WISP-1 is a Wnt-1- and β -catenin-responsive oncogene

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WISP-1 (Wnt-1 induced secreted protein 1) is a member of the CCN family of growth factors. This study identifies *WISP-1* as a β -catenin-regulated gene that can contribute to tumorigenesis. The promoter of *WISP-1* was cloned and shown to be activated by both Wnt-1 and β -catenin expression. TCF/LEF sites played a minor role, whereas the CREB site played an important role in this transcriptional activation. *WISP-1* demonstrated oncogenic activities; overexpression of *WISP-1* in normal rat kidney fibroblast cells (NRK-49F) induced morphological transformation, accelerated cell growth, and enhanced saturation density. Although these cells did not acquire anchorage-independent growth in soft agar, they readily formed tumors in nude mice, suggesting that appropriate cellular attachment is important for signaling oncogenic events downstream of *WISP-1*.

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The Wnt/Wingless signaling pathway has been extensively studied in the past several years (Kinzler and Vogelstein 1996; Cadigan and Nusse 1997). A key player in the Wnt/Wingless signaling cascade is β -catenin, whose concentration increases in response to the Wnt-1 signal. Cytoplasmic levels of β -catenin are regulated strictly by its interactions with the adenomatous polyposis coli (APC) (Munemitsu et al. 1995), axin (Zeng et al. 1997; Behrens et al. 1998) and GSK-3 β (Rubinfeld et al. 1996; Yost et al. 1996) proteins. Failure of this regulatory pathway results in stabilization and subsequent accumulation of β -catenin in the cytoplasm. The elevated levels of β -catenin in the cytoplasm promote its interactions with members of the TCF/LEF DNA-binding family (Korinek et al. 1997; Morin et al. 1997; Willert and Nusse 1998). The bipartite complexes of β -catenin and TCF/LEF translocate to the nucleus and activate transcription of downstream genes (van de Wetering et al. 1997). Because the interaction between β -catenin and TCF/LEF family members and subsequent transcriptional activation cannot account for the diverse developmental processes regulated by the Wnt/Wingless signaling pathway, there are probably other Wnt/Wingless and β -catenin-responsive transcription factors. It has been shown recently that stabilized *armadillo*, the β -catenin homolog in *Drosophila*, can also interact with the zinc finger protein Teashirt, which promotes *teashirt* phosphorylation and

accumulation in the nucleus in response to Wingless signaling (Gallet et al. 1998, 1999).

The Wnt/Wingless signaling pathway is not only developmentally important but is also implicated in multiple cancers. One member of the Wnt family, *Wnt-1*, was originally identified as an oncogene activated by the insertion of mouse mammary tumor virus in virus-induced mammary adenocarcinomas (Nusse et al. 1984). Chain-termination mutations of APC occur in most human colon carcinomas (Polakis 1997), and mutations that stabilize β -catenin have been discovered in colon carcinomas, melanomas, pilomatricomas, and hepatocellular carcinomas (Morin et al. 1997; Rubinfeld et al. 1997; de La Coste et al. 1998; Chan et al. 1999). Mutations of these Wnt/Wingless signaling pathway components all have the same consequence, the accumulation of β -catenin in the cytoplasm. It is believed that through an interaction with DNA-binding factors, increased levels of β -catenin induce uncontrolled activation of downstream genes, some of which might be involved in tumorigenesis (Gumbiner 1997; Nusse 1997; Peifer 1997). *Cyclin D1* and *c-myc* are two oncogenes that have recently been suggested to be regulated by β -catenin (He et al. 1998; Shtutman et al. 1999; Tetsu and McCormick 1999).

To identify other downstream genes in the Wnt/Wingless signaling pathway, especially those that play a role in tumor development and progression, suppression subtractive hybridization was carried out between the mouse mammary epithelial cell line C57MG and *Wnt-1*-expressing C57MG cells. The C57MG cells were chosen because they can be partially transformed by *Wnt-1*

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expression (Mason et al. 1992). Wnt-1 induced secreted protein 1 (*WISP-1*) was identified as one of the differentially expressed genes (Pennica et al. 1998). Sequence analysis revealed that *WISP-1* shares high sequence homology with the CCN family of growth factors, a family that has been implicated in playing a role in tumorigenesis (Lau and Lam 1999). This family is composed of CTGF (connective tissue growth factor), Cyr-61 (cysteine-rich 61), and nov (nephroblastoma overexpressed) (Brigstock 1999). CTGF is a downstream effector of TGF- β (Igarashi et al. 1993; Grotendorst et al. 1996; Kothapalli et al. 1997), and it can stimulate cell growth, up-regulate extracellular matrix protein expression, and induce angiogenesis (Frazier et al. 1996; Babic et al. 1999; Shimo et al. 1999). Cyr-61 is an extracellular matrix-associated signaling protein that can augment growth factor-induced DNA synthesis, stimulate cell proliferation, and promote angiogenesis and tumor growth (Kireeva et al. 1996; Babic et al. 1998). *nov* was originally identified at an integration site of the myeloblastosis associated virus (MAV-1). It is considered to be a proto-oncogene because an amino-terminal-truncated *nov* induces morphological transformation when expressed in chicken and rat embryonic fibroblasts (Joliot et al. 1992).

In this study, the promoter of the *WISP-1* gene was isolated and *WISP-1* was identified as a target gene regulated by Wnt-1 and β -catenin. A CREB-binding site was shown to be a novel site mediating the activation of

WISP-1 by β -catenin. Furthermore, characterization of the oncogenic activity of *WISP-1* demonstrated that *WISP-1* might contribute to β -catenin-mediated tumorigenesis.

Results

Construction of β -catenin mutants

β -catenin is not only a transcriptional activator in the Wnt signaling pathway, but also a component of adherens junctions (Miller and Moon 1996). To study domains required for activation of β -catenin downstream genes, as well as the effect of β -catenin subcellular localization on the activation of these genes, four β -catenin mutants were constructed (Fig. 1A). Mutant 4145 had both Thr-41 and Ser-45 at the amino-terminal GSK-3 β phosphorylation site (Yost et al. 1996) changed to alanines. These changes resulted in a more stable form of β -catenin as demonstrated by pulse-chase analysis (data not shown). Mutant 4145TV not only had the above mutations, but also had an additional double-point mutation that changed Thr-120 and Val-122 to alanines at the α -catenin-binding region (Aberle et al. 1996). These mutations abrogated the binding of β -catenin to α -catenin, as demonstrated by immunoprecipitation analysis (Fig. 1B). Mutant nuclear localization signals (NLSs) had an SV40 large T antigen NLS (Boulikas 1993) cloned

Figure 1. (A) Schematic of β -catenin mutants. (Stippled boxes) The armadillo repeats. (B) Immunoprecipitation of β -catenin mutants in C57MG cells. Myc-tagged β -catenin mutants were transfected into C57MG cells. Cells were then metabolically labeled with 35 S-methionine for 5 hr and transfected β -catenin was immunoprecipitated with anti-myc antibody 9E10 (CalBiochem). Signals were visualized by autoradiography. (C) Mutant NLSs localized in the nucleus instead of cytoplasm. Mutant 4145 (a) and mutant NLS (b) were transfected into 293 cells. Immunofluorescence staining was performed with anti-myc antibody as the first antibody, followed by sequential incubation with biotinylated goat anti-mouse IgG and FITC-conjugated streptavidin (Jackson ImmunoResearch Laboratories). (D) Response of TopFlash reporter to β -catenin mutants. The 293 cell were transfected with 0.5 μ g of TopFlash reporter together with 0.5 μ g of empty pCS2/MT vector, or different β -catenin mutants. A total of 1 μ g of pCS2/MT plasmid DNA was used to make up the total DNA to 2 μ g; 0.1 μ g of CMV- β -gal was cotransfected to normalize transfection efficiency.

in-frame at the amino terminus of 4145TV mutant of β -catenin. It was localized to the nucleus as shown by immunofluorescence staining when transfected into human embryonic kidney 293 cells (Fig. 1C). Mutant D(C) introduced a stop codon at the carboxy-terminal region of mutant 4145 and resulted in a protein product that was 88 amino acids shorter than wild type. Each of these mutants were transfected into 293 cells together with a TopFlash luciferase reporter (which measures β -catenin transcriptional activity; Korinek et al. 1997) to test their activity on a known downstream promoter element (Fig. 1D). Mutant 4145, 4145TV, and NLSs were able to induce the TopFlash reporter with comparable high levels. Compared with stabilized β -catenin, Mutant D(C) was reduced in its ability to induce the TopFlash reporter because it lacked the transcriptional activation domain (van de Wetering et al. 1997).

Elevation of *WISP-1* mRNA in C57MG cells expressing mutant β -catenin

WISP-1 was identified as one of the genes that was differentially expressed between parental C57MG cells and *Wnt-1*-expressing C57MG cells (Pennica et al. 1998). Because β -catenin is downstream of *Wnt-1* in the *Wnt* signaling pathway, it is possible that *Wnt-1* modulates *WISP-1* transcription through β -catenin. To investigate the effect of β -catenin overexpression on the endogenous *WISP-1* gene, C57MG cell lines expressing either *Wnt-1* or stabilized β -catenin mutants were generated. C57MG cells were chosen because it was shown that *Wnt-1* up-regulates the expression of *WISP-1* in these cells (Pen-

nica et al. 1998). Northern blot analysis showed that *WISP-1* mRNA was at much higher levels in C57MG cells expressing *Wnt-1*, stabilized β -catenin mutants 4145 or 4145TV (about fourfold higher than control cells), when compared with the C57MG parental cells (Fig. 2A).

WISP-1 is a downstream target gene of *Wnt-1* and β -catenin

To determine whether *Wnt-1* and β -catenin regulate the transcription of the *WISP-1* gene, a 5.9-kb human *WISP-1* promoter fragment was isolated and cloned upstream of the pGL2 basic luciferase reporter. A co-culture experiment in which *Wnt-1* protein was provided in a paracrine manner was carried out to determine whether *Wnt-1* signaling directly regulates transcriptional activation of the *WISP-1* promoter. Pools of C57MG cells transfected with the empty pGL2 basic vector (pGL2B), the *WISP-1* promoter pGL2 basic luciferase construct (pGL2B-WP), or the TopFlash luciferase reporter were co-cultured with either the parental quail fibrosarcoma QT6 cells or *Wnt-1* constitutively expressing QTWnt-1 cells (Parkin et al. 1993). Luciferase activity was measured 48 hr later. The *WISP-1* luciferase reporter was induced in response to the *Wnt-1* signal, and the induction level was almost comparable with that of the TopFlash reporter (Fig. 2B). To determine whether the activation of the *WISP-1* promoter by *Wnt-1* was mediated by β -catenin, the *WISP-1* luciferase reporter was cotransfected into 293 cells with different dosages of β -catenin mutant plasmids and luciferase analysis was

Figure 2. (A) Northern blot analysis of *WISP-1* RNA in C57MG cells expressing mutant β -catenin or *Wnt-1*. Total RNA (10 μ g) from the cells was loaded onto each lane. The blot was hybridized with a full-length mouse *WISP-1* probe. For loading control, the blot was rehybridized with a probe for mouse *GAPDH* gene. (B) Response of the *WISP-1* promoter luciferase reporter (pGL2B-WP, *WISP-1* sequence -5882 ~ -42, translation start site as +1) to *Wnt-1*. C57MG cells were cotransfected with 1 μ g of pGK-hygro and 10 μ g of pGL2B, pGL2B-WP, or TopFlash. Two days after transfection, hygromycin was added to the medium for selection. Resistant colonies were pooled as stable cell lines. In the coculture experiments, QT6 (solid bars) and QTWnt-1 (open bars) cells were plated on 10-cm plates at a density so that after 48 hr they would be ~40% confluent. The sample numbers of pGL2B,

pGL2B-WP, or TopFlash-transfected C57MG cells were then plated on tissue culture dishes containing either QT6 or QTWnt-1 cells. Luciferase analyses were carried out 48 hr after coculture. (C) *WISP-1* promoter response to increasing amounts of β -catenin. The 293 cell were transfected with 0.5 μ g, 1.0 μ g, or 1.5 μ g of empty pCS2/MT vector or different β -catenin mutants together with 0.5 μ g of pGL2B-WP. A total of 0.1 μ g of CMV- β -gal reporter was cotransfected with each sample to normalize transfection efficiency. Empty pCS2/MT vector was added to transfections to make a total of 2 μ g of DNA. Luciferase analysis was carried out 60 hr after transfection. All luciferase measurements are expressed as means $\pm$ S.D. of triplicate cultures.

carried out. The 293 cells were chosen because they had much higher transfection efficiency than C57MG cells and showed a much more sensitive response to β -catenin in TopFlash reporter assays (data not shown). Figure 2C shows that the *WISP-1* luciferase reporter was strongly transcribed in response to β -catenin, especially stabilized β -catenin mutants 4145 and 4145TV, in a dose-dependent manner. Adding TCF to the transfection does not further increase the activation of the reporter (data not shown). The carboxyl terminus of β -catenin appeared to be important in mediating this activation, as the D(C) mutant was significantly reduced in its ability to activate the *WISP-1* reporter. Interestingly, we also observed that although mutant NLS was able to induce TopFlash luciferase reporter to comparable levels as 4145 and 4145TV, it failed to induce the *WISP-1* luciferase reporter in this analysis, suggesting that cytoplasmic

accumulation of β -catenin is important for activating the *WISP-1* promoter.

The role of TCF/LEF sites and the CREB site in the activation of the WISP-1 promoter by β -catenin

DNA-binding factors of the TCF/LEF family are known to interact with β -catenin and activate many downstream genes (Behrens et al. 1996; Molenaar et al. 1996). Therefore, a sequence analysis was carried out with the *WISP-1* promoter to search for potential TCF/LEF-binding consensus sequences. As depicted in Figure 3A, five putative TCF/LEF-binding sites were found in the *WISP-1* promoter. Sites 1, 2, and 5 are imperfect matches to the consensus TCF/LEF-binding sequence that differ from the TCF/LEF core consensus sequence 5'-CTTTGA/TA/T-3' at the 3' most base pair. Sites 3 and 4, on the

Figure 3. (A) Schematic representation of *WISP-1* promoter pGL2 basic luciferase reporter constructs and deletion mutants. Putative TCF/LEF (stippled boxes) and CREB (black boxes) binding sites are indicated. Their sequences are as follows; site 1 -4882 5'-CACAAAG-3' -4876, site 2 -4677 5'-CTCAAAG-3' -4671, site 3 -2978 5'-CTTTGAT-3' -2972, site 4 -2546 5'-CTTTGTT-3' -2540, site 5 -461 5'-CTCAAAG-3' -455, CREB site -155 5'-TGACGTCA-3' -148. (B) Response of *WISP-1* promoter deletion constructs to β -catenin. The 293 cells were transfected with 0.5 μ g of *WISP-1* promoter, different deletion constructs, or empty pGL2 basic luciferase reporters, together with 1 μ g of empty pCS2/MT vector, wild type, or 4145 β -catenin. A total of 0.1 μ g of CMV- β -gal was cotransfected to normalize transfection efficiency. (C) Schematic representation of Frag3 and TCF/LEF site mutation reporter constructs. Mutated sites are as indicated. Transfections were carried out as described in B. (D) Schematic representation of Frag3, multiple TCF/LEF site mutations, or CREB site mutation. Transfections were carried out as described in B.

other hand, conform to the consensus sequence. To identify the sequences in the *WISP-1* promoter that mediate transcriptional activation by β -catenin, a series of 5' promoter deletions were constructed (Fig. 3A). Each of these deletion constructs contained one less TCF/LEF consensus-binding site. Deletion mapping shows that deleting sequences containing TCF/LEF site 3 (Frag2), site 4 (Frag1), or site 5 (Frag0), but not site 1 (Frag4) and site 2 (Frag3), leads to consecutively reduced response of the *WISP-1* promoter reporter to β -catenin (Fig. 3B). However, these deletions are all large and not only eliminate the TCF/LEF sites but also delete long stretches of surrounding sequences. To investigate the functional significance of the three promoter proximal TCF/LEF sites for the activation of the *WISP-1* promoter, multiple point mutations were introduced into Frag3 that should render each of these sites inactive (Brannon et al. 1997) (Fig. 3C). Frag3 was chosen for further study because its response to β -catenin is comparable with that of the full-length promoter. Surprisingly, mutations in any of these sites did not significantly affect the response of the *WISP-1* promoter to β -catenin. Because functional redundancy of TCF/LEF sites had been reported (Brannon et al. 1997), multiple TCF/LEF sites were also eliminated by point mutations and their effect on the *WISP-1* promoter were tested. Knockout of multiple sites did not significantly affect the transactivation of the *WISP-1* promoter by β -catenin to any higher degree than the single-site mutations (Fig. 3D).

To further determine whether β -catenin activation of the *WISP-1* promoter depends on TCF, a dominant-negative form of TCF, DN-TCF (Korinek et al. 1997), was cotransfected into 293 cells with β -catenin. Consistent with the results of site-directed mutagenesis experiments, DN-TCF had no significant effect on the transactivation of the *WISP-1* promoter by β -catenin (Fig. 4A), although it significantly reduced the activation of the TopFlash reporter by β -catenin in the positive control experiment (Fig. 4B).

Sequence analysis showed that there was a CREB-binding site at the 3' end of the *WISP-1* promoter (Fig. 3A). A CREB site has been reported to collaborate with a LEF site to regulate the transcription of TCR- α (Giese et al. 1995). Therefore, elimination of the CREB site was carried out to evaluate the effect of this site on β -catenin-mediated transactivation. Surprisingly, knockout of the CREB site (Frag3-C1) not only reduced the basal activity of the *WISP-1* promoter, but also reduced the activation by β -catenin from 9- to 10-fold to ~5-fold (Fig. 3D). The transfections were repeated multiple times, each time in triplicate, and the CREB site elimination consistently reduced the response of the *WISP-1* promoter to β -catenin. Frag3 with a deletion of 150 bp around the CREB site also yielded similar results (data not shown). The combination of a CREB site mutation with TCF/LEF site mutations did not further reduce the response of the *WISP-1* promoter to β -catenin (data not shown), suggesting that CREB did not function through TCF/LEF.

To further investigate whether activation of the *WISP-1* promoter by β -catenin depends on CREB protein,

Figure 4. (A) Effects of dominant-negative TCF on the *WISP-1* promoter reporter. The 293 cells were transfected with 0.5 μ g of pCS2/MT, wild type, 4145, or 4145TV β -catenin, and 1.0 μ g of pcDNA3 (left) or DN-TCF (right), together with 0.5 μ g of *WISP-1* promoter pGL2 basic luciferase reporter. A total of 0.1 μ g of CMV- β -gal was cotransfected to normalize transfection efficiency. (B) Effects of dominant-negative TCF on the TopFlash reporter. Transfections were carried out as described in A except that 0.5 μ g of TopFlash reporter was used.

a dominant-negative form of CREB, KCREB (Walton et al. 1992), was cotransfected into 293 cells with β -catenin. As shown in Figure 5A, KCREB reduced β -catenin activation of the *WISP-1* promoter to approximately half of its original level. KCREB did not change the response of TopFlash to β -catenin in the control experiment (Fig. 5B), suggesting that it did not suppress general transcription. Thus, this study not only identified *WISP-1* as a downstream target gene transcriptionally activated by β -catenin, but also showed that TCF/LEF sites play only a minor role, whereas the CREB site plays an important role in mediating the observed β -catenin-dependent transcriptional activation of *WISP-1*.

The oncogenic activity of *WISP-1*

WISP-1 shares high-sequence homology with growth factors of the CCN family, which are involved in growth control, embryonic development, and tumor formation (Brigstock 1999). CTGF and Cyr-61, two members of the CCN family, have mitogenic effects on some cell lines (Frazier et al. 1996; Kireeva et al. 1996). Therefore, experiments were carried out to determine whether *WISP-1* could also act as a mitogen. Human *WISP-1* (hWISP-1) protein was expressed by a baculovirus vector,

Figure 5. (A) Effects of dominant-negative CREB on the *WISP-1* promoter reporter. The 293 cells were transfected with 0.5 μ g of pCS2/MT, wild type, 4145, or 4145TV β -catenin, and 1.0 μ g of pcDNA3 (left) or KCREB (right), together with 0.5 μ g of *WISP-1* promoter pGL2 basic luciferase reporter. A total of 0.1 μ g of CMV- β -gal was cotransfected to normalize transfection efficiency. (B) Effects of dominant-negative CREB on the TopFlash reporter. A total of 1.0 μ g of pcDNA3 (left) or KCREB (right) was used. Transfections were carried out as described in A except that 0.5 μ g of TopFlash reporter was used.

purified from infected cell cultures, and tested for its mitogenic activity on normal rat kidney fibroblast (NRK-49F) cells. Different concentrations of hWISP-1 protein were added to plated NRK-49F cells. A dose-dependent stimulation of thymidine incorporation was seen when cells were treated with a minimum concentration of 1.5 nM of hWISP-1 for 24 hr (Fig. 6), suggesting that *WISP-1* could promote cell proliferation. A three to fivefold increase in the rate of DNA synthesis was also seen in normal mouse lung (MLg) cells and mouse renal adenocarcinoma (RAG) cells (data not shown).

The ability of *WISP-1* to support long-term growth of cultured cells was then investigated. The *hWISP-1* cDNA was cloned into the retroviral expression vector pBabe-puro. Retroviruses were made with the BOSC23 packaging cell line (Pear et al. 1993) and used to infect several established mouse or rat cell lines. Puromycin was added to infected cultures and resistant clones ($>10^5$) were pooled to generate *hWISP-1*-expressing cell lines and vector-expressing control cell lines. We found that NRK-49F responded to *WISP-1* overexpression by undergoing changes in morphology and growth. Figure 7A shows the expression of endogenous *WISP-1* mRNA as well as the expression of infected *hWISP-1* mRNA in NRK cells. The biological responses to *WISP-1* overexpression in NRK cells were subsequently characterized.

Upon *WISP-1* overexpression, NRK cells exhibited progressive morphological changes; they packed more densely, adopted a more refractile appearance, and tended to line up in uniform direction (Fig. 7B). An examination of saturation density showed a threefold increase in cells/cm² on the culture dish surface compared with the control cells (data not shown). *WISP-1* overexpression also accelerated NRK cell growth. When cells were plated at a density of 1×10^5 in DMEM supplemented with 10% FBS, there was an approximately twofold difference in growth rate between NRK-*WISP1* and NRK-Vector cells. When grown in DMEM supplemented with 0.5% FBS, the difference in growth rate was more dramatic (Fig. 7C). Two *WISP-1*-expressing and two vector-expressing NRK cell lines were established via independent infections. Similar phenotypic alterations and growth rate accelerations were observed in both cases.

To assess the effect of *WISP-1* overexpression on the tumorigenicity of NRK cells, the ability of NRK-vector and NRK-*WISP1* cells to form colonies in soft agar, as well as their ability to form tumors in nude mice was evaluated. The NRK-*WISP1* cells from two independent infections were tested. Experiments to determine the anchorage-independent growth of these cells in soft agar demonstrated that although 293 control cells formed prominent colonies after 2 weeks, neither NRK-Vector nor NRK-*WISP1* cells (line 1 and line 2) formed colonies after 6 weeks (data not shown). To test the capability of these cells to form tumors in nude mice, they were injected subcutaneously into 8-week-old nude mice and tumor growth was monitored every other week. Tumors first became apparent in mice injected with NRK-*WISP1* cells (line 1 and line 2) 3 weeks after injection and grew rapidly. Twelve weeks after injection, all of the mice

Figure 6. Effect of *WISP-1* on DNA synthesis. NRK-49F cells were plated in 96-well plates at 3×10^4 in HGDMEM with 10% serum. Twenty-four hours after plating, the medium was changed to HGDMEM with 0.2% serum. *WISP-1* was serially diluted in fresh medium and added at the indicated concentrations in a total volume of 70 μ l/well. After 18 hr incubation at 37°C, 5 μ Ci/ml [³H]-thymidine was added for 5 hr. Cells were harvested onto a GF/C filter using Packard's 96-well Filtermate 196 and counted on a top count, microplate scintillation counter (Packard). Values are the means of triplicate wells. The experiment was repeated at least four times with similar results.

Figure 7. (A) Expression of *hWISP-1* mRNA in NRK-49F cells. NRK-49F cells were infected with retroviruses expressing empty pBabe-puro vector or pBabe-*hWISP-1* and selected with puromycin for resistant colonies. Stable colonies ($>10^5$) were pooled. Northern blot analysis was carried out with 10 μ g of total RNA. Full-length mouse *WISP-1* cDNA was used as a probe. The blot was rehybridized with a probe for mouse *GAPDH* gene. (B) *WISP-1* expression alters the morphology of NRK-49F fibroblasts. Phase contrast microscopy of polyclonal NRK-vector and NRK-*WISP1* fibroblasts cell lines. (1,2) NRK-vector and NRK-*WISP1* cells at the same passage number after infection, respectively. (C) *WISP-1* expression alters the growth of NRK-49F cells. NRK-vector ($\bullet$) and NRK-*WISP1* ($\blacktriangle$) cells (both are of passage 5) were seeded at low density ($10^5/10$ -cm plate) and monitored for growth. Cell numbers are presented as means $\pm$ S.D. of three plates.

injected with NRK-*WISP1* cells developed tumors (line 1 ~8–15 mm, line 2 ~14–28 mm in diameter), whereas those injected with NRK-Vector cells showed no tumor growth (≤ 1 mm) (Fig. 8A). Northern blot analysis on tumor RNA showed strong expression of *hWISP-1* in the tumors (Fig. 8B).

Discussion

WISP-1 was originally identified as one of the genes expressed at higher levels in *Wnt-1*-transformed C57MG cells when compared with parental C57MG cells (Pennica et al. 1998). This study has shown that the elevated expression level of *WISP-1* is due to the fact that the *WISP-1* promoter element responds transcriptionally to *Wnt-1* and β -catenin signaling. To determine whether *WISP-1* mRNA was up-regulated by *Wnt-1* without the need for any new protein synthesis, QT6 or QTWnt-1 cells were co-cultured with C57MG cells in the presence of cycloheximide. However, the cycloheximide treatment itself induces *WISP-1* mRNA expression in C57MG cells (data not shown), eliminating this approach. The *WISP-1* promoter was strongly induced by β -catenin in a dose-dependent manner, which is consistent with the possibility that the *WISP-1* promoter might be directly regulated by β -catenin. Both the carboxyl terminus of β -catenin (i.e., the transcriptional activation domain) and the cytoplasmic localization of β -catenin appeared to be important for the induction of the *WISP-1* promoter.

Placing *WISP-1* downstream of β -catenin could explain the observation that in 25 human colon adenocarcinomas examined, *WISP-1* mRNA was overexpressed (2- to >25 -fold in ~80% of tumors) compared with normal mucosa from the same patient (Pennica et al. 1998). Be-

cause most human colon cancers have elevated levels of β -catenin either caused by loss-of-function APC mutations or stabilized β -catenin mutations, elevated *WISP-1* RNA expression might be the result of enhanced β -catenin-mediated transcription.

One surprising finding of this study was that TCF/LEF factors played only a minor role in the induction of the *WISP-1* promoter. This fact was supported by two lines of evidence. The first is that mutations of all putative TCF/LEF sites had little effect on the β -catenin-dependent induction of the *WISP-1* promoter, and the second is that a dominant-negative mutant of TCF failed to inhibit this induction. Instead, a CREB-binding site was identified as a sequence that played an important role in the activation of the *WISP-1* promoter. Not only did mutations in the CREB site reduce the activation of this promoter, but also a dominant-negative mutant of CREB significantly inhibited this activation.

On the basis of these results, a novel pathway through which β -catenin regulates some of its downstream genes may be proposed. In this pathway, accumulation of β -catenin in the cytoplasm might cause an increase in cAMP levels. Elevated levels of cAMP activate protein kinase A (PKA), which phosphorylates the CREB protein and induces transcription of downstream genes through the CREB-binding site. Precisely how β -catenin induces cAMP levels remains to be elucidated. It also remains possible that β -catenin activates an alternative kinase that phosphorylates the CREB protein. In contrast to the prevailing model in which nuclear localized β -catenin/TCF complex turns on downstream gene transcription, this model postulates that the cytoplasmic localization of β -catenin leads to the activation of a kinase, because the nuclear localized β -catenin failed to induce transcription of the *WISP-1* promoter (Fig. 2C).

Xu et al.

Figure 8. (A) WISP-1 expression induces tumorigenic potential of NRK-49F cells. NRK-vector (passage 10) and NRK-WISP1 (line 1, passage 6; line 2, passage 10; line 1 and line 2 are established from independent infections) were injected into nude mice (5×10^6) subcutaneously. None of the nude mice injected with NRK-vector (0/3) developed tumors, whereas all mice injected with either of the two lines of NRK-WISP1 (5/5 for each line) had prominent tumor growth after 12 weeks of observation. Tumors first became apparent 3 weeks after injection. (◆) Vector; (■) WISP (line 1); (▲) WISP (line 2). (B) WISP-1 mRNA expression of tumor samples harvested from nude mice. Northern blot analyses were carried out as described in Fig. 6. Tumors 1 and 2 are representative tumors from NRK-WISP1 line 1 and line 2, respectively.

Recently, a zinc finger protein Teashirt was reported to bind to Armadillo (*Drosophila* β -catenin homolog) upon Armadillo stabilization in the cytoplasm (Gallet et al. 1998, 1999). This binding promotes phosphorylation and nuclear accumulation of Teashirt, leading to enhanced transcription of Teashirt-dependent downstream genes. Taken together, these data suggest that β -catenin is able to regulate downstream events through multiple factors, in addition to the TCF/LEF family members. The heterogeneity of genetic elements regulated by Wnt-1 and β -catenin also suggests the functional diversity of the Wnt-signaling pathway.

One of the most important findings of this study is the characterization of an oncogene, *WISP-1*, as a Wnt-1-regulated gene. Overexpression of *WISP-1* induced morphological transformation, increased cellular saturation density, and promoted growth in NRK-49F fibroblast cells in culture. The cells that overexpress *WISP-1* protein formed tumors in nude mice but not colonies in soft agar, suggesting that appropriate cell attachment and extracellular matrix formation might be important for these cells to respond to *WISP-1* and become tumori-

genic. Considering that CTGF and Cyr-61 are closely related to *WISP-1* and both signal through integrins (Kireeva et al. 1998; Babic et al. 1999), it is possible that *WISP-1* also triggers downstream events via integrin signaling. It remains to be elucidated whether tumor development in these nude mice is dependent on proper integrin binding.

The murine *WISP-1* was found independently in mouse melanoma cells with a lower metastatic ability (Hashimoto et al. 1998), and called *Elm1*. Thus, this protein has been associated with tumors in both the murine and human systems (Pennica et al. 1998). It is of interest that *WISP-1* can suppress metastasis in one melanoma cell line (Hashimoto et al. 1998) and promote transformation in the experimental system studied here. This indicates a complexity that needs to be studied further.

Mammary tumors from *Wnt-1* transgenic mice have been investigated to study *WISP-1* RNA expression patterns (Pennica et al. 1998). In situ hybridization revealed strong staining in surrounding stromal cells with only focal staining in epithelial cells. The high level of *WISP-1* RNA expression in the stroma surrounding the carcinoma (epithelial cells) suggests that *WISP-1* might be a stromal factor that exerts its effect in a paracrine manner. The low level of focal expression in epithelial cells suggests that *WISP-1* might also have an autocrine effect. It has long been suggested that stromal factors play an important role during mammary tumor development and progression (Kanazawa and Hosick 1992; Hornby and Cullen 1995; Frazier and Grotendorst 1997; Sasaki et al. 1998). On the basis of these RNA expression patterns of *WISP-1* in mammary tumors induced by Wnt-1 expression, a model in which the paracrine and autocrine signals of *WISP-1* contribute to the development of mammary tumors in *Wnt-1* transgenic mice is proposed (Fig. 9). In this model, epithelial cells secrete Wnt-1 protein, which then activates the Wnt-signaling pathway in surrounding stromal cells and leads to overproduction of *WISP-1*. *WISP-1* could stimulate the mam-

Figure 9. Putative model of *WISP-1* paracrine and autocrine signaling in mammary tumors of Wnt-1 transgenic mice.

mary epithelia to proliferate in a paracrine manner. Further mutations accumulated during the uncontrolled proliferation of epithelial cells could eventually give rise to cells that constitutively produce WISP-1 and drive cell division in an autocrine manner. It is of interest that some human colon carcinoma cell lines and human colon adenocarcinomas have *WISP-1* gene amplifications (two to fourfold) (Pennica et al. 1998). Thus, a combination of first a paracrine and then an autocrine production of WISP-1 might efficiently stimulate epithelial cells to continue unregulated proliferation and eventual tumor formation.

Materials and methods

Sequences

The GenBank accession number for the *hWISP-1* promoter region is AF223404.

Cell cultures and DNA transfections

C57MG, 293, and NRK-49F cell lines were maintained in DMEM supplemented with 10% FBS. The quail fibrosarcoma cell line QT6 and QTWnt-1 were obtained from Harold Varmus (NIH, Bethesda, MD). QTWnt-1 was maintained in medium containing 400 μ g/ml Geneticin.

The Wnt-1-expressing C57MG cell line was established by retroviral infections (Pennica et al. 1998). Cells were collected 6 days after infection. Total RNA was extracted for Northern blot analysis to assess *WISP-1* mRNA expression. β -Catenin mutants expressing C57MG cell lines were established by transfecting C57MG cells with 4145 or 4145TV β -catenin plasmids together with pGK-hygro, a plasmid carrying the hygromycin resistance gene. Resistant colonies were selected and propagated in the presence of hygromycin. Two stable clones that express 4145 or 4145TV at high levels were obtained. Cells were collected and total RNA extracted for Northern blot analysis to assess *WISP-1* mRNA expression. All DNA transfections were performed using the lipofectamine reagent (GIBCO BRL) as directed by the manufacturer.

Construction of β -catenin mutants

Myc-epitope-tagged wild-type *Xenopus* β -catenin in the pCS2/MT expression vector and empty pCS2/MT vector were provided by Barry Gumbiner (Memorial Sloan Kettering Cancer Center, New York, NY). Site-directed mutagenesis experiments to make β -catenin mutants 4145 and 4145TV were carried out as described previously (Lin et al. 1994). To make β -catenin NLS mutant, SV40 large T antigen NLS was subcloned in-frame in front of the myc tag repeats. To make D(C) mutant, PCR-based site-directed mutagenesis was used to generate a stop codon in front of the β -catenin internal *Pst*I site. All mutants were sequenced to confirm that only the intended point mutations were introduced.

Northern blot analysis

Total RNA was isolated with the RNeasy Mini Kit (Qiagen) as directed by the manufacturer. A total of 10 μ g of RNA was loaded onto 1.2% agarose gel containing 6% formaldehyde and run in 1 $\times$ E buffer (18 mM Na_2HPO_4 plus 2 mM NaH_2PO_4) overnight while circulating buffer. RNA was transferred to Nytran membrane (Schleicher & Schuell) using the Turboblotter Trans-

fer System (Schleicher & Schuell). After UV cross-linking, blots were prehybridized in Church buffer (0.2 M NaH_2PO_4 , 0.3 M Na_2HPO_4 , 1% BSA, 7% SDS, 1 mM EDTA) for 2 hr at 65°C. The probes were labeled with [$\alpha^{32}\text{P}$]dCTP using the Prime-It RmT Kit (Stratagene). Blots were hybridized with labeled probe overnight in Church buffer at 65°C and washed four times at 30 min in 1 $\times$ SSC/0.1% SDS at 65°C before exposing to X-Omat film.

Isolation of genomic clones containing the WISP-1 promoter

The human genomic clone for *WISP-1* was obtained using Genome Systems, Inc. PCR-based DOWN TO THE WELL screening method, according to the manufacturer's instructions. Genomic DNA was digested with *Bam*HI and tested for hybridization to the hWISP-1 amino-terminal sequences by Southern blot analysis. One hybridizing fragment, corresponding to -5882--42 bp (numbered according to translation start site as +1) of the *WISP-1* promoter, was gel purified, subcloned into the pBlue-script vector (Stratagene), and sequenced.

Construction of luciferase reporter plasmids

To clone the isolated *WISP-1* promoter fragment to pGL2B, -5.9 kb/pBlue-script construct was cut at the *Sma*I and *Spe*I sites on pBlue-script vector and cloned directionally into the *Sma*I and *Nhe*I sites of pGL2B. The 5' deletions of *WISP-1* were generated by digesting the -5.9 kb/pBlue-script construct at internal *Nhe*I (Frag4), *Kpn*I (Frag3), *Xho*I (Frag2), and *Sac*I (Frag1) sites and cloned directionally into pGL2B. To generate Frag0, PCR-based site-directed mutagenesis was carried out to introduce a *Kpn*I site at -328, then the PCR fragment was cut by *Kpn*I and cloned directionally into pGL2B.

Site-directed mutagenesis was performed with the QuikChange Site-Directed Mutagenesis Kit (Stratagene). The mismatched oligonucleotides used to eliminate TCF sites 3-5 are as follows: site 3, 5'-GGTGAATGGTCAGCGAATTCATCATGTTTTGGGGTGCC-3'; site 4, 5'-GGCCTCTAGGATT-TATTTTTTGCTAGCTTTGTTTGCTTGTTTG-3'; site 5, 5'-CACTGGGAGCCCTCTGCTAGCCCCACACCCCGCCTG-3'. The mismatched oligonucleotides used to eliminate CREB site is as follows: 5'-GGGTTTGTCTTCACCCTGAATTCAGATCTTGCTTTAATAAAACC-3'. Each mutagenic oligonucleotide introduces a restriction site (underlined) that was used to assay for incorporation of the mutation. All constructs were sequenced to confirm that only the intended point mutations were introduced.

Luciferase assay

TopFlash luciferase reporter and DN-TCF were provided by Bert Vogelstein (Johns Hopkins School of Medicine, Baltimore, MD). KCREB was provided by Richard Goodman (Oregon Health Science Center). Promoter fragments were subcloned into pGL2 basic luciferase reporter plasmid (Promega). Luciferase analysis was performed with Dual-Light Kit (Tropix) according to the manufacturer's directions. Measurements were carried out with TR717 microplate luminometer (Tropix). Luciferase readout was always obtained from triplicate transfections and averaged.

Retroviral infections and cell culture

Retroviral expression vector pBabe-puro was used to construct and express full-length *hWISP-1*. Recombinant retroviruses were generated by transfecting the retroviral constructs into BOSC23 packaging cells with lipofectamine. Forty-eight hours post-transfection, retrovirus-containing supernatant was collected. Target cell lines were infected at ~40% confluence by

Xu et al.

replacing the culture medium with retrovirus-containing supernatant, supplemented with 1 ml of FBS and 8 µg/ml Polybrene. Infecting medium was replaced by 10 ml of DMEM plus 10% FBS 4–6 hr later. At 48 hr post-infection, cells were split into medium containing 2.5 µg/ml puromycin. Resistant clones were pooled and maintained in puromycin.

To measure growth in low serum, cells were seeded at 10^5 /10-cm plate and allowed to attach in the presence of 10% serum overnight. The following day, cells were washed once with PBS and then cultured in DMEM plus 0.5% FBS. To count cells, cells were dissociated by trypsinization and viable cells were counted after Trypan blue staining.

Growth in soft agar of NRK cells was assayed by embedding 4×10^5 cells in 0.3% Noble agar (DIFCO Laboratory) in DMEM plus 10% FBS, over a substratum of 0.5% Noble agar in DMEM plus 10% FBS. No significant colony formation was observed after 6 weeks of incubation. As a positive control, 293 cells formed visible colonies after 2 weeks of incubation. Nude mice injections were carried out by subcutaneously injecting 5×10^6 cells on the back of 8-week-old female BALB/c nude mice.

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***WISP-1* is a Wnt-1- and β -catenin-responsive oncogene**

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