

The Mouse *Wnt-1* Gene Can Act Via a Paracrine Mechanism in Transformation of Mammary Epithelial Cells

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The mouse *Wnt-1* gene plays an essential role in fetal brain development and can contribute to tumorigenesis when activated aberrantly in the mammary gland. The gene encodes secretory glycoproteins associated with the extracellular or pericellular matrix, and it has been proposed that *Wnt-1*, as well as its *Drosophila* homolog *wingless*, may function in intercellular signalling. We show here that fibroblasts expressing *Wnt-1* protein, although not transformed themselves, are able to elicit morphological transformation of neighboring C57MG mammary epithelial cells in coculture experiments. Heparin inhibits this effect, possibly by displacing *Wnt-1* protein from its normal site of action. Our results indicate that the *Wnt-1* gene can act via a paracrine mechanism in cell culture and strongly support the notion that in vivo the gene may function in cell-to-cell communication.

Wnt-1 (previously known as *int-1* [17]) is the first of a family of 10 or more closely related genes in the mouse, some of which are strongly implicated in mouse mammary tumorigenesis and all of which are thought to encode secreted factors that may normally function in embryonic development (8, 14, 16, 17, 27, 28). *Wnt-1* was first identified as an oncogene frequently activated by proviral insertions of the mouse mammary tumor virus in virally induced mammary carcinomas (19). These insertions result in expression of a wild-type gene product in a tissue in which the gene is normally silent (18, 19, 35). In cell culture, *Wnt-1* can induce transformation of certain mammary epithelial cell lines (4, 25), while the oncogenic potential of *Wnt-1* in the mammary gland has been further demonstrated by transgenic mice designed to express *Wnt-1* specifically in that tissue. Such mice develop extensive mammary hyperplasia, and many are subsequently afflicted with mammary carcinomas (32). These data are consistent with a multistep pathway of mammary tumorigenesis in which the activation of *Wnt-1* may be an early step sufficient to induce premalignant lesions.

In normal tissues, *Wnt-1* is not expressed in the mammary gland but instead is expressed in the developing neural tube of midgestational embryos and in spermatids of the adult testis (30, 37). While the significance of *Wnt-1* expression in the testis remains to be established, targeted gene disruption experiments have recently demonstrated that *Wnt-1* plays an essential role in early development of the central nervous system; embryos homozygous for a disrupted *Wnt-1* allele develop with much of their midbrain and cerebellum missing and most die within 24 h of birth (13, 31).

The *Drosophila* homolog of *Wnt-1*, *wingless*, is also known to play a vital role in development (20, 24). *Wingless* is a segment polarity gene, and its normal function is essential for correct pattern formation within each segment of the *Drosophila* embryo. Since *wingless* mutations are not cell autonomous and the mutant phenotype extends beyond

the narrow stripe of cells in each segment that express *wingless* RNA, it has been proposed that the *wingless* gene may act in cell-to-cell communication (1, 24, 36). This notion is supported by the presence of an amino-terminal signal peptide in the predicted *wingless* protein sequence and by the detection of *wingless* antigen in blastoderm cells adjacent to those in which the gene is transcribed (24, 34).

The extensive sequence conservation between mouse *Wnt-1* and *Drosophila* *wingless* suggests that their protein products may act via similar mechanisms. Moreover, the mouse *Wnt-1* product is known to be secreted. Studies of mammalian cell culture have shown that *Wnt-1* encodes secreted glycoproteins of 41 to 44 kDa which do not normally accumulate in the culture medium but instead are found associated with the extracellular or pericellular matrix (2, 21). The quantity of secreted *Wnt-1* protein detectable in conditioned media is substantially increased, however, when cells are grown in the presence of either heparin or suramin (2, 21).

We have been studying the mechanism of action of *Wnt-1* in mammalian cell culture systems. It was previously shown that expression of *Wnt-1* in the mouse mammary epithelial cell line C57MG causes a phenotypic transformation characterized by an apparent loss of contact inhibition at confluence, growth to higher cell densities, and an elongated and refractile morphology (4). Although a similar morphological change can be induced by *Wnt-1* in RAC311C cells, another line derived from mouse mammary tissue (25), no such changes are seen when *Wnt-1* is expressed in fibroblast cell lines (4, 26). It seems possible that this is due to the absence of a functional receptor signalling pathway responsive to *Wnt-1* in such cells. However, the cell-type specificity of *Wnt-1* transformation provides an opportunity to distinguish paracrine from autocrine mechanisms and to so seek evidence of intercellular communication mediated by *Wnt-1*. In this report, we describe experiments in which fibroblasts expressing *Wnt-1* are cocultured with C57MG cells that do not express the gene. Our results demonstrate that cells expressing *Wnt-1* are capable of inducing morphological transformation of neighboring C57MG cells, apparently via a

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paracrine mechanism. The effect is abolished by growing the cells in the presence of heparin. These data indicate that the *Wnt-1* gene can play a role in cell-to-cell communication.

MATERIALS AND METHODS

Cell lines and retroviral vectors. All cell lines, with the exception of C57MG, were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum and antibiotics. C57MG, a cell line derived from normal mouse mammary epithelium (33), was maintained in the same medium but with the addition of 10 μ g of insulin per ml. This medium was also used for coculture experiments. Madin-Darby canine kidney epithelial cells (MDCK) were obtained from E. Rodriguez-Boulan.

The retrovirus vectors MXIN and MV*Wnt-1* each contain mouse *Wnt-1* cDNA together with a neomycin phosphotransferase gene (*neo*) (2, 4). In both vectors, *Wnt-1* is expressed from the retroviral long terminal repeat promoter, and in MV*Wnt-1*, an internal thymidine kinase promoter directs expression of *neo*. MV7, the parental vector from which MV*Wnt-1* was constructed, expresses *neo* alone (10). MXfsIN is identical to MXIN but carries a frameshift mutation such that no functional *Wnt-1* protein is produced (4). Helper-free virus stocks of the above vectors were harvested from transfected or infected ψ 2 or PA317 packaging cells and then used to infect Rat-2 cells following procedures described by Brown and Scott (3). Infected cells were selected in the presence of 500 μ g of G418 per ml (GIBCO). Absence of helper virus in the infected populations was confirmed by the failure to detect any focus-forming, or *neo*-transducing, particles when media supernatants were harvested from at least 2×10^6 cells and used to infect appropriate target cells.

Immunoblot analysis of *Wnt-1* proteins. MV7/Rat-2 and MV*Wnt-1*/Rat-2 cells were grown in 60-mm-diameter dishes for 3 days until approximately 80% confluent. The culture medium was then replaced with Dulbecco modified Eagle medium supplemented with 1% fetal calf serum and, where stated, heparin (from porcine intestinal mucosa; Sigma) at 200 μ g/ml was included. Twenty-four hours later, the medium was harvested and cells were detached by incubation in 1 mM EDTA. After the dishes were rinsed with EDTA, extracellular material (ECM) was solubilized by being scraped in Laemmli buffer at 90°C as previously described (2). To concentrate *Wnt-1* proteins from conditioned medium, samples precleared by centrifugation at $1,000 \times g$ for 5 min were spun at $100,000 \times g$ for 3 h at 4°C in a Beckman SW50.1 rotor (29). Pellets were solubilized in 100 μ l of Laemmli buffer and, together with ECM samples, loaded onto a sodium dodecyl sulfate (SDS)-10% polyacrylamide gel. Following electrophoresis, the proteins were transferred to nitrocellulose and detected by using a monoclonal antibody against *Wnt-1* peptide A as previously described (2).

Coculture experiments. For standard coculture experiments, 100 or 500 infected Rat-2 fibroblasts were plated in 10-cm-diameter dishes together with 10^5 C57MG mammary epithelial cells on the same day. The cultures were then grown to confluence over a 5- to 6-day period without replenishing of the growth medium. Foci or rings of transformed cells were visible in dishes containing *Wnt-1*-expressing fibroblasts 24 to 48 h after the monolayers reached confluence. Cells were photographed under phase-contrast illumination with a Nikon TMS inverted microscope. In some experiments, cells from the transformed foci were trypsinized by using cloning cylinders and plated at high

dilution for examination of the morphology of individual colonies. Where stated, heparin (from porcine intestinal mucosa; Sigma) was added to the cocultures at a final concentration of 50 to 200 μ g/ml at the time the cells were plated.

Experiments involving mitomycin C followed a modified protocol in which 100 or 500 infected fibroblasts alone were first plated in 10-cm-diameter dishes and grown for 7 to 10 days until the colonies had reached a diameter of 2 to 3 mm. At this point, the cells were treated with 5 μ g of mitomycin C per ml (Sigma) in Dulbecco modified Eagle medium for 1 h and rinsed several times with phosphate-buffered saline (PBS). C57MG cells were then added to the dishes at high density (40% of confluent density) in conditioned medium harvested from confluent C57MG cells. These conditions ensured that a quiescent monolayer of C57MG cells was obtained within 2 to 3 days.

Hoechst staining and immunofluorescence. Cocultures of C57MG cells with mitomycin C-treated MV*Wnt-1*/Rat-2 colonies grown as described above were fixed in 2% paraformaldehyde in PBS at room temperature for 30 min and rinsed three times with PBS, and the cell nuclei were stained with 20 μ g of Hoechst 33258 stain per ml (Molecular Probes, Inc.) for 5 min. After being rinsed extensively in PBS, the cells were mounted in Aquamount (Lerner Laboratories, Inc.) containing 0.1% *para*-phenylene diamine (Sigma) by coverslips being laid over the cell monolayer. DNA staining patterns in nuclei were examined and photographed by using a $\times 100$ objective on a Leitz Labolux microscope equipped with epifluorescence illumination. Treatment with mitomycin C reduced the intensity of staining of the Rat-2 cell nuclei and so emphasized the differences in staining pattern between the two cell types.

RESULTS

Generation of fibroblast lines expressing *Wnt-1*. To express *Wnt-1* stably and efficiently in fibroblast cell lines, we infected two different sublines of Rat-2 cells with helper-free stocks of the murine leukemia virus-based retrovirus vectors MXIN and MV*Wnt-1* (2, 4). Both these vectors express *Wnt-1* together with a bacterial neomycin phosphotransferase gene (*neo*), and infected cell populations were therefore selected in the presence of G418. For controls, Rat-2 cells were infected either with MXfsIN, a vector bearing a frameshift mutation near the start of the *Wnt-1* open reading frame (4) or with the parent retrovirus vector MV7, which carries *neo* without a *Wnt-1* allele (10). Although MXIN and MV*Wnt-1* virus stocks were proficient at inducing morphological transformation of C57MG mouse mammary epithelial cells (4) (data not shown), the Rat-2 cells infected with these viruses showed no obvious phenotypic changes and were morphologically indistinguishable from either uninfected cells or Rat-2 cells infected with the control viruses (see below and Fig. 3A and B). Immunoblot analysis confirmed, however, that the Rat-2 cells infected with MV*Wnt-1* expressed and secreted 44- and 41-kDa species of *Wnt-1* protein associated with the ECM (Fig. 1, lane 3). As previously demonstrated for another fibroblast line expressing *Wnt-1* protein (2), growth in the presence of heparin led to a reduced amount of extracellular *Wnt-1* protein in the ECM fraction and an increase in the level detectable in conditioned culture medium (Fig. 1, lanes 3, 4, 7, and 8).

Induction of cell transformation by cocultivation of two cell types. Since fibroblasts infected with *Wnt-1* vectors produce extracellular *Wnt-1* protein but apparently do not respond to

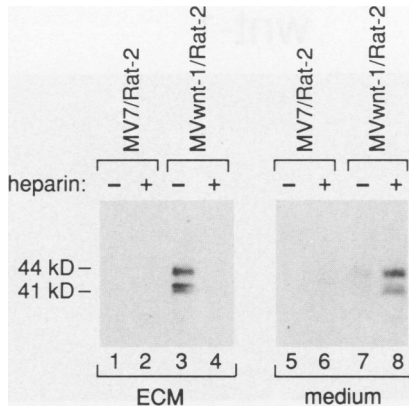


FIG. 1. Immunoblot analysis showing Wnt-1 proteins secreted by MVWnt-1/Rat-2 cells and the effect of heparin. MVWnt-1/Rat-2 cells (lanes 3, 4, 7, and 8) and control MV7/Rat-2 cells (lanes 1, 2, 5, and 6) were grown in the absence (odd-numbered lanes) or presence (even-numbered lanes) of heparin. ECM material (lanes 1 through 4) and concentrated conditioned culture medium (lanes 5 through 8) were analyzed by SDS-polyacrylamide gel electrophoresis followed by transfer to nitrocellulose. Wnt-1 proteins of 41 and 44 kDa were visualized by using a monoclonal antipeptide antibody. Under normal growth conditions, these proteins accumulate in the ECM fraction (lane 3). Addition of heparin increased the quantity detected in conditioned medium (lane 8). Note that samples of conditioned medium were concentrated from 10 times more material than that used for ECM preparations, but not all the Wnt-1 protein present was recovered; quantitative comparisons between medium and ECM samples are therefore not valid.

it phenotypically, we wished to determine the effect of coculturing such fibroblasts with cells of the C57MG line. The latter cells do not express *Wnt-1* but can be morphologically transformed by expression of the gene (4). The initial cocultivation protocol involved plating approximately 100 MXIN/Rat-2 or control MXfIN/Rat-2 cells, together with 2×10^5 C57MG cells, and allowing the mixture to grow to confluence. This is illustrated diagrammatically in Fig. 2. The majority of cells in the dishes were C57MG, which formed a flat cuboidal monolayer at confluence, and this

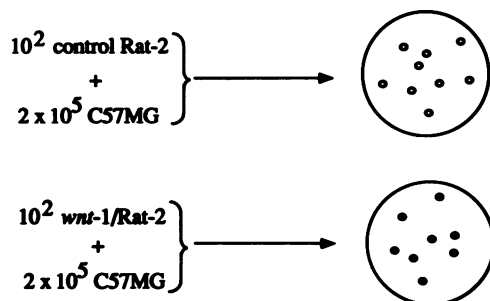


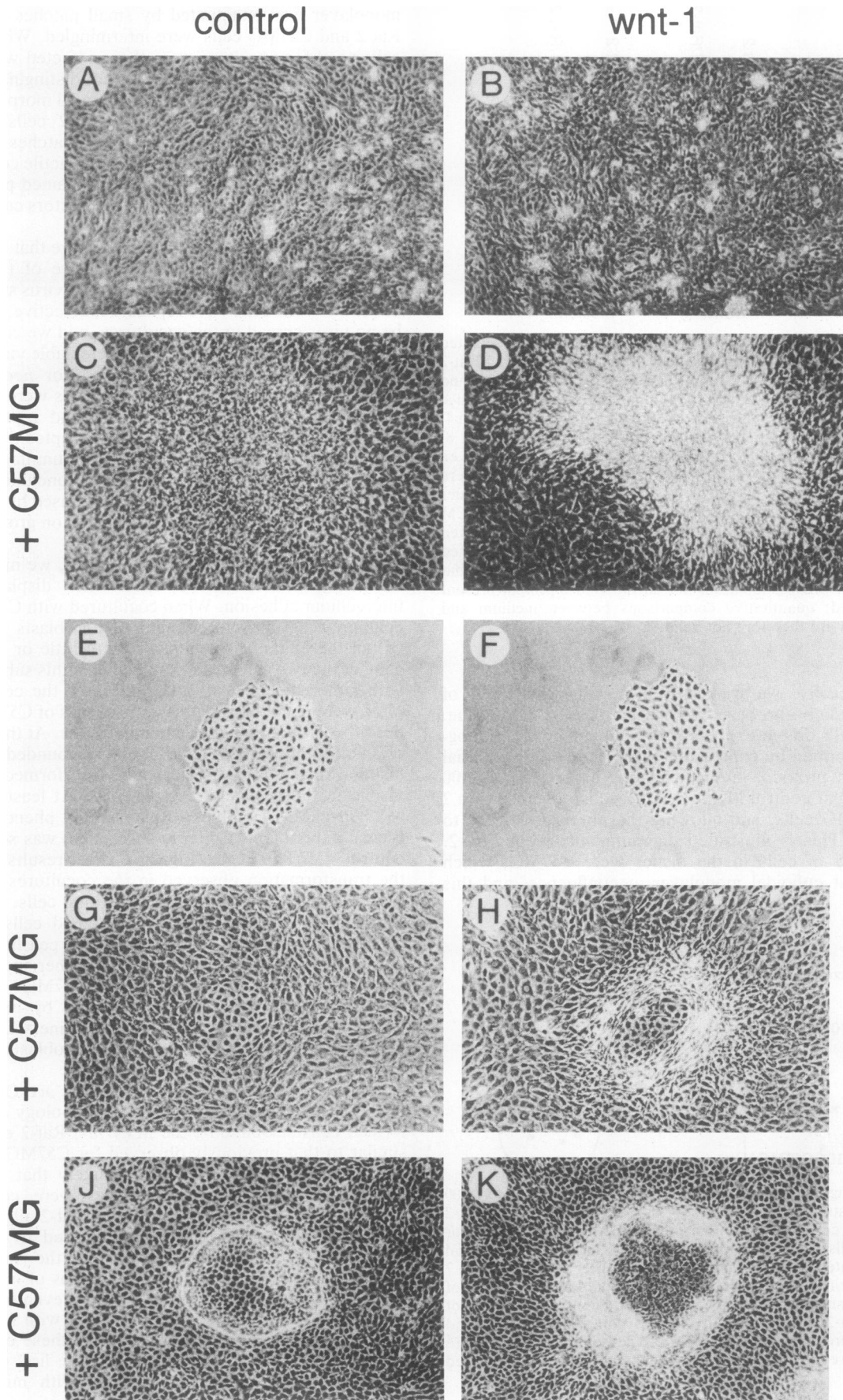
FIG. 2. Diagram of cocultivation procedure. Approximately 100 Rat-2 fibroblasts expressing *Wnt-1* (MXIN/Rat-2 or MVWnt-1/Rat-2 cells) or Rat-2 cells infected with a control vector (MXfIN/Rat-2 or MV7/Rat-2 cells), together with 2×10^5 C57MG mammary epithelial cells, were plated in 10-cm-diameter dishes. After growth for 5 to 7 days, the cultures were composed of flat confluent monolayers of C57MG cells surrounding small regions containing the progeny of individual Rat-2 derivatives. In cultures containing C57MG and fibroblasts expressing *Wnt-1*, transformed cells (closed circles) were observed where the two cell types were intermingled or juxtaposed (see Fig. 3).

monolayer was punctuated by small patches in which the Rat-2 and C57MG cells were intermingled. When the Rat-2 cells used for coculture were those infected with a control virus, these patches were hard to distinguish but were identifiable by a distortion in the cuboidal morphology of the monolayer (Fig. 3C). When the Rat-2 cells were those infected with MXIN, however, these patches appeared as morphologically transformed foci of refractile cells that were virtually indistinguishable from foci obtained previously by infecting C57MG cells with retroviral vectors carrying *Wnt-1* (4) (Fig. 3D).

Several steps were then taken to ensure that this apparent paracrine effect resulted from coculture of the cell lines rather than from gene transfer. Since the virus stocks used to infect the Rat-2 cells were replication defective, there should be no virus spread in the cocultures, and we confirmed that the infected cell lines produced no detectable virus by testing their conditioned medium for *Wnt-1*- or *neo*-transducing particles. Moreover, when refractile cells were picked from transformed foci in the cocultures and replated at low densities, all of the resulting colonies adopted the normal flat morphology of either MXIN/Rat-2 or uninfected C57MG cells (data not shown). Therefore, we concluded that focus formation in these cocultures was not caused by virus spread or by cell fusion but appeared to depend on growing the two cell types in proximity.

To elucidate this phenomenon further, we made use of a different subline of Rat-2 cells which displayed greater intercellular adhesion. When cocultured with C57MG, these cells grew as discrete colonies of fibroblasts recognizable within the epithelial monolayer, with little or no intermingling of the two cell types. Rat-2 cells of this subline infected with either the MVWnt-1 retrovirus or the control vector MV7 were cocultured with a large excess of C57MG cells as described above and grown to confluence. At the perimeters of colonies of MVWnt-1/Rat-2 cells surrounded by C57MG, distinct rings of morphologically transformed cells were visible in these cocultures (Fig. 3H). At least 90% of the MVWnt-1/Rat-2 colonies displayed this phenomenon in a typical experiment, while no such effect was seen with the control MV7/Rat-2 cells (Fig. 3G). These results confirm that the transformation observed in the cocultures was dependent on expression of *Wnt-1* in the Rat-2 cells. Similar foci, or rings of morphologically transformed cells, were also observed when *Wnt-1* was expressed in cell lines of either mesenchymal or epithelial origin from other species and the resulting cells were cocultured with C57MG. These cells, whose own morphology was unaltered by expression of *Wnt-1*, include the canine epithelial cell line MDCK (Fig. 3J and K), as well as murine NIH 3T3 fibroblasts and human HeLa cells (data not shown).

The morphologically transformed cells are C57MG cells. Since the elongated and refractile morphology of the transformed cells surrounding the MVWnt-1/Rat-2 colonies was similar to that previously observed for C57MG cells transformed by *Wnt-1*, it seemed very likely that the rings of transformed cells in the cocultures were composed of C57MG cells rather than MVWnt-1/Rat-2 fibroblasts expressing *Wnt-1*. Nevertheless, we wished to confirm the identity of the transformed cells. Since the rings of transformed cells were associated with regions of increased cell density, we first tested the effect of preventing cell division of the Rat-2 cells by treating them with mitomycin C prior to cocultivation with C57MG. In these experiments, MVWnt-1/Rat-2 or MV7/Rat-2 cells were first grown separately as colonies and then treated with mitomycin C.



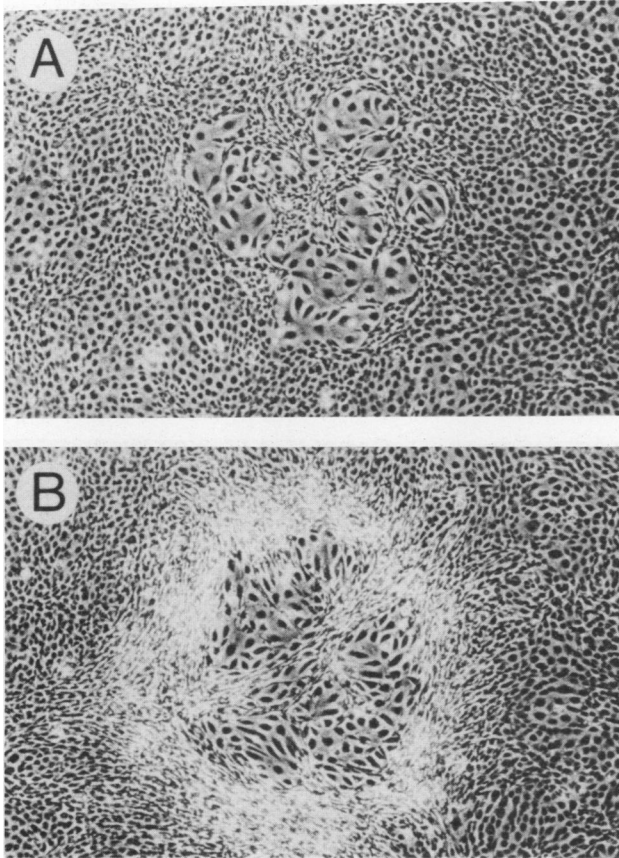


FIG. 4. Coculture of C57MG cells with MVWnt-1/Rat-2 cells treated with mitomycin C still results in transformation. MV7/Rat-2 (A) or MVWnt-1/Rat-2 (B) cells were grown as colonies in the absence of C57MG cells and treated with mitomycin C to prevent further cell division. Untreated C57MG cells were then added to the dishes, and the cultures were photographed 2 days after reaching confluence.

Untreated C57MG cells were subsequently added to the same dishes, and the cultures were left to grow to confluence. Typical results are shown in Fig. 4. Although some of the fibroblasts detached from the dish following mitomycin C treatment (allowing infiltration of the colonies by C57MG cells), rings of transformed cells surrounding the MVWnt-1/Rat-2 colonies were still evident (Fig. 4B). This reinforced the suggestion that the transformed cells were C57MG since only those cells were capable of continued division.

In order to resolve the identity of the transformed cells more definitively, we took advantage of species-specific nuclear staining patterns to distinguish Rat-2 derivatives from C57MG after treating the cells with Hoechst 33258

fluorescent DNA stain. The nuclei of Rat-2 derivatives show a largely uniform Hoechst staining pattern, and after mitomycin C treatment, they become more extended and lightly stained (Fig. 5A). In contrast, the mouse cell nuclei in C57MG cells display a punctate staining pattern that is readily distinguishable from that of MVWnt-1/Rat-2 nuclei (Fig. 5A and B). When cocultures containing C57MG and mitomycin C-treated MVWnt-1/Rat-2 cells were stained with Hoechst stain, the elongated transformed cells surrounding the fibroblast colonies clearly exhibited the punctate staining pattern characteristic of C57MG (Fig. 5C and D). This confirmed that it is the C57MG cells that become transformed in the cocultivations.

Heparin prevents cell transformation in cocultures. The above results suggested that extracellular Wnt-1 protein might be directly involved in the observed paracrine transformation. While we have so far been unable to confirm this (see Discussion), we wished to determine the effect of heparin on the coculture assays, since secreted Wnt-1 protein has been shown to associate with this glycosaminoglycan and heparin is capable of changing the extracellular distribution of Wnt-1 protein (2) (Fig. 1). Accordingly, we repeated the cocultivations with the addition of 50 to 200 μ g of heparin per ml. As shown in Fig. 6, the presence of heparin in the medium dramatically inhibited the transformation of C57MG cells surrounding the MVWnt-1/Rat-2 colonies. Concentrations of heparin sufficient to inhibit paracrine transformation did not alter the appearance of control cocultures and had little or no effect on the growth rate of C57MG cells (data not shown). Since heparin reduces the amount of Wnt-1 protein associated with the extracellular matrix fraction of MVWnt-1/Rat-2 cells (Fig. 1), it seems possible that the heparin inhibition of paracrine transformation might result from a displacement of Wnt-1 protein from its normal site of action.

DISCUSSION

In this report, we have exploited the cell-type specificity of Wnt-1 action to show that fibroblasts expressing Wnt-1, although not phenotypically altered themselves, are able to induce morphological transformation of neighboring C57MG mammary epithelial cells in coculture experiments. Our results indicate that the Wnt-1 gene can act via a paracrine mechanism in a cell culture system. Thus, they provide strong support for the idea that Wnt-1 can function in cell-to-cell communication in mammary tumorigenesis and central nervous system development.

The induction of transformed C57MG cells in these experiments was dependent on expression of a functional Wnt-1 allele in Rat-2 fibroblasts, and similar results were also observed by coculturing C57MG cells with other cell lines expressing a cloned Wnt-1 gene. These include NIH 3T3, HeLa, and MDCK cell lines, none of which displayed an

FIG. 3. Morphological transformation induced by coculture of C57MG cells with cells expressing Wnt-1. Phase-contrast photomicrographs of homogeneous confluent cultures of MXfsIN/Rat-2 (A) and MXIN/Rat-2 (B) cells show that a functional Wnt-1 allele has no effect on the morphology of Rat-2 cells. Cocultures of MXfsIN/Rat-2 (C) or MXIN/Rat-2 (D) cells with C57MG mammary epithelial cells show regions in which the two cell types are intermingled. For cocultures containing MXIN/Rat-2 cells, this results in the appearance of transformed foci. Individual colonies of MV7/Rat-2 (E) and MVWnt-1/Rat-2 (F) cells again show no change in cell morphology as a result of Wnt-1 expression. (G and H) Coculture of these cells with C57MG. In panel G, a colony of MV7/Rat-2 cells (center) surrounded by the flat C57MG cell monolayer is visible, and in panel H, a ring of morphologically transformed cells surrounds a colony of MVWnt-1/Rat-2 cells. Cocultures of uninfected MDCK cells (J) or a clone of MVWnt-1/MDCK cells (K) with C57MG cells are shown. Some of the refractile cells in panel K appeared to overgrow the underlying epithelial cell colony.

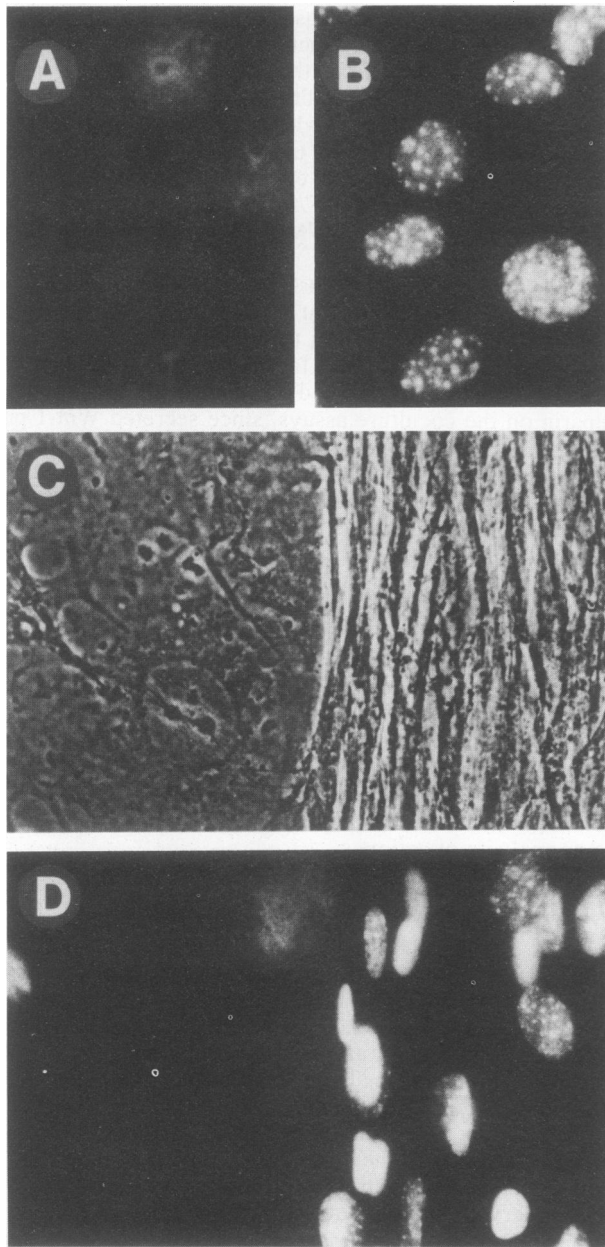


FIG. 5. Fluorescent DNA staining of cocultures demonstrates that the transformed cells are C57MG and not MVWnt-1/Rat-2 cells. (A and B) Mitomycin C-treated MVWnt-1/Rat-2 cells (A) and untreated mouse C57MG cells (B) stained with Hoechst 33258 stain and photographed under epifluorescence to show the characteristic punctate staining pattern of C57MG cell nuclei. (C and D) C57MG cells were cocultured with mitomycin C-treated MVWnt-1/Rat-2 cells as described in the legend to Fig. 4 and stained with Hoechst 33258 stain. (C) High-magnification phase-contrast photomicrograph showing the margin between a central colony of flat fibroblasts (left) and the surrounding elongated transformed cells (right); (D) the same field as shown in panel C but photographed under epifluorescence. The nuclei of transformed cells exhibit the punctate staining pattern of C57MG. All the transformed cells in this field were similarly identified by photography in other planes of focus (data not shown).

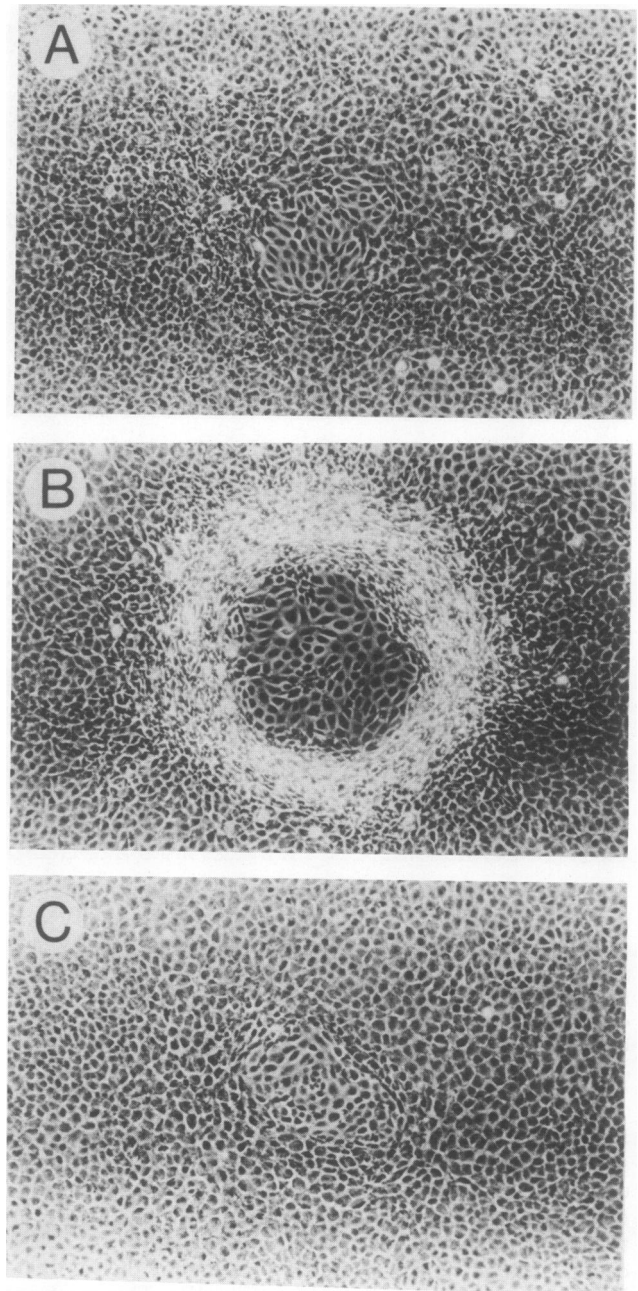


FIG. 6. Transformation of C57MG cells by cocultivation is inhibited by heparin. Cells were cocultured as before but with the addition of 200 μ g of heparin per ml to some dishes at the time of plating the two cell types. C57MG cells were cocultured with control MV7/Rat-2 cells (A), MVWnt-1/Rat-2 cells (B), or MVWnt-1/Rat-2 cells in the presence of heparin (C).

altered morphology as a result of *Wnt-1* expression. The cocultivation procedure therefore provides an effective assay for expression of a biologically active *Wnt-1* product in a variety of cell types.

Since the Rat-2 derivatives described in this report express *Wnt-1* from retroviral vectors, it was important to exclude virus spread as an explanation of the observed paracrine transformation. Several observations allow this conclusion. Firstly, the virus stocks used were helper free

and the infected Rat-2 populations were tested to confirm their lack of virus production. Secondly, the transformation observed in cocultures was reversible when cells from the foci were replated at low density and grown as separate colonies. Thirdly, a similar paracrine transformation phenomenon has recently been observed when C57MG cells are cocultured with quail QT6 fibroblasts transiently transfected with expression plasmids bearing *Wnt-1* alleles (21a). In the latter case, no viral vectors are involved.

Together with definitive identification of the transformed cells by their species-specific nuclear staining pattern (Fig. 5), these arguments confirm that expression of *Wnt-1* in Rat-2 cells can elicit transformation of C57MG cells in the immediate vicinity. This implies that *Wnt-1* could act in either an autocrine or paracrine manner, depending on the cell type in which the gene is activated. It is likely that both mechanisms may pertain to the transformation of C57MG cells expressing *Wnt-1* (4) and to the genesis of mammary tumors following insertional activation of *Wnt-1* by mouse mammary tumor virus proviral DNA. Mouse mammary tumor virus-induced tumors with activated *Wnt-1* alleles in C3H mice are usually clonally derived from a single cell (5), and in such cases *Wnt-1* presumably operates via an autocrine mechanism. The tumors of GR mice, however, are frequently oligoclonal in their early stages and can be composed of two or more mutually interdependent cell populations, only one of which contains an activated *Wnt-1* allele (15). It seems likely that in these cases the *Wnt-1*-expressing cells exert a paracrine effect on the other cells of the tumor mass.

An effect on neighboring cells mediated by *Wnt-1* is also reminiscent of the behavior of wingless in *Drosophila* embryos. At the extended germ band stage, wingless RNA is expressed in each segment in a narrow stripe of cells adjacent to another stripe expressing the homeobox gene engrailed (see reference 6 for review). Genetic evidence indicates that for a certain period the maintenance of engrailed expression in the latter stripe depends on expression of the wingless gene in the adjacent cells (7, 9, 11). In addition, the protein product of another segment polarity gene, armadillo, shows an altered distribution in a broad stripe surrounding the cells expressing wingless RNA, and this effect is also dependent on wingless function (22, 23). Together with the detection of wingless antigen in cells expressing engrailed (34), these observations can be explained by a model in which wingless protein diffuses locally within the embryo and elicits specific changes in gene expression within neighboring cells. In this regard, it will be interesting to determine whether any genes related to engrailed or armadillo in C57MG cells might exhibit changes in expression in response to *Wnt-1*.

Since *Wnt-1* is known to encode a secreted glycoprotein, and by analogy with wingless, the simplest model to account for the paracrine transformation effect we have observed here would be that extracellular *Wnt-1* protein itself acts as a signalling molecule diffusing locally between the cells, perhaps via interactions with the ECM. Although we favor this model, compelling evidence in support of it is lacking, as attempts to demonstrate transforming potential associated with either conditioned medium or ECM fractions from MVWnt-1/Rat-2 cells have so far been unsuccessful and we cannot, therefore, exclude the possibility that cell-cell contact is required. Attempts to block the paracrine effect by adding *Wnt-1* antibodies to the coculture dishes have also been ineffectual, although the existing antibodies may not present a particularly rigorous challenge. An alternative

explanation for the paracrine effect could be that another signalling mechanism is activated as a secondary consequence of *Wnt-1* expression in Rat-2 cells. In principle, this might involve release of an unrelated secreted factor with transforming potential. However, the paracrine effect has now been observed by using *Wnt-1*-expressing cell lines derived from five different species and of both mesenchymal and epithelial origin. It would be surprising if in each case *Wnt-1* induced release of a factor that was unable to affect the cells producing it but was nevertheless capable of transforming C57MG. Such a factor would have to display the same restricted specificity of target cell transformation as shown by the *Wnt-1* gene itself in the various cell lines tested.

Certain observations are nevertheless compatible with a direct role for *Wnt-1* protein in the observed paracrine transformation. Firstly, we have demonstrated that under normal growth conditions secreted *Wnt-1* proteins are associated with the extracellular matrix of the MVWnt-1/Rat-2 cells used in these coculture experiments and are barely detectable in the culture medium (Fig. 1). The radius of action of the paracrine effect in cocultures typically extended across the diameters of 5 to 10 transformed cells, consistent with a factor diffusing over short distances in the ECM but not freely soluble in the medium. Secondly, it has recently been shown that a mutated *Wnt-1* allele that encodes a protein lacking its secretory signal peptide is incapable of eliciting transformation in coculture assays similar to those described here (11a). Thirdly, the presence of heparin in the culture medium both reduces the amount of *Wnt-1* protein in the ECM fraction (Fig. 1) and inhibits paracrine transformation (Fig. 6), suggesting that these phenomena could be causally linked. Secreted *Wnt-1* proteins bind heparin-agarose with moderate affinity (2), and it is therefore likely that heparin in the medium may compete with *Wnt-1*-binding sites on the ECM or cell surface. Such sites could represent signal-transducing receptors or possibly proteoglycans necessary for presenting *Wnt-1* protein to its receptor (12, 38). Alternatively, association with heparin might inactivate *Wnt-1* protein. We cannot exclude the possibility, however, that heparin is affecting other secretory factors relevant to these biological effects.

In summary, our data indicate that phenotypic transformation of C57MG cells can occur by a paracrine mechanism elicited by expression of the *Wnt-1* gene in neighboring cells. Once sufficient quantities of *Wnt-1* protein can be obtained in soluble form, it should be possible to investigate more precisely the mechanism by which the *Wnt-1* gene or its protein product achieves this intercellular signalling effect.

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REFERENCES

1. Baker, N. 1987. Molecular cloning of sequences from *wingless*, a segment polarity gene in *Drosophila*: the spatial distribution of a transcript in embryos. *EMBO J.* 6:1765-1773.
2. Bradley, R. S., and A. M. C. Brown. 1990. The proto-oncogene *int-1* encodes a secreted protein associated with the extracellular

- lar matrix. *EMBO J.* **9**:1569–1575.
3. Brown, A. M. C., and M. R. D. Scott. 1987. Retroviral vectors, p. 189–212. In D. M. Glover (ed.), *DNA cloning—a practical approach*, vol. III. IRL Press, Oxford.
 4. Brown, A. M. C., R. A. Wildin, 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.
 5. Cohen, J. C., P. R. Shank, V. L. Morris, R. Cardiff, and H. Varmus. 1979. Integration of the DNA of mouse mammary tumor virus in virus-infected normal and neoplastic tissues of the mouse. *Cell* **16**:333–345.
 6. DiNardo, S., and J. Heemskerk. 1990. Molecular and cellular interactions responsible for intrasegmental patterning during *Drosophila* embryogenesis. *Semin. Cell Biol.* **1**:173–183.
 7. DiNardo, S., E. Sher, J. Heemskerk-Jongens, J. A. Kassis, and P. H. O'Farrel. 1988. Two-tiered regulation of spatially patterned *engrailed* gene expression during *Drosophila* embryogenesis. *Nature (London)* **332**:604–609.
 8. Gavin, B. J., J. A. McMahon, and A. P. McMahon. 1990. Expression of multiple novel Wnt-1/int-1-related genes during fetal and adult mouse development. *Genes Dev.* **4**:2319–2332.
 9. Heemskerk, J., S. DiNardo, R. Kostriken, and P. H. O'Farrell. 1991. Multiple modes of *engrailed* regulation in the progression towards cell fate. *Nature (London)* **352**:404–410.
 10. Kirschmeier, P. T., G. M. Housey, M. D. Johnson, A. S. Perkins, and I. B. Weinstein. 1988. Construction and characterization of a retroviral vector demonstrating efficient expression of cloned cDNA sequences. *DNA* **7**:219–225.
 11. Martinez-Arias, A., N. Baker, and P. Ingham. 1988. Role of the segment polarity genes in the definition and maintenance of cell states in the *Drosophila* embryo. *Development* **103**:157–170.
 - 11a. Mason, J., J. Kitajewski, N. Parkin, and H. E. Varmus. Unpublished results.
 12. Massague, J. 1991. A helping hand from proteoglycans. *Curr. Biol.* **1**:117–119.
 13. 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**:1073–1085.
 14. McMahon, J. A., and A. P. McMahon. 1989. Nucleotide sequence, chromosomal localization and developmental expression of the mouse *int-1*-related gene. *Development* **107**:643–650.
 15. Mester, J., E. Wagenaar, M. Sluysen, and R. Nusse. 1987. Activation of *int-1* and *int-2* mammary oncogenes in hormone-dependent and -independent mammary tumors of GR mice. *J. Virol.* **61**:1073–1078.
 16. Nusse, R. 1988. The *int* genes in mammary tumorigenesis and in normal development. *Trends Genet.* **4**:291–295.
 17. Nusse, R., A. Brown, J. Papkoff, P. Scambler, G. Shackleford, A. McMahon, R. Moon, and H. Varmus. 1991. A new nomenclature for *int-1* and related genes: the *Wnt* gene family. *Cell* **64**:231.
 18. 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 (London)* **307**:131–136.
 19. 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.
 20. Nusslein-Volhard, C., and E. Wieschaus. 1980. Mutations affecting segment number and polarity in *Drosophila*. *Nature (London)* **287**:795–801.
 21. Papkoff, J., and B. Schryver. 1990. Secreted *int-1* protein is associated with the cell surface. *Mol. Cell. Biol.* **10**:2723–2730.
 - 21a. Parkin, N., J. Kitajewski, J. Mason, and H. E. Varmus. Unpublished data.
 22. 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.
 23. 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.
 24. Rijsewijk, F., M. Schuerman, E. Wagenaar, P. Parren, D. Weigel, and R. Nusse. 1987. The *Drosophila* homolog of the mouse mammary oncogene *int-1* is identical to the segment polarity gene *wingless*. *Cell* **50**:649–657.
 25. Rijsewijk, F., L. van Deemter, E. Wagenaar, A. Sonnenburg, and R. Nusse. 1987. Transfection of the *int-1* mammary oncogene in cuboidal RAC mammary cell line results in morphological transformation and tumorigenicity. *EMBO J.* **6**:127–131.
 26. Rijsewijk, F. A. M., M. van Lohuizen, A. van Ooyen, and R. Nusse. 1986. Construction of a retroviral cDNA version of the *int-1* mammary oncogene and its expression *in vitro*. *Nucleic Acids Res.* **14**:693–702.
 27. Roelink, H., and R. Nusse. 1991. Expression of two members of the *Wnt* family during mouse development—restricted temporal and spatial patterns in the developing neural tube. *Genes Dev.* **5**:381–388.
 28. Roelink, H., E. Wagenaar, S. Lopes da Silva, and R. Nusse. 1990. *Wnt-3*, a gene activated by proviral insertion in mouse mammary tumors, is homologous to *int-1/Wnt-1* and is normally expressed in mouse embryos and adult brain. *Proc. Natl. Acad. Sci. USA* **87**:4519–4523.
 29. Schubert, D., M. LaCorbiere, F. G. Klier, and C. Birdwell. 1983. A role for adherens in neural retina cell adhesion. *J. Cell Biol.* **96**:990–998.
 30. Shackleford, G. M., and H. E. Varmus. 1987. Expression of the proto-oncogene *int-1* is restricted to postmeiotic male germ cells and the neural tube of mid-gestational embryos. *Cell* **50**:89–95.
 31. 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 (London)* **346**:847–850.
 32. 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 adenocarcinomas in male and female mice. *Cell* **55**:619–625.
 33. Vaidya, A. K., E. Y. Lasfargues, J. B. Sheffield, and W. G. Coutinho. 1978. Murine mammary tumor virus (MuMTV) infection of an epithelial cell line established from C57BL/6 mouse mammary glands. *Virology* **90**:12–22.
 34. 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.
 35. van Ooyen, A., and R. Nusse. 1984. Structure and nucleotide sequence of the putative mammary oncogene *int-1*; proviral insertions leave the protein coding domain intact. *Cell* **39**:233–240.
 36. Wieschaus, E., and R. Riggelman. 1987. Autonomous requirement for the segment polarity gene *armadillo* during *Drosophila* embryogenesis. *Cell* **49**:177–184.
 37. Wilkinson, D. G., J. A. Bailes, and A. P. McMahon. 1987. Expression of the proto-oncogene *int-1* is limited to specific neural cells in the developing mouse embryo. *Cell* **50**:79–88.
 38. Yayon, A., M. Klagsbrun, J. D. Esko, P. Leder, and D. M. Ornitz. 1991. Cell surface heparin-like molecules are required for binding of basic fibroblast growth factor to its high affinity receptor. *Cell* **64**:841–849.