. (C) Wnt-1 inhibited c-Myc–induced cell death. Cells were treated with OHT (100 nM) to activate c-Myc or the vehicle control for 48 h in a low-serum condition (1%). Cell viability was determined with the trypan blue exclusion assay. The assays were performed in triplicate, and the results represent the mean value from the three independent experiments. (D) Suppression of c-Myc–induced DNA fragmentation by Wnt-1 in Rat-1 cells. The attached and detached cells were collected at the indicated time points following OHT treatment. DNA was isolated and separated on a 1.2% agarose gel.

et al., 2000; Bafico et al., 2001; Mao et al., 2001a,b; Sun et al., 2001). The elevated β-catenin can translocate to the nucleus where it forms a complex with Tcf (T cell factor) to stimulate the expression of Wnt-responsive genes (Behrens et al., 1996; Korinek et al., 1997; Morin et al., 1997; Riese et al., 1997; Hecht et al., 2000; Takemaru and Moon, 2000).

Growing evidence has demonstrated that the Wnt signaling pathway is associated with tumor development and/or progression (Gat et al., 1998; Bienz and Clevers, 2000; Peifer and Polakis, 2000; Polakis, 2000). Aberrant activation of the Wnt signaling pathway is associated with a variety of human cancers, correlating with the overexpression or amplification of c-Myc (de la Coste et al., 1998; He et al., 1998; Miller et al., 1999; Bienz and Clevers, 2000; Polakis, 2000; Brown, 2001). Interestingly, c-Myc was identified as a transcriptional target of the APC/β-catenin/Tcf pathway in colorectal cancer cells (He et al., 1998), suggesting that one way Wnt signaling functions in oncogenesis is through the growth promoting activity of c-Myc (de la Coste et al., 1998; Miller et al., 1999). However, because c-Myc is also an established inducer of apoptosis, oncogenic transformation mediated by c-Myc must therefore require a survival signal to overcome its proapoptotic activity (Amati and Land, 1994; Hueber et al., 1997; Schreiber-Agus and DePinho, 1998; Zindy et al., 1998; Cole and McMahon, 1999; Dang, 1999; Obaya et al., 1999; Prendergast, 1999; Grandori et al., 2000). Interestingly, it has been observed that c-Myc–induced hepatocellular carcinoma is associated with a "second hit" mutation in the β-catenin gene, suggesting that compensating mutations in β-catenin may serve to protect cells from apoptosis and thereby facilitate transformation (de la Coste et al., 1998).

Apoptosis is characterized by caspase activation, condensation of the nucleus, cleavage of specific protein, and DNA fragmentation (Wang et al., 1996, 1999a; Cryns and Yuan, 1998; Green and Reed, 1998; Wang, 2001). We and others have shown that Wnt/β-catenin signaling promotes cell survival in various cell types (Morin et al., 1995; Orford et al., 1999; Cox et al., 2000; Reya et al., 2000; Satoh et al., 2000; Shih et al., 2000; Chen et al., 2001; Ioannidis et al., 2001; Mukhopadhyay et al., 2001). Using an inducible expression system, Morin et al. (1995) found that overexpression of APC in human colorectal cancer cells suppressed cell growth by induction of apoptosis. Orford et al. (1999) reported that overexpression of β-catenin inhibited anoikis. We found that Wnt signaling inhibited chemotherapeutic drug-mediated apoptosis by suppressing the cytochrome c release and caspase-9 activation. Inhibition of β-catenin/Tcf transcription blocked Wnt-mediated cell survival (Chen et al., 2001). The present study was undertaken to explore whether Wnt signaling inhibited c-Myc–mediated apoptosis, and if so, to define the molecular mechanism underlying this activity. Using an inducible c-MycER expression system, we found that the Wnt signaling potently inhibited c-Myc–mediated apoptosis by suppressing c-Myc–induced release of cyto-

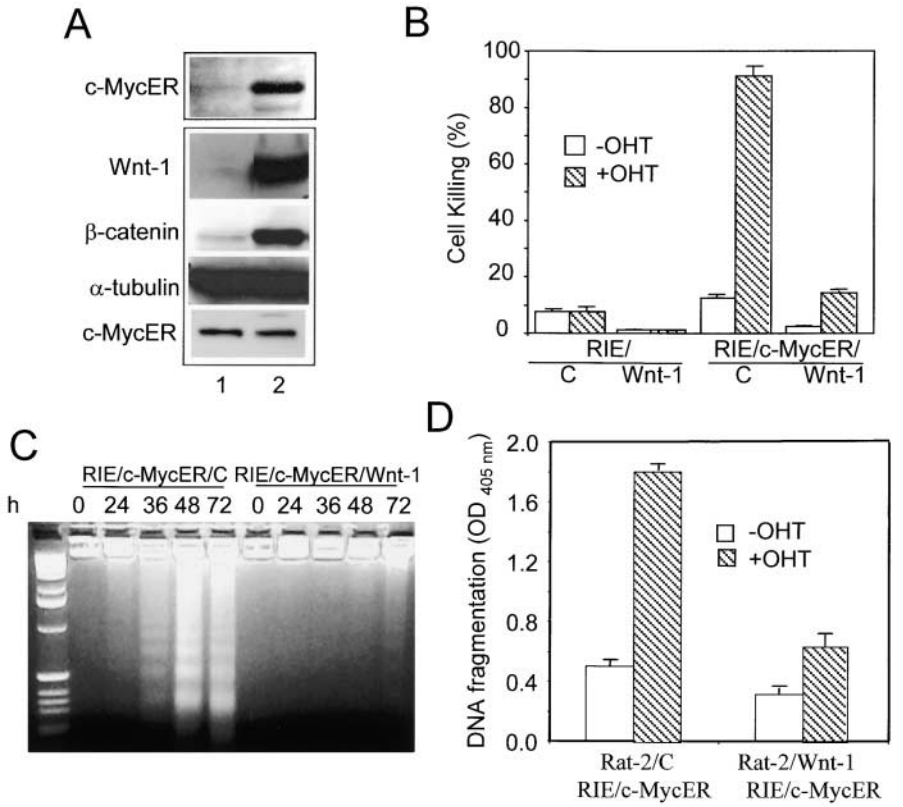

Figure 2. **Wnt-1 inhibits c-Myc–mediated apoptosis in RIE cells.** (A) Establishment of RIE/c-MycER cells and RIE/c-MycER cells expressing Wnt-1. RIE cells were first transduced with retroviruses encoding the c-MycER expression vector or a control vector and selected with puromycin (1.5 μg/ml) for 1 wk. The expression of c-MycER in RIE cells was detected with the Western blot analysis (top, lane 2). Lane 1 represented RIE cells expressing empty control vector. Subsequently, RIE/c-MycER cells were infected with retroviruses encoding the Wnt-1 expression vector or a control vector, and selected with hygromycin (600 μg/ml). RIE/c-MycER/Wnt-1 cells expressing Wnt-1 were confirmed with the Western blot analysis (second panel, lane 2). Both cell fractionations and the detection of β-catenin were performed as described in Fig. 1 A. For loading control, membrane was stripped and reprobed with monoclonal antibodies against α-tubulin (fourth panel). Both RIE/c-MycER/C and RIE/c-MycER/Wnt-1 cells expressing c-MycER proteins were confirmed with monoclonal antibodies against c-Myc (bottom). (B) Wnt-1 inhibited c-Myc-induced cell death. Cells were treated with OHT (100 nM) or the vehicle control for 72 h in a low-serum condition (1%). The assays were performed in triplicate, and the results represent the mean value from three independent experiments. (C) Wnt-1 inhibited c-Myc–induced DNA fragmentation in RIE cells. DNA fragmentation analyses were performed as described in Fig. 1 D. (D) Wnt-1 inhibited c-Myc–induced apoptosis in the coculture assay. RIE/c-MycER cells were cocultured with Rat-2/Wnt-1 or control cells. Cell treatment was performed as described in (B). Twenty μl of cell supernatants were incubated with anti-histone and -DNA antibodies for 2 h. The reaction was measured with a microplate reader at 405 nm.

chrome c and caspase activation. Both Cox-2 and WISP-1 were identified as effectors of the Wnt-mediated survival signal. Moreover, we found that Wnt-1 potentiated c-Myc–mediated oncogenic transformation in vitro and in vivo by antiapoptotic mechanisms. These results provide new insight into Wnt-mediated oncogenesis.

Results

Wnt signaling inhibits c-Myc–induced apoptosis

To determine whether Wnt signaling inhibited c-Myc–induced apoptosis, we utilized an established Rat-1 fibroblast cell line expressing the inducible c-Myc protein (Rat-1/c-MycER) (Hueber et al., 1997). In this system, c-Myc polypeptide is fused with a portion of the human estrogen receptor (c-MycER), which is activated only in the presence of the synthetic steroid 4-hydroxytamoxifen (OHT). OHT treatment alone is not cytotoxic to cells, and activation of c-Myc by OHT addition leads to severe apoptotic cell death in Rat-1/c-MycER cells in low-serum condition (Hueber et al., 1997). A Wnt-1 expression vector or control vector was transduced into Rat-1/c-MycER cells with retroviruses, allowing the whole cell population to be assessed. Both Rat-1/c-MycER cells expressing Wnt-1 (Rat-1/c-MycER/Wnt-1) and control cells (Rat-1/c-MycER/C) were generated and ectopic protein expression was demonstrated by Western blot analysis (Fig. 1 A, second panel). As predicted, expression of Wnt-1 led to an elevated cytosolic level of β-catenin (Fig. 1 A, third panel, lane 2) and activated β-catenin/Tcf-dependent transcription (Fig. 1 B). To assess Wnt-1 antiapoptotic activity, c-Myc was induced in both control and Wnt-1–expressing cells. In contrast to control cells, Wnt-1 expression was observed to significantly reduce c-Myc–induced cell death (Fig. 1 C). Apoptosis is a programmed cell death with a hallmark of DNA fragmentation (Salvesen and Dixit, 1997; Nagata, 1999). As shown in Fig. 1 D, DNA fragmentation analysis showed that c-Myc–induced apoptosis occurred in a time-dependent fashion in Rat-1/c-MycER/C cells, but not in Rat-1/c-MycER/Wnt-1 cells, demonstrating that Wnt-1 mediated cell survival by suppressing c-Myc–induced apoptosis.

c-Myc is frequently amplified or overexpressed in epithelial-derived cancers (Amati and Land, 1994; He et al., 1998; Schreiber-Agus and DePinho, 1998). Next, we utilized rat intestinal epithelial cells (RIE), which are widely used for the study of oncogenic transformation, as a model to test the activity of Wnt-1 in c-Myc–mediated apoptosis. Analogous to Rat-1 cells, a c-Myc–inducible system was generated in RIE cells (Fig. 2 A). Activation of c-Myc by OHT induced apoptosis in RIE/c-MycER cells, but not in control cells (RIE/C), after growth factor depletion (Fig. 2 B). RIE/c-MycER cells were infected with retroviruses encoding the Wnt-1 expression vector and a control vector, and both RIE/c-MycER/Wnt-1 and RIE-c-MycER/C cell lines were generated after

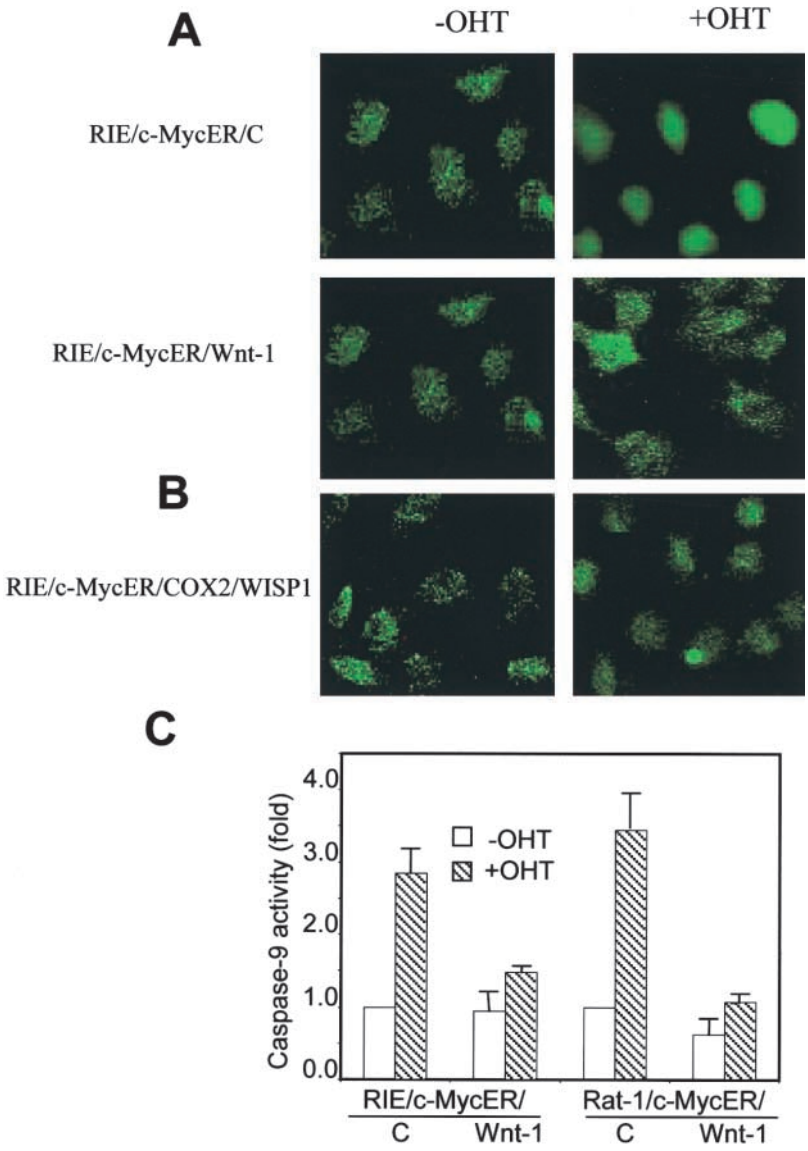

Figure 3. **Wnt-1 inhibits the release of cytochrome c induced by c-Myc.** (A) The cytosolic release of cytochrome c was inhibited by Wnt-1. RIE/c-MycER/C and RIE/c-MycER/Wnt-1 cells were treated with OHT (100 nM) for 24 h and fixed with 4% paraformaldehyde. Cells were incubated with primary monoclonal antibodies against cytochrome c and with isothiocyanate-conjugated secondary antibody. The results were examined and photographed under a fluorescence microscope. (B) Inhibition of the release of cytochrome c by the expression of Cox-2 and WISP-1. Cell staining was performed as described in A. (C) Wnt-1 suppressed c-Myc–induced caspase-9 activities. Cells were treated with OHT for 36 h The attached and detached cells were harvested and the whole cell proteins were extracted. 200 μg aliquots of protein extracts were incubated with caspase-9 substrate LEHD-pNA (100 μM) for 3 h at 37°C. The reaction was recorded with a plate reader at 405 nm. The results represent the average value from the three independent experiments.

hygromycin selection, respectively (Fig. 2 A, second panel). Like in Rat-1 cells, Wnt-1 expression increased the cytosolic level of β-catenin in RIE cells (Fig. 2 A, third panel, lane 2). 72 h after OHT treatment, ~90% of RIE/c-MycER/C cells were dead, whereas strikingly, >70% of RIE/c-MycER/Wnt-1 cells remained viable (Fig. 2 B). DNA fragmentation analysis confirmed that Wnt-1 inhibited c-Myc–induced apoptosis in RIE cells (Fig. 2 C). The inhibition of c-Myc–mediated apoptosis by Wnt-1 was also confirmed by a long-term clonogenicity assay (unpublished data). One of critical barriers for studying Wnt signaling is that there are no biologically active forms of Wnt proteins available. To confirm our results from Wnt-expressing RIE cells, we also applied a paracrine coculture assay to demonstrate that Wnt antiapoptotic activity can be conferred in a paracrine fashion (Jue et al., 1992; Mao et al., 2001a,b). RIE/c-MycER cells were cocultured with Rat-2 fibroblasts secreting Wnt-1 proteins or control cells, and c-Myc–induced apoptosis was determined with a cell death ELISA (enzyme-linked immunosorbent assay). Of note, at the late stage of apoptosis, the fragmented DNA and histones are released to the cell culture medium and can be detected by the cell death ELISA (Chen et al., 2001). As shown in Fig. 2 D, after OHT addition, DNA fragmentations were significantly induced in RIE/c-MycER cells cocultured with Rat-2 control cells, but not with Rat-2/Wnt-1 cells, indicating that Wnt-1 could suppress c-Myc–mediated apoptosis by a paracrine manner.

Activation of c-Myc has been found to trigger the release of cytochrome c from mitochondria to the cytosol during the early stage of apoptosis (Juin et al., 1999). The cytosolic cytochrome c binds to apoptotic protease-activating factor-1 to activate caspase-9 to induce apoptosis (Wang, 2001). Studies from caspase-9−/− embryonic fibroblasts found that caspase-9, an initial caspase, plays an essential role in c-Myc–mediated apoptosis (Soengas et al., 1999). Thus, we determined whether Wnt-1 inhibited c-Myc–induced release of cytochrome c by immunofluorescence staining as described previously (You et al., 2001). RIE cells were utilized because they had relatively flat morphology. As shown in Fig. 3 A, untreated cells exhibited a punctate cytoplasmic staining of cytochrome c, indicating mitochondrial localization. After OHT induction, RIE/c-MycER/C cells had very

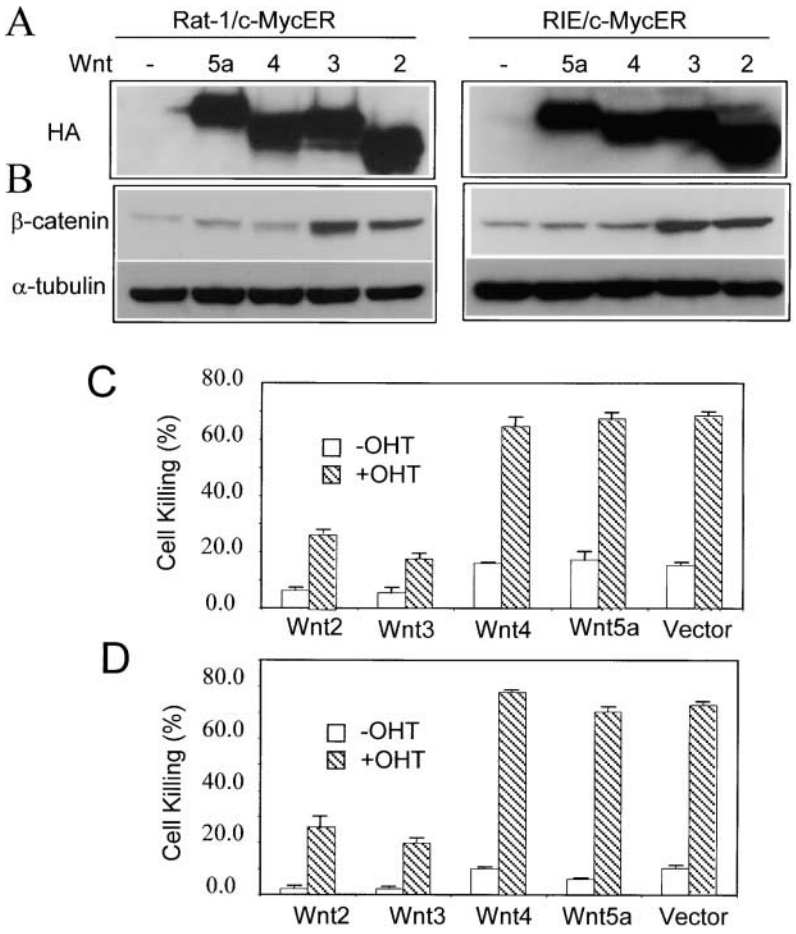

Figure 4. **Wnt-mediated anti-apoptosis is associated with an elevated level of β-catenin.** (A) Ectopic expression of Wnt-2, -3, -4, and -5a in Rat-1/c-Myc-ER and RIE/c-MycER cells. Rat-1 cells or RIE cells were transduced with retroviruses encoding Wnt-2, -3, -4, or -5a or a control vector. The expression of Wnt was detected as described in Fig. 1 A. (B) Stabilization of β-catenin by Wnts. Cell fractionation and Western blot analyses were performed as described in Fig. 1 A. For the internal controls, the blots were stripped and reprobed with monoclonal antibodies against α-tubulin. (C) Inhibition of c-Myc–induced apoptosis by Wnts in Rat-1 cells. The experiments were performed as described in Fig. 1 C. (D) Inhibition of c-Myc–induced apoptosis by Wnts in RIE cells. The experiments were performed as described in Fig. 2 B.

diffuse staining patterns in the entire cells, indicative of the release of cytochrome c from mitochondria to the cytosol. In contrast, RIE/c-MycER/Wnt-1 cells retained a punctate staining pattern, suggesting that Wnt-1 blocked the release of cytochrome c. Consistent with this observation is the finding that c-Myc–induced caspase-9 activities were suppressed by Wnt-1 (Fig. 3 C).

Wnt family members are unable to transform cells in vitro as measured by the soft agar assay. However, some Wnt members can induce so-called "partial transformation" of cultured cells, characterized by morphologic changes such as high-density growth and an elongated refractile cell shape (Brown et al., 1986; Wong et al., 1994; Bradley and Brown, 1995; Shimizu et al., 1997). Interestingly, the morphologic transformation by Wnt family proteins is associated with their ability to stabilize β-catenin and tumorigenesis (Wong et al., 1994; Bradley and Brown, 1995; Shimizu et al., 1997; Michaelson and Leder, 2001). Additionally, some members of Wnt family proteins such as Wnt-2 are highly expressed in colorectal cancer and breast carcinoma (Vider et al., 1996; Peifer and Polakis, 2000; Polakis, 2000). Thus, experiments were performed to determine whether the morphological transforming potential of Wnt family members was correlated with their ability to inhibit c-myc–mediated apoptosis. Transforming Wnt-2 and -3 as well as nontransforming Wnt-4 and -5a expression vectors were transduced into Rat-1/c-MycER cells or RIE/c-MycER cells by retroviral delivery, respectively (Fig. 4 A). Consistent with previous studies (Wong et al., 1994; Bradley and Brown, 1995; Shimizu et al., 1997), Wnt-2 and -3, but not Wnt-4 and -5a, caused morphological transformation (unpublished data). By cell fractionation analysis Wnt-2 and -3 significantly increased levels of free cytoplasmic β-catenin, whereas Wnt-4 and -5a had a minimal effect (Fig. 4 B). Histone H1 or E-cadherin was undetectable in the cytosolic fraction, indicating that the cytosolic fraction was not contaminated by the nuclear or membrane fraction (unpublished data). As shown in Fig. 4, C and D, Wnt-2 and -3 potently inhibited c-Myc–induced apoptosis in both Rat-1/c-MycER and RIE/c-MycER cells, respectively. Although Wnt-4 and -5a slightly increased the level of free cytosolic β-catenin, the level of induction might not be sufficient to provide protection against c-Myc–induced apoptosis. The results suggested that only the transforming Wnts possessed antiapoptotic activities that correlated with stabilized levels of β-catenin. Components of the Wnt signaling pathways have been found to be mutated in various human cancers (Miller et al., 1999; Bienz and Clevers, 2000; Peifer and Polakis, 2000; Polakis, 2000; Brown, 2001). Common to these mutations is the accumulation of free cytoplasmic β-catenin and the constitutive activation of β-catenin/Tcf-mediated transcription (Behrens et al., 1996; Korinek et al., 1997; Morin et al., 1997; Rubinfeld et al., 1997; de la Coste et al., 1998; Hetman et al., 2000). To mimic the oncogenic effects of elevated β-catenin, we also determined whether the ectopic expression of β-catenin could inhibit c-Myc–induced apoptosis. RIE/c-MycER cells

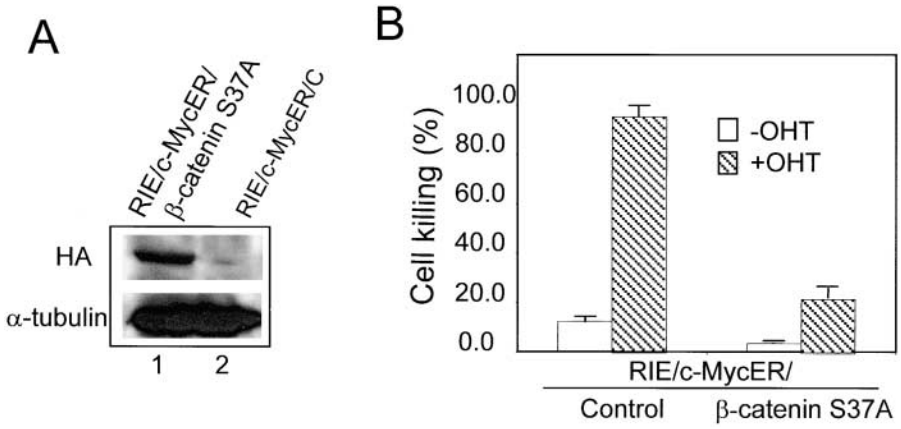

Figure 5. **β-catenin inhibits c-Myc–mediated apoptosis.** (A) Establishment of RIE/c-MycER cells expressing β-catenin (S37A). RIE/c-mycER cells were transduced with retroviruses encoding the HA-β-catenin (S37A) expression vector or a control vector and selected with neomycin (600 μg/ml) for 1 wk. The expression of β-catenin (S37A) was detected with monoclonal antibodies against HA (top, lane 1). For the internal controls, the blots were stripped and reprobed with monoclonal antibodies against α-tubulin (bottom). (B) β-Catenin suppressed c-myc-induced apoptosis. The experiments were performed as described in Fig. 2 B.

were transduced with retroviruses encoding a mutant β-catenin (S37A) expression vector or a control vector (Fig. 5 A). β-Catenin (S37A) has a serine to alanine point mutation at residue 37 which cannot be phosphorylated by GSK-3β and subsequently cannot be degraded by the ubiquitin-proteasome pathway (Chen et al., 2001). As shown, overexpression of β-catenin (S37A) potently suppressed c-Myc–mediated apoptosis, suggesting that Wnt-mediated survival was dependent on β-catenin (Fig. 5 B).

Wnt-inducible genes Cox-2 and WISP-1 suppress c-Myc–induced apoptosis

To identify the potential antiapoptotic genes induced by Wnt/β-catenin signaling, Western blot analysis was performed to analyze known antiapoptotic genes such as the bcl-2 family proteins and the inhibitors of apoptosis (Duckett et al., 1996; Wang et al., 1998, 1999b; Vaux and Korsmeyer, 1999). Our analysis indicated that such genes were not regulated by Wnt signaling. Given that Cox-2 is a known target gene of the Wnt signaling pathway (Howe et al., 1999; Haertel-Wiesmann et al., 2000) and has been shown to play a functional role in colorectal cancers (Tsujii et al., 1998), the level of Cox-2 expression was examined. Western blot analysis and semiquantitative reverse transcription-PCR (RT-PCR) found that the expression of Cox-2 was induced by Wnt-1 in our model system (Fig. 6 A; unpublished data). Overexpression of Cox-2 suppressed c-Myc–induced apoptosis in RIE/c-Myc cells (Fig. 6, B and C). To determine whether Cox-2 was an antiapoptotic gene responsible for Wnt-mediated antiapoptotic function, cells were treated with the specific Cox-2 inhibitor NS-398 at physiologic concentrations (Tsujii et al., 1998). As shown in Fig. 6 D, inhibition of Cox-2 activity partially aborted the Wnt-1–mediated prosurvival function in RIE/c-MycER/Wnt-1 cells, indicating that other gene products, in addition to Cox-2, function to suppress c-Myc–mediated apoptosis.

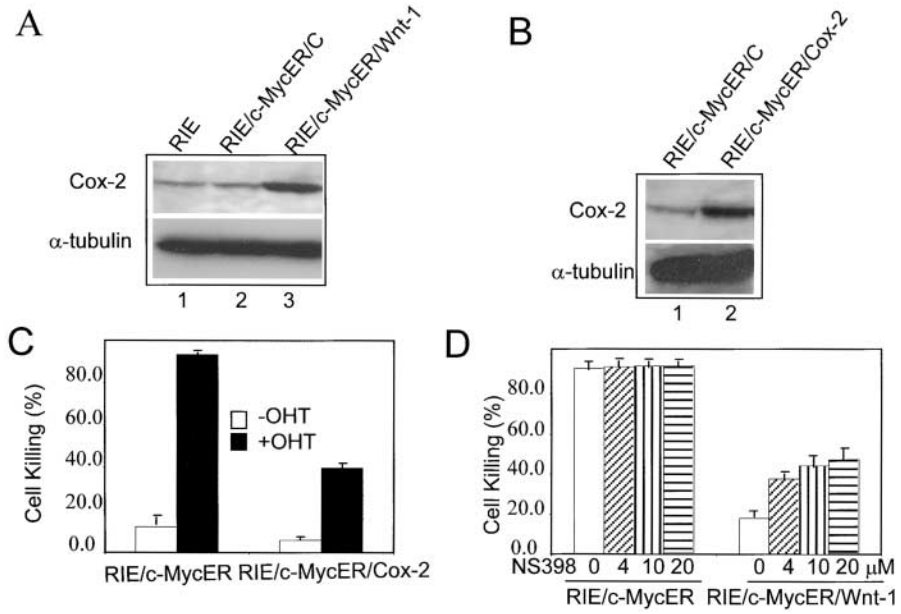

Figure 6. **Wnt-1–inducible gene Cox-2 suppresses c-Myc–induced apoptosis.** (A) Induction of Cox-2 by Wnt-1. The whole-cell extracts were prepared and 50 μg of aliquots of proteins were probed with polyclonal antibodies against Cox-2 (top). For the internal controls, the blots were stripped and reprobed with monoclonal antibodies against α-tubulin. (B) Establishment of RIE/c-MycER cells expressing Cox-2. Cells were transduced with retroviruses encoding the human Cox-2 expression vector and a control vector. The ectopic expression of Cox-2 was detected by Western blot analysis. (C) Cox-2 partially suppressed c-Myc–induced cell death. Cell viability was performed as described in Fig. 2 B. (D) NS-398, a specific Cox-2 inhibitor, partially aborted Wnt-1–mediated antiapoptosis. Cells were treated with OHT and the indicated concentrations of NS-398. Cell viability was performed as described in Fig. 2 B. The assay was performed in triplicate and the results represent three independent experiments.

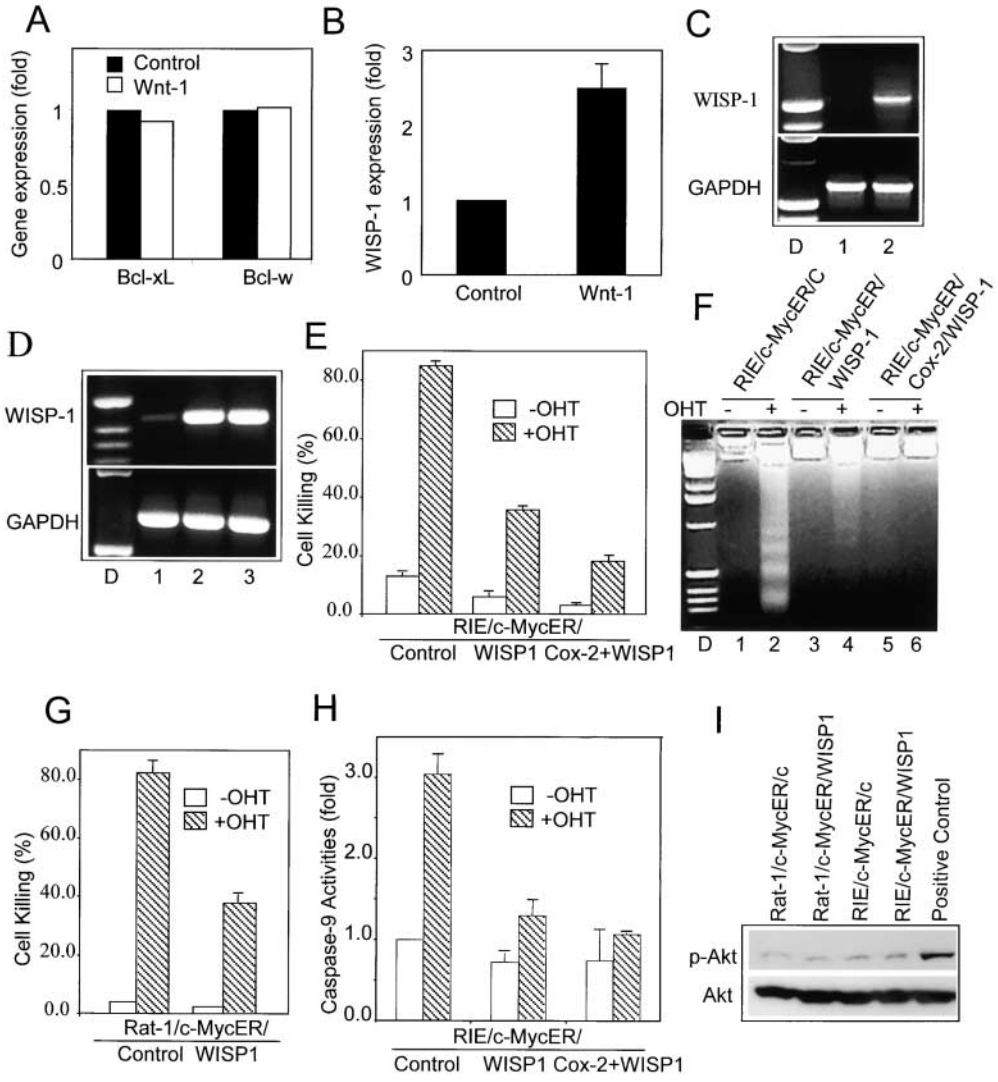

Figure 7. **Wnt-1–inducible gene WISP-1 suppresses c-Myc–induced apoptosis.** (A) Both Bcl-x_L and Bcl-w were not induced by Wnt-1, as detected by the microarray chips. The total RNA was prepared from cells expressing Wnt-1 or control cells. The profile of gene expression was systemically analyzed with Affymetrix U94A microarray chips. (B) The gene expression of WISP-1 was induced by Wnt-1. The level of WISP-1 mRNA is presented as fold induction by comparing cells expressing Wnt-1 with control cells. (C) The detection of WISP-1 expression by RT-PCR. The total RNA was isolated and 0.5 μg of total RNA from each sample was amplified by RT-PCR with specific primers for mouse *WISP-1*. The expression of GAPDH mRNA in each sample was measured as an internal control for RT-PCR. D, 1 kb DNA ladder; lane 1, RIE/c-MycER/C cells; lane 2, RIE/c-MycER/Wnt-1. (D) Establishment of RIE/c-MycER cells expressing WISP-1. Cells were transduced with retroviruses encoding the human WISP-1 expression vector and a control vector. The ectopic expression of human WISP-1 was detected with the specific primers for human WISP-1 by RT-PCR. Parallel amplification of GAPDH served as an internal control. D, 1 kb DNA ladder; lane 1, RIE/c-MycER cells expressing the control vector; lane 2, RIE-c-MycER cells expressing human WISP-1; lane 3, RIE-c-MycER cells expressing human WISP-1 and Cox-2. (E) WISP-1 potently suppressed c-Myc–induced cell death in RIE cells. Cell viability was performed as described in Fig. 2 B. (F) WISP-1 potently inhibited DNA fragmentation. Cells were treated with OHT for 48 h, and DNA fragmentation analysis was performed as described in Fig. 2 C. (G) WISP-1 inhibited c-Myc–induced cell death in Rat-1 cells. Rat-1 cells were transduced with retroviruses encoding the human WISP-1 expression vector and a control vector. The ectopic expression of human WISP-1 was confirmed as described in (D). Cell viability was performed as described in Fig. 1 B. (H) WISP-1 suppressed c-Myc-induced caspase-9 activation. Cells were treated with OHT for 36 h and the attached and detached cells were harvested. The whole cell protein extracts were prepared. The caspase-9 assay was performed as described in Fig. 2 D. (I) WISP-1 did not activate Akt in Rat-1 and RIE cells. The whole cell extracts were incubated with polyclonal antibodies against phospho-specific Akt (1:1,000). For internal controls, the blots were stripped and reprobed with polyclonal antibodies against Akt (1:1,000).

To explore more fully the nature of Wnt gene regulation in our model, a gene array analysis was performed. Consistent with the Western blot analysis discussed above, the array analysis demonstrated that antiapoptotic genes such as Bcl-x_L and Bcl-w were not regulated by Wnt-1 (Fig. 7 A). Interestingly, a recently cloned Wnt/β-catenin target gene, WISP-1 (Pennica et al., 1998; Xu et al., 2000), was identified by this analysis (Fig. 7 B). Previous characterization of the WISP-1 promoter showed that WISP-1 is transcriptionally induced by Wnt-1/β-catenin, but is independent of Tcf-mediated transcription (Xu et al., 2000). In addition, examination of 25 human colon adenocarcinomas revealed overexpression of

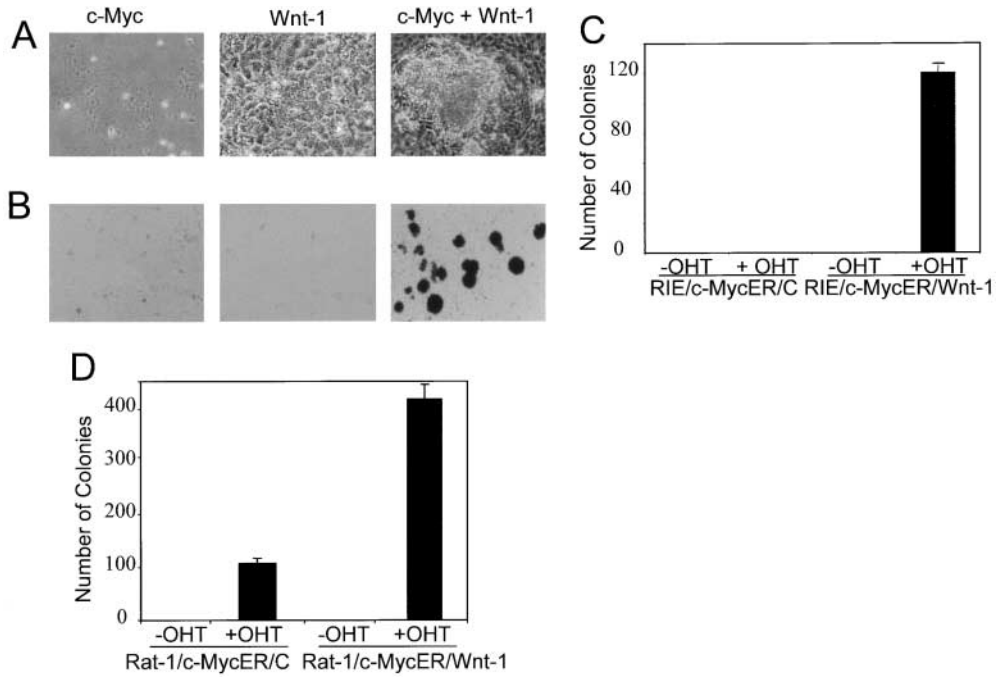

Figure 8. **Wnt-1 cooperates with c-Myc in cell transformation.** (A) Induction of focus formation by coexpression of Wnt-1 and c-Myc. RIE/c-MycER cells were transduced with retroviruses encoding the Wnt-1 expression vector or a control vector. Cells were untreated or treated with OHT for activation of c-Myc and selected with hygromycin (600 μg/ml) for three weeks. No foci were formed in cells expressing Wnt-1 or c-Myc alone. c-Myc, RIE/c-MycER cells treated with OHT; Wnt-1, RIE/c-MycER/Wnt-1 cells treated with the vehicle control; c-Myc+Wnt-1, RIE/c-MycER/Wnt-1 cells treated with OHT. (B) The photograph of colonies formed in soft agar. Both RIE/c-MycER/C cells and RIE/c-MycER/Wnt-1 cells (5×10^3 cells each) were plated in 0.3% agar medium over 0.6% agar medium underlayers in six-well plates. Three weeks after without or with the OHT induction, the plates were stained with methylthiazol tetrazolium (MTT) and photographed. (C) Wnt-1 coordinated with c-Myc in cell transformation. The assay was performed in triplicate and the results represent the mean values from three independent experiments. (D) Wnt-1 cooperated with c-Myc in cell transformation in Rat-1 cells. Both Rat-1/c-MycER/C cells and Rat-1/c-MycER/Wnt-1 cells (2×10^3 cells each) were used for the soft agar assay as described in (B).

WISP-1 mRNA (Pennica et al., 1998). Because most human colon cancers have elevated levels of β-catenin either caused by the loss of function of APC mutations or by stabilizing β-catenin mutations (Miller et al., 1999; Bienz and Clevers, 2000; Peifer and Polakis, 2000; Polakis, 2000), the elevated WISP-1 mRNA expression in vivo is likely to result from enhanced β-catenin–mediated transcription. As shown in Fig. 7 C, RT-PCR analysis confirmed that WISP-1 was induced by Wnt-1 in RIE cells. Similar results were obtained in Rat-1 cells (unpublished data). Because the ectopic expression of WISP-1 has been shown to induce tumorigenesis in normal rat kidney fibroblast cells (Xu et al., 2000), we hypothesized that WISP-1 had an antiapoptotic function. WISP-1 was stably transduced into RIE/c-MycER cells with retroviruses. The ectopic expression of WISP-1 was detected by RT-PCR (Fig. 7 D). As shown in Fig. 7 E, after the addition of OHT, ~85% of RIE/c-MycER cells were dead, compared with only 40% of the cells expressing WISP-1. The results were also confirmed with DNA fragmentation analysis (Fig. 7 F). Ectopic expression of WISP-1 also potently inhibited c-Myc-induced apoptosis in Rat-1 cells (Fig. 7 G). To determine whether Cox-2 and WISP-1 could cooperatively inhibit c-Myc–induced apoptosis, both Cox-2 and WISP-1 were stably coexpressed in RIE/c-MycER cells. As shown in Fig. 7, E and F, coexpression of Cox-2 and WISP-1 more efficiently inhibited c-Myc–induced apoptosis than either WISP-1 or Cox-2 alone. Moreover, coexpression of Cox-2 and WISP-1 suppressed the release of cytochrome c (Fig. 3 B) and caspase-9 activation (Fig. 7 H) induced by c-Myc. During the preparation of this manuscript, Su et al. (2002) reported that WISP-1 suppressed p53-mediated apoptosis through activation of Akt. Rat-1 or RIE cells express wild-type p53 and earlier work demonstrated that c-Myc–mediated apoptosis, partially dependent on p53, was inhibited by Akt (Kauffmann-Zeh et al., 1997). Based on these findings, we examined whether WISP-1 activated Akt in Rat-1 or RIE cells. As shown in Fig. 7 I, overexpression of WISP-1 did not induce Akt phosphorylation, suggesting that WISP-1–mediated antiapoptosis was independent of Akt signaling in these cells.

Wnt signaling cooperates with c-Myc in oncogenic transformation by inhibiting apoptosis

Studies by He et al. (1998) demonstrated that the expression of c-Myc was dependent on the APC/β-catenin/Tcf pathway in transformed colorectal cancer cells. Because c-Myc expression is tightly linked to the constitutive activation of β-catenin/Tcf transcription (He et al., 1998), it is difficult to assess the biological role of Wnt-mediated prosurvival in cell transformation. In Rat-1 or RIE cells, we have been unable to detect Wnt-1–mediated induction of c-Myc expression. Considering that several oncogenes including Ras and Bcl-2 have been shown to functionally cooperate with c-Myc in oncogenic transforma-

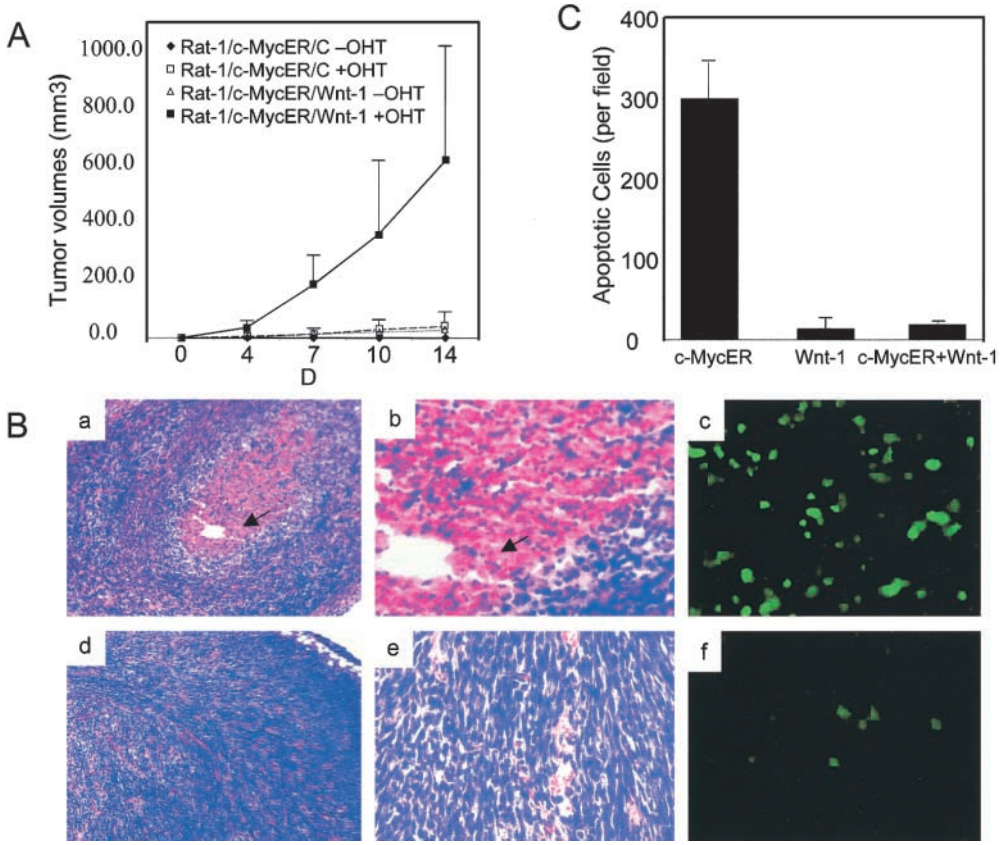

Figure 9. **Wnt-1 cooperates with c-Myc to induce tumor formation and progression by inhibiting apoptosis in vivo.** (A) Coexpression of Wnt-1 and c-Myc stimulated rapid tumor growth in nude mice. Rat-1/c-MycER/C or Rat-1/c-MycER/Wnt-1 cells were injected subcutaneously into the back of athymic nude mice. Animals were treated with the intraperitoneal injection of OHT or vehicle control every two days for two weeks. None of the nude mice injected with Rat-1/c-MycER/C developed tumors after administration of vehicle control. The experiments were performed twice and a total of 40 mice were used. (B) Histological examination of tumors. Tumors were dissected and subjected to HE staining or TUNEL assay. A mass of dead tissue (arrow) was present in the tumor derived from cells expressing c-Myc (a, low magnification; b, high magnification). No tissue breakdown was found in the tumor derived from cells expressing c-Myc and Wnt-1 (d, low magnification; e, high magnification). c, TUNEL-positive cells in the tumor derived from cells expressing c-Myc; f, TUNEL-positive cells in the tumor derived from cells expressing c-Myc and Wnt-1. (C) Wnt-1 inhibited c-Myc-mediated apoptosis in vivo. The sections were examined under a fluorescence microscope and the TUNEL-positive cells were counted and averaged from three fields per sample. The results represent average values from five tumors from each group.

tion (Land et al., 1982; Bissonnette et al., 1992; Cory et al., 1999), an assessment was made whether Wnt-1 coordinated with c-Myc in oncogenic transformation by inhibiting apoptosis. Coexpression of Wnt-1 and c-Myc together, but not alone, induced RIE cells to form foci in a monolayer culture (Fig. 8 A). To further delineate this cooperative function, we performed soft agar assays to measure anchorage-independent growth that typically correlates with a tumorigenic phenotype (Land et al., 1982; Orford et al., 1999). Consistent with above focus formation assays, expression of either c-Myc or Wnt-1 alone was not sufficient to induce colony formation. In contrast, substantial colony formation was seen upon coexpression of both c-Myc and Wnt-1 in RIE cells (Fig. 8, B and C). In the Rat-1 cells, Wnt-1 expression alone could not induce formation of colonies, and activation of c-Myc had a background level of colony formation. However, coexpression of c-Myc and Wnt-1 produced four times more colonies in soft agar than the induction of c-Myc alone (Fig. 8 D).

To determine whether Wnt-1 cooperated with c-Myc in tumor development or progression, we injected both Rat-1/c-MycER/C cells and Rat-1/c-MycER/Wnt-1 cells subcutaneously into nude mice. Animals were treated by intraperitoneal injection of OHT or vehicle control every two days, respectively. In the absence of OHT treatment, Rat-1/c-MycER/C cells were unable to form tumors, whereas only small tumors were observed 2 wk after injections of Rat-1/c-MycER/Wnt-1 cells, most likely resulting from leaky expression of the c-Myc transgene. Inducible expression of c-Myc with OHT treatment also caused small tumors to develop from Rat-1/c-MycER/C cells. In sharp contrast, mice injected with Rat-1/c-MycER/Wnt-1 cells had a rapid tumor growth and progression in which tumors were visible 4–5 d after OHT stimulation (Fig. 9 A). Western blot analysis confirmed that tumors expressed c-Myc or both c-Myc and Wnt-1, respectively (unpublished data). To investigate the molecular mechanisms that underlie the synergetic action between c-Myc and Wnt-1, tumors were subjected to pathological analyses. Interestingly, hematoxylin-eosin staining revealed that a mass of dead tissue was frequently present in c-Myc–induced tumors (Fig. 9 B, panel a and b), but not in c-Myc– and Wnt-1–induced

tumors (Fig. 9, panel d and e). In addition, the deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assays found that c-Myc–induced tumors contained widespread apoptotic cells (Fig. 9, B, panel c, and C). In contrast, relatively few TUNEL-positive cells were monitored in c-Myc– and Wnt-1–induced tumors (Fig. 9, B, panel f, and C). In summary, these results suggest that Wnt-1 is capable of promoting tumor growth and progression by inhibiting c-Myc–induced apoptosis in vivo.

Discussion

Because c-Myc was identified as a target gene of the APC/β-catenin/Tcf pathway, it has been hypothesized that Wnt signaling may inhibit c-Myc–mediated apoptosis (Miller et al., 1999; Peifer and Polakis, 2000; Polakis, 2000). In this report, we provide the first proof that Wnt signaling inhibits c-Myc–induced apoptosis and uncover two effectors of the Wnt antiapoptotic signal. The first is the Cox-2 gene, which is somewhat not surprising since Cox-2 was reported to maintain the ability suppress apoptosis (Tsujii et al., 1998). The second effector is WISP-1, a novel Wnt-1/β-catenin–regulated gene. WISP-1 was very recently reported to protect cells from p53-mediated apoptosis, a mechanism involving the activation of the Akt survival pathway (Su et al., 2002). Although Rat-1 and RIE cells express wild-type p53, we were unable to detect activated Akt in these cells in response to WISP-1 signaling, which indicates that WISP-1 functions in multiple antiapoptotic pathways. Previously, WISP-1 was found to promote cell growth in low serum condition and cells expressing WISP-1 developed prominent tumors in nude mice, but the mechanisms of this regulation are unknown. Consistent with its antiapoptotic function, WISP-1 was found to promote c-Myc–mediated cell transformation (unpublished data). Considering that WISP-1 is highly expressed in colorectal carcinoma cells in vivo (Pennica et al., 1998; Xu et al., 2000), our results support the notion that WISP-1 is an additional gene target that plays critical roles in tumorigenesis by regulating apoptosis.

Recently, several studies demonstrated that overexpression of β-catenin leads to accumulation and activation of p53 tumor suppressor protein (Damalas et al., 1999, 2001). The accumulation of p53 was due to induction of expression of an alternative reading frame (ARF) product of the INK4A tumor suppressor locus (Damalas et al., 2001). Overexpression of β-catenin resulted in a senescence-like phenotype in normal primary mouse embryo fibroblasts, which was dependent on both ARF and p53 function. However, these studies did not report that apoptosis was induced following the induction of both ARF and p53. The results suggested that β-catenin probably activated cell survival pathways to suppress the proapoptotic effects of both ARF and p53 (Zindy et al., 1998). In future studies, it will be important to determine whether WISP-1 and/or Cox-2 can inhibit ARF-mediated apoptosis.

We demonstrated here that the Wnt antiapoptotic function promotes c-Myc–mediated oncogenic transformation in vitro and in vivo. Considering that c-Myc is a potent activator of apoptosis, these results may provide an explanation for high levels of both c-Myc and β-catenin found in many human cancer cells, such as colorectal cancers and hepatocellular carcinoma (Amati and Land, 1994; de la Coste et al., 1998; Miller et al., 1999; Schreiber-Agus and DePinho, 1998; Harada et al., 1999; Obaya et al., 1999; Bienz and Clevers, 2000). Cancer cells with the constitutive activation of Wnt/β-catenin signaling may tolerate the apoptotic function of c-Myc. Another significant implication of our study is a rationale for the molecular basis for loss of APC at the early stage of colon cancer (Morin et al., 1995; Bienz and Clevers, 2000; Polakis, 2000; Shih et al., 2000). Activation of the Wnt signaling pathway by loss of APC function may have dual effects on cell transformation or tumor progression. One is to promote cell growth and proliferation by induction of the growth promoting genes such as cyclin D1 and c-Myc (He et al., 1998; Tetsu and McCormick, 1999). A second is to provide the antiapoptotic function to suppress oncogenic activation-mediated apoptosis. In other words, inhibition of apoptosis by the Wnt/β-catenin signal pathway may render cells more susceptible to the actions of oncogenes.

Materials and methods

Cell culture, plasmids, and retroviral infection

Rat-1 and RIE cell lines were maintained in DME supplemented with 10% FBS. A retrovirus pLNCX vector encoding Wnt-1, Wnt-2, Wnt-3, Wnt-4, or Wnt-5a, and a retrovirus pBabe-c-MycER vector were utilized in our experiments, as described previously (Hueber et al., 1997; Shimizu et al., 1997). A retrovirus pBabe vector encoding the full-length human WISP-1 cDNA was provided by Dr. Arnold Levine (Rockefeller University, New York, NY) (Xu et al., 2000). The human full-length Cox-2 cDNA was provided by Dr. Leslie Crofford (The University of Michigan, Ann Arbor, MI) and subcloned into a retrovirus pLNCX vector. To establish cell lines stably expressing Wnt protein, the high-titer retroviruses were utilized, allowing whole populations of cells to be examined with minimal expansion in culture. Retroviruses were generated by transfecting the retroviral constructs into 293T cells and retrovirus-containing supernatant was collected and stored at –70°C. Cells were infected with retroviruses in the presence of 6 μg/ml polybrene. 48 h after infection, cells were selected with puromycin (1.5 μg/ml), hygromycin (600 μg/ml), or neomycin (600 μg/ml) for 1 wk, respectively. The resistant clones were pooled and confirmed by the Western blot analysis.

Cell fractionation and Western blot analysis

The cell fractionations were performed as described previously (Shimizu et al., 1997). The proteins were separated in a 10% SDS-PAGE and transferred to PVDF membrane with a semidry transferrer apparatus (Bio-Rad Laboratories). The membranes were probed with monoclonal antibodies against β-catenin (1:2,000; Transduction Laboratory), c-Myc (1:1000; Santa Cruz), or Cox-2 (1:500; Santa Cruz Biotechnology). For internal controls, the blots were stripped and reprobed with monoclonal antibodies against α-tubulin (1:7,500; Sigma-Aldrich).

Cell viability, caspase activity assay, and DNA fragmentation

Cells were untreated or treated with the synthetic steroid OHT (100 nM) for 48–72 h. The detached and attached cells were collected and cell viability was determined by trypan blue exclusion or Cell Death ELISA. To determine caspase-9 activity, cells were treated as described above. Cells were lysed in 200 μl of ice cold hypotonic lysis buffer. Approximately 200-μg aliquots of cell extracts were incubated with caspase-9 substrate, LEHD-NA (CLONTECH Laboratories, Inc). The samples were assessed with a plate reader by measuring the OD at 405 nm. To examine DNA laddering, the attached and detached cells were collected at the indicated time points after OHT treatment. DNA was isolated and separated on a 1.2% agarose gel as described previously (Wang et al., 1996).

Soft agar assay and tumor growth in vivo

To assess anchorage-independent growth, 10^3 Rat-1 cells or 5×10^3 RIE cells were plated in 0.3% low melting point agarose/growth medium onto six-well plates with a 0.6% agarose underlay. About 1.5 ml growth medium uncontaining or containing OHT (100 nM) was added on the top of agarose and the medium was changed every 3–4 d. To determine tumor growth, 5×10^6 Rat-1/c-Myc/C cells or Rat-1/c-MycER/Wnt-1 cells were injected subcutaneously into the back of 8-wk-old male nude mice (Taconic). Animals were injected intraperitoneally with OHT (2 mg/

mouse) or vehicle control every 2 d for 2 wk, respectively. These procedures were approved by the University of Michigan Committee on Use and Care of Animals (Ann Arbor, MI).

Histology and TUNEL staining

Animals were killed by asphyxiation in a CO_2 chamber. The tumors were dissected, fixed in 10% formalin, and embedded in paraffin blocks. The specimens were cut into 4-μm thick sections and stained with hematoxylin and eosin. For TUNEL staining, the sections were dewaxed, rehydrated, and treated with proteinase K. The labeling was performed according to the manufacturer's instruction (Roche).

Immunofluorescence staining.

RIE cells were seeded in 12-well plates the day before stimulation and then treated with OHT for 36 h. Cells were fixed with 4% paraformaldehyde and washed with PBS. After blocking with normal goat serum for 1 h, the cells were incubated with primary monoclonal antibodies against cytochrome c for 1 h, and immunocomplexes were detected with a fluorescein isothiocyanate-conjugated secondary antibody against mouse IgG. The results were photographed by a fluorescence microscope using a filter set for fluorescein isothiocyanate (You et al., 2001).

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