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### Authors

Hinck, L  
Nelson, WJ  
Papkoff, J

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# Wnt-1 Modulates Cell-Cell Adhesion in Mammalian Cells by Stabilizing $\beta$ -Catenin Binding to the Cell Adhesion Protein Cadherin

Lindsay Hinck,<sup>\*‡</sup> W. James Nelson,<sup>\*‡</sup> and Jackie Papkoff<sup>‡§</sup>

<sup>\*</sup>Department of Molecular and Cellular Physiology, Stanford University School of Medicine, Stanford, California 94305-5426; <sup>‡</sup>Cancer Biology Program, Stanford University, Stanford, California 94305-5482; and <sup>§</sup>SUGEN, Inc., Redwood City, California 94063

**Abstract.** Wnt-1 homologs have been identified in invertebrates and vertebrates and play important roles in cellular differentiation and organization. In *Drosophila*, the products of the segment polarity genes *wingless* (the Wnt-1 homolog) and *armadillo* participate in a signal transduction pathway important for cellular boundary formation in embryonic development, but functional interactions between the proteins are unknown. We have examined Wnt-1 function in mammalian cells in which armadillo ( $\beta$ -catenin and plako-

globin) is known to bind to and regulate cadherin cell adhesion proteins. We show that Wnt-1 expression results in the accumulation of  $\beta$ -catenin and plakoglobin. In addition, binding of  $\beta$ -catenin to the cell adhesion protein, cadherin, is stabilized, resulting in a concomitant increase in the strength of calcium-dependent cell-cell adhesion. Thus, a consequence of the functional interaction between Wnt-1 and armadillo family members is the strengthening of cell-cell adhesion, which may lead to the specification of cellular boundaries.

WNT-1 is a striking example of a protein involved in both normal development and oncogenic transformation (Nusse and Varmus, 1992). In mammalian cells, Wnt-1 is thought to function as a growth factor that is secreted and then becomes associated with the cell surface (Bradley and Brown, 1990; Papkoff and Schryver, 1990); in *Drosophila*, the Wnt-1 homolog, *wingless*, is thought to function as a locally restricted morphogen (van den Heuvel et al., 1989). *Wingless* plays an essential, but poorly defined role in a signal transduction pathway that determines segmental patterning in early embryogenesis (Morata and Lawrence, 1977). In vertebrates, regulated *Wnt-1* expression is required for murine neural development (McMahon and Bradley, 1990; Thomas and Capecchi, 1990), and its overexpression in *Xenopus* results in anterior duplication of the embryonic axis (McMahon and Moon, 1989). However, *Wnt-1* was originally identified in vertebrates as a proto-oncogene (Nusse and Varmus, 1982; Nusse et al., 1984). Unregulated expression of *Wnt-1* was found to participate in the induction of mammary hyperplasias in mice (Tsukamoto et al., 1988), and morphological transformation of some cultured cell lines (Brown et al., 1986; Rijsewijk et al., 1987). These apparently disparate effects of *Wnt-1* expression have been difficult to reconcile in part because the function(s) of the protein is unknown.

*Wingless* expression in *Drosophila* modulates the accumulation of a cytoplasmic protein, armadillo, which is also required for segmental patterning (Riggelman et al., 1990). Recent studies have demonstrated that the mammalian homologs of armadillo protein,  $\beta$ -catenin, and plakoglobin (Peifer and Wieschaus, 1990; McCrea et al., 1991; Butz et al., 1992), bind to the cytoplasmic domain of a family of  $\text{Ca}^{2+}$ -dependent cell adhesion molecules, the cadherins (Nagafuchi and Takeichi, 1988; Ozawa et al., 1989; Knudsen and Wheelock, 1992; Peifer et al., 1992; Piepenhagen and Nelson, 1993). Like Wnt-1, regulation of cadherin expression affects both normal development and oncogenic transformation. Cadherins determine homotypic interactions between cells in the formation of solid tissues, and establish boundaries between different cell types (Nagafuchi et al., 1987; Takeichi, 1991). However, abnormal expression of cadherins disrupts tissue morphogenesis (Detrick et al., 1990), and inhibition of cadherin function correlates with tumor cell invasion (Behrens et al., 1989; Navarro et al., 1991; Vleminckx et al., 1991).

Beta-catenin and plakoglobin, together with  $\alpha$ -catenin, play important roles in regulating cadherin function. Deficiency of  $\alpha$ -catenin inhibits cadherin function (Hirano et al., 1992), and aberrant phosphorylation of the  $\beta$ -catenin/cadherin complex by *v-src* correlates with decreased cell-cell adhesion and increased cell invasiveness (Matsuyoshi et al., 1992; Behrens et al., 1993). Taken together, these observations indicate that the efficacy of cadherin-mediated cell-

Address all correspondence to Jackie Papkoff, SUGEN, Inc., 515 Galveston Drive, Redwood City, CA 94063.

cell adhesion is regulated by the interactions of  $\alpha$ -catenin and armadillo family members with cadherin.

Given that members of the armadillo protein family play key roles in both the wingless signaling pathway in segmental patterning and in cadherin mediated cell adhesion, a fundamental question arises: does Wnt-1 function through  $\beta$ -catenin and plakoglobin to modulate cell-cell adhesion? In *Drosophila*, wingless expression leads to the accumulation of armadillo protein, but it is not known whether cell-cell interactions are altered. Here, we have examined the effects of Wnt-1 expression on armadillo-cadherin complex formation and the consequences on cell-cell adhesion in mammalian cells. We show that Wnt-1 expression increases the levels of armadillo family members in two different mammalian cell types and the consequence is increased strength of cell-cell adhesion. The mechanism through which Wnt-1 regulates cell-cell adhesion appears to be stabilization of the cadherin/ $\beta$ -catenin complex at the cell surface. These effects are specific to armadillo family members as the expression and binding of  $\alpha$ -catenin to cadherin is unaffected by Wnt-1 expression. We suggest that one function of Wnt-1 is to regulate cell adhesion and, thereby, influence both cell boundary formation and cell proliferation.

## Materials and Methods

### Cell Lines and Antisera

Control and Wnt-1-expressing A1T20 (Papkoff and Schryver, 1990) and C57MG cell lines (Blasband et al., 1992) and the MDCK cell line (Nelson and Veshnock, 1986) have been described previously.

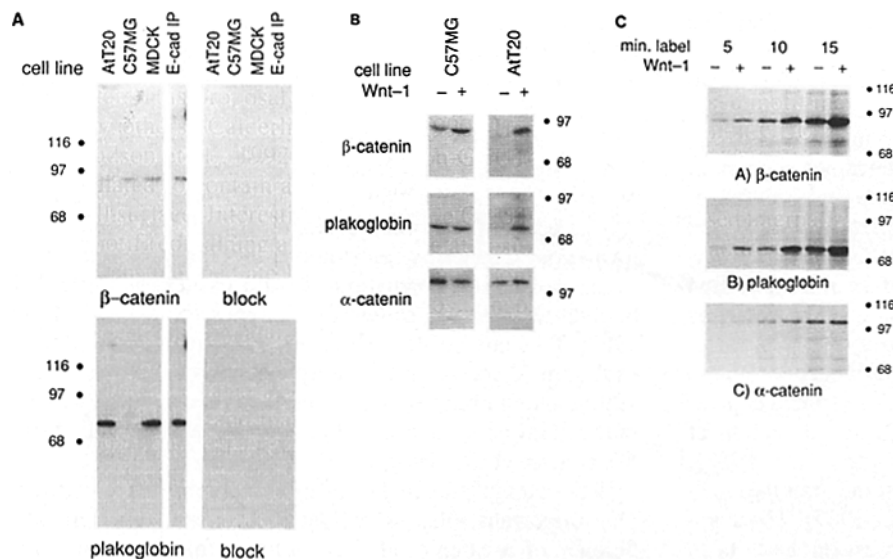
A pan-cadherin antibody that recognizes E-cadherin has previously been described (a gift from Dr. James Marrs, Stanford University, Stanford, CA) (Marrs et al., 1993). An antibody directed against the cytoplasmic domain of murine N-cadherin was a gift from Dr. Dietmar Vestweber, Max-Planck-Institut für Immunobiologie, Freiburg, Germany. Antibodies directed against  $\beta$ -catenin, plakoglobin, and  $\alpha$ -catenin were raised in rabbits

against peptides with the sequences, PGDSNQLAWFDITDLC, CIDTYS-DGLRPPYPTADH, and KKHVNPVQALSEFKAC, respectively, conjugated through the cysteine to KLH using the Inject kit (Pierce Chemical Co., Rockford, IL).

### Immunoprecipitation and Immunoblots

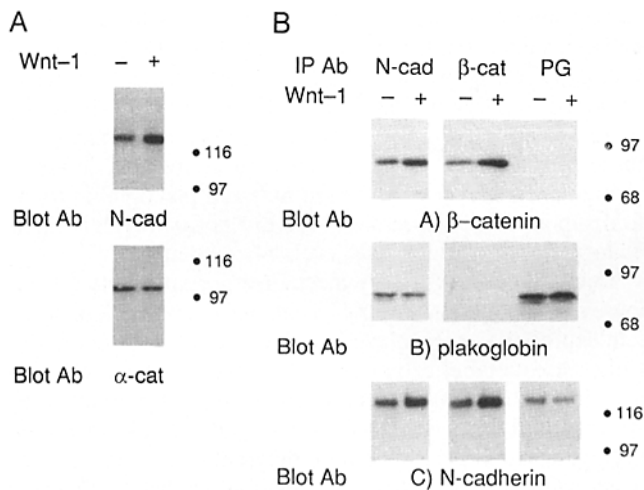
For the immunoprecipitation and immunoblots (Figs. 1, A and B and 2 A), replicate cultures of cells were extracted with radioimmunoprecipitation assay (RIPA) buffer (Papkoff and Schryver, 1990) for 15 min at 4°C, scraped from the tissue culture dish, and centrifuged at 12,000 g for 15 min at 4°C. The total protein concentration of the clarified extracts was determined using BCA reagents (Pierce Chemical Co.). An equivalent amount of two-fold concentrated Laemmli sample buffer was added to aliquots of each extract, which were normalized to total protein. For the E-cadherin immunoprecipitate (Fig. 1 A, *E-cad IP*), an MDCK cell extract was incubated for 2 h on ice with a pan-cadherin antibody that recognizes E-cadherin. The antibody was prepared for immunoprecipitation by preincubation with a 10% suspension of protein A-Sepharose 4B (Pharmacia Fine Chemicals, GaitHERSBURG, MD) for 1 h on ice followed by washing the complex once with PBS. The immunoprecipitate was washed as previously described (Wollner et al., 1992). The samples were boiled for 5 min in Laemmli sample buffer, separated in a SDS 7.5% polyacrylamide gel (Laemmli, 1970), and electrophoretically transferred (Towbin et al., 1979) to Immobilon-P filters (Millipore Corp., Bedford, MA). The molecular weight standards are  $\beta$ -galactosidase ( $M_r$  116,000), phosphorylase b ( $M_r$  97,000), and bovine serum albumin ( $M_r$  68,000). The filters were incubated in gelatin wash buffer (GWB)<sup>1</sup> (Wollner et al., 1992) containing 5% nonfat dry milk followed by addition of antibodies at a 1:500 dilution in GWB for 2 h. Antibody incubation was followed by extensive washing in GWB, incubation with [<sup>125</sup>I]-protein A (0.1  $\mu$ Ci/ml) (Dupont New England Nuclear, Cambridge, MA) for 1 h, extensive washing in GWB and exposure to x-ray (XAR-5) film at -80°C. For Figs. 1 A and B and 5,  $\beta$ -catenin, plakoglobin, and  $\alpha$ -catenin were specifically detected by incubating the filters with polyclonal anti-peptide sera. Reactivity of each antibody with protein was completely blocked by preincubation with excess cognate peptide. For Fig. 2 A, N-cadherin was specifically detected by incubating the filter with an antibody generated against the cytoplasmic domain of murine N-cadherin. For the

1. *Abbreviations used in this paper:* ECL, enhanced chemiluminescence; GWB, gelatin wash buffer; HGF, hepatocyte growth factor; RIPA, radioimmunoprecipitation assay.



**Figure 1.** (A) Distinguishing armadillo family members,  $\beta$ -catenin and plakoglobin. Extracts of total cell protein were prepared from C57MG, A1T20, and MDCK cells, separated by SDS-PAGE, and immunoblotted with anti-peptide antibodies generated against unique COOH-terminal amino acid sequences of either  $\beta$ -catenin (top) or plakoglobin (bottom). An E-cadherin immunoprecipitate from MDCK cell extracts was included in the same immunoblot (*E-cad IP*) to verify the identity of these proteins by demonstrating that  $\beta$ -catenin and plakoglobin co-immunoprecipitate in a complex with E-cadherin. Reactivity of each antibody with protein was completely blocked by adding excess cognate peptide (*block*). (B) Wnt-1 expression results in increased levels of  $\beta$ -catenin and plakoglobin proteins in C57MG and A1T20 cells. Protein levels

of  $\beta$ -catenin, plakoglobin, and  $\alpha$ -catenin in control (-Wnt-1) and Wnt-1-expressing (+Wnt-1) C57MG and A1T20 cells were determined by immunoblotting equivalent amounts of total cell protein with specific antibodies directed against  $\beta$ -catenin, plakoglobin and  $\alpha$ -catenin. (C) Wnt-1 expression results in increased rates of synthesis of  $\beta$ -catenin and plakoglobin, but not  $\alpha$ -catenin proteins. Control (-Wnt-1) and Wnt-1-expressing (+Wnt-1) A1T20 cells were pulse-labeled with [<sup>35</sup>S]methionine for either 5, 10, or 15 min. Extracts were prepared and immunoprecipitated with antibodies directed against  $\beta$ -catenin (A), plakoglobin (B), or  $\alpha$ -catenin (C).

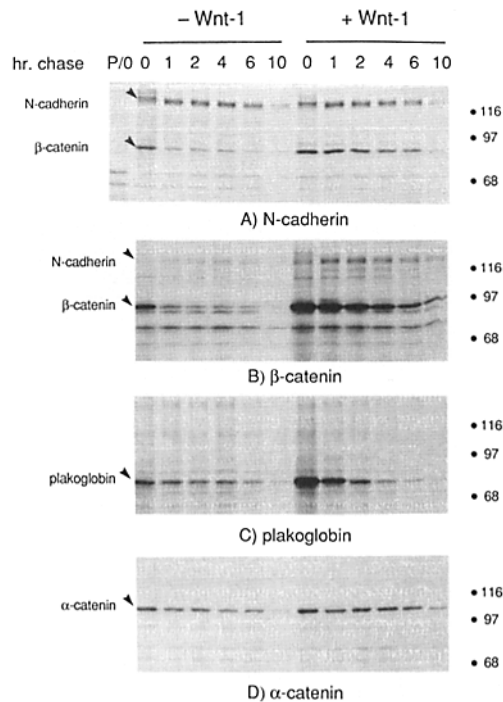


**Figure 2.** (A) Wnt-1 expression results in an increase in the level of N-cadherin protein. Relative levels of N-cadherin and  $\alpha$ -catenin in control (-Wnt-1) and Wnt-1-expressing (+Wnt-1) AtT20 cells were determined by immunoblotting equivalent amounts of total cell protein with an antibodies directed against N-cadherin and  $\alpha$ -catenin. (B) Complex formation between N-cadherin,  $\beta$ -catenin, and plakoglobin. Cell extracts from control (-Wnt-1) and Wnt-1-expressing (+Wnt-1) AtT20 cells were prepared with immunoprecipitated (IP Ab) with either N-cadherin (N-cad),  $\beta$ -catenin ( $\beta$ -cat), or plakoglobin (PG) antibodies. The immunoprecipitates were separated by SDS-PAGE, and immunoblotted (Blot Ab) with antibodies directed against  $\beta$ -catenin (A), plakoglobin (B), or N-cadherin (C).

sequential immunoprecipitation/immunoblot (Fig. 2 B), replicate cultures of control (AtT20-) and Wnt-1-expressing (AtT20+) cells were extracted with RIPA buffer and protein equivalent aliquots of the extracts were immunoprecipitated with specific antibodies (IP Ab) as described in Fig. 1. The immunoprecipitates were separated in a SDS 7.5% polyacrylamide gel followed by immunoblotting with antibodies specific for  $\beta$ -catenin, plakoglobin and N-cadherin as described in Fig. 1. The immunoblot was exposed to x-ray (XAR-5) film at  $-80^{\circ}\text{C}$ . The films were analyzed using a scanning densitometer (Molecular Devices Corp., Menlo Park, CA). For the steady state biotinylation, replicate cultures of AtT20- and AtT20+ cells were biotinylated on the cell surface with S-NHS-biotin as previously described (Wöllner et al., 1992). Cells were extracted with RIPA buffer and protein equivalent aliquots of each extract were immunoprecipitated, as described above, with the indicated antibodies. The immunoprecipitates were separated in a SDS 7.5% polyacrylamide gel followed by immunoblotting with antibodies specific for  $\beta$ -catenin or  $\alpha$ -catenin as described in Fig. 1. To detect the biotinylated N-cadherin, the blot was blocked overnight in 5% ovalbumin, washed in Tris-buffered saline (10 mM Tris, pH 8.0, 150 mM NaCl) containing 0.1% Tween-20 and incubated with a complex of Avidin DH: biotinylated horseradish peroxidase H (Vectastain ABC kit; Vector Laboratories, Inc., Burlingame, CA) as directed by the manufacturer. The blot was processed with enhanced chemiluminescence (ECL) detection system (Amersham Corp., Arlington Heights, IL).

### Metabolic Labeling and Immunoprecipitation

For metabolic labeling (Fig. 1 D), replicate cultures of control (AtT20-) and Wnt-1-expressing (AtT20+) cells were incubated in the absence of methionine for 20 min, pulse-labeled in the presence of  $150 \mu\text{Ci/ml}$  [ $^{35}\text{S}$ ]methionine (Dupont NEN) for either 5, 10, or 15 min and extracts prepared as described above. Protein equivalent aliquots of each extract were immunoprecipitated with antibodies and washed as described above. The immunoprecipitates were boiled for 5 min in Laemmli sample buffer and separated in a SDS 7.5% polyacrylamide gel (Laemmli, 1970). The gel was subsequently immersed in Amplify as directed by the manufacturer (Amersham Corp.), dried under vacuum, and exposed to x-ray (XAR-5) film at  $-80^{\circ}\text{C}$ . The films were analyzed using a Molecular Devices scanning den-



**Figure 3.** Wnt-1 expression results in the increased stability of  $\beta$ -catenin/N-cadherin complexes, and increased levels of newly synthesized  $\beta$ -catenin and plakoglobin proteins. Replicate cultures of control (-Wnt-1) and Wnt-1-expressing (+Wnt-1) AtT20 cells were pulse-labeled (0 h chase) with [ $^{35}\text{S}$ ]methionine and chased in the absence of label for the indicated time periods (1, 2, 4, 6, 10 h chase). At each time point, cell extracts were prepared, and protein equivalent aliquots were immunoprecipitated with specific antibodies directed against N-cadherin (A),  $\beta$ -catenin (B), plakoglobin (C), or  $\alpha$ -catenin (D). As a control, preimmune serum was used to immunoprecipitate an aliquot of extract from the 0 time point (A, P/0).

sitometer. The molecular weight standards are  $\beta$ -galactosidase ( $M_r$  116,000), phosphorylase b ( $M_r$  97,000) and bovine serum albumin ( $M_r$  68,000).

For the pulse-chase analysis (Fig. 3) replicate cultures of control (AtT20-) and Wnt-1-expressing (AtT20+) cells were incubated in the absence of methionine for 20 min, pulse-labeled with  $150 \mu\text{Ci/ml}$  [ $^{35}\text{S}$ ]methionine for 30 min, washed once, and incubated in medium containing excess unlabeled methionine for 1, 2, 4, 6, and 10 h. At the indicated times, cells were extracted in RIPA buffer and protein equivalent aliquots of each extract were immunoprecipitated with specific antibodies as described above. The immunoprecipitates were boiled for 5 min in Laemmli sample buffer and separated in a SDS 7.5% polyacrylamide gel (Laemmli, 1970) which was subsequently treated as described above. For analysis of cell surface N-cadherin, replicate cultures of control (AtT20-) and Wnt-1-expressing (AtT20+) cells were incubated in the absence of methionine for 20 min, pulse-labeled with [ $^{35}\text{S}$ ]methionine for 45 min, washed once, and incubated in medium containing excess unlabeled methionine for 1, 2, 4, and 8 h. At the indicated times, a single culture of AtT20- and AtT20+ cells was biotinylated on the cell surface with NHS-S-S-biotin as previously described (Wöllner et al., 1992). Cells were extracted with RIPA buffer and protein equivalent aliquots of each extract were immunoprecipitated, as described above, with a pan-cadherin rabbit antiserum which recognizes N-cadherin (Marrs et al., 1993). The immunoprecipitates were washed and the immune complex dissociated in 0.1 M glycine, pH 2.5, as previously described (Wöllner et al., 1992). Biotinylated proteins were precipitated from neutralized supernatant by the addition of avidin-agarose (Pierce Chemical Co.) for 1 h. The immunoprecipitates were washed as above, the pellets were boiled for 5 min in Laemmli sample buffer and analyzed in a

SDS 7.5% polyacrylamide gel that was treated for fluorography as described above.

### Cell Adhesion Assay

Control (AtT20<sup>-</sup>, C57MG<sup>-</sup>) and Wnt-1-expressing (AtT20<sup>+</sup>, C57MG<sup>+</sup>) cells were plated at very low density ( $2.5 \times 10^6$  cells/15-cm dish) and allowed to grow for 12 h (Nelson and Veshnock, 1986). The cultures were rinsed two times with PBS/5 mM EDTA (Nelson and Veshnock, 1986), incubated in this solution for 10 min at 37°C, washed from the tissue culture dishes, and pelleted for 1 min at 500 g. The cells were resuspended in 0.5 ml of DME containing no Ca<sup>2+</sup> and  $5 \times 10^5$  cells were plated onto a 3.5-cm dish, precoated with a 2%-agarose solution, containing 3 ml of either DME (1.8 mM Ca<sup>2+</sup>) or calcium-free DME/2 mM EDTA (without calcium). After 2-h incubation, the cells were triturated 10 times with a 2-ml pipette (Schmidt et al., 1992). A 300- $\mu$ l aliquot was pipetted onto a glass slide and viewed with an Axiovert 35 Microscope using a 10 $\times$  objective (both from Carl Zeiss, Inc., Thornwood, NY) and photographed on Tri-X pan-400 film. The assay was performed double blind and quantified by counting the aggregates in prints. For each cell type, three separate experiments were performed; random fields were photographed and counted from each experiment.

## Results

### Specific Antibodies Distinguish Armadillo Protein Family Members in Mammalian Cells

To distinguish closely related members of the *armadillo* family of proteins, specific antipeptide antibodies were generated against unique COOH-terminal amino acid sequences of  $\beta$ -catenin and plakoglobin. Immunoblots of extracts from C57MG and AtT20 cell lines demonstrated that proteins with distinct electrophoretic mobilities were recognized by these antibodies (Fig. 1 A). The apparent molecular weights of these proteins were similar to those of  $\beta$ -catenin (~94 kD) and plakoglobin (~83 kD), which were recognized by the antipeptide antibodies in both immunoblots of MDCK cell extracts (Fig. 1 A) and in complexes coimmunoprecipitated with E-cadherin antibodies from MDCK cells (Fig. 1 A: *E-cad IP*); this E-cadherin/catenin complex has been previously well-characterized (Ozawa et al., 1989; McCrea and Gumbiner, 1991). Antibody specificity was demonstrated by blocking reactivity with excess cognate peptide (Fig. 1 A; *block*).

### Wnt-1 Regulates the Accumulation of $\beta$ -catenin and Plakoglobin Proteins in Mammalian Cells

The effects of Wnt-1 expression on  $\beta$ -catenin and plakoglobin protein levels were examined in C57MG and AtT20 cells transfected with Wnt-1 cDNA. The C57MG murine, mammary epithelial cell line is routinely used to analyze the transforming properties of Wnt genes (Brown et al., 1986; Blasband et al., 1992). The AtT20 murine, neuroendocrine cell line is highly efficient at processing and secreting Wnt-1 protein which has facilitated its biochemical characterization (Papkoff and Schryver, 1990). Both control (transfected with neo<sup>r</sup> gene only) and Wnt-1-expressing clonal cell lines used in this analysis were randomly obtained from populations of transfected cells that had been selected in G418; none of the clones were selected by any morphological criteria.

Extracts from control (C57MG<sup>-</sup>, AtT20<sup>-</sup>) and Wnt-1-expressing (C57MG<sup>+</sup>, AtT20<sup>+</sup>) cell lines were analyzed by immunoblotting with  $\beta$ -catenin-, plakoglobin- and  $\alpha$ -catenin-specific antibodies. The immunoblots revealed increased

steady state amounts of  $\beta$ -catenin and plakoglobin in both cell types upon Wnt-1 expression (Fig. 1 B). Data from several experiments revealed that the most dramatic increase occurred in  $\beta$ -catenin levels in AtT20<sup>+</sup> cells (20–30-fold); there was also a 10–15-fold increase in plakoglobin levels in these cells. In C57MG cells, there was an increase in levels of both  $\beta$ -catenin (four- to eightfold) and plakoglobin (two-fold) upon Wnt-1 expression. We did not detect consistent changes in the steady state levels of  $\alpha$ -catenin.

To investigate regulatory mechanisms responsible for the increased steady state levels of  $\beta$ -catenin and plakoglobin protein expression, we analyzed the levels of protein synthesis. Cells were metabolically pulse-labeled for short periods and proteins were immunoprecipitated from cell extracts with specific antibodies (Fig. 1 C). Two- and fourfold increases in  $\beta$ -catenin and plakoglobin synthesis after 10 min, respectively, were detected in AtT20<sup>+</sup> cells (Fig. 1 C) and C57MG<sup>+</sup> cells (data not shown), compared to control cells. This result raises the possibility that Wnt-1 regulates a factor(s) that locally influences the translation of *armadillo* protein family member mRNAs. However, these small changes are not sufficient to account for the increases in steady state levels of  $\beta$ -catenin and plakoglobin in AtT20 cells expressing Wnt-1. We did not detect changes in the rate of synthesis of  $\alpha$ -catenin upon Wnt-1 expression (Fig. 1 C).

### Wnt-1 Expression Results in Accumulation of N-cadherin in AtT20 Cells

Previous studies in mammalian epithelial cells and fibroblasts have demonstrated that  $\beta$ -catenin and a significant proportion of plakoglobin are not present in a free pool, but are bound to members of the cadherin superfamily of Ca<sup>2+</sup>-dependent cell adhesion proteins (Korman et al., 1989; Knudsen and Wheelock, 1992; Peifer et al., 1992; Piepenhagen and Nelson, 1993). To examine effects of Wnt-1 expression and  $\beta$ -catenin/plakoglobin accumulation on cadherin/catenin complex formation, we needed to identify cadherins expressed in the C57MG and AtT20 cell lines. PCR analysis using oligonucleotide primers to the conserved cytoplasmic domain of cadherins revealed the presence of a single amplified sequence in cDNA from both C57MG and AtT20 cells lines (unpublished results). Extracts from these cells were screened with a panel of cadherin antibodies. A cadherin is recognized in AtT20 cells by an antibody generated against murine N-cadherin (Fig. 2 A). Since the antibody was raised against the conserved cytoplasmic domain, it is possible that it recognizes cadherins other than N-cadherin; however, for ease of reference we designated the cadherin recognized by this antibody in AtT20 cells as N-cadherin. Comparison of steady state levels of N-cadherin by immunoblotting extracts from AtT20<sup>-</sup> and AtT20<sup>+</sup> cells revealed an increase (twofold) in the amount of N-cadherin protein in AtT20<sup>+</sup> cells (Fig. 2 A). Note that there is little or no difference in the amount of  $\alpha$ -catenin between the Wnt-1 expressing and control AtT20 cells. We were unable to identify the cadherin expressed in C57MG cells with antibodies due either to differences in antigenicity, or to low levels of cadherin expression. However, functional studies of C57MG<sup>+</sup> cells, described below, revealed the presence in these cells of calcium-dependent cell-cell adhesion that has characteristics expected of a cadherin-mediated mechanism.

### ***$\beta$ -catenin and Plakoglobin Form Mutually Exclusive Complexes with Cadherin in Mammalian Cells***

Complexes between N-cadherin,  $\beta$ -catenin, and plakoglobin were examined by high stringency immunoprecipitation of AtT20<sup>-</sup> and AtT20<sup>+</sup> cell extracts with either N-cadherin (*N-cad*),  $\beta$ -catenin ( $\beta$ -*cat*), or plakoglobin (*PG*)-specific antibodies, followed by SDS-PAGE analysis and immunoblotting with one of the other antibodies (Fig. 2 B). Complexes comprising N-cadherin and both  $\beta$ -catenin (Fig. 2 B, A and B) and plakoglobin (Fig. 2 B, B and C) were detected. It appears that N-cadherin,  $\beta$ -catenin, and plakoglobin interact with high affinity because the complexes are stable in buffers containing 1 M NaCl and 0.1% SDS. Note that in both AtT20<sup>-</sup> and AtT20<sup>+</sup> cells, plakoglobin was not detected in the  $\beta$ -catenin immunoprecipitate (Fig. 2 B, B), and vice versa (Fig. 2 B, A), demonstrating that  $\beta$ -catenin and plakoglobin form mutually exclusive complexes with N-cadherin. We do not know the functional significance of these different complexes, but it raises the possibility that cadherin function is regulated by binding either  $\beta$ -catenin or plakoglobin (see below).

Examination of complexes formed between N-cadherin and  $\beta$ -catenin or plakoglobin confirmed the increased steady state levels of N-cadherin (Fig. 2 B, C) and  $\beta$ -catenin (Fig. 2 B, A) in AtT20<sup>+</sup> cells. Note that fourfold more  $\beta$ -catenin was coimmunoprecipitated in a complex with N-cadherin from AtT20<sup>+</sup> cells than from AtT20<sup>-</sup> cells, (Fig. 2 B, A and C; see also Fig. 3, A and B). This indicates that a consequence of Wnt-1-induced accumulation of  $\beta$ -catenin and N-cadherin in the AtT20<sup>+</sup> cells is a corresponding increase in the absolute amount of N-cadherin/ $\beta$ -catenin complexes in the cell. However, similar amounts of plakoglobin were coimmunoprecipitated in a complex with N-cadherin from AtT20<sup>+</sup> and from AtT20<sup>-</sup> cells (Fig. 2 B, B). This difference in loading capacity of cadherins for  $\beta$ -catenin and plakoglobin may reflect differences in binding affinities. In MDCK cells, for example, only a small fraction of total plakoglobin is detected in a cadherin-associated complex (Kapprell et al., 1987) (unpublished results), whereas most of the  $\beta$ -catenin is bound to a cadherin complex (unpublished results).

### ***Wnt-1 Expression Results in the Stabilization of Complex Formation Between $\beta$ -catenin and N-cadherin***

The effect of Wnt-1 expression on the formation and stability of complexes of N-cadherin and  $\beta$ -catenin or plakoglobin was examined by pulse-chase analyses. Replicate cell cultures were pulse-labeled with [<sup>35</sup>S]methionine for 30 min, and then chased in the absence of label for various times. Cells were harvested and aliquots of the extracts immunoprecipitated with antibodies directed against N-cadherin,  $\beta$ -catenin, plakoglobin, or  $\alpha$ -catenin. Fig. 3 A shows that the level of synthesis and rate of turnover of newly synthesized N-cadherin was not affected by Wnt-1 expression.

In contrast, there were significant Wnt-1-dependent differences in the stability of the pool of newly synthesized  $\beta$ -catenin complexed with newly synthesized N-cadherin; this complex was immunoprecipitated with antibodies specific for either N-cadherin or  $\beta$ -catenin (Fig. 3, A and B). At the

end of the pulse label (time 0), immunoprecipitation with N-cadherin antibodies revealed that the ratio of N-cadherin to  $\beta$ -catenin in the complexes was similar in both AtT20<sup>-</sup> (1:1.3) and AtT20<sup>+</sup> (1:1.1) cells (Fig. 3 A). However, within 1 h of the chase period in AtT20<sup>-</sup> cells, 60% of the newly synthesized  $\beta$ -catenin was lost from the N-cadherin/catenin complex; the remaining 40% decayed more slowly (Fig. 3 A). This biphasic turnover of  $\beta$ -catenin in N-cadherin complexes was confirmed by direct immunoprecipitation of  $\beta$ -catenin (Fig. 3 B). In contrast to these results, a distinctly different profile of  $\beta$ -catenin turnover from the N-cadherin complex was found in AtT20<sup>+</sup> cells. The ratio of N-cadherin/ $\beta$ -catenin in the complexes was approximately constant over the time course of the experiment, and both N-cadherin and  $\beta$ -catenin were degraded with similar kinetics (Fig. 3 A). Therefore, in AtT20<sup>+</sup> cells, N-cadherin and  $\beta$ -catenin assemble and turn over coordinately as a protein complex similar to the degradation profile of these proteins in other cells (McCrea and Gumbiner, 1991; Shore and Nelson, 1991; Ozawa and Kemler, 1992). Note that in AtT20<sup>+</sup> cells the ratio of N-cadherin/ $\beta$ -catenin in the complexes was different depending on the immunoprecipitating antibody (Fig. 3, A and B). More  $\beta$ -catenin was immunoprecipitated with the  $\beta$ -catenin antibody (Fig. 3 B), than was coimmunoprecipitated with the N-cadherin antibody (Fig. 3 A), although both antibodies were used in excess of protein. These results reveal a N-cadherin-independent pool of  $\beta$ -catenin.

These data demonstrate that Wnt-1 expression in AtT20<sup>+</sup> cells results in the stabilization of  $\beta$ -catenin in the N-cadherin complex. It is possible that Wnt-1 expression results in a posttranslational modification of  $\beta$ -catenin, increasing its avidity for N-cadherin and resulting in a more stable association. Alternatively,  $\beta$ -catenin may exchange between an N-cadherin assembled and unassembled pool of  $\beta$ -catenin; in AtT20<sup>-</sup> cells, the free pool of  $\beta$ -catenin is very small, whereas in AtT20<sup>+</sup> cells it is relatively large (Fig. 3 B).

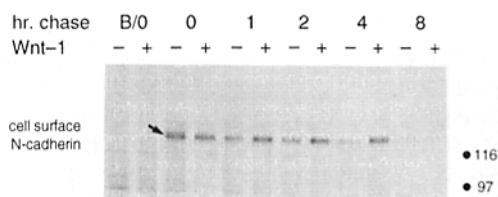
Newly synthesized N-cadherin did not appear to coimmunoprecipitate with newly synthesized plakoglobin (Fig. 3, A and C). That this complex forms at steady state, however, was established in the sequential immunoprecipitation/immunoblots shown in Fig. 2 B, B and C. The absence of a detectable complex after a pulse label may reflect noncoordinate assembly or the weak association of N-cadherin and plakoglobin, supporting the idea that differences in the N-cadherin complexes between  $\beta$ -catenin and plakoglobin may be functionally significant (see above). Nonetheless, the results confirmed the Wnt-1-dependent increase in plakoglobin accumulation. In both the AtT20<sup>-</sup> and AtT20<sup>+</sup> cells, plakoglobin decayed exponentially.

Changes were not detected in the synthesis and turnover of  $\alpha$ -catenin as a consequence of Wnt-1 expression (Fig. 3 D). Although the association of  $\alpha$ -catenin with cadherin was disrupted under the conditions used to extract the complexes shown here (Fig. 3 D) (McCrea and Gumbiner, 1991), other extraction conditions (Wollner et al., 1992; Piepenhagen and Nelson, 1993) revealed complexes of these proteins in both AtT20<sup>-</sup> and AtT20<sup>+</sup> cells (data not shown). That there were no changes in  $\alpha$ -catenin synthesis and turnover, or in the synthesis of total cellular protein (data not shown), indicates that the Wnt-1 effects on  $\beta$ -catenin synthesis and stabilization in cadherin complexes do not reflect a Wnt-1-dependent, global alteration of protein synthesis and stability.

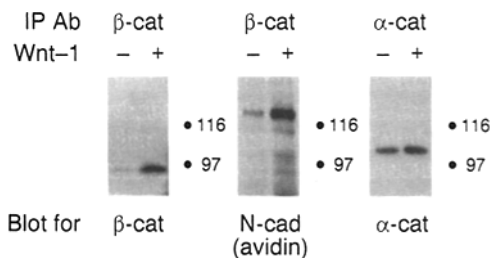
### Wnt-1 Expression Results in Increased Stability of Cell Surface N-cadherin

We sought to investigate the effects of increased loading and stability of  $\beta$ -catenin/cadherin complexes in Wnt-1-expressing cells on cell surface expression and function of N-cadherin. The time course of cell surface expression of N-cadherin was determined by pulse-labeling proteins with [<sup>35</sup>S]methionine, followed by a chase period in the absence of labeled methionine, and detection of newly synthesized protein at the plasma membrane by cell surface biotinylation (Fig. 4). The results showed a short term increase in stability of the population of N-cadherin expressed on the surface of AtT20+ cells (Fig. 4), which could be sufficient to account for the increased steady state levels of N-cadherin (Fig. 2 A). The increased stability of the cell surface pool of cadherin is not reflected in an increased stabilization of the total population of newly synthesized cadherin (Fig. 3 A). This may reflect that only a fraction of total cadherin is stabilized. Nevertheless, our results show that the increased stability of the cell surface population and the increased steady state accumulation of N-cadherin correlates with the accumulation of  $\beta$ -catenin and the stabilized assembly of  $\beta$ -catenin in the N-cadherin complex.

Under the experimental procedures used to isolate the biotinylated pool of N-cadherin,  $\beta$ -catenin was dissociated from the N-cadherin complex (Fig. 4). Therefore, we performed this experiment in a different way to confirm that the additional N-cadherin molecules on the cell surface of Wnt-1-expressing cells were complexed with  $\beta$ -catenin. To detect these complexes, proteins on the cell surface were biotinylated, cells were extracted as before, and protein complexes were immunoprecipitated with either  $\beta$ -catenin or  $\alpha$ -catenin antibodies. After SDS-PAGE, proteins were blotted with either  $\beta$ -catenin or  $\alpha$ -catenin antibodies, or with HRP-avidin (Fig. 5). A dramatic increase in  $\beta$ -catenin levels was detected in AtT20+ cells compared to control cells as expected (see Fig. 1 B). In addition, a corresponding increase in cell surface biotinylated cadherin was immunoprecipitated in a



**Figure 4.** Wnt-1 expression results in increased stability of N-cadherin at the surface of AtT20 cells. The kinetics of synthesis and turnover of the cell surface population of N-cadherin were determined by cell surface biotinylation of newly synthesized protein in control (-Wnt-1) and Wnt-1-expressing (+Wnt-1) AtT20 cells. Replicate cultures of cells were pulse-labeled (0 h chase) with [<sup>35</sup>S]methionine and chased in the absence of label for the indicated times (1, 2, 4, 8 h chase). At each time point, cell surface proteins were biotinylated and cell extracts prepared. Protein equivalent aliquots were immunoprecipitated with a pan-cadherin antibody and, following dissociation of the immune complex, the biotinylated population of N-cadherin was isolated by precipitation with avidin-agarose. As a control, the immunoprecipitation was specifically blocked by preincubating the antiserum with the cognate fusion protein (B/O).



**Figure 5.** Wnt-1 expression results in increased steady state levels of N-cadherin/ $\beta$ -catenin complexes at the surface of AtT20 cells. The steady state protein levels of cell surface N-cadherin complexed with  $\beta$ -catenin were determined by biotinylation of cell surface proteins in control (-Wnt-1) and Wnt-1-expressing (+Wnt-1) AtT20 cells. Cell extracts were prepared and protein equivalent aliquots were immunoprecipitated with an antibody directed against  $\beta$ -catenin or  $\alpha$ -catenin. The immunoprecipitates were separated by SDS-PAGE and biotinylated N-cadherin, immunoprecipitated in association with  $\beta$ -catenin, was detected by blotting with HRP-avidin. The levels of  $\beta$ -catenin and  $\alpha$ -catenin were determined by immunoblotting with antibodies directed against  $\beta$ -catenin and  $\alpha$ -catenin.

complex with  $\beta$ -catenin from the Wnt-1-expressing cells. Note that there is little difference in the amount of  $\alpha$ -catenin between the Wnt-1-expressing and control cells. These results confirm that there is increased  $\beta$ -catenin and cadherin in Wnt-1-expressing cells and, significantly, that the increased cadherin at the cell surface is complexed with  $\beta$ -catenin.

### Wnt-1 Expression Correlates with Increased Strength of Cell-Cell Adhesion in AtT20 Cells

We sought to determine directly whether increased N-cadherin stability and N-cadherin/ $\beta$ -catenin complex formation affected cell-cell adhesion. For these studies, we used a previously characterized assay for cell adhesion in which cell aggregates, formed in suspension culture, were sheared by vigorous trituration (Schmidt et al., 1992). This assay was chosen because it provides a measure of the strength of cell-cell adhesion. Since both Wnt-1+ and Wnt-1- AtT20 cell lines express cell surface cadherins, we expected that both cell lines would adhere; however, we considered that the increased stability of the catenin/cadherin complex in Wnt-1+ cells might result in an increase in the strength of the interaction between cells.

Both AtT20- and AtT20+ cells formed equally large cell aggregates (>200 cells/aggregate) within 2 h (Fig. 6 A) as expected since both cell lines express cell surface N-cadherin. However, trituration of the aggregates resulted in the dissociation of ~90% of control aggregates (-Wnt-1) into groups of <15 cells (Fig. 6, A and B). In contrast, ~45% of Wnt-1-expressing cells (AtT20+) survived this treatment with larger aggregates intact (>15 cells/aggregate) (Fig. 6, A and B). Analysis of the AtT20+ cell aggregates at higher magnification revealed a greater degree of compaction compared to the AtT20- cells. These results demonstrate an increased adhesiveness of Wnt-1-expressing AtT20 cells. We also performed the trituration assay on Wnt-1-expressing and control cells suspended in calcium-free growth media (Fig. 6 C). Aggregates formed in calcium-free growth media, indicating the presence of calcium-independent cell-cell adhesion mech-



anisms. However, trituration of the Wnt-1-expressing and control cell aggregates formed in calcium-free media resulted in their complete dissociation. This suggests that calcium is required for strong cell-cell adhesion in both cell lines, and for the resistance to trituration exhibited by the Wnt-1-expressing cell line.

### ***Wnt-1 Expression Increases Calcium-dependent Cell-Cell Adhesion in C57MG Cells***

Previous studies in C57MG mammary epithelial cells have shown that ectopic expression of Wnt-1 causes morphological transformation (Brown et al., 1986; Blasband et al., 1992; Mason et al., 1992), although the cells did not show a change in growth rate, significant anchorage independence, nor increased tumorigenicity in syngeneic or athymic mice (Brown et al., 1986). Here, we examined the effects of ectopic Wnt-1 expression on the degree of cell-cell adhesion in the same clone of cells used in those previous studies (Fig. 7, A and B). In the presence of extracellular calcium, the Wnt-1-expressing and control cells formed large cell aggregates. Trituration of the Wnt-1-expressing cells demonstrated that these cells were resistant to disruption as compared to control cells (Fig. 7, A and B). We quantified the representative micrographs shown in Fig. 7 A by counting the number of cells in an aggregate (Fig. 7 B). After trituration, 40% of the Wnt-1-expressing cells remained in aggregates larger than 11 cells compared to 8% of the control cells. Aggregates formed by the control C57MG cells were easily disrupted (Fig. 7 A and B); after trituration 91% of the control cell aggregates were dissociated to aggregates of <10 cells. When cells were suspended in calcium-free growth medium, both C57MG cells expressing Wnt-1 and control cells exhibited little or no propensity to form aggregates (Fig. 7 A, *w/o Calcium*). This suggests that in these cell lines, a calcium-dependent cell adhesion mechanism is the primary mechanism responsible for cell-cell adhesion and the strength of this adhesion is enhanced by Wnt-1 expression.

## ***Discussion***

### ***Wnt-1 and Formation of the Catenin/Cadherin Complex***

The results of this study indicate a causative role of Wnt-1 expression in regulating cell-cell adhesion in mammalian cells. Strikingly, regulation of cell adhesion correlates with Wnt-1-dependent accumulation of  $\beta$ -catenin and plakoglobin and, specifically, increased loading of  $\beta$ -catenin onto cadherin complexes. Accumulation of armadillo protein family members may be controlled at a posttranscriptional level in these mammalian cells. Preliminary analysis of  $\beta$ -catenin and plakoglobin mRNA levels indicate no change in Wnt-1-expressing compared to control AtT20 cells. Significantly, previous studies in *Drosophila* also demonstrated that wingless expression regulates the accumulation of armadillo protein by a post-transcriptional mechanism during embryogenesis (Riggelman et al., 1990). Those studies in *Drosophila*, however, were not able to shed light on the consequences of posttranslational accumulation of armadillo family members that are known to interact with cadherin cell adhesion pro-

teins in vertebrates. In the present study, we show that the accumulation of  $\beta$ -catenin in mammalian cells correlates directly with a stabilization of the  $\beta$ -catenin/cadherin complex at the cell surface and increased strength of calcium-dependent cell-cell adhesion.

We observed increases in the steady state levels of  $\beta$ -catenin and plakoglobin, and increased stability of  $\beta$ -catenin with cadherin, but we did not detect changes in the steady state level of  $\alpha$ -catenin or binding of  $\alpha$ -catenin to cadherin as a consequence of Wnt-1 expression. This result indicates that  $\alpha$ -catenin is not part of the same pathway involving Wnt-1 regulation of  $\beta$ -catenin levels and, hence, cadherin function in mammalian cells. In this context, it is interesting to note that the distribution of *Drosophila*  $\alpha$ -catenin is rather homogeneous compared to the discrete segmental patterns of wingless and armadillo proteins (Oda et al., 1993). This indicates that the regulatory mechanisms involved in determining the distributions of  $\alpha$ -catenin and armadillo protein in *Drosophila* are also different.

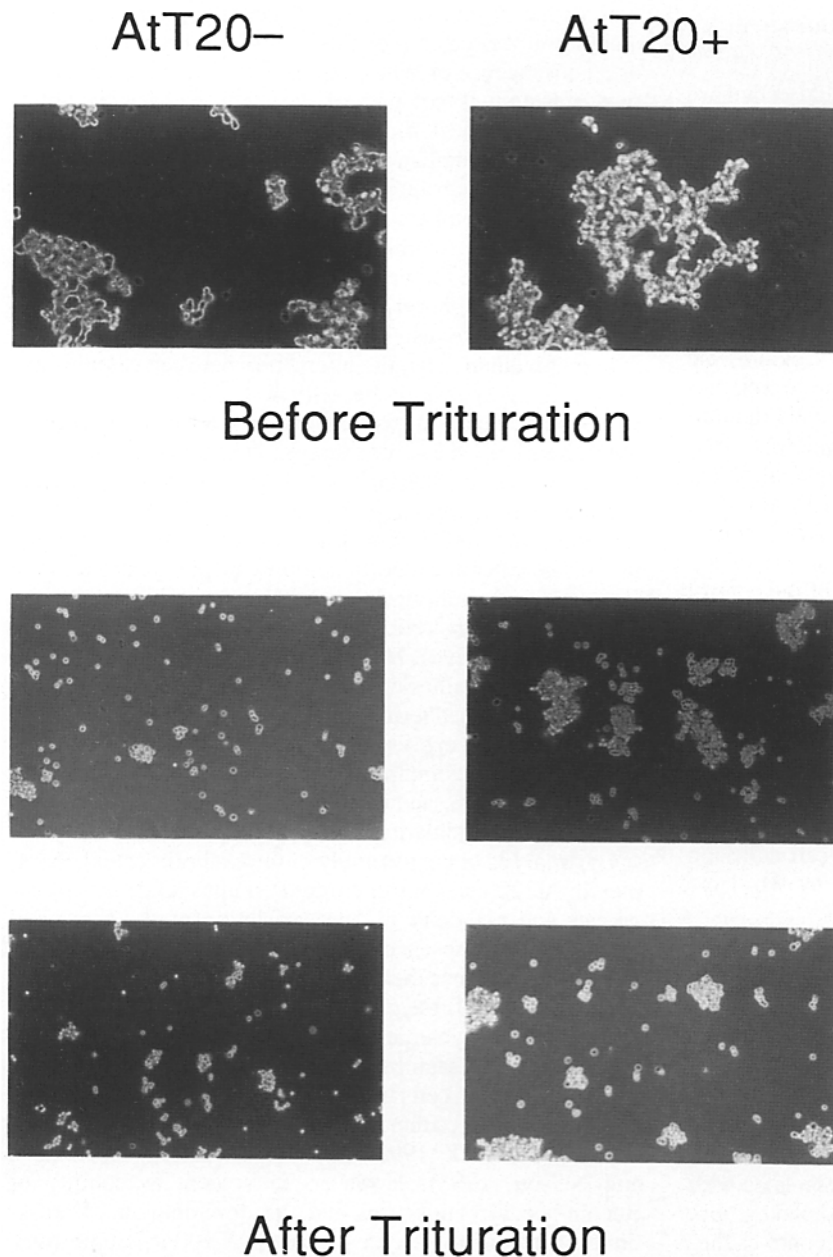
In mammalian cells, the interaction between catenins and cadherins is known to be critical for the formation and strength of calcium-dependent cell-cell adhesion (Takeichi, 1990; Kemler, 1992). For example, cells that express cadherin and  $\beta$ -catenin, but lack  $\alpha$ -catenin, do not form cell aggregates until  $\alpha$ -catenin is expressed by cDNA transfection (Hirano et al., 1992). In addition, posttranslational modulation of the catenin/cadherin complex by phosphorylation of  $\beta$ -catenin and cadherin by *v-src* tyrosine kinase coincides with a decrease in cell adhesion (Matsuyoshi et al., 1992; Behrens et al., 1993). Here, we have shown that strengthening of cell-cell adhesion occurs after stabilized loading of  $\beta$ -catenin onto cadherin/ $\alpha$ -catenin complexes as a consequence of Wnt-1 expression. Thus, our results provide the first evidence that binding of  $\beta$ -catenin to cadherin promotes cell-cell adhesion, and thus  $\beta$ -catenin, specifically, plays a direct role in modulating cadherin function.

Our analysis of the assembly of the cadherin/catenin complex in AtT20 cells has uncovered an unusual degree of dynamics and plasticity in the association of  $\beta$ -catenin and cadherin. In the absence of Wnt-1 expression,  $\beta$ -catenin initially bound to, but then dissociated from cadherin; in the presence of Wnt-1, the  $\beta$ -catenin/cadherin complex was stabilized. These dynamics are different from the prevailing view of cadherin/catenin complex assembly in MDCK cells, fibroblasts and A6 cells in which  $\alpha$ - and  $\beta$ -catenin form a stable complex with cadherin (Ozawa and Kemler, 1992; Knudsen and Wheelock, 1993; Peifer et al., 1993; Piepenhagen and Nelson, 1993). However, the recent availability of catenin-specific antibodies and the development of cross-linking methods to analyze the Triton X-100 insoluble complex has revealed a remarkable plasticity in the pathway of complex formation in MDCK cells, and uncovered temporal and spatial regulation of assembly, disassembly, and exchange of  $\alpha/\beta$ -catenin and cadherin complexes (Hinck, L., I. S. Näthke, J. Papkoff, and W. J. Nelson, manuscript submitted for publication; Näthke, I. S., L. Hinck, J. Swedlow, J. Papkoff, and W. J. Nelson, manuscript submitted for publication). These new insights into cadherin/catenin complex assembly indicate that there are multiple steps in the assembly and exchange of proteins in the complex each of which are potential targets for regulation. Our present studies in AtT20 cells, therefore, show that Wnt-1 expression regulates

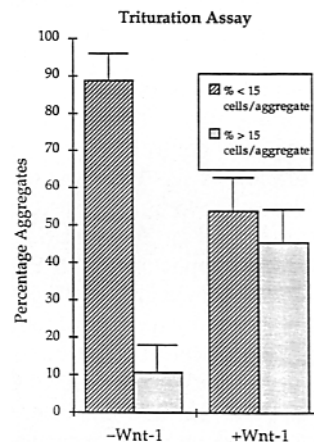


A

## Calcium



B



**Figure 6.** Wnt-1 expression increases the adhesiveness of AtT20 cells. (A) Micrographs showing the morphology of control (-Wnt-1) and Wnt-1-expressing (+Wnt-1) AtT20 cells before and after trituration following aggregation in suspension culture. (B) Histogram representing the data collected by counting the control (-Wnt-1) and Wnt-1-expressing (+Wnt-1) AtT20 cell aggregates that remain following trituration. Three separate experiments, representing 18 micrographs, were quantified by counting the number of aggregates containing less than 15 cells (% < 15 cells/aggregate: -Wnt-1 = 89%, SD = 7; +Wnt-1 = 54%, SD = 9) and more than 15 cells (% > 15 cells/aggregate: -Wnt-1 = 11%, SD = 7; +Wnt-1 = 46%, SD = 9). (C) Micrograph showing the morphology of control (-Wnt-1) and Wnt-1-expressing (+Wnt-1) AtT20 cells before and after trituration following aggregation in calcium free suspension culture (*w/o Calcium*).

one aspect of this dynamic assembly pathway at the level of stabilizing  $\beta$ -catenin loading on cadherin.

### *Wnt-1 and Regulation of Cell-Cell Adhesion*

Since both control and Wnt-1-expressing cell lines have cadherin on the cell surface, we reasoned that both cell types would adhere, but that the changes in the cadherin/catenin complex of Wnt-1-expressing cells might modulate the strength of adhesion. A stringent test of the strength of cell-cell adhe-

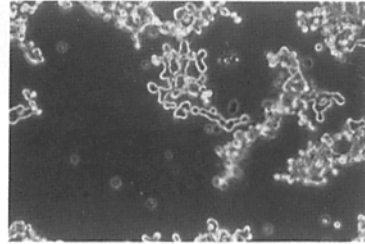
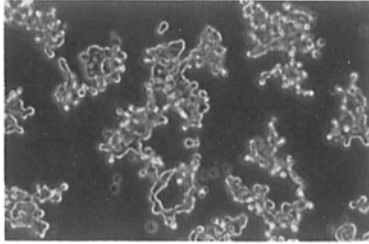
sion is vigorous trituration of cells following their aggregation in suspension culture (Schmidt et al., 1992). Initial aggregation of AtT20 cells expressing Wnt-1 and control cells was not dependent on the presence of extracellular calcium, indicating that these cells expressed a cell adhesion mechanism in addition to cadherin. It is important to note, however, that in the absence of extracellular calcium, trituration of either AtT20- or AtT20+ cell aggregates resulted in rapid disruption of the aggregates and the generation of a population of single cells. In contrast, when cells were allowed to

C

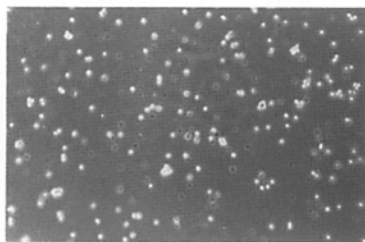
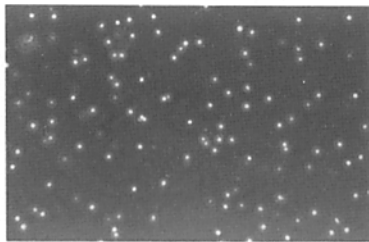
w/o Calcium

AtT20-

AtT20+



Before Trituration



After Trituration

Figure 6.

aggregate in the presence of a normal concentration of calcium in the growth medium (1.8 mM), the cell aggregates formed from Wnt-1-expressing cells were dramatically more resistant to disruption by trituration than control cells. Together, these results indicate that calcium-independent adhesion in these cells does not contribute significantly to the increased strength of adhesion. These results show a direct correlation between increases in steady state levels of N-cadherin, stabilized binding of  $\beta$ -catenin to the cadherin complex, and strength of calcium-dependent cell-cell adhesion in Wnt-1-expressing AtT20 cells.

Unlike AtT20 cells, initial cell-cell adhesion of C57MG cells was dependent on the presence of extracellular calcium. Both the Wnt-1-expressing and control cells formed large cell aggregates in the presence of extracellular calcium. Similar to the results with the AtT20 cells, the Wnt-1-expressing C57MG cells survived trituration with larger aggregates intact. This indicates that the strength of cell-cell adhesion in this cell line also requires a calcium dependent cell-cell adhesion mechanism.

It is interesting to note that while we found Wnt-1 effects in C57MG and AtT20 cells, we have not detected significant Wnt-1-dependent modulation of cadherin/catenin complexes or increased cell adhesion in MDCK cells (unpublished results). In MDCK cells, which adhere rapidly and strongly in the presence of calcium (Gumbiner and Simons, 1986), the Wnt-1 signal may already be complemented by another factor, perhaps another Wnt family member. Based on these results, we hypothesize that Wnt-1 acts as a signal for cell adhesion in cells expressing a receptive cell adhesion mechanism that can be modulated by an increase in  $\beta$ -catenin and plakoglobin protein levels.

#### ***Wnt-1 and Control of Cell Proliferation and Formation of Cell Boundaries***

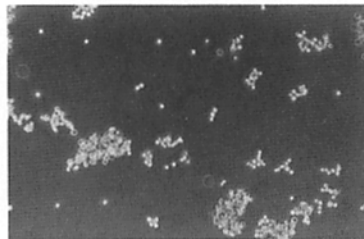
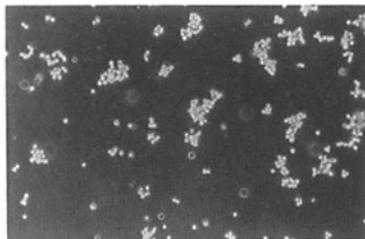
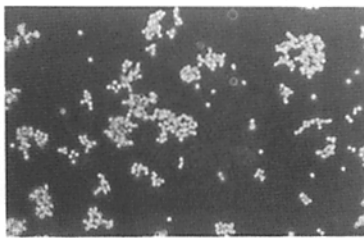
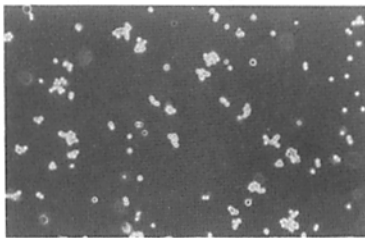
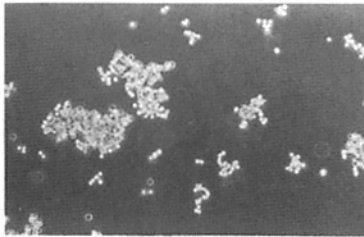
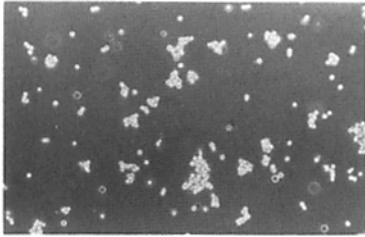
The data presented in this paper demonstrate, in two independent cell types, that one consequence of Wnt-1 expression is increased strength of cell-cell adhesion. This new phenotype of Wnt-1 may be construed to be at odds with previous

A

Calcium

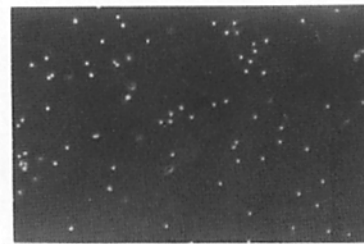
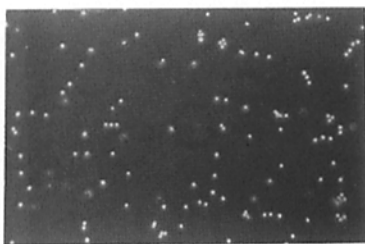
C57MG-

C57MG+



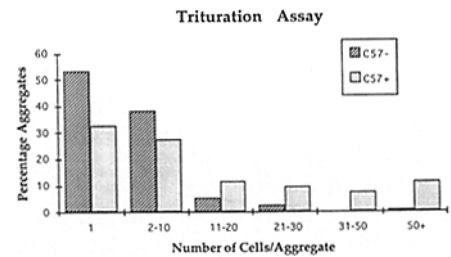
After Trituration

w/o Calcium



No Trituration

B



**Figure 7.** Wnt-1 expression increases the adhesiveness of C57MG cells. (A) Micrographs showing the morphology of control (-Wnt-1) and Wnt-1-expressing (+Wnt-1) C57MG cells after trituration following aggregation in suspension culture containing 1.8 mM calcium (Calcium), and after aggregation in calcium-free media (w/o Calcium). (B) Histogram representing the data collected by counting the control (-Wnt-1) and Wnt-1-expressing (+Wnt-1) C57MG cell aggregates that remain following trituration. Representative micrographs were selected (shown in A) from three separate experiments and the number of single cells and aggregates containing 2-10, 11-20, 21-30, 31-50, and 50+ cells were counted.

ideas about the function of Wnt-1 as a regulator of cell proliferation and differentiation. However, reanalysis of previous studies indicates that the effects of Wnt-1 on cell proliferation and cell-cell adhesion are not mutually exclusive. We will briefly discuss those studies in the context of the new Wnt-1 phenotype.

The C57MG mammary epithelial cell line has been used extensively as a model system to analyze Wnt-1 effects in vitro. Initial studies showed that Wnt-1 expression in these cells caused partial transformation (Brown et al., 1986). Unlike the control cell lines, Wnt-1-expressing C57MG cells continue to divide beyond confluence. They exhibit a phenotypic alteration characterized by elongated cell morphology and multilayered growth. Of note is the observation that there is no change in the growth rate of Wnt-1-expressing cells in subconfluent culture compared to control cells. Also, by established criteria, these cells are not fully transformed; they do not grow in soft agar or form tumors in syngeneic or athymic mice. In an independent study, we confirmed, using the same C57MG cell line, that Wnt-1 expression leads to a similar phenotype (Blasband et al., 1992). Significantly, however, we found in the present study, that this same cell line also exhibits an increase in calcium-dependent cell-cell adhesion. Together, these data demonstrate that the effects of Wnt-1 expression in these cells on cell proliferation and cell-cell adhesion are not mutually exclusive events.

In invertebrates, the Wnt-1 homolog, wingless plays a critical role in establishing segmental boundaries. It has been suggested that wingless acts to modify the character of the segmental boundary in order to prevent diffusion of morphogens (Sampedro et al., 1993). The sealing of boundaries between groups of cells could be accomplished by a Wnt-1-induced increase in cell-cell adhesion as suggested by the results of our experiments. It has also been shown in other studies that wingless acts as a morphogen in the regulation of cell proliferation and differentiation of Malpighian tubules (Skaer and Martinez-Arias, 1992). It is interesting to note that a null mutant of *wingless* is dwarfish, but rescued mutants that ubiquitously express wingless in the embryo are nearly returned to wildtype size (Sampedro et al., 1993). Thus, one interpretation of these data is that wingless expression affects cell proliferation. Together, these data could be interpreted as evidence that in *Drosophila* Wnt-1 has an effect on both cell proliferation and boundary formation/cell-cell adhesion.

In vertebrates, Wnt-1 was originally identified as a target for MMTV insertional activation that leads to mammary hyperplasias (Nusse and Varmus, 1982; Nusse et al., 1984). In *Xenopus* development, ectopic expression of Wnt-1 causes duplication of the embryonic axis (McMahon and Moon, 1989), although it is not known whether this effect is at the level of cell proliferation, cell adhesion, or cell differentiation. In contrast, however, expression of Wnt-1 in early *Xenopus* embryos results in increased gap junctional communication (Olson et al., 1991), which in mammalian cell has been shown to require calcium-dependent cell-cell adhesion (Musil et al., 1990; Jongen et al., 1991).

The duality of effects of Wnt-1 on both cell proliferation and cell-cell adhesion observed in our studies fit the paradigm of other growth factors that affect both morphogenetic movements and cell proliferation. One example is hepatocyte growth factor (HGF). This factor was originally de-

scribed as "scatter factor" because it caused MDCK cells to migrate away from each other and divide (Stoker et al., 1987). However, HGF was shown in the same cell line to cause branching morphogenesis of epithelial tubules formed in vitro (Montesano et al., 1991a,b). Activin, a mesoderm inducer in *Xenopus* embryos, causes cell proliferation and elongation of *Xenopus* animal caps in vitro (Smith et al., 1990), which mimics a normal morphogenetic movement that occurs during gastrulation. At present, it is not clear how Wnt-1, or other growth factors (see above), function in the regulation of both cell adhesion and cell proliferation. One possibility is that Wnt-1 may regulate cell proliferation independent of its effects on  $\beta$ -catenin. Alternatively, Wnt-1 may regulate the interaction of  $\beta$ -catenin with other proteins that play a role in the regulation of cell proliferation. In this context it is interesting to note that  $\beta$ -catenin has recently been found to interact with the protein encoded by the tumor suppressor gene, APC (Rubinfeld et al., 1993; Su et al., 1993). The identification of other proteins that interact with  $\beta$ -catenin may provide new insights into targets of Wnt-1 regulation.

In general, the formation of different tissues requires coordinate cell proliferation and morphogenetic movements of groups of cells (e.g., radial intercalation and convergent-extension during gastrulation) (Gerhart and Keller, 1986). During these events, the integrity of groups of cells is maintained by cell-cell adhesion, but at the same time, the cells proliferate and slide past or dissociate themselves from other groups of cells (Trinkaus, 1984; Gumbiner, 1992). Based upon the results of this study, we speculate that Wnt family members could play a general role in modulating cell-cell interactions during these morphogenetic movements. Wnt-1 signaling could be mediated by its own specific cell surface receptor, as is the case of HGF (Naldini et al., 1991) and activin (Mathews and Vale, 1991; Attisano et al., 1992). That both C57MG and AtT20 cells respond to Wnt-1 expression indicates that these cells carry an active receptor and signaling pathway. In these cells, the binding of Wnt-1 to, and activation of, its specific receptor may indirectly regulate the stability and accumulation of  $\beta$ -catenin, plakoglobin and cadherin leading to the increased strength of cell-cell adhesion. However, it remains a possibility that these effects are through direct binding of Wnt-1 to cadherin or a protein closely associated with cadherin. In either case, such a post-transcriptional mechanism for modulating catenin/cadherin complex formation and cadherin function in cell adhesion means that the cell would be able to respond rapidly to coordinate transient cell-cell contacts in the formation of cellular boundaries during development.

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## References

- Attisano, L., J. L. Wrana, S. Cheifetz, and J. Massagué. 1992. Novel activin receptors: distinct genes and alternative mRNA splicing generate a repertoire of serine/threonine kinase receptors. *Cell*. 68:97-108.
- Behrens, J., M. M. Mareel, R. F. Van, and W. Birchmeier. 1989. Dissecting tumor cell invasion: epithelial cells acquire invasive properties after the loss of uvomorulin-mediated cell-cell adhesion. *J. Cell Biol.* 108:2435-2447.
- Behrens, J., L. Vakaet, R. Friis, E. Winterhager, F. Van Roy, M. M. Mareel, and W. Birchmeier. 1993. Loss of epithelial differentiation and gain of invasiveness correlates with tyrosine phosphorylation of the E-cadherin/ $\beta$ -catenin complex in cells transformed with a temperature-sensitive v-SRC gene. *J. Cell Biol.* 120:757-766.
- Blasband, A., B. Schryver, and J. Papkoff. 1992. The biochemical properties and transforming potential of human Wnt-2 are similar to Wnt-1. *Oncogene*. 7:153-161.
- Bradley, R. S., and A. M. C. Brown. 1990. The proto-oncogene int-1 encodes a secreted protein associated with the extracellular matrix. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:1569-1575.
- Brown, A. M. C., R. S. Wildon, T. J. Prendergast, and H. E. Varmus. 1986. A retrovirus vector expressing the putative mammary oncogene int-1 causes partial transformation of a mammary epithelial cell line. *Cell*. 46:1001-1009.
- Butz, S., J. Stappert, H. Weissig, and R. Kemler. 1992. Plakoglobin and  $\beta$ -catenin: distinct but closely related [letter]. *Science (Wash. DC)*. 257:1013-1016.
- Detrick, R. J., D. Dickey, and C. R. Kintner. 1990. The effects of N-cadherin misexpression on morphogenesis in *Xenopus* embryos. *Neuron*. 4:493-506.
- Gerhart, J., and R. Keller. 1986. Region-specific cell activities in amphibian gastrulation. *Annu. Rev. Cell Biol.* 2:201-229.
- Gumbiner, B. 1992. Epithelial morphogenesis. *Cell*. 69:385-387.
- Gumbiner, B., and K. Simons. 1986. A functional assay for proteins involved in establishing an epithelial occluding barrier: identification of a uvomorulin-like polypeptide. *J. Cell Biol.* 102:457-468.
- Hirano, S., N. Kimoto, Y. Shimoyama, S. Hirohashi, and M. Takeichi. 1992. Identification of a neural  $\alpha$ -catenin as a key regulator of cadherin function and multicellular organization. *Cell*. 70:293-301.
- Jongen, W. M., D. J. Fitzgerald, M. Asamoto, C. Piccoli, T. J. Slaga, D. Gros, M. Takeichi, and H. Yamasaki. 1991. Regulation of connexin 43-mediated gap junctional intercellular communication by  $Ca^{2+}$  in mouse epidermal cells is controlled by E-cadherin. *J. Cell Biol.* 114:545-555.
- Kapprell, H. P., P. Cowin, and W. W. Franke. 1987. Biochemical characterization of the soluble form of the functional plaque protein, plakoglobin, from different cell types. *Eur. J. Biochem.* 166:505-517.
- Kemler, R. 1992. Classical cadherins. *Semin. Cell Biol.* 3:149-155.
- Knudsen, K. A., and M. J. Wheelock. 1992. Plakoglobin, or an 83-kD homologue distinct from  $\beta$ -catenin, interacts with E-cadherin and N-cadherin. *J. Cell Biol.* 118:671-679.
- Korman, N. J., W. E. Russell, M. D. Eyre, V. Klaus-Kovtun, and J. R. Stanley. 1989. Demonstration of an adhering-junction molecule (plakoglobin) in the autoantigens of pemphigus foliaceus and pemphigus vulgaris. *N. Eng. J. Med.* 321:631-635.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)*. 227:680-687.
- Marrs, J. A., E. W. Napolitano, C. Murphy-Erdosh, R. W. Mays, L. F. Reichardt, and W. J. Nelson. 1993. Distinguishing roles of the membrane-cytoskeleton and cadherin mediated cell-cell adhesion in generating different  $Na^{+}, K^{+}$ -ATPase distribution in polarized epithelia. *J. Cell Biol.* 123:149-164.
- Mason, J. O., J. Kitajewski, and H. E. Varmus. 1992. Mutational analysis of mouse Wnt-1 identifies two temperature-sensitive alleles and attributes of Wnt-1 protein essential for transformation of a mammary cell line. *Mol. Biol. Cell*. 3:521-533.
- Mathews, L. S., and W. W. Vale. 1991. Expression cloning of an activin receptor, a predicted transmembrane serine kinase. *Cell*. 65:973-982.
- Matsuyoshi, N., M. Hamaguchi, S. Taniguchi, A. Nagafuchi, S. Tsukita, and M. Takeichi. 1992. Cadherin-mediated cell-cell adhesion is perturbed by v-src tyrosine phosphorylation in metastatic fibroblasts. *J. Cell Biol.* 118:703-714.
- McCrea, P. D., and B. Gumbiner. 1991. Purification of a 92 kDa cytoplasmic protein tightly associated with the cell-cell adhesion molecule E-cadherin (uvomorulin). *J. Biol. Chem.* 266:4514-4520.
- McCrea, P. D., C. W. Turck, and B. Gumbiner. 1991. A homolog of the *armadillo* protein in *Drosophila* (plakoglobin) associated with E-cadherin. *Science (Wash. DC)*. 254:1359-1361.
- McMahon, A. P., and A. Bradley. 1990. The Wnt-1 (int-1) proto-oncogene is required for development of a large region of the mouse brain. *Cell*. 62:1073-1082.
- McMahon, A. P., and R. T. Moon. 1989. Ectopic expression of the proto-oncogene int-1 in *Xenopus* embryos leads to duplication of the embryonic axis. *Cell*. 58:1075-1084.
- Montesano, R., K. Matsumoto, T. Nakamura, and L. Orci. 1991a. Identification of a fibroblast-derived epithelial morphogen as hepatocyte growth factor. *Cell*. 67:901-908.
- Montesano, R., G. Scaller, and L. Orci. 1991b. Induction of epithelial tubular morphogenesis in vitro by fibroblast-derived soluble factors. *Cell*. 66:697-711.
- Morata, G., and P. A. Lawrence. 1977. The development of *wingless*, a homeotic mutation in *Drosophila*. *Dev. Biol.* 56:227-240.
- Musil, L. S., B. A. Cunningham, G. M. Edelman, and D. A. Goodenough. 1990. Differential phosphorylation of the gap junction protein Connexin43 in junctional communication-competent and -deficient cell lines. *J. Cell Biol.* 111:2077-2088.
- Nagafuchi, A., and M. Takeichi. 1988. Cell binding function of E-cadherin is regulated by the cytoplasmic domain. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:3679-3684.
- Nagafuchi, A., Y. Shirayoshi, K. Okazaki, K. Yasuda, and M. Takeichi. 1987. Transformation of cell adhesion properties by exogenously introduced E-cadherin cDNA. *Nature (Lond.)*. 329:340-343.
- Naldini, L., K. M. Weidner, E. Vigna, G. Gaudino, A. Bardelli, C. Ponzetto, R. P. Narsimhan, G. Hartmann, R. Zarnegar, G. K. Michalopoulos, W. Birchmeier, and P. M. Comoglio. 1991. Scatter factor and hepatocyte growth are indistinguishable ligands for the MET oncogene. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:2867-2878.
- Navarro, P., M. Gomez, A. Pizarro, C. Gamallo, M. Quintanilla, and A. Cano. 1991. A role for E-cadherin cell-cell adhesion molecule during tumor progression of mouse epidermal carcinogenesis. *J. Cell Biol.* 110:349-357.
- Nelson, W. J., and P. J. Veshnock. 1986. Dynamics of membrane-skeleton (fodrin) organization during development of polarity in Madin-Darby canine kidney epithelial cells. *J. Cell Biol.* 103:1751-1765.
- Nusse, R., and H. E. Varmus. 1982. Many tumors induced by the mouse mammary tumor virus contain a provirus integrated in the same region of the host genome. *Cell*. 31:99-109.
- Nusse, R., and H. E. Varmus. 1992. Wnt genes. *Cell*. 69:1073-1087.
- Nusse, R., A. van Ooyen, D. Cox, Y. K. Fung, and H. E. Varmus. 1984. Mode of proviral activation of a putative mammary oncogene (int-1) on mouse chromosome 15. *Nature (Lond.)*. 307:131-136.
- Oda, H., T. Uemura, K. Shiomi, A. Nagafuchi, S. Tsukita, and M. Takeichi. 1993. Identification of a *Drosophila* homologue of  $\alpha$ -catenin and its association with *armadillo* protein. *J. Cell Biol.* 121:1133-1140.
- Olson, D. J., J. A. Christian, and R. T. Moon. 1991. Effect of Wnt1 and related proteins on gap junctional communication in *Xenopus* embryos. *Science (Wash. DC)*. 252:1173-1176.
- Ozawa, M., and R. Kemler. 1992. Molecular organization of the uvomorulin-catenin complex. *J. Cell Biol.* 116:989-996.
- Ozawa, M., H. Baribault, and R. Kemler. 1989. The cytoplasmic domain of the cell adhesion molecule uvomorulin associates with three independent proteins structurally related in different species. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:1711-1717.
- Papkoff, J., and B. Schryver. 1990. Secreted int-1 protein is associated with the cell surface. *Mol. Cell Biol.* 10:2723-2730.
- Peifer, M., and E. Wieschaus. 1990. The segment polarity gene *armadillo* encodes a functionally modular protein that is the *Drosophila* homolog of human plakoglobin. *Cell*. 63:1167-1178.
- Peifer, M., P. D. McCrea, K. J. Green, E. Wieschaus, and B. M. Gumbiner. 1992. The vertebrate adhesive junction proteins  $\beta$ -catenin and plakoglobin and the *Drosophila* segment polarity gene *armadillo* form a multigene family with similar properties. *J. Cell Biol.* 118:681-691.
- Piepenhagen, P. A., and W. J. Nelson. 1993. Defining E-cadherin-associated protein complexes in epithelial cells: plakoglobin,  $\beta$ -catenin and  $\gamma$ -catenin are distinct components. *J. Cell Sci.* 104:751-762.
- Riggelman, B., P. Schedl, and E. Wieschaus. 1990. Spatial expression of the *Drosophila* segment polarity gene *armadillo* is posttranscriptionally regulated by *wingless*. *Cell*. 63:549-560.
- Rijsewijk, F., L. van Deemter, E. Wagenaar, A. Sonnenberg, and R. Nusse. 1987. Transfection of the int-1 mammary oncogene in cuboidal RAC mammary cell line results in morphological transformation and tumorigenicity. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:127-131.
- Rubinfeld, B., B. Souza, I. Albert, O. Muller, S. C. Chamberlain, F. R. Masiarz, S. Munemitsu, and P. Polakis. 1993. The APC gene product associates with  $\beta$ -catenin. *Science (Wash. DC)*. 262:1731-1734.
- Sampedro, J., P. Johnston, and P. A. Lawrence. 1993. A role for *wingless* in the segmental gradient of *Drosophila*? *Development (Camb.)*. 117:677-687.
- Schmidt, J. W., J. S. Brugge, and W. J. Nelson. 1992. pp60<sup>src</sup> tyrosine kinase modulates P19 embryonal carcinoma cell fate by inhibiting neural but not epithelial differentiation. *J. Cell Biol.* 116:1019-1033.
- Shore, E. M., and W. J. Nelson. 1991. Biosynthesis of the cell adhesion molecule uvomorulin (E-cadherin) in Madin-Darby canine kidney (MDCK) epithelial cells. *J. Biol. Chem.* 266:1-9.
- Skaer, H., and A. Martinez-Arias. 1992. The *wingless* product is required for cell proliferation in the Malpighian tubule anlage of *Drosophila melanogaster*. *Development (Camb.)*. 116:745-752.
- Smith, J. C., K. Symes, R. O. Hynes, and D. Desimone. 1990. Mesoderm induction and the control of gastrulation in *Xenopus laevis*: the roles of fibronectin and integrins. *Development (Camb.)*. 108:229-238.
- Stoker, M., E. Gherardi, M. Perryman, and J. Gray. 1987. Scatter factor is

- a fibroblast-derived modulator of epithelial cell mobility. *Nature (Lond.)*. 327:239-242.
- Su, L.-K., B. Vogelstein, and K. W. Kinzler. 1993. Association of the APC tumor suppressor protein with catenins. *Science (Wash. DC)*. 262:1734-1737.
- Takeichi, M. 1990. Cadherins: a molecular family important in selective cell-cell adhesion. *Annu. Rev. Biochem.* 59:237-252.
- Takeichi, M. 1991. Cadherin cell adhesion receptors as a morphogenetic regulator. *Science (Wash. DC)*. 251:1451-1455.
- Thomas, K. R., and M. R. Capecchi. 1990. Targeted disruption of the murine int-1 proto-oncogene resulting in severe abnormalities in midbrain and cerebellar development. *Nature (Lond.)*. 346:847-850.
- Towbin, H. T., T. Staehlin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA*. 76:4350-4354.
- Trinkaus, J. P. 1984. *Cells into Organs*. Prentice Hall, Englewood Cliffs, N.J.
- Tsukamoto, A. S., R. Grosschedl, R. C. Guzman, T. Parslow, and H. E. Varmus. 1988. Expression of the int-1 gene in transgenic mice is associated with mammary gland hyperplasia and adenocarcinoma in male and female mice. *Cell*. 55:619-625.
- van den Heuvel, M., R. Nusse, P. Johnston, and P. A. Lawrence. 1989. Distribution of the *wingless* gene product in *Drosophila* embryos: a protein involved in cell-cell communication. *Cell*. 59:739-749.
- Vleminckx, K., L. Vakaet, M. Mareel, W. Fiers, and F. Van Roy. 1991. Genetic manipulation of E-cadherin expression by epithelial tumor cells reveals an invasion suppressor role. *Cell*. 66:107-120.
- Wollner, D. A., K. A. Krzeminski, and W. J. Nelson. 1992. Remodeling the cell surface distribution of membrane proteins during the development of epithelial cell polarity. *J. Cell Biol.* 116:889-899.