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We have assessed the ability of *Wnt*-1, *Wnt*-2, *Wnt*-3, *Wnt*-3A, *Wnt*-4, *Wnt*-5A, *Wnt*-5B, *Wnt*-6, *Wnt*-7A, and *Wnt*-7B to transform mammary epithelial cells and found that Wnt-1, Wnt-2, Wnt-3 and Wnt-3A proteins transform mammary epithelial cells; Wnt-7A and Wnt-7B proteins partially transform; and Wnt-4, Wnt-5A, Wnt-5B, and Wnt-6 proteins does not affect mammary epithelial cells. Transformation correlated with Wnt-mediated increases in the cytosolic pool of β-catenin. By generating chimeric Wnt proteins and Wnt-1 deletions we have defined regions of Wnt-1 that are critical for transformation potential, signal transduction and frzb association. We have demonstrated that a secreted protein, frzb, blocks Wnt signaling and that a cytosolic protein, fused, is a negative regulator of Wnt signaling in mammalian cells. Wnt-1 acts as a mitogen in cultured Rat-1 fibroblasts, allowing growth of cells in serum-free medium. Using the TAC-2 mammary epithelial cell line, we found that Wnt-1 induces branching morphogenesis of mammary epithelial cells and can act as a morphogen in this capacity.

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WNT PROTEINS IN MAMMARY EPITHELIAL TRANSFORMATION
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INTRODUCTION

I. NATURE OF THE PROBLEM

There is strong evidence that Wnt proteins function as peptide growth factors that regulate the mammary gland growth cycle. Some of these proteins have already been shown to contribute to experimental mammary gland tumorigenesis in the mouse. Several groups are currently assessing whether *Wnt* genes play a role in the pathology of human mammary tumors, as might be predicted. Despite this evidence, work focused on the role of *Wnt* genes in breast cancer is in its infancy. The proposed studies are directly aimed at testing the hypothesis that *Wnt* genes encode regulators of normal and neoplastic mammary gland development.

The Wnt-1 protein is recognized as a mediator of cell-cell signaling events that can contribute to mammary tumorigenesis in the mouse. Despite accumulating evidence that Wnt-1 proteins act as growth factors, in the past it has been extremely difficult to purify Wnt-1 proteins in a soluble, cell-free form. For this reason, very little is known about Wnt specific cell surface receptors, which are proposed to be responsible for receiving signals from extracellular Wnt proteins. There is a pressing need to produce soluble, active Wnt ligands in order to understand the nature and regulation of Wnt-mediated growth control. In this proposal we will evaluate the hypothesis that *Wnt* genes encode a family of proteins that act as secreted growth factors that affect mammary epithelial cell physiology by interacting with cell surface receptors. It is expected that several of the Wnt proteins will demonstrably affect the growth properties of mammary epithelial cells, that these proteins act as secreted factors, and that they carry out their functions by stimulating specific cell-surface receptors on mammary epithelial cells.

II. BACKGROUND

The development of the mammary gland is a poorly understood process that consists of cycles of growth, morphogenesis, differentiation, and involution under the control of a variety of hormones and growth factors. Peptide growth factors have been implicated as effectors of mammary gland development (reviewed in (1)). Deregulation of growth factor-stimulated signaling pathways can contribute to the pathobiology of breast cancer (2, 3, 4). Our current knowledge of growth factor involvement in mammary tumorigenesis has focused on the activation of tyrosine kinase signaling cascades. The *Wnt* gene family encodes growth factors that were originally identified by their ability to induce mammary gland tumors in mouse model systems. Wnts are involved in cell growth and cell fate determination during embryogenesis, organogenesis, and oncogenesis. Wnt proteins utilize a novel signal transduction pathway that may involve the frizzled, dishevelled, glycogen synthase kinase-3, catenins, and the tumor suppressor APC. Aberrant Wnt signaling is found in human colon cancers and melanomas. We are interested in the role of the Wnt signaling cascade in mammary tumorigenesis.

Wnt family genes

The first *Wnt* genes to be cloned were identified based on their oncogenic effects in the mouse mammary gland. The *Wnt*-1 gene (originally *int*-1(5))was identified as a frequent target for insertional activation by mouse mammary tumor virus (MMTV) proviral DNA in MMTV-induced mammary gland tumors(6, 7). Inappropriate expression of the *Wnt*-1 gene leads to mammary gland tumorigenesis(8, 9). *Wnt*-3, was also originally identified as a transcriptionally activated oncogene in MMTV-induced mammary tumors(10). Most murine *Wnt* genes were isolated by cloning genes homologous to *Wnt*-1 [11, 12, 13] and encode cysteine-rich, secretory glycoproteins ranging from 350-380 amino acids. A comparison of the predicted amino acid sequences among murine *Wnt* gene family members reveal over 100 conserved residues fairly evenly distributed across the entire sequence and striking conservation of roughly 23 cysteines in nearly parallel positions. Different Wnt proteins are generally 40-60% identical at the amino acid level.

The normal functions of *Wnt* genes have been analyzed in organisms tractable to genetic or biochemical analysis of early development. The *Wnt*-1 orthologue in *Drosophila* is the segment polarity gene *wingless* (11, 12). Genetic and biochemical analyses suggest that the wg protein functions as a

local-acting, secreted factor that triggers a cascade of molecular events leading to the specification of segment polarity in the *Drosophila* embryo(reviewed in (13)). In the frog, *Xenopus laevis,* several different *Wnt* genes have been shown to contribute to the experimental induction of dorsal mesoderm tissue and subsequent establishment of the body axis (14, 15, 16, 17). Current models of early embryonic patterning events in the frog invoke one or several Wnt proteins as determinants of dorsal-axial position(17, 18).

The murine *Wnt* genes cloned to date are expressed in spatially restricted patterns during gastrulation, neurulation, or early organogenesis. On the basis of the analysis of *Wnt*-1 gene deficiencies, the normal function of the murine *Wnt*-1 gene is in proper development of the cerebellum and midbrain (19, 20). Wnt family proteins are also implicated in limb development(21, 22), kidney development(23, 24), and uterine development(25). In collaborative work with Dr. Andrew Lassar (Harvard University), we have also demonstrated that Wnt proteins, in combination with Sonic hedgehog (Shh), can induce myogenesis in somitic tissue *in vitro* (26). This work indicates that myotome formation *in vivo* is directed by the combinatorial activity of Shh secreted by the floor plate and notochord and Wnt ligands secreted by the dorsal neural tube (Appendix paper-Muensterberg *etal.*).

Wnt proteins and their mechanism of action

Wnt-1 encodes a secreted polypeptide characterized by a hydrophobic signal peptide, N-linked glycosylation sites, cysteine residues, and the absence of an identifiable membrane anchor domain (27). Work on the biochemical properties of Wnt proteins has been carried out with cells programmed to express exogenous *Wnt* cDNAs. In such cells, Wnt-1 behaves as a secretory glycoprotein, undergoing entry into the endoplasmic reticulum (ER), leader cleavage, and asparagine(N)-linked glycosylation at several sites (28, 29). Despite entry into the ER, Wnt-1 proteins are very poorly secreted. Intracellular Wnt-1 is predominantly bound to BiP; a chaperonin-like protein found in the ER (30). The appearance of extracellular Wnt-1 proteins is enhanced by addition of heparin sulfate (31) or suramin (32) to the media. Wnt-1 proteins thus are not freely diffusible once outside of cells. This lack of diffusibility is thought to be a result of tight association with either the cell surface (32) or the extracellular matrix (31). Wnt proteins can act in a paracrine fashion as cell transformation assays have been used to define paracrine effects of Wnt-1(33, 34). Analysis of wg protein function, the *Drosophila* homologue of *Wnt*-1, suggests that it acts in a paracrine fashion (35). These observations have led to the model that Wnt proteins are local-acting factors that function to signal cells adjacent or near the site of Wnt production but do not affect cells distant from the site of production. In fact, Wnt-1 proteins tethered to the cell surface by addition of a transmembrane tail still exhibit autocrine and paracrine transforming activities(36). Wnt-specific activity can be detected in the medium of mammary epithelial cells programmed to express *Wnt*-1 cDNA ((37)and J. Kitajewski, unpublished observations). Biological activity of soluble wingless protein from cultured *Drosophila* imaginal disc cells has also been reported(38) . These studies suggest that Wnt proteins can act as diffusible secreted factors; however, the levels of soluble Wnt proteins are low. Despite such progress the purification of Wnt proteins has not yet been accomplished.

Wnt signal transduction events

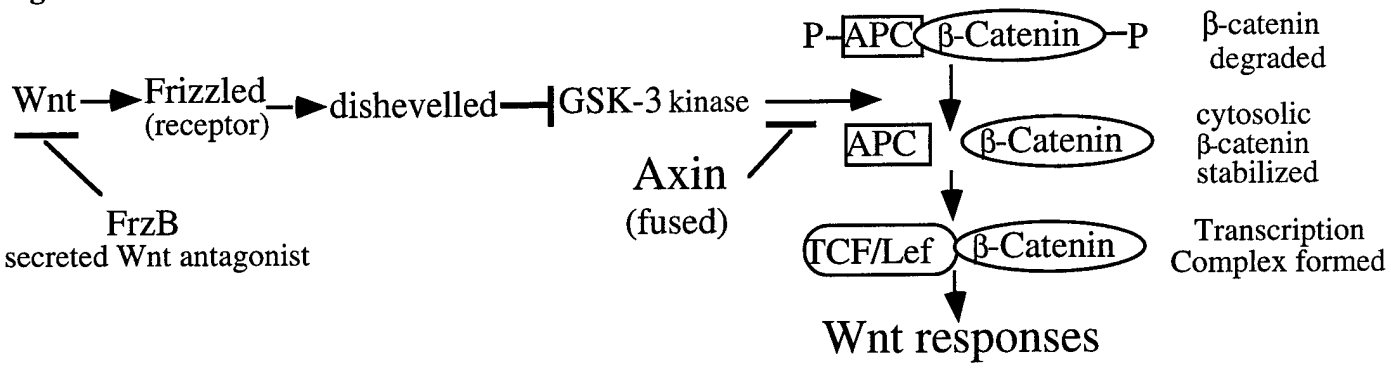
The nature of the signaling events triggered by Wnt proteins is now becoming apparent (39). Genetic and biochemical analysis of the *wg* signal transduction pathway in *Drosophila* embryos and cultured insect cells suggest a cascade of events distinct from any previously described signal transduction pathway (13, 40, 41). A model for the *wg*-mediated signaling pathway (schematized in Figure 1) has been proposed to involve the frizzled cell surface receptor (42). Frizzled proteins contain a conserved amino-terminal cysteine-rich domain (the CRD domain) and seven putative transmembrane segments. Within the target cell, the cytosolic dishevelled protein is the first known component in *wg*-mediated signaling, however, its function is unclear. Dishevelled (dsh) may undergo hyperphosphorylation in response to wg protein (Roel Nusse, personal communication). Downstream of dsh is a protein kinase, zeste-white 3 (zw-3), whose activity must be suppressed to transmit wg signals. Suppression of the zw-3 kinase leads directly, or indirectly, to dephosphorylation and increased stability of the armadillo protein(43). Increased cytosolic armadillo is then thought to form a complex with members of the Tcf/LEF-1 family of transcription factor which then move to the nucleus to regulate expression of wg target genes (44). The *Drosophila* homologue of RhoA p21 GTPase has been genetically tied to frizzled function but it is not known how RhoA may function in Wnt signaling (45).

The components of the *Drosophila* wg signaling pathway have been conserved in vertebrates. Eight frizzled genes (46) and three *Dsh* genes (47) have been identified in the mouse or rat. *Drosophila zeste-white 3* (*zw-3*) encodes a serine/threonine kinase that is the homologue of mammalian Glycogen Synthase Kinase-3β (48). Amadillo is the orthologue of vertebrate cadherin-associated molecules known as β-catenin and plakoglobin (γ-catenin) (49). Catenins exist in three isoforms α, β, and γ, which form a complex with cadherins. The wg signaling cascade is conserved in vertebrates. Ectopically expressing Wnt signaling components in *Xenopus* embryos induces axis duplication, as previously reported for *Wnt-1*(14). Expression of *Xenopus dsh* gives phenotypes that are similar to those conferred by Wnts (50), as does dominant-negative mutants of vertebrate GSK-3 (51, 52). Ectopic expression of β-catenin (53) or plakoglobin(54) mimics Wnt-like activity. Increased cytosolic levels of catenins are thought to be critical for Wnt signaling in *Xenopus* (55).

Recently, several components of the Wnt signaling pathway, not previously identified in *Drosophila*, have been described in mouse. These include axin and APC. Axin is a protein that is structurally similar to RGS (Regulators of G-Protein Signaling) proteins and to dishevelled. Disruption of this gene in the mouse germ line causes embryonic axis duplication suggesting that it functions as a negative regulator of Wnt signaling. In fact, axin blocks Wnt-mediated axis duplication in *Xenopus*. Axin proteins that lack the RGS domain function like dominant-negative proteins as they induce axis duplication [58].

The adenomatous polyposis coli protein, APC, has been implicated in the Wnt signaling pathway based on its ability to associate with β-catenin and GSK-3 (56, 57, 58). APC protein normally functions as a growth suppressor in colon cancer(59) as loss of APC is correlated with cancer progression. In addition, loss of APC is associated with mammary tumorigenesis in mice(60, 61). APC is thought to function by maintaining low levels of cytosolic β-catenin(62), possibly through ubiquitination of β-catenin. The APC protein itself may be regulated by GSK-3 (57). Although APC is thought to downregulate β-catenin, a recent report demonstrated that APC expression induces axis duplication (63). This finding is in contrast to proposed activity of APC as a suppressor of Wnt signaling and may suggest a distinct function for APC in signal transmission as well as signal suppression.

Figure 1. WNT SIGNAL TRANSDUCTION PATHWAY.



Frzb-a secreted antagonist of Wnt signaling

Frzb is a secretory polypeptide that is homologous to the frizzled family of proteins (64). Frzb, however, has no membrane spanning domain. The region of highest homology between Frzb and frizzled is that of the cysteine rich domain. This structure suggests that Frzb functions as a secreted antagonist of frizzled receptors. In fact, Frzb expression interferes with Wnt-mediated axis duplication in *Xenopus* (65). Frzb and Wnt proteins co-immunoprecipitate, providing direct biochemical evidence for Frzb-Wnt interactions (65). No direct binding of Wnt to frizzled proteins has yet been demonstrated, although frizzled expression can induce cell surface association of Wnt proteins (42).

***Wnt* genes and mammary tumorigenesis**

Abnormal expression of the *Wnt*-1 gene contributes to the development of mammary tumors (66). Transgenic mice expressing the *Wnt*-1 gene in the mammary gland develop mammary tumors with high levels of *Wnt*-1 mRNA (8). Additional transgenic experiments also suggest Wnts cooperate with

fibroblast growth factor (FGF) members to induce mammary tumorigenesis (9, 67). Expression of the *Wnt*-1 gene in two established mammary epithelial cell lines, C57MG cells (68) or RAC311C cells (69) leads to morphological transformation from flat cuboidal cells to highly refractile, elongated cells that continue to grow post-confluence. *Wnt*-1 expression also leads to increased tumorigenicity in RAC311C cells. Thus it is well established that the *Wnt* genes are potent oncogenes in mouse mammary tumorigenesis.

Several human malignancies have been documented to have aberrant expression of *Wnt* genes. Human *WNT* genes 2, 3, 4, and 7b have been found overexpressed in breast cell lines and human breast tumors, as compared to normal breast tissue(70). Aberrant expression of *WNT*-5a was also reported in lung, breast, and prostate carcinomas and melanomas(71).

<u>*Wnt* genes in normal mammary gland developments</u>

Several *Wnt* genes are expressed during post-natal development of the mammary gland (72, 73), see Table 1. *Wnt*-2, *Wnt*-4, *Wnt*-5a, *Wnt*-5b, *Wnt*-6, and *Wnt*-7a are expressed in the mammary gland during growth and differentiation (in virgin and pregnant mice) but they are not expressed in lactating glands, when the gland is no longer growing. Furthermore, the expression of several Wnt genes appears to be hormonally regulated (74). These two findings, taken together, suggest that regulated expression of *Wnt* gene products play a role in the normal expansion or differentiation of the mammary epithelium before lactation. Several lines of evidence suggest that *Wnt* proteins may affect mammary gland development. Mice bearing a MMTV driven *Wnt*-1 transgene exhibit hormone-independent hyperplasia of mammary epithelium(8). In these mice, the glands of both virgin females and male animals resemble those of pregnant animals. Hormone deprivation by ovarectomy has no obvious effect on the morphology of the mammary hyperplasias in *Wnt*-1 transgenics (75). Both *Wnt*-1 and *Wnt*-3 expression can affect mammary gland growth; however, neither gene is expressed in the normal mammary gland. Thus, it has been proposed that Wnt-1 and Wnt-3 act through Wnt receptors that respond to *Wnt* family proteins normally expressed in the mammary gland. The oncogenic effects of the *Wnt*-1 and *Wnt*-3 genes may thus interfere with the normal *Wnt*-mediated regulation of mammary gland growth.

<u>Wnt-Frizzled signaling in human carcinogenesis</u>

Several genes involved in Wnt signal transduction, most notably β-catenin and APC, act as oncogenes or tumor suppressor genes, respectively. Mutated forms of β-catenin occur in gastric cancer cells (76) and a truncated form of β-catenin was identified as a transforming oncogene (77). The human β-catenin gene (CTNNB1) is localized to a region of chromosome 3p21 that is implicated in tumor development (78). Mutations in the APC gene are associated with human colon cancer. It is noteworthy that the Apc min mouse model, which has a germline APC mutation, is predisposed to develop mammary carcinomas (60, 61).

The most compelling case for a role of aberrant Wnt signaling in pathobiology of human tumors has comes from studies defining APC and β-catenin mutations in colon and melanoma cancer cell lines. Colorectal tumors with intact APC genes contain activating mutations of β-catenin that alter phosphorylation sites critical to APC's ability to bind and regulate β-catenin (79). In addition, a Tcf family member (Tcf-4), which is found to be expressed in colonic epithelium, activates transcription only when associated with β-catenin. Colon carcinoma cells devoid of APC contained a β–catenin-Tcf complex that is constitutively active (80). Thus, constitutive transcription of Tcf target genes can be caused by loss of APC function suggesting activation of the Wnt signaling pathway is a key event in the transformation of colonic epithelium.

Genetic defects that result in regulation of β-catenin play a role in melanoma progression (62). Abnormally high levels of β-catenin due to mis-splicing or missense mutations of the β-catenin gene were detected in several human melanoma cell lines. Other melanoma lines are missing APC or contain structurally altered APC proteins and these alterations are associated with constitutive activation of β-catenin-Tcf transcription complexes.

III. PURPOSE

The *overall objective* of the work proposed here is to determine how Wnt proteins modulate the growth of mammary epithelial cells, with the *long term goal* of understanding the role of *Wnt* genes in mammary tumorigenesis.

IV. METHODS OF APPROACH

The *overall objective* of the work proposed here is to determine how Wnt proteins modulate the growth of mammary epithelial cells, with the *long term goal* of understanding the role of *Wnt* genes in mammary tumorigenesis. Our *general strategy* will to carry out a study of the proteins encoded by ten different *Wnt* genes (*Wnt*-1, *Wnt*-2, *Wnt*-3, *Wnt*-3A, *Wnt*-4, *Wnt*-5A, *Wnt*-5B, *Wnt*-6, *Wnt*-7A, *Wnt*-7B) that will address the following *specific aims:*

1. *Examine the biochemical and secretory properties of Wnt proteins.* The coding potential for an antigenic epitope will be added to full length cDNAs encoding *Wnt*-1, *Wnt*-2, *Wnt*-3, *Wnt*-3A, *Wnt*-4, *Wnt*-5A, *Wnt*-5B, *Wnt*-6, *Wnt*-7A, and *Wnt*-7B. We will prepare stable cell lines expressing epitope-tagged Wnt proteins in order to determine how the biochemical properties of the proteins encoded by newly described *Wnt* genes compare to those described for Wnt-1 proteins. We will evaluate if these proteins enter the secretory pathway, how efficiently are they secreted, and once outside the cell are these proteins freely soluble, bound tightly to the extracellular matrix, or bound to the cell surface? Our goal is to identify Wnt proteins that can be purified for use as ligands.

2. *Determine the transforming potential of Wnt genes.* Using retroviral vectors to express the proteins encoded by these cDNAs in cultured cell lines, we will determine whether: (a) expression of these genes in cultured mammary epithelial cells leads to transformation, (b) these proteins transmit signals in a paracrine fashion, and (c) these proteins are secreted in a soluble, biologically active form.

3. *Map domains of Wnt proteins required for transforming potential.* We propose to make chimeric proteins composed of regions from active Wnt-1 proteins and regions from inactive Wnt proteins. These chimeric proteins will be used to map peptide sequences or domains of Wnt-1 that are required for biological activity. The transforming potential of the chimeric proteins will be assessed by using a rapid transformation assay, which we have recently developed. We are interested in the minimum set of sequences derived from Wnt-1 that are required for transforming potential.

4. *Characterization of Wnt proteins as ligands.* We will determine if purified Wnt proteins, act as soluble ligands to affect mammary epithelial cell physiology. Wnt proteins will be overexpressed, purified, and used to determine if they behave as agonists or antagonists of Wnt-1 activity. Biological and biochemical responses to treatment with Wnt ligands will be analyzed.

5. *Identification of Wnt-specific cell surface receptors.* Purified, active Wnt proteins will be used to probe for Wnt specific receptors on the surface of cells and to biochemically identify Wnt-specific cell surface receptors by chemical crosslinking of radiolabeled ligands. The long term goal is to identify genes encoding Wnt specific receptors.

BODY

The progress of each specific aim will described in the following sections.

I. Specific aim 1.

TO EXAMINE THE BIOCHEMICAL AND SECRETORY PROPERTIES OF WNT PROTEINS.

We proposed to assess the characteristics of eleven family members; including, *Wnt*-1, *Wnt*-2, *Wnt*-3, *Wnt*-3a, *Wnt*-4, *Wnt*-5a, *Wnt*-5b, *Wnt*-6, *Wnt*-7a, and *Wnt*-7b and *Wnt*-8c. Toward this goal, we have generated cDNAs encoding all eleven of these *Wnt* gene family members fused to an influenza hemagluttinin (HA) epitope to allow detection of the gene products with an anti-HA monoclonal antibody. All eleven of the HA epitope-tagged Wnt proteins have been expressed in transiently transfected 293T cells can be detected by immunoblot analysis with anti-HA antibodies. The *Wnt*-8c clone expresses protein very poorly in tissue culture cells; therefore, we continued the analysis with the other ten family members. Biochemical analysis has been initiated to determine the secretory properties of the ten different Wnt proteins. Immunofluorescence analysis carried out on Rat-1 cell lines expressing epitope-tagged Wnt proteins display prominent staining in the endoplasmic reticulum and weak staining at the cell surface. This pattern is similar to that seen for Wnt-1, indicating that the ten Wnt proteins enter the secretory pathway but are poorly secreted. Multiple forms of the various Wnt proteins are detected using immunoblot analysis, suggesting that these proteins are glycosylated. The molecular size of the different Wnt proteins correlates roughly to that predicted based upon the predicted open reading frame and potential N-linked glycosylation sites of the different Wnt genes. In conclusion, we have demonstrated that the ten Wnt proteins we have analyzed are secretory in nature and are associated with extracellular material in a fashion similar to that seen for Wnt-1. Thus, we were unable to find an efficiently secreted and highly diffusible Wnt protein family member.

II. Specific Aim 2.

TO DETERMINE THE TRANSFORMING POTENTIAL OF WNT GENES.

We have completed a study of the transforming potential of Wnt gene family members. We evaluated the transforming potential of *Wnt*-1, *Wnt*-2, *Wnt*-3, *Wnt*-3a, *Wnt*-4, *Wnt*-5a, *Wnt*-5b, *Wnt*-6, *Wnt*-7a, and *Wnt*-7b. We also compared the transfoming activity of Wnt proteins to their ability to upregulate β-catenin. This study utilized cDNAs encoding all ten of these *Wnt* gene family members fused to an HA epitope to allow detection of Wnt proteins with an anti-HA monoclonal antibody (12CA5, Boehringer Mannheim).

The transforming potential of the Wnt proteins was tested by using retroviral vectors (LNCX vectors, (81)) to express these genes in the murine C57MG mammary epithelial cell line. Paracrine transforming capability of *Wnt* genes has been assessed by co-cultivating mammary epithelial cells with *Wnt*-expressing Rat-B1a fibroblasts (82) (which themselves show no response to *Wnt*-1 and act as donors of Wnt proteins to adjacent C57MG cells). Our results indicate that HA-tagged Wnt-1, Wnt-2, Wnt-3, and Wnt 3a transform mammary epithelial cells. Wnt-4, Wnt-5a, Wnt-5b, Wnt-6, and Wnt-7b do not detectably transform C57MG mammary epithelial cells. Wnt-7a may show very weak transforming potential. Paracrine assays were carried out for all ten *Wnt* genes using Rat fibroblast cell lines (Rat-B1a (82)) as donors of Wnt activity. The results of the paracrine assays coincide with results obtained by direct expression in mammary epithelial cells, summarized in Table 1. Included in this table is a comparison of the expression patterns of *Wnt* genes in the murine mammary gland (72). We conclude that there are two classes of Wnt proteins; transforming Wnts and non-transforming Wnts. *Wnt* gene family members thus differ in their potential to morphologically transform mammary epithelial cells,

suggesting several distinct receptors or quantitative differences in the signals different Wnt proteins provide

Table 1. Summary of Mammary Gland Expression & Transforming Activities of Wnt Proteins

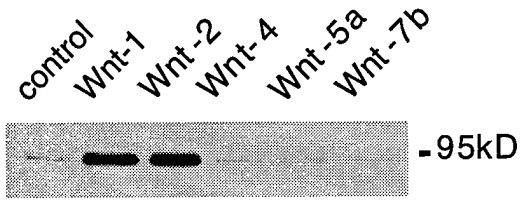
	Expression in Mammary Gland	Transforming -direct	-paracrine
Wnt-1	-	+	+
Wnt-2	+ virgin	+	+
Wnt-3	-	+	+
Wnt-3a	-	+	+
Wnt-4	+ virgin, pregnant	-	-
Wnt-5a	+ pregnant	-	-
Wnt-5b	+ pregnant	-	-
Wnt-6	+ pregnant	-	-
Wnt-7a	-	-/+	-/+
Wnt-7b	+ pregnant	-	-

Several rapid biochemical events in response to Wnt signals have been reported, see *Background.* These include phosphorylation of dishevelled, suppression of GSK-3 kinase activity, and regulation of cytosolic levels of catenins. Recent work suggests that the cytosolic form of β–catenin is stabilized in response to Wnt signals.

Cytosolic β-catenin assay: Catenins are predominantly found in the membranous fraction of cells associated with cadherins. We have evaluated membrane bound and cytosolic forms of β–catenin in C57MG cells programmed to express Wnt proteins. C57MG cells expressing Wnt proteins were Dounce homogenized in a buffered physiological solution containing protease inhibitors. A low speed spin of 500g X 5 minutes was used to pellet nuclei and unbroken cells. The supernatants were then subjected to a high speed spin of 100,000g X 90 minutes to pellet membranous material. The supernatant of this spin is designated the cytosolic fraction, the pellet is designated membranous. Both membranous and cytosolic fractions have been subjected to immunoblot analysis, using a monoclonal antibody against mouse β-catenin (Transduction Laboratories, Lexington, KY). In Wnt-1 transformed cells the membranous fraction contains equivalent amounts of β–catenin as non-transformed control cells (data not shown). Figure 2 displays the cytosolic forms of β-catenin. The cytosolic fraction from Wnt-1 and Wnt-2 transformed cells contains 10-20 fold greater amounts of β-catenin than do control cells. To date, this is one of the most sensitive assays for Wnt-signaling in cultured cells.

Wnt causes dramatic changes to the pool of cytosolic β–catenin. The evidence presented in Figure 2 suggests that upregulation of cytosolic catenins correlates with the transformed phenotype of C57MG cells. Levels of cytosolic β-catenin are increased by Wnt-1 and Wnt-2, both of which transform C57MG cells (see Table 1). C57MG cells programmed to express non-transforming Wnt-4, Wnt-5a, and Wnt-7b (Table 1) do not contain higher levels of cytosolic β-catenin. These findings indicate that upregulation of cytosolic β-catenin is dependent on expression of the transforming class of Wnt proteins, providing the first evidence that increased cytosolic β-catenin correlates with cellular transformation.

Figure 2. Immunoblot analysis of the cytosolic pool of β-catenin from C57MG expressing Wnt proteins. Lysates of C57MG cells were separated into cytosolic and membranous fractions, the cytosolic fraction was analyzed by immunoblot using anti-β-catenin antibodies.



III. Specific aim 3

TO MAP DOMAINS OF WNT PROTEINS REQUIRED FOR TRANSFORMING POTENTIAL.

Little is known about the specific amino acid regions of Wnt proteins required for biological activity. Wnt proteins display anywhere from 30% to 60% amino acid identity. Conservation between different Wnts is found throughout the length of the proteins and a comparison of the sequences does not reveal highly conserved subdomains. Therefore, it is difficult to infer regions important for biological activity or regions conferring different properties among the different Wnt proteins. Identifying functional domains of Wnt proteins, therefore, must be done experimentally by modifying the structure of these proteins and testing how these modifications effect biological activity. We have documented that Wnt-1HA transforms C57MG cells whereas Wnt-5aHA does not. This observation serves as the basis to use chimeric proteins composed of regions of Wnt-1 and Wnt-5a to define the region(s) specifying transformation.

We have prepared a set of chimeric proteins composed of the N-terminus of Wnt-1 and the C-terminus of Wnt-5aHA or vice-versa. These chimeras were generated using the PCR based technique "gene splicing by overlap extension" (83). Oligonucleotides were used to amplify a fragment of the *Wnt*-1 gene and to amplify a fragment of the *Wnt*-5a gene. The oligos were designed to share homology at the desired junction site to allowed the two PCR products to anneal. External primers were used to amplify a gene composed of the two fragments spliced together. These chimeras are designated N-terminal Wnt$^{\text{amino acid \#}}$/C-terminal Wnt$^{\text{amino acid \#}}$; note that Wnt-1 consists of residues 1-359 and Wnt-5a consists of residues 1-378. As an example, a set of chimeras with increasing N-terminal Wnt-1 and decreasing amounts of C-terminal Wnt-5a was derived from the following junctions: Wnt-1$^{1\text{-}99}$/5a$^{111\text{-}378}$(**A**), Wnt-1$^{1\text{-}186}$/5a$^{198\text{-}378}$(**B**), Wnt-1$^{1\text{-}199}$/5a$^{219\text{-}378}$(**C**), and Wnt-1$^{1\text{-}291}$/5a$^{311\text{-}378}$(**D**). A complementary set has been constructed consisting of increasing contributions of N-terminal Wnt-5a. The chimeric proteins are schematized in Figure 3.

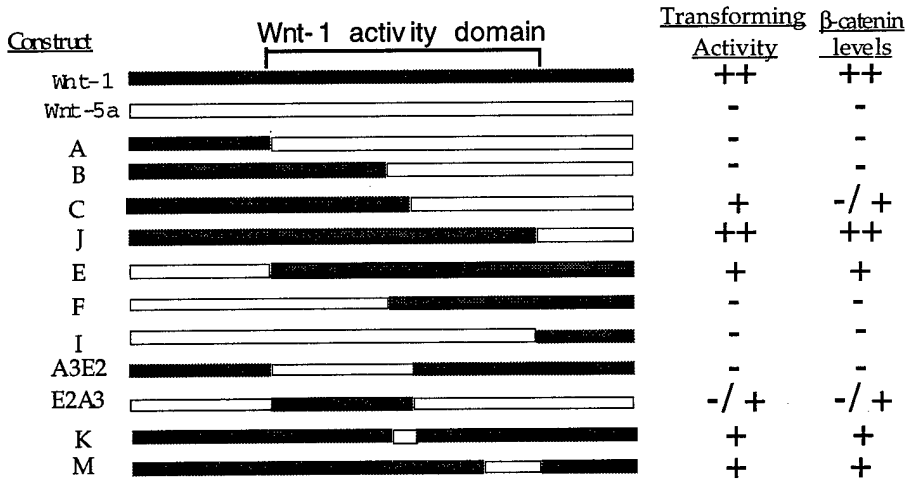


Figure 3. Transforming Potential of Wnt-1/Wnt-5a chimeras. Schematic diagram of the structure of chimeric Wnt-1/ Wnt-5a proteins. Transforming activity was judged by morphological transformation of C57MG cells programmed to express chimeric Wnt proteins. The levels of cytosolic β-catenin were evaluated by immunoblot of extracts from C57MG cells expressing Wnt chimeras.

To control for differences in protein levels, lysates from C57MG cells or transiently transfected 293 cells expressing chimeric Wnt proteins have been analyzed (Figure 4). Cell extracts were fractionated on SDS-PAGE, electroblotted, and probed by anti-HA antibodies (all chimeric proteins are HA-tagged). Figure 4 shows that equivalent levels of all chimeric Wnts are detected. The size and glycosylation patterns of these chimeras are consistent with that predicted for the chimeric proteins.

Figure 4. Immunoblot Analysis of Wnt-1/5a chimeric proteins. 293 cells were transfected with plasmids encoding Wnt chimeric proteins. Two days later, protein levels were evaluated by immunoblot using anti HA antibodies.

These constructs have been tested for transforming potential in mammary epithelial cells (C57MG) and the levels of cytosolic β-catenin were measured, as described in *cytosolic β-catenin assay*. Results of these experiments are schematized in Figure 3. Our preliminary results suggest the transformation and signaling specific determinants of Wnt-1 proteins map to the same region encompassing the central 120 amino acids of Wnt-1. Modest activity was found for chimeras C and E2A3, this analysis may allow futher localization of the minimal domain required for Wnt-1 signaling to the Wnt-1 domain present in E2A3.

IV. Specific aim 4. *CHARACTERIZATION OF WNT PROTEINS AS LIGANDS.* Specific aim 5. *IDENTIFICATION OF WNT-SPECIFIC CELL SURFACE RECEPTORS.*

These aims were originally focused on the eventual identification of Wnt specific receptors. Recently, the Frizzled family of proteins have been identified as Wnt signaling receptors. These proteins are characterized by a cysteine rich extracellular domain and seven membrane spanning segments. No group has yet demonstrated that frizzled proteins bind directly to Wnt proteins. In collaboration with Dr. Frank Luyten, we have recently shown that Wnt-1 will bind to a frizzled-like protein, Frzb-1. To take advantage of this finding we propose to focus these aims on the interaction between Frzb and Wnt as a means of analyzing receptor interaction with Wnt proteins. The following section describes progress made in this area.

Frzb-1 contains an N-terminal domain with 50% identity to the cysteine-rich domain (CRD) of *Drosophila* frizzled (64), proposed to be the ligand binding domain. In fact, the frizzled CRDs are as homologous to the CRD of frzb as they are to each other. Recently, the Luyten laboratory found that Frzb-1 is a secreted antagonist of Wnt-8 mediated axis duplication in *Xenopus* (65). We investigated if the inhibitory effect of Frzb-1 on Wnt-1 signaling, as observed in *Xenopus*, could also be demonstrated in mammalian cells. Ectopic Wnt-1 expression in 293 cells induces the accumulation of ß-catenin within the cytosol, whereas membrane-associated levels of ß-catenin remain virtually unchanged (Fig. 5A). Figure 5B shows that the induction of cytosolic ß-catenin by Wnt-1 is attenuated in the presence of increasing amounts of Frzb-1 encoding plasmid. Frzb-1 expression alone had no affect on cytosolic ß-catenin levels. This data is described in detail in the Appendix paper (Lin *et.al.*) where we also demonstrate that the CRD domain of Frzb-1 is required for Wnt-1 binding and that both Wnt-1 and Wnt-5a bind Frzb.

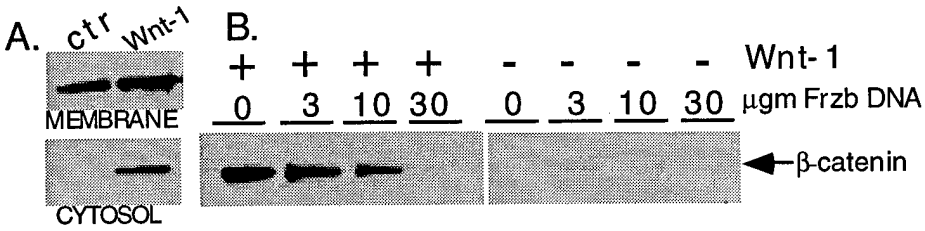
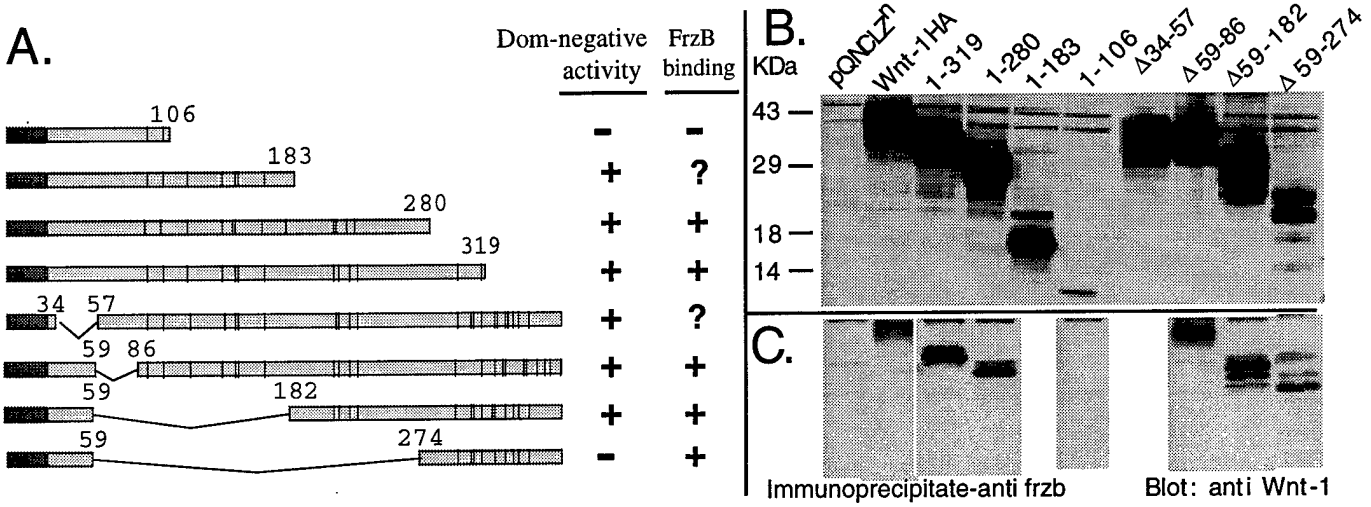


Figure 5. FrzB antagonizes Wnt-1 signaling in 293 cells. **A.** Wnt-1 expression in 293 cells regulates cytosolic β-catenin. **B.** Transfection of Frzb expression vector blocks Wnt-1-mediated regulation of cytosolic β-catenin.

We have begun a detailed examination of regions of Wnt-1 that are required for frzb association. A panel of HA-tagged Wnt-1 deletion mutants were prepared and tested for expression in transiently transfected 293 cells (see Figure 6 A,B). Frzb/Wnt-1 interactions were detected within lysates derived from 293 cells co-transfected by frzb and Wnt deletions. Complexes were recovered by anti-frzb antibody and detected by immuno-blotting using anti-HA antibodies (Figure 6C). The data indicate, thus far, at least two discrete binding domains downstream of Wnt-1 codon 182.

In addition, co-expression of Wnts with full length Wnt-1 identified several Wnt deletions that behaved as dominant negative alleles, as judged by their ability to block Wnt regulation of β-catenin. These studies start to define the domains of Wnt-1 proteins required for receptor interaction.

Figure 6. Expression and Functional analysis of Wnt-1 deletion mutants.
A. schematic diagram of Wnt-1 deletions, Dominant-negative activity and Frzb binding are noted.
B. Immunoblot analysis of Wnt-1 deletions; **C**. Frzb-Wnt-1 deletion co-immunoprecipitations.

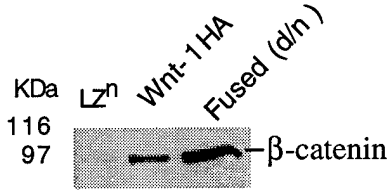


V. Wnt Signal Transduction.

In an effort to develop rapid assays of Wnt signaling, we found that transient transfection of Wnt-1 into human 293 cells (embryonic kidney cells) led to increased levels of cytosolic β-catenin (see Fig. 5 and Appendix paper-Lin *et al.* for description). Using this assay to define the activities of Wnt family proteins and chimeric Wnts, we found that our data concurred with that found for regulation of β-catenin in C57MG cell lines (data not shown). Thus, we believe this regulation reflects Wnt receptor mediated events in these cell types.

Fused suppresses Wnt signaling. To probe the utility of this assay we evaluated the regulatory potential of the fused (axin) protein. Please note the fused protein product has been named axin, to avoid confusion we will refer in the text to this gene as fused or fused(axin). Fused is a negative regulator of Wnt signaling in *Xenopus* (84). A dominant-negative allele of fused (Fused-dom/neg), lacking the RGS domain, was provided by Frank Costantini (Columbia University). 293 cells were transfected with Fused-dom/neg and protein expression was confirmed by immunoblot (data not shown). Expression of Fused dom/neg led to markedly increased levels of cytosolic β–catenin (Fig. 7). The regulation was consistently several fold more pronounced than found for Wnt-1 . We thus conclude that Fused functions in the mammalian Wnt signaling pathway, presumably as a potent negative regulator.

Figure 7. Expression of dominant-negative Fused (axin) leads to increased cytosolic levels of β-catenin. Cytosolic β-catenin was evaluated by immunoblot of extracts derived from 293 cells transfected with control (LZ^n), Wnt-1HA, or dominant negative fused (d/n).



Adenoviral vector development. We propose to express Wnt signaling proteins using adenovirus (ad) vectors in cultured cells. This system has the advantage of delivering a high copy number genome to cultured cells and eliminates the need to generate cell lines. In our hands, ad vectors can express heterologous proteins at high levels after infection of human or rat cells but not mouse cells. cDNAs encoding Wnt signaling proteins were cloned into ad transfer vectors (kindly provided by Dr. Stephen Hardy, UCSF). This vector places the gene to be expressed under the control of the CMV promoter. Using standard procedures (85), the ad transfer vector and ad genomic DNA were co-transfected into the human 293 cell line which provides E1a and E1b proteins critical for adenovirus replication. Recombinant ad containing the genes of interest were isolated and plaque purified. These vectors lack the adenovirus E1a and E1b genes, which may affect the biology of infected target cells, and thus they do not replicate in E1a/E1b deficient cells.

We have generated a series of adenovirus vectors expressing Wnt signaling components. The list of vectors currently completed is described in Table 2. Stocks for all of vectors listed in Table 2 are being made and we are in the process of evaluating the ectopic expression of proteins using these vectors.

Table 2. Adenovirus expression vectors currently generated

AD VECTOR	EXPRESSED GENE
Ad-LacZ	control virus expressing LacZ
Ad-Wnt-1HA	murine Wnt-1 HA tagged
Ad-Wnt-5aHA	murine Wnt-5a HA tagged (non-transforming Wnt)
Ad-GSK-3 w/t	rat GSK-3β wild type
Ad-GSK-3dom/neg	rat GSK-3β dominant negative, mutated ATP binding lysine
Ad-β-catenin	human β-catenin
Ad-β-cateninS37A	human β-catenin with phosphorylation site mutation S37A

We have utilized ad vectors expressing Wnt-1HA (Ad-Wnt-1HA) and a dominant negative Glycogen Synthase Kinase-3β (Ad-GSK-3dom/neg), this mutant lacks the ATP binding lysine. The dominant negative GSK-3 cDNA was also cloned into the ZNCX retroviral vector. Immunoblot analysis was used to demonstrate the expression potential of these two vectors (Fig. 8). Very high levels of Wnt-1 proteins have been produced in RatB1a fibroblast, Rat-2 fibroblast , PC12 cells, MCF-7 cells, and several human breast cancer cell lines. Wnt-1 proteins can be detected 24 hours post infection of Rat-2 cells (Fig. 8A) and continue to be produced at high levels for several days. Ad-GSK-3dom/neg expression was analyzed by immunoblotting using anti-GSK-3β antibodies that recognize both rat and human GSK-3β. Endogenous human GSK-3β is detected in control and infected cells (Fig. 8B). The ectopically expressed rat GSK-3βdom/neg migrated more slowly and was found most efficiently expressed in Ad-GSK-3β infected 293 cells. This experiment demonstrated the power of the Ad vector to express dominant negative GSK-3β at levels much higher than the endogenous enzyme, as would be required for a dominant negative acting gene product.

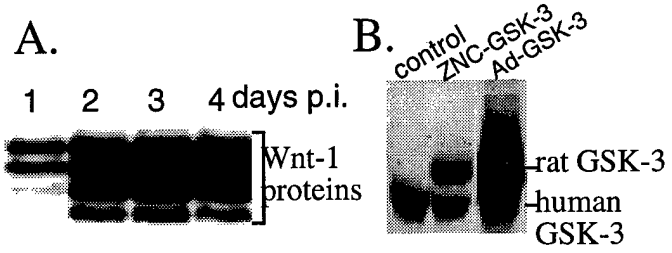


Figure 8. Wnt-1 and GSK-3 proteins produced by ad-infection.
Infected cell lysates were immuno-blotted with anti-HA or anti-GSK-3 antibodies.
A. Rat-2 fibroblasts infected with Ad-Wnt1HA, analyzed at several days post infection (p.i.)
B. GSK-3βdom/neg expressed in cultured 293 cells. Cells were transfected with control vector, GSK-3dom/neg, or infected by Ad-GSK-3dom/neg.

VI. Wnt as a Mitogen

In order to develop new model systems to study biological Wnt responses, we evaluated the effect of overexpressing Wnt-1 using adenovirus vectors in a variety of cell lines. Our goal was to develop cell culture systems that reflected Wnt biological activity. These assays could then be used to confirm and extend findings on Wnt signal transduction evaluation using C57MG mammary epithelial cells.

Rat-1 fibroblasts respond mitogenically to Wnt-1. A Rat-1 fibroblast line (kindly provided by Dr. Riccardo Dalla-Favera, Columbia University) displayed a modestly elongated morphology to Wnt-1. More importantly, Wnt-1 expression allowed these cells to proliferate in the absence of serum; that is, they displayed a mitogenic response. It must be noted that Wnt proteins have never previously been shown to promote serum-independent growth. This finding provides evidence that Wnt-1 can act as a mitogen. Figure 9 shows a growth curve for Rat-1 fibroblasts seeded at low density and then allowed to grow in serum-free conditions (DMEM with no added components). Control Rat-1 cells slowly quiesce, doubling after 2 weeks in culture. Rat-1 cells expressing Wnt-1 grow in serum free media at approximately 3-4 fold greater rate than do controls, eventually achieving confluence between one and two weeks in culture. Pictures of cultures are provided in Figure 10. Rat-1 cells fail to grow in serum-free media (Rat-1) whereas Rat-1-Wnt-1 cells continue to grow to confluence.

Figure 9. Growth curve for Wnt-1 expressing Rat-1 fibroblasts in serum-free media.

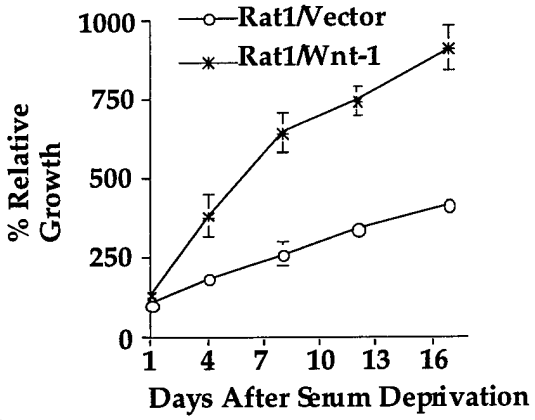
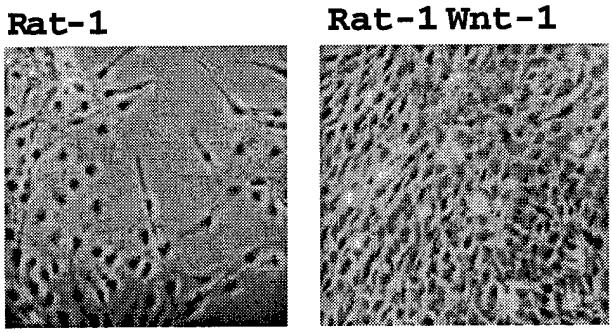
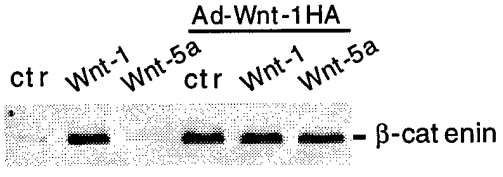


Figure 10. Morphology of Rat-1 and Rat-1:Wnt-1 fibroblasts after two weeks in serum-free media.



Rat-1 Fibroblasts display Wnt-1 dependent signaling. We have established that Wnt-1 transformation of cultured mammary epithelial cells correlates with increased levels of the cytosolic form of β-catenin. We next conducted experiments to determine if ectopic expression of Wnt-1 proteins similarly affects β-catenin levels in Rat-1 fibroblasts. Control, Wnt-1, and Wnt-5a expressing Rat-1 fibroblasts were made using retroviral vectors. Figure 11 shows that the cytosolic β-catenin is significantly increased in response to Wnt-1 expression, but not to Wnt-5a expression. Similarly, Ad-Wnt-1 expression induces increased cytosolic β-catenin levels. As a control for all experiments, we have utilized a comparable adenovirus vector expressing LacZ, referred to as Ad-LacZ. Wnt-1 expression had very little effect on the levels of β-catenin found in the membranous fraction (data not shown). These experiments display the utility in analyzing Wnt-signal transduction with adenovirus expression vectors. In addition, Wnt-5a expression did not affect the ability of Wnt-1 to regulate β-catenin. We found that Wnt-1 expression increased mitogenicity and β-catenin levels in Rat-1 fibroblasts whether the gene was introduced by retroviral or adenoviral vector.

Figure 11. Wnt-1, but not Wnt-5a, expression leads to increased levels of cytosolic β–catenin in Rat-1 cells. Rat-1 cells programmed to express Wnt-1 or Wnt-5a or infected with Ad-Wnt-1HA vectors were evaluated for the levels of cytosolic β–catenin by immunoblot analysis.

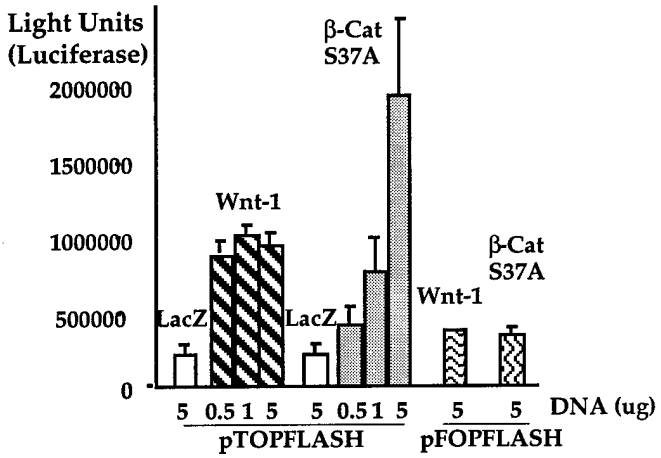


Wnt-1 activates the Tcf/Lef-1 transcription complex in Rat-1 fibroblasts. To investigate Wnt signaling in Rat-1 cells we used two sets of reporter constructs in a β-catenin-Tcf reporter gene assay. Reporter constructs were kindly provided by Dr. Hans Clever, Utrecht, Netherlands. pTOPFLASH contains three copies of the optimal Tcf motif CCTTTGATC, and pFOPFLASH contains three copies of a mutant motif CCTTTGGCC, upstream of a minimal *c-Fos* promoter driving luciferase expression. These constructs function as Tcf responsive reporters and previous work had established that β-catenin in combination with Tcf4 could activate such elements. (80).

Wnt-1 expression led to increased Tcf transcriptional activity in Rat-1 fibroblasts (Fig. 12), thus providing the first demonstration that signaling via Wnt-1 can lead to transcriptional activation of Tcf responsive elements. A mutant version of β-catenin, found in colon cancers, replaces residue S37 with A (S37A). This mutation is thought to interfere with β-catenin phosphorylation, thus stabilizing and activating the protein. We tested whether β-cateninS37A behaved as a gain of function mutation. Transfection of β-cateninS37A led to increased Tcf transcriptional activity. Unlike Wnt-1, increasing amounts of plasmid led to increased activity. This finding is consistent with Wnt acting as a growth factor whose activity is amplified by a signaling cascade, whereas, β-catenin levels are directly proportional to the amount of transcriptional complex made and thus to activity. As controls, LacZ expression lead to basal activity comparable to that seen with mutant reporter elements (FOPFLASH). Wild type β-catenin also displayed transcriptional activity but less than that found for β-cateninS37A (data not shown). Wnt-5a, which does not transform Rat-1 fibroblasts, does not activate the Tcf reporter element (data not shown). We conclude that Wnt-1 activates a signaling cascade in Rat-1 cells that can lead to activation of Tcf responsive elements.

Figure 12. Wnt-1 and β-catenin expression activates TCF-mediated transcription.
Rat-1 fibroblasts were co-transfected with either Wnt-1 or activated β-catenin expression constructs and Tcf-luciferase constructs. Luciferase activity was measured two days post transfection.

Luciferase Reporter Constructs:
pTOPFLASH -- 3X optimal Tcf CCTTTGATC.
pFOPFLASH -- 3X mutant Tcf CCTTTGGCC.
Binding sites are placed upstream of a minimal c-Fos promoter driving luciferase gene expression.



In summary, the Rat-1 cell line displays biological responses to Wnt-1 and biochemical activation of the Wnt signaling pathway from the cell surface to the nuclear transcription. These cells are readily infectable with adenovirus or retroviral vectors, thus Wnt signaling protein expression can be easily modulated. Our data suggests that this cell line provides an excellent tool to study Wnt signaling events.

VII. Wnt as a Morphogen

Because *Wnt* genes are expressed in the mammary gland during growth only, it is likely that they contribute to its morphogenesis. Elongation and branching of epithelial ducts is a major part of this process. Utilizing the mouse mammary epithelial TAC-2 cell line, we examined the role of the Wnt-1, HGF, TGFß2 and the Notch4 receptor on branching morphogenesis. Wnt-1, HGF and TGFß2 induce elongation and branching of epithelial tubules. Wnt-1 strongly cooperates with HGF or TGFß2 in this activity. In contrast, an activated form of the Notch4 receptor completely blocks HGF- and TGFß2-induced branching morphogenesis. Furthermore, Wnt-1 overcomes the Notch-mediated block of ductal morphogenesis. This work is described in detail in the Appendix manuscript- Uyttendaele, Soriano, *et al.*, which has been submitted for publication, and provides evidence that Wnt-1 induces branching morphogenesis. In addition, this assay for Wnt-1 function provides an alternate means to study Wnt genes and can be used to corroborate findings derived from the C57MG or Rat-1 cell systems.

CONCLUSIONS

The data presented in this annual report represents results of experiments outlined in specific aim 1-5 of the research proposal. We feel we have completed all of the major objectives in the first three aims. The former specific aims 4-5 are now focused on Wnt signal transduction and on the interaction between the frizzled-like protein, frzb, and Wnt proteins. Significant progress has been made in this area. We have documented a physical interaction between frzb and Wnt-1 and have begun to map the domains required for Wnt-frzb association. In addition, we have begun to define the events that lead to regulation of β-catenin levels and activity.

In conclusion, we have segregated Wnt proteins into functional classes based upon their ability to transform mammary epithelial cells. This segregation may represent classes of Wnt proteins that interact with distinct Wnt-cell surface receptors. This may represent the first type of evidence that their may be distinct Wnt-cell surface receptors. Alternatively, one class may be involved in mitogenic stimulus and are thus are transforming, whereas the other class may be involved in differentiation of the mammary epithelium. Two interesting aspects of the segregation come out of this analysis. First, it appears that those Wnt genes either not normally expressed in the mammary gland (Wnt-1, Wnt-3, Wnt-3A, and Wnt-7B) or expressed at very low levels in the mammary gland (Wnt-2) are the most transforming. Whereas, those Wnt genes that are well expressed in the mammary gland (Wnt-4, Wnt-5A, Wnt-5B, Wnt-6) do not exhibit transformation activity. Second, when one compares the activity of the transforming genes to those reported to be overexpressed in mammary tumors (70) only one Wnt gene that is not transforming is overexpressed in this study. Wnt-2, Wnt-3, and Wnt-7B were all found to be transforming in our hands and have been found to be overexpressed in several mammary tumors; however, Wnt-4 never displayed transforming activity in our experiments but was found to be overexpressed in mammary tumors. The transforming potential correlates with the ability of Wnt family proteins to increase levels of cytosolic β-catenin. Thus, Transformation may depend on the ability of Wnt proteins to alter β-catenin levels. These findings indicate that upregulation of cytosolic β-catenin is dependent on expression of the transforming class of Wnt proteins, providing the first evidence that increased cytosolic β-catenin correlates with cellular transformation.

By generating a panel of Wnt-1/Wnt-5a chimeric proteins, we have narrowed down a region of Wnt-1 that confers transforming ability. These constructs have been tested for transforming potential in mammary epithelial cells (C57MG) and the levels of cytosolic β-catenin were measured. Results of these experiments are schematized in Figure 3. We conclude that the transformation and signaling specific determinants of Wnt-1 proteins map to the same region encompassing the central 120 amino acids of Wnt-1. Modest activity was found for chimeras C and E2A3, this analysis may allow further localization of the minimal domain required for Wnt-1 signaling to the Wnt-1 domain present in E2A3.

We demonstrate that Frzb-1 blocks Wnt-1 induced cytosolic accumulation of ß-catenin, a key component of the Wnt signaling pathway, in human embryonic kidney cells. Structure/function analysis reveals that complete removal or partial deletions of the frizzled domain of Frzb-1 abolishes its inhibition of Wnt-1 activity. Deletion of the C-terminal domain of Frzb decreases its blocking efficiency.

Expression of Fused dom/neg leads to markedly increased levels of cytosolic β–catenin. The regulation was consistently several fold more pronounced than found for Wnt-1 . We thus conclude that Fused functions in the mammalian Wnt signaling pathway, presumably as a potent negative regulator.

Wnt-1 expression in Rat-1 fibroblasts allows these cells to proliferate in the absence of serum; that is, they displayed a mitogenic response. It must be noted that Wnt proteins have never previously been shown to promote serum-independent growth. These finding provides evidence that Wnt-1 can act as a mitogen. Wnt-1 activity in these cells correlates with increased levels of β-catenin and increased transcriptional activity of a Tcf responsive element.

Utilizing the mouse mammary epithelial TAC-2 cell line, we examined the role of the Wnt-1, HGF, TGFß2 and the Notch4 receptor on branching morphogenesis. Wnt-1, HGF and TGFß2 induce elongation and branching of epithelial tubules. Wnt-1 strongly cooperates with HGF or TGFß2 in this activity. In contrast, an activated form of the Notch4 receptor completely blocks HGF- and TGFß2-induced branching morphogenesis. Furthermore, Wnt-1 overcomes the Notch-mediated block of ductal morphogenesis.

REFERENCES

1. M. C. Neville, C. W. Daniel, *The Mammary Gland: Development, Regulation, and Function.* (Plenum Press, New York, 1987).
2. M. E. Lippman, R. E. Dickson, *Breast Cancer: Cellular and Molecular Biology* (Kluwer Academic Publishers, Boston/Dordrecht/London, 1988).
3. R. B. Dickson, et al., Growth factors in breast cancer: mitogenesis to transformation., Proceedings of the Fourth International Congress on Hormones and Cancer (Pergamon Press, 1992).
4. R. A. Jensen, et al., *Nature Genetics* **12**, 303-08 (1996).
5. R. Nusse, et al., *Cell* **64**, 231 (1991).
6. R. Nusse, H. E. Varmus, *Cell* **31**, 99-109 (1982).
7. R. Nusse, A. van Ooyen, D. Cox, Y. K. Fung, H. Varmus, *Nature* **307**, 131-136 (1984).
8. A. S. Tsukamoto, R. Grosschedl, R. C. Guzman, T. Parslow, H. E. Varmus, *Cell* **55**, 619-625 (1988).
9. G. M. Shackleford, C. A. Macarthur, H. C. Kwan, H. E. Varmus, *Proc. Nat. Acad. Sci USA* **90**, 740-744 (1993).
10. H. Roelink, E. Wagenaar, S. Lopes Da Silva, R. Nusse, *Proc. Natl. Acad. Sci. USA* **87**, 4519-4523 (1990).
11. N. E. Baker, *EMBO J.* **6**, 1765-1773 (1987).
12. F. Rijsewijk, et al., *Cell* **50**, 649-657 (1987a).
13. N. Perrimon, *Cell* **76**, 781-784 (1994).
14. A. P. McMahon, R. T. Moon, *Cell* **58**, 1075-1084 (1989).
15. J. L. Christian, J. A. McMahon, A. P. McMahon, R. T. Moon, *Development* **111**, 1045-1055 (1991).
16. S. Sokol, J. L. Christian, R. T. Moon, D. A. Melton, *Cell* **67**, 741-752 (1991).
17. W. C. Smith, R. M. Harland, *Cell* **67**, 753-765 (1991).
18. S. Y. Sokol, D. A. Melton, *Dev. Biol.* **154**, 348-356 (1992).
19. A. P. McMahon, A. Bradley, *Cell* **62**, 1073-1085 (1990).
20. K. R. Thomas, M. R. Capecchi, *Nature* **346**, 847-850 (1990).
21. B. A. Parr, M. J. Shea, G. Vassileva, A. P. McMahon, *Dev.* **119**, 247-261 (1993).
22. Y. Yang, L. Niswander, *Cell* **80**, 939-947 (1995).
23. K. Stark, S. Vainio, G. Vassileva, A. P. McMahon, *Nature* **372**, 679-83 (1994).
24. D. Herzlinger, J. Qiao, D. Cohen, N. Ramakrishna, A. M. Brown, *Dev. Biol.* **166**, 815-8 (1994).
25. A. Pavlova, E. Boutin, G. Cunha, D. Sassoon, *Development* **120**, 335-346 (1994).
26. A. E. Munsterberg, J. Kitajewski, D. A. Bumcrot, A. P. McMahon, A. B. Lassar, *Genes Dev.* **9**, 2911-12 (1995).
27. Y. K. Fung, G. M. Shackleford, A. M. Brown, G. S. Sanders, H. E. Varmus, *Mol. Cell. Biol.* **5**, 3337-3344 (1985).
28. A. M. C. Brown, J. Papkoff, Y. K. Fung, G. M. Shackleford, H. E. Varmus, *Mol. Cell. Biol.* **7**, 3971-3977 (1987).
29. J. Papkoff, A. M. C. Brown, H. E. Varmus, *Mol. Cell. Biol.* **7**, 3978-3984 (1987).
30. J. Kitajewski, J. O. Mason, H. E. Varmus, *Mol. Cell. Biol.* **12**, 784-790 (1992).
31. R. S. Bradley, A. M. C. Brown, *EMBO J.* **9**, 1569-1575 (1990).
32. J. Papkoff, B. Schryver, *Mol. Cell. Biol.* **10**, 2723-2730 (1990).
33. S. F. Jue, R. S. Bradley, J. A. Rudnicki, H. E. Varmus, A. M. C. Brown, *Mol. Cell. Biol.* **12**, 321-328 (1992).
34. J. O. Mason, J. Kitajewski, H. E. Varmus, *Mol. Biol. Cell* **3**, 521-533 (1992).
35. N. E. Baker, *Dev. Biol.* **125**, 96-108 (1988).
36. N. Parkin, J. Kitajewski, H. E. Varmus, *Genes Dev.* **7**, 2181-2193 (1993).
37. R. S. Bradley, A. M. Brown, *Mol. Cell. Biol.* **15**, 4616-22 (1995).
38. F. van Leeuwen, C. H. Samos, R. Nusse, *Nature* **368**, 342-4 (1994).
39. N. Perrimon, *Cell* **86**, 513-516 (1996).
40. J. Klingensmith, R. Nusse, *Dev. Biol.* **166**, 396-414 (1994).
41. M. Peifer, S. Orsulic, L. Pai, J. Loureiro, *Development* **Supplement**, 163-176 (1993).
42. P. Bhanot, et al., *Nature* **382**, 225-230 (1996).
43. M. Peifer, L. M. Pai, M. Casey, *Dev. Biol.* **166**, 543-56 (1994).
44. J. Riese, et al., *Cell* **88**, 777-87 (1997).

45. D. I. Strutt, U. Weber, M. Mlodzik, *Nature* **387**, 292-5 (1997).
46. Y. Wang, et al., *J. Biol. Chem.* **271**, 4468-76 (1996).
47. D. J. Sussman, et al., *Dev. Biol.* **166**, 73-86 (1994).
48. E. Siegfried, T. B. Chou, N. Perrimon, *Cell* **71**, 1167-1179 (1992).
49. M. Peifer, E. Wieschaus, *Cell* **63**, 1167-1178 (1990).
50. U. Rothbacher, et al., *Dev. Biol.* **170**, 717-21 (1995).
51. X. He, J. P. Saint-Jeannet, J. R. Woodgett, H. E. Varmus, I. B. Dawid, *Nature* **374**, 617-22 (1995).
52. S. B. Pierce, D. Kimelman, *Development* **121**, 755-65 (1995).
53. K. A. Guger, B. M. Gumbiner, *Dev. Biol.* **172**, 115-25 (1995).
54. A. Karnovsky, M. W. Klymkowsky, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 4522-6 (1995).
55. B. M. Gumbiner, *Cell* **84**, 345-357 (1996).
56. B. Rubinfeld, et al., *Science* **262**, 1731-1734 (1993).
57. B. Rubinfeld, et al., *Science* **272**, 1023-6 (1996).
58. L.-K. Su, B. Vogelstein, K. W. Kinzler, *Science* **262**, 1734-1737 (1993).
59. K. J. Smith, et al., *Proc. Natl. Acad. Sci. U.S.A.* **90**, 2846-50 (1993).
60. A. R. Moser, et al., *Proc. Natl. Acad. Sci. U.S.A.* **90**, 8977-81 (1993).
61. A. R. Moser, et al., *European Journal of Cancer* **31A**, 1061-4 (1995).
62. B. Rubinfeld, et al., *Science* **275**, 1790-2 (1997).
63. K. Vleminckx, et al., *J. Cell Biol.* **136**, 411-20 (1997).
64. B. Hoang, M. Moos, Jr., S. Vukicevic, F. P. Luyten, *J. Biol. Chem.* **271**, 26131-7 (1996).
65. S. Wang, M. Krinks, K. Lin, F. P. Luyten, M. Moos, Jr., *Cell* **88**, 757-66 (1997).
66. R. Nusse, H. E. Varmus, *Cell* **69**, 1073-1087 (1992).
67. F. S. Lee, T. F. Lane, A. Kuo, G. M. Shackleford, P. Leder, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 2268-72 (1995).
68. A. M. C. Brown, R. S. Wilden, T. J. Prendergast, H. E. Varmus, *Cell* **46**, 1001-1009 (1986).
69. F. Rijsewijk, L. Deemter, E. Wagenaar, A. Sonneberg, R. Nusse, *EMBO J.* **6**, 127-131 (1987b).
70. E. L. Huguet, J. A. McMahon, A. P. McMahon, R. Bicknell, A. L. Harris, *Cancer Res.* **54**, 2615-2621 (1994).
71. R. V. Iozzo, I. Eichstetter, K. G. Danielson, *Cancer Res.* **55**, 3495-9 (1995).
72. B. J. Gavin, A. P. McMahon, *Mol. Cell. Biol.* **12**, 2418-2423 (1992).
73. T. A. Buhler, T. C. Dale, C. Kieback, R. C. Humphreys, J. M. Rosen, *Dev. Biol.* **155**, 87-96 (1993).
74. S. J. Weber-Hall, D. J. Phippard, C. C. Niemeyer, T. C. Dale, *Differentiation* **57**, 205-14 (1994).
75. T. Lin, R. C. Guzman, R. C. Osborn, G. Thordarson, S. Nandi, *Cancer Res.* **52**, 4413-4419 (1992).
76. J. Kawanishi, et al., *Mol. Cell. Biol.* **15**, 1175-81 (1995).
77. I. Whitehead, H. Kirk, R. Kay, *Mol. Cell. Biol.* **15**, 704-10 (1995).
78. C. Kraus, et al., *Genomics* **23**, 272-4 (1994).
79. P. J. Morin, et al., *Science* **275**, 1787-90 (1997).
80. V. Korinek, et al., *Science* **275**, 1784-7 (1997).
81. A. D. Miller, G. J. Rosman, *Bio. Techniques* **7**, 980-990 (1989).
82. R. E. Finney, J. M. Bishop, *Science* **260**, 1524-7 (1993).
83. R. M. Horton, Z. Cai, S. N. Ho, L. R. Pease, *BioTech.* **8**, 528-535 (1990).
84. L. Zeng, et al., *Cell,* **in press** (1997).
85. K. L. Berkner, *BioTech.* **6**, 616-29 (1988).

Biological Sciences:Biochemistry

The cysteine-rich frizzled domain of Frzb-1 is required and sufficient for modulation of Wnt signaling.

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Abstract: Convincing evidence has accumulated to identify the Frizzled proteins as receptors for the Wnt growth factors (1, 2, 3). In parallel, a number of secreted frizzled-like proteins with a conserved N-terminal frizzled motif have been identified (4, 5, 6). One of these proteins, Frzb-1, binds Wnt-1 and Xwnt-8 proteins (7) and antagonizes Xwnt-8 signaling in *Xenopus* embryos (7, 8). We now report that Frzb-1 also blocks Wnt-1 induced cytosolic accumulation of ß-catenin, a key component of the Wnt signaling pathway, in human embryonic kidney cells. Structure/function analysis reveals that complete removal or partial deletions of the frizzled domain of Frzb-1 abolishes its inhibition of Wnt-1 activity. Deletion of the C-terminal domain of Frzb-1 decreases its blocking efficiency. Interestingly, whereas co-immunoprecipitation experiments demonstrate a direct Frzb-1/Wnt-5A interaction, Frzb-1 does not block Wnt-5A signaling in a functional assay in *Xenopus* embryos. This provides evidence that a Frzb-1/Wnt protein interaction does not necessarily imply inhibition of Wnt function.

The Wnt family of signaling molecules are of great interest because of their roles in developmental processes and in oncogenesis (9, 10). The recent implication of ß-catenin, one effector protein in the Wnt signaling pathway, in colon cancers and melanomas, has underscored the importance of this signaling pathway in human cancers(11, 12, 13). A major advance in the understanding of Wnt signaling was provided by the discovery that Dfz2, a member of the class of Frizzled proteins, functions as a receptor for Drosophila Wingless (Wg), the homologue of vertebrate Wnt-1 (1). In addition, overexpression of rat fz-1 in *Xenopus* embryos recruits Dishevelled, another component of the Wnt signaling pathway (2), to the plasma membrane. These findings led to the proposal that the frizzled group within the G-protein receptor superfamily comprises receptors for the Wnt proteins (1).

The Wnts have been divided into two operationally defined classes. Class I Wnts (e.g. Wnt-1) induce both transformation of cultured mammalian cells and axis duplication in Xenopus embryos; class II Wnts (e.g. Wnt-5A) do not. The observation that several, but not all, mammalian frizzled proteins are able to confer Wg binding in cell biological assays, suggests that some Frizzled proteins may be selective for particular members of the Wnt family (1). This is supported by the demonstration that rat Fz-1 is involved in Wnt signaling in a manner which discriminates between the functionally distinct Xwnt-8 (Class I) and Xwnt-5A (Class II) (2).

Frzb-1, originally discovered by primary protein sequencing of highly purified cartilage-derived protein preparations, contains an N-terminal domain with about 50 % identity to the cysteine-rich domain (CRD) of *Drosophila* Frizzled (5). Because this domain has been proposed to be the ligand binding domain of the Frizzled proteins (1), we explored the possibility of structural and functional interactions between Frzb-1 and members of the Wnt family. The observation of complementary expression patterns of Xfrzb-1 and Xwnt-8 in developing embryos and the demonstration of their direct interaction led to the finding that Frzb-1 is a Wnt -8 antagonist (7, 8, 14).

In this paper, we demonstrate that Frzb-1 prevents Wnt-1 induced cytosolic accumulation of ß-catenin in human embryonic kidney cells. Structure/function studies indicate that the N-terminal CRD of Frzb-1 is critical for the inhibition of Wnt-1 signaling, and suggest a supportive role for the C-terminus. We further show that Frzb-1 also binds to murine Wnt-5A in immunoprecipitation experiments. However, this interaction does not necessarily predict inhibition of Wnt function, as our findings indicate that Frzb-1 does not block Wnt-5A signaling.

Materials and Methods

Constructs and plasmids The bovine (B)Frzb-1 plasmid was described previously (5). Δ C-term (Fig 2A) was made by deletion of amino acids 160 to 316; Δ CRD, by deletion of amino acids 39 - 145; Δ 7C, by deletion in the CRD of amino acids 79 - 149; Δ 2C, by deletion of amino acids 124 to 149; and Δ 57-95,

by deletion of amino acids 57 - 95, which contains the hydrophobic domain (Fig. 2A). The pFrzb-1-FLAG was made by replacement of the last seven residues of Frzb-1 by the Flag-tag (DYKDDDDK). All constructs were subcloned into pcDNA3 (Invitrogen Corporation, Carlsbad, CA). The plasmids pLNCW1-HA and pLNCW5A-HA carrying the respective Wnt gene family members have been reported previously (15).

Transient transfections

COS7 cells (1.6 X 10^6 initial seeding density) were transfected either with 5 µg of plasmid DNA, or co-transfected with 4 µg for each plasmid DNA per 100-mm dish by using 30 µl LipofectAMINE™ reagent (Life Technologies, Gaithersburg, MD). Transfections were carried out for 6 hr in serum-free Opti-MEM I® (Life Technologies, Gaithersburg, MD). Subsequently, equal amounts of 10% fetal bovine serum in Opti-MEM I® were added to the transfections and the cultures were continued for 18 hr. The cells were further incubated at 37°C for 24 hr in serum-free Opti-MEM I®. The cultures were then extracted for 30 min on ice with RIPA buffer (50 mM Tris, 150 mM NaCl, 1.0% NP-40, 0.5% Deoxycholic acid and 0.1% SDS). 293 cells were transfected as described (16) with varying amounts of plasmid as shown in the legend of Figure 1. After two days, cells were lysed in an hypotonic buffer and insoluble (membrane-associated) and soluble (cytosol-associated) material was recovered by preparative ultracentrifugation at 100,000 x g. Protein contents were determined using a protein assay (Bio-Rad, Richmond, CA). Samples

were adjusted to 1x Laemmli sample buffer prior to denaturation by boiling (17).

Co-immunoprecipitation:

Immunoprecipitations with HA antiserum: 50 µl of protein A-Agarose (Boehringer Mannheim, Indianapolis, IN) were incubated with 100 µl of hybridoma supernatant of anti-HA antibody 12CA5 in 450 µl of 50 mM Tris-HCl, pH 7.4/150 mM NaCl by rotating overnight. Cell extracts (100-400 µl) and RIPA buffer (0-300 µl) were added to a final volume of 1 ml and the incubation was continued for another hour. The agarose beads were washed twice 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, twice in 50 mM Tris-HCl, pH 7.4, 500 mM NaCl, and once in 50 mM Tris-HCl, pH 7.4 . After the last wash, the beads were suspended in 30-50 µl of 2 x Laemmli sample buffer with 4% ß-mercaptoethanol, boiled and separated on denaturing 4-20% gradient Tris-Glycine gels (Novex, San Diego, CA). Samples were blotted onto Immobilon™-P membrane (Millipore Corporation, Bedford, MA) by using a GENIE™ electrophoretic blotter (Idea Scientific Company, Minneapolis, MN) and analyzed by immunoblotting. Membranes were blocked 30 min in blocking buffer (BF) consisting of 1 M Tris, pH 7.5, 0.9% NaCl, 0.05% Tween 20 and 4% BSA. The primary antiserum (N374-PEP)(5) was incubated with the membranes in 1/10 BF and 9/10 TBST buffer (10 mM Tris, pH 7.5; 0.1% Tween; 150 mM NaCl) at a dilution of 1: 2,500. The membranes were then washed four times for 5 min in TBST. The membranes were subsequently incubated with the secondary antibody at a dilution of 1:10,000 for 60 min.

Blots were developed by using the SuperSignal™CL Substrate System (Pierce, Rockford, IL) and exposed to Kodak XAR-5 films for 1 to 10 min

Immunoprecipitation with anti-FLAG M2 antiserum. We used 50 µl of protein G-Agarose (Boehringer Mannheim, Indianapolis, IN) and 5 µg anti-FLAG M2 antibody (Eastman Kodak Company, New Haven, CT) for immunoprecipitation, 1: 1, 000 dilution of anti-HA-peroxidase antibody (HRP-HA antibody) (Boehringer Mannheim, Indianapolis, IN) for immunoblotting, and SuperSignal™ ULTRA Substrate (Pierce, Rockford, IL) for the chemiluminescent detection.

Detection of ß-catenin in transiently transfected 293 cells. Membrane or cytosol preparations were generated as described as described above with some modifications (Brown, A.M.C, personal communication). 50 µg protein from cell extracts were separated on denaturing 8% polyacrylamide-SDS gels. Proteins were transferred from gels onto nitrocellulose filters by electroblotting and blocked overnight at 4 °C in TBST containing 1% bovine serum albumin (fraction V). Blots were then incubated in anti- mouse ß-catenin monoclonal antibody (Transduction Laboratories, Lexington, KY) diluted 1:4000 in TBST, at room temperature for 2 hr. The primary antibody was then removed by washing in TBST at room temperature three times for 5 min each. Blots were exposed to a 1:5,000 dilution of horseradish peroxidase-conjugated sheep anti-mouse immunoglobulin G (Amersham, Arlington Heights, IL). Blots were then incubated 5 min in HRP substrate and exposed to Kodak XAR-5 films.

Xenopus embryo manipulations:

Frogs and their embryos were maintained and manipulated using standard methods (18). mRNA injection experiments were performed by standard procedures as described (19). The *Xwnt-5A* assay was scored as reported previously (20).

Results

Frzb-1 blocks Wnt-1 signaling in mammalian cells

We determined whether the inhibitory effect of Frzb-1 on Wnt-1 signaling, observed initially in *Xenopus* embryos, could also be demonstrated in mammalian cells. It has been shown that Wnt-1 expression can lead to stabilization of the cytosolic pool of ß-catenin, a key component in the Wnt signaling pathway (21). Ectopic Wnt-1 expression in human embryonic kidney cells induces the accumulation of ß-catenin within the cytosol, whereas membrane-associated levels of ß-catenin remain virtually unchanged (Fig. 1A). Figure 1B shows that the induction of cytosolic ß-catenin by Wnt-1 is attenuated in the presence of increasing amounts of plasmid encoding Frzb-1. Partial inhibition is observed when 3 µg Frzb-1 plasmid were transfected and complete inhibition is achieved with 30 µg Frzb-1 expression, in the absence of Wnt-1, had no effect on cytosolic ß-catenin levels.

The CRD of Frzb-1 is required for Wnt-1 binding and inhibition of Wnt-1 function

The observation that the extracellular domain of Dfz2 containing the CRD confers Wg binding (1) suggests that the CRD of Frzb-1 may be sufficient for Wnt binding. We confirmed this by performing immunoprecipitation experiments with lysates of COS7 cells co-transfected with *wnt-1* cDNA and several Frzb-1 deletion constructs retaining the signal peptide (Fig. 2A). Interestingly whereas removal of the entire CRD indeed resulted in loss of co-immunoprecipitation with the Wnts (Fig. 2B), several of the other CRD deletions had little or no effect on the outcome of the immunoprecipitations (Fig. 2B). Only the deletion of 7 of the 10 cysteines (Δ 7C, see Materials and Methods) appeared to reduce the efficiency of the immunoprecipitation (Fig. 2B).

We further evaluated the structural requirements for inhibition of Wnt-1 signaling *in vivo*. Injection of Wnt-1 mRNA into Xenopus embryos results in duplication of the dorsal axis (22, 23) and can easily be scored by direct inspection (Fig. 3 A, B). Co-injection of Wnt-1 with Frzb-1 results in the complete inhibition of secondary axis formation (8). Previous reports suggested that this effect was due to blockade of Wnt signaling (7, 8). Substantial inhibition of Wnt-1 mediated axis duplication was also observed when the CRD of Frzb-1 only was co-injected with Wnt-1 (Fig. 3C). Conversely, removal of the entire CRD abolished the inhibitory activity of Frzb-1 (Fig. 3C). It is noteworthy that the C-terminal domain does play some functional role with regard to Frzb-1 activity, as inhibition appeared to be more efficient in the presence of this domain (Fig. 3C). In contrast to the co-

immunoprecipitation data however, inhibition of Wnt signaling was not observed in the *in vivo* assay by any of the deletion constructs affecting the CRD domain (Fig. 3C). Even a limited deletion of a small domain (Δ 2C, 27 amino acids, see Materials and Methods, Fig. 3C) containing the last two of the 10 conserved cysteines, resulted in an almost complete loss of Wnt-1 inhibition. These data support the critical role of a preserved cysteine core of the CRD for inhibition of Wnt-1 activity.

Specifity of Frzb-1/Wnt interactions

To further investigate the specificity of Frzb-1/Wnt interactions, COS7 cells were co-transfected with Frzb-1 and Wnt-5A. Wnt-1 was used as a positive control (7). Frzb-1 co-immunoprecipitated with Wnt-5A (Fig. 4A), and using a Flag-tagged Frzb-1 protein, Wnt-5A co-immunoprecipitated with Frzb-1 (Fig 4B). These findings demonstrate that Frzb-1 has sufficient affinity to allow co-immunoprecipitation with both Wnt-1 and Wnt-5A. Lack of soluble Wnt proteins precluded classical binding studies. However, variation of the washing conditions after immunoprecipitation did not result in any noticable differences in the association between Frzb-1 and Wnt-1 or Wnt-5A (data not shown). We next analyzed whether Frzb-1 functioned as an antagonist to Wnt-5A signaling for which a *Xenopus* assay has been described (20). Overexpression of Wnt-5A in Xenopus embryos produces a characteristic phenotype with head and/or tail malformations (20). Co-injections of Wnt-5A with preprolactin resulted in about 75 % of the injected embryos in an abnormal phenotype (two independent experiments, 40/53 and 27/36), and

co-injection with Frzb-1 showed similar results (34/34 and 30/36). Therefore Frzb-1 does not appear to suppress the Wnt-5A induced phenotypic changes, and may even enhance the development of the head and/or tail abnormalities. This finding is consistent with recent reports that a dominant negative Xwnt-8 is also unable to block Wnt-5A activity in the same model (24).

Discussion

This report provides the first evidence that Frzb-1 can directly block the Wnt signaling cascade in a mammalian cell line, as measured by the inhibition of the Wnt-1 induced cytosolic accumulation of ß-catenin. It was previously described that Fzb-1 blocks Wnt-1 and Xwnt-8 signaling in *Xenopus* embryos (7, 8). However, this blocking activity was assessed by its inhibition of Wnt-1/Xwnt-8 axis duplication or the induction of Xwnt-8 response genes *Xnr3* and *Siamois* in animal cap assays, all relatively late read-out systems in the Wnt signaling patwhay (7). ß-catenin, an early component in the Wnt signaling cascade, has been implicated in the development of either colon cancer or melanomas (11, 12, 13). Therefore it is possible that Frzb-1 could potentially act as a tumor suppressor by inhibiting Wnt mediated cell proliferation. Based on its mapping to chromosome 2q31-33, it was previously suggested that Frzb-1 may be a potential tumor suppressor gene (8). Loss of one copy of the 2q arm has indeed been associated with a high incidence of lung carcinomas, colorectal carcinomas, and neuroblastomas (25, 26).

Our deletion studies showed that the CRD of Frzb-1 is required and sufficient for its interaction with the Wnt proteins. Functionally, all the deletion constructs in the CRD resulted in the loss of the inhibitory activity of Fzb-1. Our co-immunoprecipitation experiments did not reveal a specific motif to be critical for the Frzb-1/Wnt interaction. Disruption of the cysteine core and/or removal of the hydrophobic domain within the CRD (57-95), potentially important for the Frzb-1/Wnt interaction, did not significantly affect the outcome of the immunoprecipitation experiments. Our functional data suggest that the affinity of the interactions between Frzb-1 and Wnts may be critical for modulation of Wnt signaling. However, immunoprecipitations are not quantitative and therefore do not provide information on binding affinity. Classical quantitative binding studies cannot be performed at this point, because soluble Wnt proteins are not available.

Interestingly, our data indicate a possible role for the C-terminal domain in the effectiveness of the inhibition of Wnt-1 signaling. This may suggest a role for the C-terminus in the stabilization of the tertiary structure of Frzb-1, in the binding affinity to Wnts, a possible role in Frzb-1 turnover, or increased solubility of the protein.

Frzb-1 belongs to a novel family of secreted proteins containing a cysteine-rich N-terminal domain with high homology to the ligand binding domain of frizzled proteins. Indeed, other secreted proteins containing frizzled-like CRDs have been reported (4, 6, 27). It is of note that each of these have less amino acid sequence identity to the frizzleds than does Frzb-1. Nevertheless,

the data indicate that besides Frzb-1, another secreted frizzled-like protein, sFRP-2, also confers binding of Wg in transfected 293 cells (6). In addition, sequence alignment between the CRD of sFRP-2 and Frzb-1 reveals an identity of only about 25 %. Taken together, this suggests that many frizzled-like proteins will potentially interact with the Wnts. However, as demonstrated by the lack of inhibition of Wnt-5A signaling by Frzb-1, it remains to be determined what the functional consequences of this interaction might be. In this regard it can not be excluded that Frzb-1 and related proteins have additional yet undiscovered regulatory functions, independent of the Wnt signaling pathway.

Figure Legends

Figure 1

Frzb-1 expression blocks Wnt-1 mediated accumulation of cytosolic ß-catenin. 293 cells were transiently transfected cells with Frzb-1 or Wnt-1 expressing plasmids. After transfection, equal amounts of denatured pellet (membrane) and supernatant (cytosol)-derived protein were electrophoresed and blotted onto nitrocellulose membranes and exposed to anti-ß-catenin antibody (Transduction Laboratories, Lexington, Ky.).

A, Distribution of ß-catenin in membrane and cytosolic fractions transfected by a control CMV promoter-containing vector (control) or vector containing Wnt-1 cDNA.

B, The accumulation of cytosolic ß-catenin by Wnt-1 is blocked by Frzb-1 expression. Cells were transfected by variable amounts of a CMV promoter-containing plasmid carrying the *frzb-1* gene in the presence of a control expression vector containing a lac z gene (right panel) or *Wnt*-1 gene (left panel). The total amount of DNA (30 µg) transfected was maintained by supplementation with vector DNA.

Figure 2

Co-immunoprecipitation of Wnt-1 with Frzb-1 deletion constructs

A, Schematic drawing of the BFrzb-1 deletion constructs (Δ). The numbers above the bars indicate the amino acid residue numbers and the junction sites (zigzag lines), the numbers below and vertical bars in the CRD (cysteine-rich domain) indicate the position of the conserved cysteines.

B, COS7 cells were (co-)transfected with Wnt-1 and BFrzb-1 deletion constructs. Cell lysates were immunoprecipitated with anti-HA antibody and probed with N374 PEP antiserum. Brackets indicate immuno-detected Frzb-1 protein.

Figure 3:

Inhibition of Wnt-1 induced axis duplication by Frzb-1 deletion constructs

A-B. Induction of secondary axis by Wnt-1. Embryos injected with 25 pg preprolactin mRNA (A), or 5 pg Wnt-1 mRNA (B)

C. Embryos were co- injected with Wnt-1 and different constructs as indicated. They were then scored for secondary axis formation. The number between brackets indicates the number of embryos injected.

Figure 4:

Frzb-1/Wnt co-immunoprecipitation experiments

COS7 cells were (co-)transfected with HA-tagged Wnt-1 and Wnt-5A and Frzb-1 (Flag-tagged) as indicated. The cell lysates were immunoprecipitated (see Methods) with anti-HA antibody (A) or anti-FLAG M2 antibody (B), and probed by Western blot with N374-PEP antiserum (A) or HRP-HA antibody (B).

References

1. Bhanot, P., Brink, M., Samos, C. H., Hsieh, J. C., Wang, Y., Macke, J. P., Andrew, D., Nathans, J. & Nusse, R. (1996) *Nature* 382, 225-230.

2. Yang-Snyder, J., Miller, J. R., Brown, J. D., Lai, C. J. & Moon, R. T. (1996) *Curr Biol* 6, 1302-1306.

3. He, X., Saint-Jeannet, J. P., Wang, Y., Nathans, J., Dawid, I. & Varmus, H. (1997) *Science* 275, 1652-1654.

4. Shirozu, M., Tada, H., Tashiro, K., Nakamura, T., Lopez, N. D., Nazarea, M., Hamada, T., Sato, T., Nakano, T. & Honjo, T. (1996) *Genomics* 37, 273-280.

5. Hoang, B., Moos Jr, M., Vukicevic, S. & Luyten, F. P. (1996) *J Biol Chem* 271, 26131-26137.

6. Rattner, A., Hsieh, J.-H., Smallwood, P. M., Gilbert, D. J., Copeland, N. G., Jenkins, N. A. & Nathans, J. (1997) *Proc. Natl. Acad. Sci. USA* 94, 2859-2863.

7. Wang, S., Krinks, M., Lin, K., Luyten, F. P. & Moos, M. J. (1997) *Cell* 88, 757-766.

8. Leyns, L., Bouwmeester, T., Kim, S.-H., Piccolo, S. & De Robertis, E. M. (1997) *Cell* 88, 747-756.

9. Nusse, R. & Varmus, H. E. (1992) *Cell* 69, 1073-1087.

10. Parr, B. A. & McMahon, A. P. (1994) *Curr Biol* 4, 523-528.

11. Korinek, V., Barker, N., Morin, P. J., van Wichen, D., de Weger, R., Kinzler, K. W., Vogelstein, B. & Clevers, H. (1997) *Science* 275, 1784-1787.

12. Morin, P. J., Sparks, A. B., Korinek, V., Barker, N., Clevers, H., Vogelstein, B. & Kinzler, K. W. (1997) *Science* 275, 1787-1790.

13. Rubinfeld, B., Robbins, P., El-Gamil, M., Albert, I., Porfiri, E. & Polakis, P. (1997) *Science* 275, 1790-1792.

14. Moon, R. T., Brown, J. D., Yang-Snyder, J. A. & Miller, J. (1997) *Cell* 88, 725-728.

15. Munsterberg, A. E., Kitajewski, J., Bumcrot, D. A., McMahon, A. P. & Lassar, A. B. (1995) *Genes Dev* 9, 2911-2922.

16. Pear, W. S., Nolan, G. P., Scott, M. L. & Baltimore, D. (1993) *Proc Natl Acad Sci U S A* 90, 8392-8396.

17. Laemmli, U. K. (1970) *Nature* 227, 680-685.

18. Gurdon, J. B. (1977) *Methods Cell Biol* 16, 125-139.

19. Moos Jr, M., Wang, S. & Krinks, M. (1995) *Development* 121, 4293-4301.

20. Moon, R. T., Campbell, R. M., Christian, J. L., McGrew, L. L., Shih, J. & Fraser, S. (1993) *Development* 119, 97-111.

21. Papkoff, J., Rubinfeld, B., Schryver, B. & Polakis, P. (1996) *Mol Cell Biol* 16, 2128-2134.

22. Smith, W. C. & Harland, R. M. (1991) *Cell* 67, 753-765.

23. Sokol, S., Christian, J. L., Moon, R. T. & Melton, D. A. (1991) *Cell* 67, 741-752.

24. Hoppler, S., Brown, J. D. & Moon, R. T. (1996) *Genes Develop* 10, 2805-2817.

25. Tsuchiya, E., Nakamura, Y., Weng, S. Y., Nakagawa, K., Tsuchiya, S., Sugano, H. & Kitagawa, T. (1992) *Cancer Res* 52, 2478-2481.

26. Kohno, T., Morishita, K., Takano, H., Shapiro, D. N. & Yokota, J. (1994) *Oncogene* 9, 103-108.

27. Rehn, M. & Pihlajaniemi, T. (1995) *J Biol Chem* 270, 4705-4711.

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Figure 1

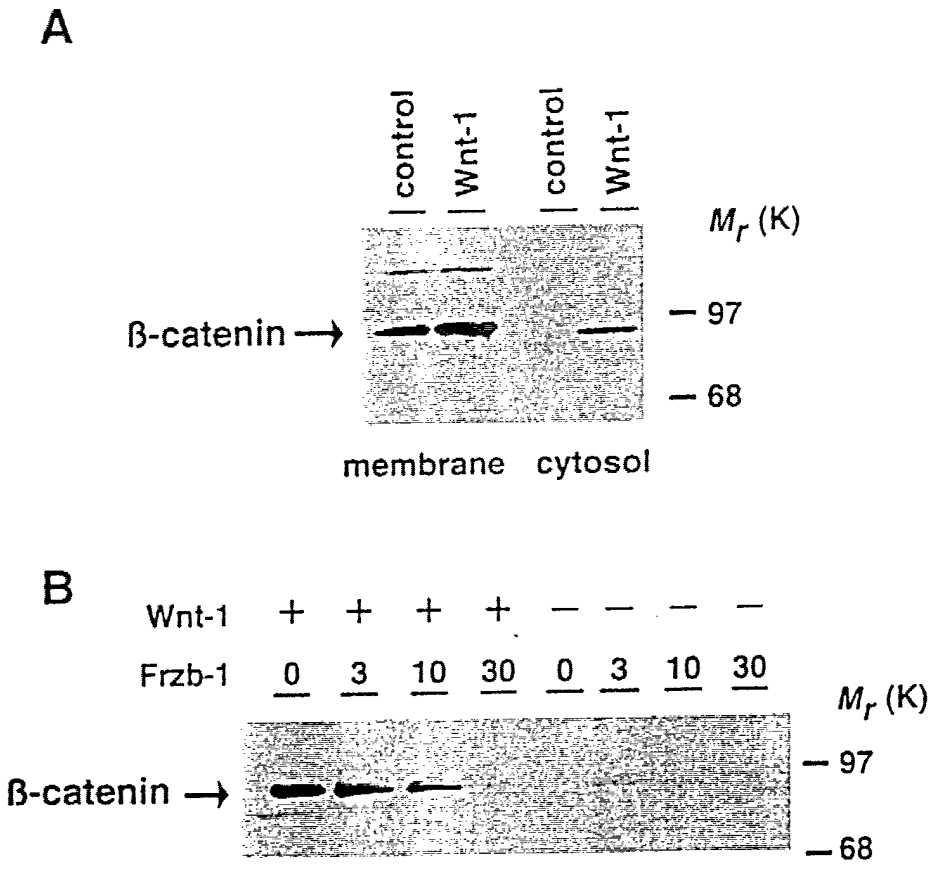
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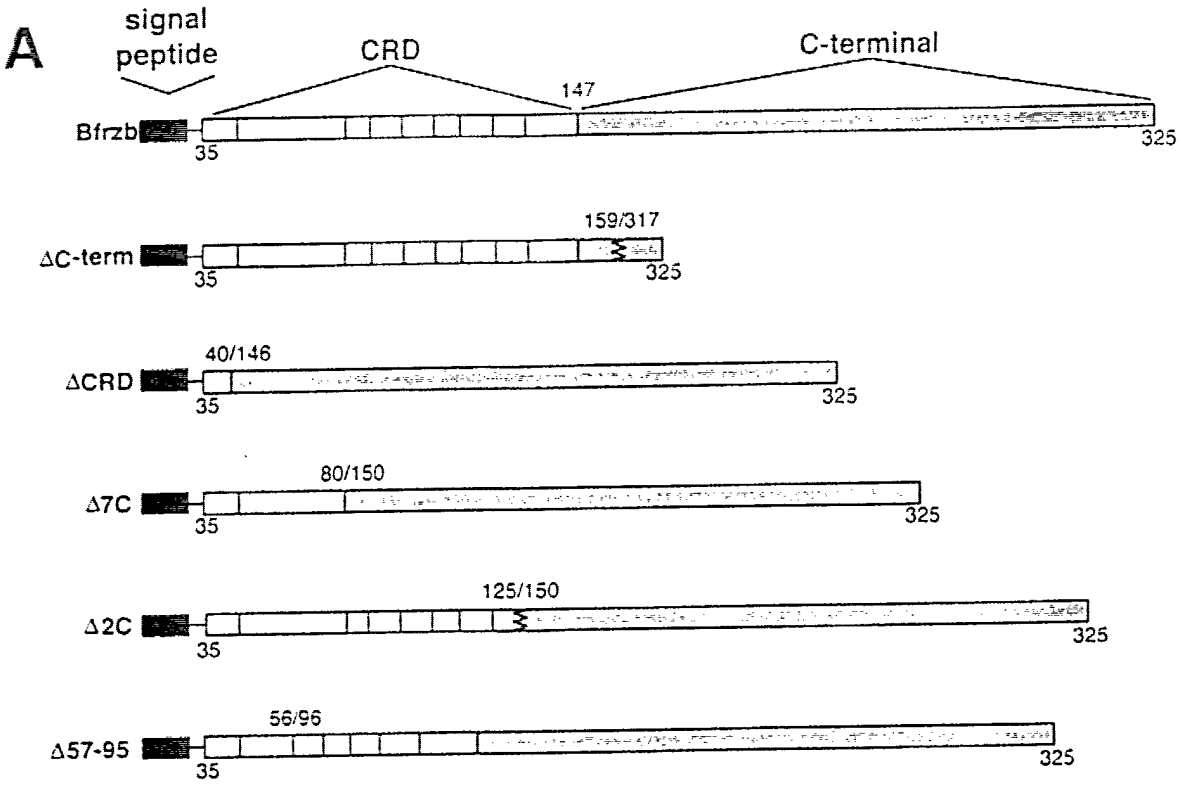
Figure 2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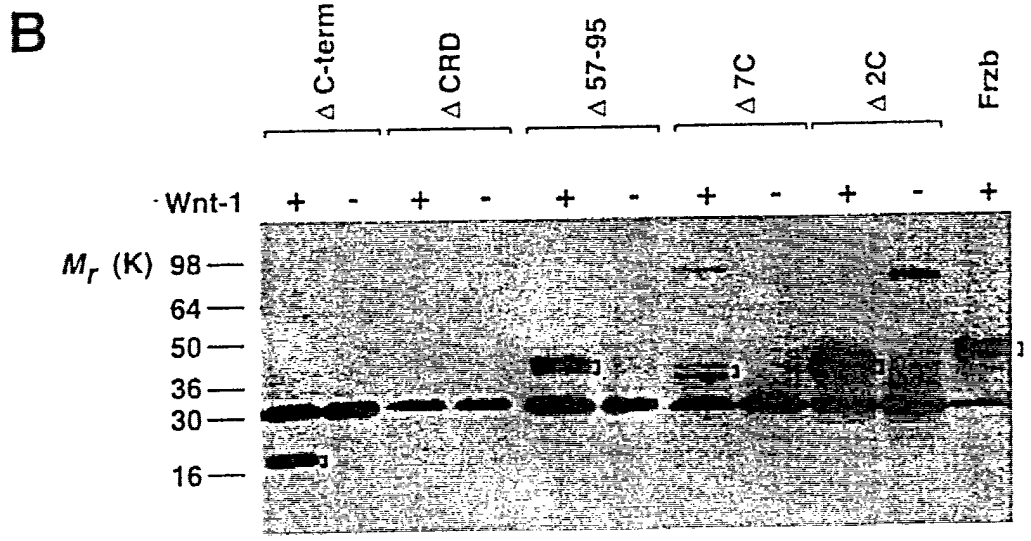
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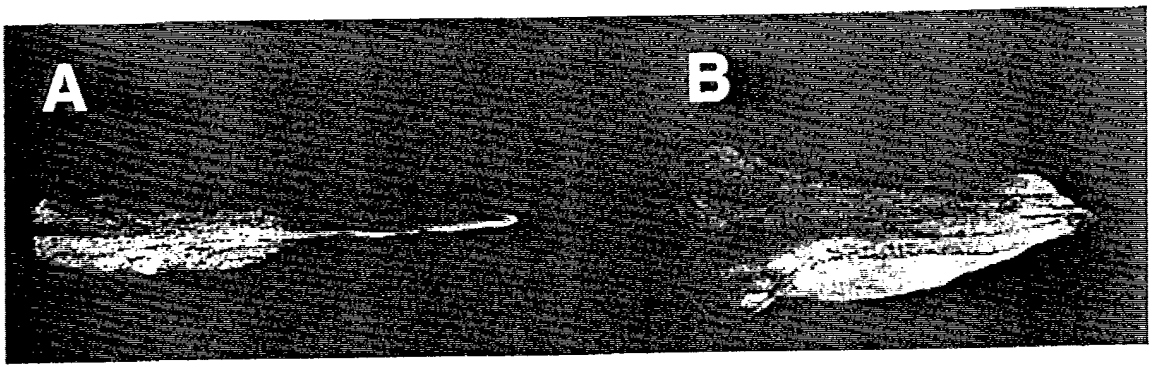
Figure 3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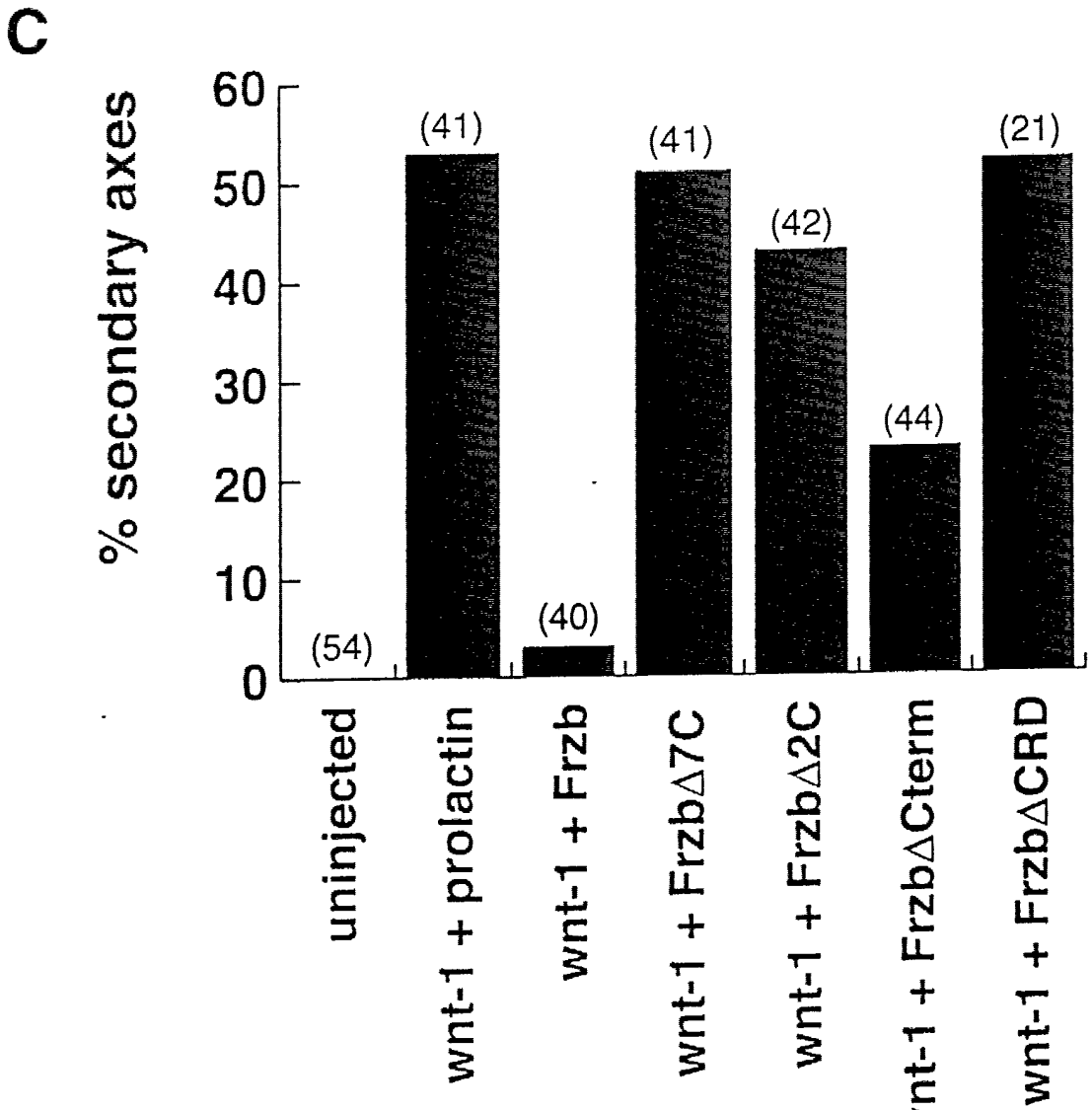
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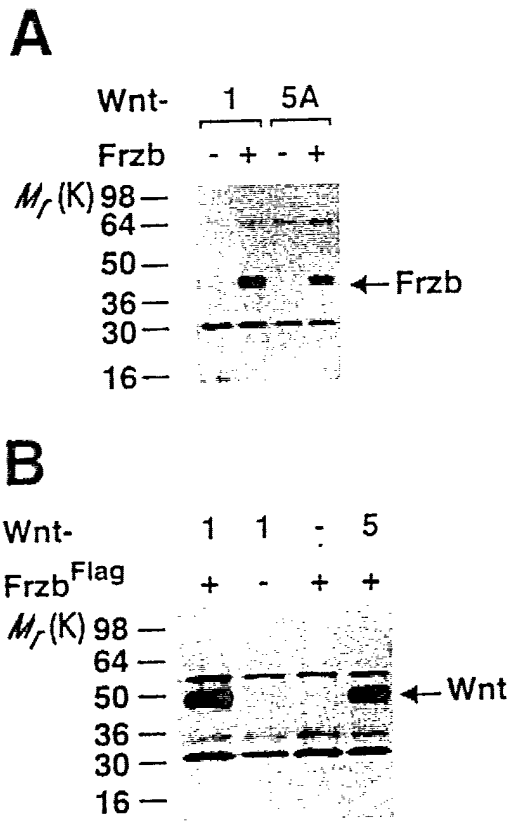
Figure 4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Notch and Wnt proteins function to regulate branching morphogenesis of mammary epithelial cells in opposing fashion.

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Key words: Notch4, int-3, Wnt-1, TAC-2, mammary oncogene, branching morphogenesis

Abstract

Elongation and branching of epithelial ducts is a crucial event during the development of the mammary gland. An in vitro assay, utilizing the mouse mammary epithelial TAC-2 cell line, was used to examine the role of the Wnt-1, HGF, TGFß2 and the Notch4 receptor on the branching morphogenesis. Wnt-1, HGF and TGFß2 induce the elongation and branching of epithelial tubules, and Wnt-1 strongly cooperates with either HGF or TGFß2 in this activity. The int-3 mammary oncoprotein is an activated form of the Notch4 receptor and can completely block HGF and TGFß2 induced branching morphogenesis. The minimal domain within the int-3 protein required to block morphogenesis consists of the CBF-1 interaction domain and the cdc10 repeat domain. Co-expression of Wnt-1 and int-3 demonstrates that Wnt-1 can overcome the int-3 mediated block of ductal morphogenesis. This data are consistent with the opposing roles of the wingless and Notch signaling pathways reported in Drosophila and suggest that Wnt and Notch signaling may play opposite roles in mammary gland development.

Introduction

The development of the murine mammary gland involves an intricate sequence of proliferative, morphogenic and differentiative events, which gradually results in the formation of an arborized tree-like structure of epithelial ducts. In adult animals, under the influence of gonadal hormones, additional developmental stages in the mammary gland occur during puberty, estrous, pregnancy and lactation. At birth, the mammary epithelial ducts have few side-branches. However during puberty, the epithelial ducts rapidly elongate and branch, and give rise to a highly organized epithelial structure with terminal end buds and lateral buds. The terminal end buds are the major sites of proliferation, where as the lateral buds will differentiate into alveoli during each estrous cycle. During pregnancy, the alveoli rapidly increase in size and number resulting in the development of fully differentiated lobules, which under the influence of lactogenic hormones will produce milk at lactation. When lactation ceases, the mammary gland remodels, which results in the involution of the secretory lobules and regression to the ductal tree observed at puberty (for review see[1-3]

Mesenchymal-epithelial and epithelial-epithelial interactions have been demonstrated to be essential to regulate the growth and development of the murine mammary gland, during embryogenesis as well as through adult life. Peptide growth factors have been implicated as regulators of mammary gland development based on their expression patterns and their ability to affect the development of the mammary gland. Hepatocyte growth factor (HGF, or scatter factor) is expressed in the mammary mesenchyme during ductal branching, whereas its tyrosine kinase receptor c-met is expressed in the mammary epithelial ducts at all stages. It has been demonstrated that HGF promotes branching morphogenesis of the mammary ductal tree[4-6]. Other mediators of receptor tyrosine kinase signaling, such as Epidermal growth factor (EGF)[7], Fibroblast growth factors (FGF's)[8], and Insulin-like growth factor II (IGF-II)[9] have been implicated as regulators of mammary gland development. Neuregulin (NRG), a member of the EGF gene superfamily, is expressed in the mesenchyme during lobulo-alveolar development, where its receptors (c-erbB3 and c-erbB4) are expressed in mammary epithelial ducts at all stages. NRG can stimulate lobulo-alveolar budding and the production of milk proteins[4]. Transforming growth factor-ß's (TGFß1, TGFß2, TGFß3) and other members of this gene superfamily can affect and are expressed in many mesenchymal-epithelial interactions. TGFß1 is expressed in the epithelial compartment of the mammary gland at all stages, except during lactation[10, 11]. In vivo, TGFß1 has been shown to inhibit ductal out-growth from the mammary end buds[12, 13]. In vitro however, TGFß1 has been shown to induce opposite effects depending on its concentration. Thus, TGFß1 at high concentrations (0.5-5 ng/ml) inhibit ductal

elongation and branching of TAC-2 mammary epithelial cells, whereas at low concentrations (5-100 pg/ml) it is able to stimulate these biological processes[14].

The Wnt family of secreted growth factors have also been proposed to function as regulators of the developing mouse mammary gland[15]. Wnt genes are expressed during ductal development of the gland (Wnt-2, Wnt-5a, Wnt-7 and Wnt-10b) and during lobular development at pregnancy (Wnt-4, Wnt-5b and Wnt-6), and the expression of most Wnt transcripts is down regulated during lactation[16, 17]. Wnt gene expression is maximal during periods of morphogenesis and is lost during terminal differentiation, such as lactation, suggesting that Wnt genes may control mammary gland morphogenesis. Wnt genes are expressed in both the stromal and epithelial compartments of the mammary gland, raising the possibility that Wnt genes could be involved in both stromal-epithelial and epithelial-epithelial interactions[17, 18]. Wnt-1 is not normally expressed within the mouse mammary gland, however it has been demonstrated to contribute to tumorigenesis when activated by insertion of mouse mammary tumor virus (MMTV) proviral DNA in MMTV-induced mammary tumors[19]. Furthermore, mammary gland tumors arise in transgenic mice where ectopic Wnt-1 gene expression is controlled by the inducible, glucocorticoid-sensitive MMTV promoter. Such mice frequently develop hyperplasia of the mammary gland with increased branching of the ductal tree, prior to the development of frank tumors, again suggesting that Wnt proteins function as peptide growth factors[20]. The Notch4/int-3 gene was also identified as a frequent target for insertional activation by MMTV proviral DNA in MMTV-induced mammary gland tumors[21, 22]. The Notch4 gene encodes for a large transmembrane receptor protein. The int-3 oncoprotein corresponds to a truncated form of Notch4 which has most of its extracellular domain deleted[23]. In contrast to the Wnt-1 transgenic mammary gland, expression of the int-3 oncogene as a transgene in the mouse mammary gland results in impaired development of the mammary gland which no longer generates a tree-like structure of epithelial ducts. Instead, a hyperproliferative mass of undifferentiated epithelial cells is observed near the nipple, from which undifferentiated mammary carcinomas rapidly develop[24]. The Notch4 protein is normally expressed in all tissues of the mammary gland, including stromal cells, adipocytes, endothelial cells and epithelial cells[25]. Both the Wnt-1 and int-3 oncoproteins have been demonstrated to transform mammary epithelial cells[21].

Using a previously described model in which TAC-2 mammary epithelial cells grown in collagen gels form branching cords or tubules in response to HGF or TGF-β_1[14, 26], we demonstrate that Wnt-1 and int-3 have opposite effects on branching morphogenesis. Wnt-1 acts to induce branching morphogenesis whereas int-3 blocks branching morphogenesis by either HGF or TGF-β. In addition, Wnt-1 overcomes the int-3 block to morphogenesis. We also define the domains of the int-3 oncoprotein that are required for its biological activity.

Results

When suspended in collagen gels, TAC-2 mammary epithelial cells form small slowly growing colonies with a morphology ranging from irregular shaped cell aggregates to poorly branched structures. However, when TAC-2 cells are grown under these conditions in the presence of either HGF or TGFß1, TAC-2 colonies develop an extensive network of branching cords consisting of elongated epithelial cords or tubules with multiple branch points[14, 26]. Since the above described TAC-2 phenotype is reminiscent of key developmental process of the mammary gland, the TAC-2 cell culture assay is a valuable experimental model to study the roles of growth factors in the development of mammary epithelial cells. To investigate the role of *int-3* and *Wnt-1* oncogenes in mammary epithelial cell development and morphogenesis, we have generated TAC-2 cell lines programmed to express the Wnt-1 and int-3 oncoproteins and analyzed their effects in the TAC-2 branching morphogenesis assay.

Wnt-1 enhances TAC-2 cell branching morphogenesis

TAC-2 cell lines programmed to express the Wnt-1 oncoprotein were generated using the retroviral vector pLNC which drives Wnt-1 expression from the CMV promoter. In order to detect the Wnt-1 oncoprotein, the Wnt-1 cDNA was fused at the carboxy terminus to the haemagglutinin-epitope (HA), allowing us to detect Wnt-1 proteins in immunoblot analysis using the anti-HA monoclonal antibody (Fig. 1). As expected, the Wnt-1 oncoprotein migrates with a molecular weight between 41 and 45kd which is due to differential glycosylation. Moderate levels of Wnt-1 expression are detected in the TAC-2/Wnt-1 cell line generated. This expression level can be significantly increased by treating the TAC-2/Wnt-1 cell line with Sodium Butyrate (2mM) which enhances transcription of the CMV promoter (Fig.1). Addition of Sodium Butyrate to the TAC-2 branching morphogenesis assay did not alter or enhance all the observed phenotypes described below, and therefore Sodium Butyrate was omitted from all TAC-2 cell biological assays. As a control, TAC-2 cells were generated that were programmed to express LacZ.

TAC-2 cells programmed to express LacZ give rise to small colonies with poorly branched cords when grown in collagen gels for four days (Fig. 2A). Addition of either HGF (20ng/ml) or TGFß2 (50pg/ml) to the culture induces pronounced changes in colony morphology, resulting in the formation of long branching cords or tubules (Fig. 2B and 2C). This experiment demonstrates that TGFß2 has an identical activity as TGFß1 in the induction of branching morphogenesis of TAC-2 cells. When TAC-2 cells are programmed to express the Wnt-1 oncoprotein, cell colonies form cords with moderate branching even in the absence of

exogenous growth factors (compare Fig. 2A and 2D). When TAC-2/Wnt-1 cells are grown in the presence of either HGF or TGFß2 (Fig. 2E and 2F), an extensive branching network is observed. This network of epithelial tubules is more extensive when compared to control TAC-2/LacZ cells grown under identical conditions. It appears that Wnt-1 cooperates with either HGF or TGFß2 in the induction of branching morphogenesis by TAC-2 cells, since the extent of the branching network formed by TAC-2/Wnt-1 cells grown in the presence of either HGF or TGFß2 is far greater than the combined branching networks of TAC-2/Wnt-1 cells grown under control conditions and TAC-2/LacZ cells grown in the presence of either HGF or TGFß2. An identical phenotype was observed in TAC-2 cell lines programmed to express either a non-epitope tagged Wnt-1 cDNA or a HA epitope tagged Wnt-1 cDNA transcribed from a SV40 based retroviral vector (data not shown).

Notch4/int-3 blocks TAC-2 cell branching morphogenesis

TAC-2 cell lines programmed to express the Notch4/int-3 oncoprotein were generated using the retroviral vector pLNC. A similar strategy of HA-epitope tagging at the carboxy terminus was used to detect the int-3 oncoprotein in immunoblot analysis using the anti-HA monoclonal antibody (Fig. 1). The int-3 oncoprotein migrates with an approximately molecular weight of 60 kd which corresponds to its predicted molecular weight. Low levels of int-3 expression are detected in the TAC-2/int-3 cell line which can be significantly increased by treatment with Sodium Butyrate (Fig.1). When TAC-2 cells programmed to express int-3 are grown in collagen gels and incubated in the presence of either HGF or TGFß2 (Fig 2H and 2I), cell colonies no longer form elongated cords. Instead, HGF- or TGFß2-treated TAC-2/int-3 cell colonies form small aggregates or structures with rudimentary branches which are similar in appearance to those formed by either TAC-2/LacZ or TAC-2/int-3 colonies grown in the absence of HGF or TGFß2 (Fig 2A and 2G). An identical phenotype was observed in either TAC-2 cell lines programmed to express a non-epitope tagged int-3 cDNA or a HA epitope tagged int-3 cDNA transcribed from a SV40 based retroviral vector (data not shown).

Wnt-1 and Notch4/int-3 do not alter the growth properties of TAC-2 cells.

To determine whether the differential effects of the Wnt-1 and int-3 oncoproteins on TAC-2 cell branching morphogenesis are due to their differential effects on the growth characteristics of the TAC-2 cells, we compared the growth characteristics of the different TAC-2 cell lines generated. TAC-2 cells were plated at different densities on collagen coated dishes, either in the presence or absence of HGF. Media and growth factors were replaced every other

day and viable cell numbers were determined either two or six days after plating. Two days after plating, when TAC-2 cells plated at lower densities are actively growing, no significant differences in cell number were found between the different TAC-2 cell lines either in the presence or absence of HGF (Fig. 3A). Six days after plating, all TAC-2 cell lines, initially plated at different densities, reached confluence and equal cell numbers were detected in all cell lines, either in the presence or absence of HGF (Fig. 3B). Thus, six days after plating the TAC-2 cell lines become quiescent due to contact/density inhibition and none of the TAC-2 cell lines were able to grow post confluence. These experiments demonstrate that TAC-2 cells programmed to express either Wnt-1 or int-3, in the presence or absence of HGF, do not display different growth characteristics. Hence, the differential effects of Wnt-1 and int-3 on TAC-2 branching morphogenesis are not due to their mitogenic or anti-mitogenic action. Identical results were obtained with TAC-2 cell lines that were programmed to express LacZ, Wnt-1 or int-3 using a SV40 based retroviral vector (data not shown).

The carboxy terminus of the Notch4/int-3 oncogene is not required for activity

The *int-3* oncogene corresponds to a truncated form of the *Notch4* gene which encodes for a large transmembrane receptor protein. The int-3 oncoprotein has most of the extracellular domain deleted and consists of the transmembrane and intracellular domain of Notch4. To investigate which region(s) of the int-3 oncoprotein is required and sufficient for int-3 activity, a series of int-3 deletion mutants were generated (Fig. 4A). The four int-3 deletion mutants were named ΔNT (deletion of the amino terminal domain), ΔCDC (deletion of cdc10 repeat domain), ΔCT (deletion of the carboxy terminal domain) and ΔNTΔCT (N-terminal and C-terminal deletion) (Fig. 4A). All four int-3 cDNA's were HA-epitope tagged at their carboxy termini and TAC-2 cell lines programmed to express each deletion mutant were generated using the retroviral vector pLNC. Immunoblot analysis using anti-HA monoclonal antibodies demonstrated expression of int-3 deletion proteins of appropriate molecular weight in each respective cell line; this expression can be increased by Sodium Butyrate treatment (Fig. 4B). The ΔNTΔCT int-3 deletion protein with predicted molecular weight of 251 kd co-migrates with a non specific anti-HA antibody background band, but is clearly visible over background when these TAC-2 cells are treated with sodium Butyrate (Fig. 4B). TAC-2 cell lines expressing the four different int-3 deletion mutants were grown in collagen gels as described above, and the ability of each int-3 deletion mutant to block HGF induced branching morphogenesis of TAC-2 cells was analyzed. As shown in Fig. 5, TAC-2 cells expressing either ΔNT (Fig. 5A and 5B), ΔCDC (Fig. 5C and 5D) or ΔNTΔCT (Fig. 5G and 5H) are responsive to HGF induced branching morphogenesis. In contrast, when grown in the presence of ΔCT expressing TAC-2 cells (Fig.

5E and 5F) display an identical phenotype as the TAC-2/int-3 cells. Thus, these experiments demonstrate that the carboxy terminus of the int-3 oncoprotein is not required for int-3 mediated inhibition of TAC-2 branching morphogenesis, and the biological activity of the int-3 oncoprotein resides in its amino terminus and cdc10 repeats.

Epistatic interaction between the Wnt-1 and Int-3 oncoproteins

Since the Wnt-1 and int-3 mammary oncoproteins have opposite activities on HGF or TGFß2 induced branching morphogenesis of TAC-2 cells, we investigated the effect of simultaneous expression of both Wnt-1 and int-3 proteins on TAC-2 branching morphogenesis. The above described TAC-2/LacZ and TAC-2/int-3 cell lines which were generated with the pLNC expression vector were now also programmed to express Wnt-1 using the retroviral vector pLHTCX. This vector contains the hygromycin resistance gene and drives gene expression from the CMV promoter as well. In this way, four additional TAC-2 cell lines were generated that were named TAC-2/LacZ/X, TAC-2/LacZ/Wnt-1, TAC-2/int-3/X and TAC-2/int-3/Wnt-1 (where X denotes empty pLHTCX vector). The Wnt-1 cDNA that was used in these experiments was not tagged with the HA epitope. To determine appropriate protein expression in each of these four cell lines, immunoblot analysis was done using either the anti-HA antibodies to detect HA tagged int-3 proteins, or anti-Wnt-1 monoclonal antibodies to detect the Wnt-1 proteins (Fig. 6A, B). Again, treatment of the TAC-2 cells with Sodium Butyrate significantly increases the level of protein expression. The TAC-2/int-3HA/Wnt-1 cell line expresses both Wnt-1 and int-3HA oncoproteins. All four cell lines were grown in collagen gels and the ability of each TAC-2 cell line to undergo HGF or TGFß2 induced branching morphogenesis was analyzed (Fig. 7). As observed previously, TAC-2/LacZ cells that are now programmed to express Wnt-1 (Fig. 7D, E, F) form small colonies that undergo modest branching even in the absence of HGF; in addition, these cells form extensive elongated branches when grown in the presence of HGF (compare to TAC-2/LacZ/X in Fig. 7A, B, C). The previously observed int-3 activity, that is the block of HGF- and TGFß2-induced branching morphogenesis, remains identical in the TAC-2/int-3HA/X cell line (Fig. 7G, H, I), demonstrating that hygromycin selection did not affect the phenotype of the TAC-2 cell lines. The TAC-2/int-3/Wnt-1 cell line is able to form colonies displaying extensive branching and elongation when grown in the presence of HGF and have an appearance indistinguishable to that of TAC-2/LacZ/Wnt-1 cells (Fig. 7J, K, L). This experiment demonstrates that Wnt-1 activity can override int-3 activity in TAC-2 cells, therefore Wnt-1 activity must function downstream of int-3 activity.

Discussion

In this study, we have used an in vitro model to evaluate the role of the Wnt-1 and int-3 proteins in mammary gland ductal morphogenesis. Formation of branching cords is induced in collagen gel cultures of TAC-2 cells by the addition of either HGF or low concentrations of TGFß. We demonstrate that expression of Wnt-1 and int-3 in TAC-2 cells has opposing effects on branching morphogenesis: Wnt-1 synergize with HGF or TGF-β in inducing formation of branching cords whereas int-3 inhibits the effect of either HGF or TGF-β. The opposite biological activities of Wnt-1 and int-3 observed in the TAC-2 cell assay correlates well with the mammary gland phenotype observed in Wnt-1 and int-3 transgenic mice that ectopically express these proteins in the mammary gland[24]. Although both oncogenes induce tumor development, Wnt-1 stimulates a hyperplastic phenotype whereas int-3 blocks ductal development. Moreover, Wnt-1 can override the int-3-mediated block to branching morphogenesis. This dominance of Wnt-1 over the activated Notch affect is analogous the epistasis reported for Drosophila Wnt (wingless) and Notch functions during development[27]. Hence, the TAC-2 assay may be a valuable experimental system, and one that is more tractable to experimental manipulation, to study the role of multiple distinct signaling pathways in the development of the murine mammary gland.

Wnt-1 stimulates branching morphogenesis of TAC-2 cells

Wnt-1 proteins induces moderate branching and elongation of TAC-2 cell tubules in the absence of added HGF or TGFß2. The extent of Wnt-1 induced branching morphogenesis of TAC-2 cells is comparable to the induction by either HGF or TGFß2. The Wnt gene family encodes factors involved in cell growth, organogenesis, oncogenesis, and cell fate determination during embryogenesis. Wnt proteins have also been proposed to function as peptide growth factors within the developing mouse mammary gland, as several Wnt genes are expressed exclusively when the gland is in growth phase, and the Wnt-1 protein can stimulate the growth and morphological transformation of certain mammary epithelial cell lines. The Wnt signal transduction pathway is mediated in part through ß-catenin, a cytoplasmic protein that can be associated with cadherins, and which is necessary for the adhesive functions of adherens junctions[28]. Wnt-1 signaling results in stabilization of the cytoplasmic pool of ß-catenin, which then can associate with downstream targets in the cytoplasm to transduce signals that lead to regulation of target gene expression. Recent evidence has demonstrated the importance of ß-catenin/cadherin interactions in regulating cell adhesion, cell migration and epithelial phenotype in embryonic development. The activation of ß-catenin by Wnt-1 induced signaling may result

in changes of the adhesive and migratory characteristics of mammary epithelial cells and consequently affect ductal morphogenesis of TAC-2 cells. It is also possible that Wnt-1 signaling leads to a block of an endogenous Notch pathway in TAC-2 cells, for which we do not have any evidence, and that the Wnt-1 activity may only consist of suppressing a yet unidentified endogenous inhibitory Notch pathway.

Int-3 blocks HGF and TGFβ2 induced branching morphogenesis of TAC-2 cells

We demonstrate that the int-3 oncoprotein blocks both the HGF and TGFß2 induced branching morphogenesis of TAC-2 cells. The exact mechanism of this inhibition is unclear. However, activated Notch genes have been demonstrated to inhibit the cell fate commitment or differentiation of many different cell types. For instance, Drosophila activated Notch inhibits neuroblast and photoreceptor cell differentiation. Xenopus activated Notch inhibits the early expression of epidermal and neural crest markers, and activated mouse Notch1 inhibits myogenesis and neurogenesis of cultured mouse cells. Transgenic mice expressing the int-3 oncoprotein from the MMTV viral promoter, display severely impaired mammary ductal growth[24]. When int-3 is expressed from the whey acidic protein promoter, whose expression is restricted to the secretory mammary epithelial cells, the differentiation of the secretory lobules of the transgenic animals is profoundly inhibited. These experiments demonstrate that int-3, like many other activated Notch genes, is a potent inhibitor of differentiative processes. Since several Notch genes are expressed in vivo in the mammary epithelial cells, including Notch4, Notch genes may inhibit or regulate the differentiative processes occurring during the mammary gland developmental cycle. Recently, the intracellular domain of LIN-12, a C. elegans Notch, has been demonstrated to associate with EMB-5, which encodes for a cytoplasmic protein containing a SH-2 domain[29]. This finding raises the possibility that the Notch signaling proteins may interact directly with those elicited by tyrosine kinase receptors, such as the HGF receptor (c-met).

Cooperation between Wnt-1 and HGF or TGFβ2

In response to both Wnt-1 and HGF, TAC-2 cells form a network of elongated and branching tubules that is far more extensive than the branching cords observed when TAC-2 cells are grown in the presence of Wnt-1 or HGF singularly. Therefore, we believe that the combined effect of HGF addition and Wnt-1 expression is not the result of the sum of their independent activities on branching morphogenesis, but that the Wnt signaling may cooperate with the HGF/c-met tyrosine kinase pathways. This cooperation between Wnt-1 and HGF may be

explained by their combined activation of ß-catenin. TAC-2 cells programmed to express Wnt-1 have indeed increased levels of cytosolic ß-catenin, when compared to TAC-2/LacZ cells (our unpublished data). ß-catenin has been demonstrated to be a substrate for tyrosine kinases and to become tyrosine phosphorylated in cells expressing activated Src and Ras[30]. Another catenin, p120, which was identified as a substrate of Src and several receptor tyrosine kinases interact with the cadherin-ß-catenin complex and may participate in regulating the adhesive function of cadherins. ß-catenin has been detected in a complex with the EGF receptor and can be phosphorylated in response to EGF and HGF[31, 32]. The Ras pathway is essential for the biological activity induced by HGF/c-met[33]. Interestingly, EGF is also able to stimulate branching morphogenesis of TAC-2 cells, although not to the same extent as HGF, whereas NGF, bFGF, IGF-II and KGF can not, which may be due to the specific activation of ß-catenin by the EGF and HGF signal transduction pathway[32]. It is yet unclear how phosphorylation of ß-catenin might regulate its activity. However ß-catenin, tyrosine phosphorylated in response to EGF or Ras transformation, accumulates in the detergent soluble pool of ß-catenin, which is believed to be the actively signaling ß-catenin. Since both Wnt-1 and HGF signaling can converge on ß-catenin, it is therefore possible that the observed cooperation between HGF and Wnt-1 is due to their combined activation on ß-catenin.

These findings thus raise the possibility that Wnt-1, HGF, and TGFß2 induce branching morphogenesis by regulating the adhesive and migratory properties of TAC-2 cells through modulation of extracellular matrix components and their interaction with their receptors. Since HGF has been demonstrated to decrease adhesion of TAC-2 cells to collagen and to enhance the deposition of type IV collagen it is also possible that the observed cooperation between HGF and Wnt-1 is due to their combined effect on cell-substrate adhesion. The molecular mechanisms of observed cooperation between Wnt-1 and TGFß2 in the induction of branching morphogenesis in TAC-2 cells is not known. TGFß signaling involves receptors with serine/threonine kinase activity. TGFß's are known to regulate the synthesis and degradation of extracellular matrix molecules and to induce matrix organization. Induction of branching morphogenesis by TGFß2 could be mediated by a remodeling of extracellular matrix components and cell-substrate interactions. The Wnt signal transduction pathway may also regulate cell-substrate interactions, and the combined activity of both Wnt-1 and TGFß2 may explain their cooperative activities on the branching morphogenesis of TAC-2 cells.

Finally, the possibility that Wnt proteins also cooperate in vivo with the HGF/c-met pathway in the regulation of mammary morphogenesis is supported by the finding of a coordinate temporal pattern of expression of both Wnt family members and HGF/c-met.

The opposite biological activities of Wnt-1 and int-3 are not due to changes in growth characteristics of TAC-2 cells

The opposite effects of the Wnt-1 and int-3 oncoproteins on branching morphogenesis of TAC-2 cells are unlikely due to their action as mitogens or anti-mitogens, as determined by the growth characteristics of these cells when grown on collagen coated dishes. HGF has been reported to increase TAC-2 proliferation twofold when grown on collagen coated dishes, which we did not observe. However when TAC-2 cells are grown in the presence of HGF in collagen coated gels, cells can grow to higher densities. The observed difference of HGF on TAC-2 cell proliferation may be explained that in collagen gels, HGF is able to stimulate the morphogenic differentiation of TAC-2 cells, and as a consequence can prevent density / contact inhibition. This HGF activity may thus allow TAC-2 cells to grow to a higher density as compared when cells are grown on collagen coated dishes and supports the notion that geometry of cell-substrate interactions is a crucial determinant of the cellular response to a growth factor. Similarly, TAC-2/Wnt-1 cells grow to a higher density when grown in collagen gels when compared with control cells, even though no difference in proliferation is seen when these cells are grown on collagen coated dishes. So, the Wnt-1 and int-3 oncogenes do not alter the growth characteristics of TAC-2 cells intrinsically, however their opposite effects on the morphogenic differentiation of TAC-2 cells may result indirectly in differences in cell densities when TAC-2 cells are grown in collagen gels.

Wnt-1 can overcome int-3 mediated block of BM

When TAC-2 cells are programmed to express both Wnt-1 and int-3, the cells are able to undergo branching morphogenesis which can be increased in the presence of either HGF or TGFß2. This observed phenotype is indistinguishable from TAC-2 cells expressing only Wnt-1. We believe that this observed phenotype is not due to differential expression of these two oncoproteins, since TAC-2/Wnt-1/int-3 cells produce approximately similar amounts of each protein and small amounts of int-3 expression are sufficient to block HGF or TGFß2 induced branching morphogenesis. From genetic studies in Drosophila, it has been known that dishevelled, a cytoplasmic protein that is also a positive mediator in the Wnt-1 signal transduction pathway, is activated upon Wingless stimulation and can block Notch signaling. It has also been demonstrated that dishevelled can physically associate with the intracellular domain of Notch. It is therefor likely that Wnt-1 can block the int-3 activity through dishevelled,

in TAC-2 cells expressing both oncoproteins, and may explain the observed Wnt-1 phenotype in those cells.

Minimal domains within the int-3 protein required to block morphogenesis .

We have demonstrated that the domain, carboxy terminal to the cdc10 repeats, of the int-3 oncoprotein is not required for int-3 biological activity. However, the amino terminal domain and the cdc10 repeats are required for int-3 activity. These findings are consistent with previous observed data for other Notch genes. The RAM23 domain which is localized between the transmembrane and cdc10 repeats has been demonstrated to be the binding site of CBF-1, a downstream and essential element in Notch signaling[34]. Deletion of the amino terminal domain of int-3, which contains the RAM23 domain, may obliterate the binding with CBF-1, and hence destroy int-3 activity. The cdc10 repeats have been demonstrated to interact with another downstream and positive regulator, EMB5[29]. Point mutations and deletions within the cdc10 repeats result in loss of function of Notch. Our data thus indicates that Notch4 might interact and be regulated through similar mechanisms.

During mammary gland development, the growth and differentiation of the gland is regulated by mesenchymal-epithelial and epithelial-epithelial interactions. Cells often receive different signals simultaneously and must integrate them in order to take on the correct proliferative or differentiative response. Our study has revealed the complex interactions between the signal transduction pathways of Wnt, Notch, HGF and TGFß, in regulating the branching morphogenesis of mammary epithelial cells.

Materials and Methods

Reagents

Recombinant human HGF (rhHGF) was kindly provided by Genentech, Inc. (San Francisco, CA). Recombinant TGFß2 was kindly provided by Dr. G. Gunderson (Columbia University, New York, NY). Rat tail collagen solution was obtained from Upstate Biotechnology Inc. (Lake Placid, NY). Anti-HA monoclonal antibody (12CA5) was obtained from Berkeley Antibody Co. (Richmond, CA) and horseradish peroxidase-conjugated sheep anti-mouse immunoglobulin G from Amersham (Arlington Heights, IL). Anti-Wnt1 monoclonal antibody (Mc123) as described in [35] and FITC conjugated Goat anti-mouse immunoglobulin G from Boehringer Mannheim Corp.. All restriction and DNA modifying enzymes were purchased from New England Biolab Inc. (Beverly, MA).

cDNA's

The murine int-3 cDNA corresponds to a truncated Notch4 cDNA as described previously. The int-3 nucleotide sequence corresponds to nucleotide 4551 to nucleotide 6244 of Notch4. The murine Wnt-1 cDNA is as previously described. An oligonucleotide sequence encoding the haemagglutinin (HA) antigenic determinant was appended to the 3' end of the int-3 and Wnt1 cDNA's. These eighteen codons specified the amino acid sequence SMAYPYDVPDYASLGPGP, including the nine amino acid-long HA epitope, as underlined. HA-tagged int-3 and Wnt1 cDNAs were created by subcloning each cDNA into Bluescript KS (Stratagene) that contained the coding region of the HA epitope situated downstream of the newly inserted cDNA, separated from it by polylinker sequence. These two sequences were made co-linear by the "loop-out" mutagenesis procedure using oligonucleotides designed to eliminate the stop codon and non-coding 3' sequence of the int-3 and Wnt1 cDNAs and flanking polylinker sequence. The sequences of the oligonucleotides used to loop out int-3 and Wnt specific sequences are CGG TTG TAA GAA ATC TGA ACT CCA TGG CCT ACC CAT ATG and CGC GCG TTC TGC ACG AGT GTC TAT CCA TGG CCT ACC C respectively. The 5' end of each oligonucleotide is complementary to the C-terminus of int-3 or Wnt1 cDNA and their 3' ends anneal to the beginning of the HA epitope-encoding sequence (underlined in oligonucleotide). Mutagenesis was accomplished using the Muta-Gene phagemid *in vitro* mutagenesis kit (Bio Rad, Richmond, CA). The presence of each fusion was confirmed by DNA sequencing. Four different int-3 cDNA deletion mutants were generated from the above described epitope tagged int-3 construct by restriction enzyme cloning, and were named ΔNT,

ΔCDC, ΔCT and ΔNTΔCT. The ΔNT deletion mutant corresponds to nucleotides 4921 to 6244 of the Notch4 sequence. The ΔCDC deletion mutant corresponds to nucleotides 4551 to 4864 and to nucleotides 5706 to 6244 of the Notch4 sequence. The ΔCT deletion mutant corresponds to nucleotides 4551 to 5718 of the Notch4 sequence. The ΔNTΔCT deletion mutant corresponds to nucleotides 4921 to 5718 of the Notch4 sequence.

Cells

The TAC-2 cell line was derived from NMuMG cells as described previously. TAC-2 cell were grown on collagen-coated dishes in Dulbecco's modified Eagle's medium (DMEM, GIBCO) supplemented with 10% fetal calf serum (FCS, GIBCO). The Bosc 23 retrovirus packaging cell line (Pear, et al., 1993) was obtained from Dr. W. Pear (MIT, MA) and grown in DMEM containing 10% FCS. Both culture media were supplemented with penicillin (500 i.u./ml) and streptomycin (100ug/ml). Both cell lines were grown at 37°C in 8% CO_2.

Cell line generation

HA-tagged cDNAs were inserted into the retroviral vector pLNCX [36] wherein neomycin phosphotransferase gene expression is controlled by the murine leukemia virus (MLV) long terminal repeat (LTR), and cDNA transcription is controlled by an internal cytomegalovirus (CMV) enhancer/promoter. The retroviral vector pLHTCX was derived from pLNCX, however the neomycin phosphotransferase gene is replaced by the hygromycin-resistence/thymidine kinase fusion gene. Distinct populations of TAC-2 cells, each expressing a unique HA-tagged or non tagged int-3 or Wnt1 cDNA, were prepared by retroviral infection. Recombinant retroviruses were generated by transiently transfecting pLNCX constructs into the BOSC 23 packaging cell line by calcium phosphate co-precipitation, as previously described [37]. Retroviral infection of TAC-2 cells was accomplished by culturing these cells in the presence of viral supernatants obtained from the transfected packaging cells two day post-transfection. Infections were carried out in the presence of 4 μg/ml polybrene for 12 hours after which the medium was replaced to regular medium (DMEM + 10% FCS). One day post-infection the culture medium was replaced to DMEM + 10% FCS containing 500 μg/ml Geneticin (GIBCO BRL Life Technologies, Grand Island, NY) or 200 μg/ml hygromycin B (Sigma Chemical Co.). Colonies appeared 5 days later and were pooled into medium containing 250 μg/ml Geneticin or 200 μg/ml hygromycin B. These resultant populations, each comprised of at least 50 clones, were used in cellular and biochemical assays described below.

Collagen cell culture

TAC-2 cell lines were harvested using trypsin-EDTA, centrifuged, and embedded in three-dimensional collagen gels as described. Briefly, 8 volumes of rat tail collagen solution (approximately 1.5 mg/ml) were mixed with 1 volume of 10x minimal essential medium (GIBCO) and 1 volume of sodium bicarbonate (11.76 mg/ml) in a sterile tube kept on ice to prevent premature collagen gellation. TAC-2 cells were resuspended in the cold mixture at cell densities of 2 or 4 x 10^4 cells/ml and 0.5 ml aliquots were dispensed into 16-mm wells of 24 multiwell plates (Becton Dickinson Labware). After the collagen mixture had gelled, 1ml of complete medium (DMEM + 10% FCS) with or without HGF or TGFß2 was added to each well. TAC-2 collagen gel cultures were initially done in the presence and absence of 2mM Sodium Butyrate, but since no difference in phenotypes was observed, the Sodium Butyrate was omitted in all experiments. Media were changed every 2 days, and after 3 to 5 days, cell cultures were photographed with a Nikon ELWD 0.3 phase contrast microscope on Kodak T-Max film (100 X magnification).

Immunoblot analysis

HA epitope tagged int-3, int-3 deletion mutants and Wnt1 proteins, as well as non-epitope tagged Wnt-1 proteins from lysates of TAC-2 cell populations were analyzed by immunoblotting. To maximize protein expression, TAC-2 cells were treated with 2mM sodium butyrate for 16 hours prior to lysis. Cells were washed twice with cold PBS and, subsequently, removed from the dish in 1.5 ml PBS using a rubber policeman. Cells were pelleted by centrifugation at 2,000x g at 4°C for 5 min. and lysed in 90 µl TENT buffer (20 mM Tris, pH 8.0, 2 mM EDTA, 150 mM NaCl, 1% Triton-X100) containing 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml aprotinin, 2 mg/ml leupeptin, 1 mg/ml pepstatin, at 4°C for 30 min. Lysates were clarified by centrifugation at 10,000xg at 4°C for 10 min., and protein contents were determined using the BioRad Protein determination kit. Lysates containing 40 µg protein were electrophoresed in 10% SDS-polyacrylamide gels. Proteins were transferred from gels onto nitrocellulose filters by electroblotting, and subsequently, blocked overnight at 4°C in TBST(10 mM Tris, pH 8.0, 150 mM NaCl, 0.2% Tween-20) containing 1% bovine serum albumin (fraction V). Blots were then either incubated in anti-HA monoclonal antibody (12CA5) diluted 1:100 in TBST at room temperature, or in anti-Wnt-1 monoclonal antibody (Mc123) diluted 1:10 in TBST at 4°C. After four hours, the primary antibody was removed by washing three times for 5 min. each in TBST at temperatures identical to the primary antibody incubation. Blots were exposed to a 1:16,000 dilution of horseradish peroxidase-conjugated sheep anti-mouse immunoglobulin G. Excess secondary antibody was removed in the same manner as the primary antibody. Blots were then

incubated 1-2 min. in enhanced chemiluminescence detection reagents (Amersham Inc, Arlington Heights, IL) and exposed to X-ray film (Fujifilm, Fuji Photo Film Co., LTD., Tokyo).

Cell proliferation assay

To measure cell proliferation rates of the different TAC-2 cell lines generated, cells were seeded at different densities in collagen coated 96 well plates (Corning Costar Co., Cambridge, MA) and grown in DMEM + 10% FCS either in the presence or absence of rhHGF (20ng/ml). After 2 and 6 days, the number of viable cells was measured using the CellTiter 96^{TM} AQueous Non-Radioactive Cell proliferation Assay (Promega) following the manufactures instructions. The microelisa auto reader (Dynatech) was used to measure the absorbance ratio at 490/570 nm which correlates with the number of viable cells.

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References

1. Neville, M.C. and C.W. Daniel, *The Mammary Gland: Development, Regulation, and Function.* 1987, New York: Plenum Press.

2. Russo, I.H., M. Tewari, and J. Russo, *Morphology and Development of the Rat Mammary Gland*, in *Integument and Mammary Glands*, T.C. Jones, V. Mohr, and R.D. Hunt, Editors. 1989, Springer-Verlag: Berlin. p. 233-252.

3. Pitelka, D.R., S.T. Hamamoto, J.G. Duafala, and M.K. Nemanic. (1973). Cell contacts in the mouse mammary gland. I. Normal gland in postnatal development and the secretory cycle. *Journal of Cell Biology* **56**(3): p. 797-818.

4. Yang, Y., E. Spitzer, D. Meyer, M. Sachs, C. Niemann, G. Hartmann, K.M. Weidner, C. Birchmeier, and W. Birchmeier. (1995). Sequential requirement of hepatocyte growth factor and neuregulin in the morphogenesis and differentiation of the mammary gland. *Journal of Cell Biology* **131**(1): p. 215-26.

5. Niranjan, B., L. Buluwela, J. Yant, N. Perusinghe, A. Atherton, D. Phippard, T. Dale, B. Gusterson, and T. Kamalati. (1995). HGF/SF: a potent cytokine for mammary growth, morphogenesis and development. *Development* **121**(9): p. 2897-908.

6. Pepper, M.S., J.V. Soriano, P.A. Menoud, A.P. Sappino, L. Orci, and R. Montesano. (1995). Modulation of hepatocyte growth factor and c-met in the rat mammary gland during pregnancy, lactation, and involution. *Experimental Cell Research* **219**(1): p. 204-10.

7. Haslam, S.Z., L.J. Counterman, and K.A. Nummy. (1992). EGF receptor regulation in normal mouse mammary gland. *Journal of Cellular Physiology* **152**(3): p. 553-7.

8. Coleman-Krnacik, S. and J.M. Rosen. (1994). Differential temporal and spatial gene expression of fibroblast growth factor family members during mouse mammary gland development. *Mol. Endocrinol.* 8: p. 218-229.

9. Bates, P., R. Fisher, A. Ward, L. Richardson, D.J. Hill, and C.F. Graham. (1995). Mammary cancer in transgenic mice expressing insulin-like growth factor II (IGF-II) [see comments]. *British Journal of Cancer* **72**(5): p. 1189-93.

10. Daniel, C.W., S. Robinson, and G.B. Silberstein. (1996). The Role of TGF-β in Patterning and Growth of the Mammary Ductal Tree. *J. of Mamm. Gland Biol. and Neoplasia* **1**(4): p. 331-341.

11. Smith, G.H. (1996). TGF-β and Functional Differentiation. *J. of Mamm. Gland Biol. and Neoplasia* **1**(4): p. 343-352.

12. Pierce, D.F., Jr., M.D. Johnson, Y. Matsui, S.D. Robinson, L.I. Gold, A.F. Purchio, C.W. Daniel, B.L. Hogan, and H.L. Moses. (1993). Inhibition of mammary duct development but not alveolar outgrowth during pregnancy in transgenic mice expressing active TGF-beta 1. *Genes & Development* **7**(12A): p. 2308-17.

13. Kordon, E.C., R.A. McKnight, C. Jhappan, L. Hennighausen, G. Merlino, and G.H. Smith. (1995). Ectopic TGF beta 1 expression in the secretory mammary epithelium induces early senescence of the epithelial stem cell population. *Developmental Biology* **168**(1): p. 47-61.

14. Soriano, J.V., L. Orci, and R. Montesano. (1996). TGF-beta1 induces morphogenesis of branching cords by cloned mammary epithelial cells at subpicomolar concentrations. *Biochemical & Biophysical Research Communications* **220**(3): p. 879-85.

15. Nusse, R. and H.E. Varmus. (1992). *Wnt* genes. *Cell* **69**: p. 1073-1087.

16. Gavin, B.J. and A.P. McMahon. (1992). Differential regulation of the *Wnt*-gene family during pregnancy and lactation suggests a role in postnatal development of the mammary gland. *Mol. Cell. Biol.* **12**: p. 2418-2423.

17. Weber-Hall, S.J., D.J. Phippard, C.C. Niemeyer, and T.C. Dale. (1994). Developmental and hormonal regulation of Wnt gene expression in the mouse mammary gland. *Differentiation* **57**(3): p. 205-14.

18. Buhler, T.A., T.C. Dale, C. Kieback, R.C. Humphreys, and J.M. Rosen. (1993). Localization and quantification of wnt-2 gene expression in mouse mammary development. *Dev. Biol.* **155**: p. 87-96.

19. 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: p. 619-625.

20. Edwards, P.A.W., S.E. Hiby, J. Papkoff, and J.M. Bradbury. (1992). Hyperplasia of mouse mammary epithelium induced by expression of the Wnt-1 (int-1) oncogene in reconstituted mammary gland,. *Oncogene* 7(10): p. 2041-2051.

21. Robbins, J., B.J. Blondel, D. Gallahan, and R. Callahan. (1992). Mouse mammary tumor gene *int-3*, a member of the *notch* gene family transforms mammary epithelial cells. *J. Virol.* **66**: p. 2594-2599.

22. Sarkar, N.H., S. Haga, A.F. Lehner, W. Zhao, S. Imai, and K. Moriwaki. (1994). Insertional mutation of int protooncogenes in the mammary tumors of a new strain of mice derived from the wild in China: normal- and tumor-tissue-specific expression of int-3 transcripts. *Virology* **203**(1): p. 52-62.

23. Uyttendaele, H., G. Marazzi, G. Wu, Q. Yan, D. Sassoon, and J. Kitajewski. (1996). Notch4/int-3, a mammary proto-oncogene, is an endothelial cell-specific mammalian Notch gene. *Development* **122**(7): p. 2251-9.

24. Jhappan, C., D. Gallahan, C. Stahle, E. Chu, G.H. Smith, G. Merline, and R. Callahan. (1992). Expression of an activated *Notch*-related *int-3* transgene interferes with cell differentiation and induces neoplastic transformation in mammary and salivary glands. *Genes Devel.* **6**: p. 345-355.

25. Smith, G.H., D. Gallahan, F. Diella, C. Jhappan, G. Merlino, and R. Callahan. (1995). Constitutive expression of a truncated INT-3 gene in mouse mammary epithelium impairs differentiation and functional development. *Cell Growth & Differentiation* **6**: p. 563-577.

26. Soriano, J.V., M.S. Pepper, T. Nakamura, L. Orci, and R. Montesano. (1995). Hepatocyte growth factor stimulates extensive development of branching duct-like structures by cloned mammary gland epithelial cells. *Journal of Cell Science* **108**: p. 413-430.

27. Axelrod, J.D., K. Matsuno, S. Artavanis-Tsakonas, and N. Perrimon. (1996). Interaction between Wingless and Notch signaling pathways mediated by dishevelled [see comments]. *Science* **271**(5257): p. 1826-32.

28. Miller, J.R. and R.T. Moon. (1996). Signal transduction through beta-catenin and specification of cell fate during embryogenesis. [Review] [118 refs]. *Genes & Development* **10**(20): p. 2527-39.

29. Hubbard, E.J., Q. Dong, and I. Greenwald. (1996). Evidence for physical and functional association between EMB-5 and LIN-12 in Caenorhabditis elegans. *Science* **273**(5271): p. 112-5.

30. 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/β -catenin complex in cells transformed with a temperature-sensitive v-Src gene. *J. Cell Biol* **120**: p. 757-766.

31. Hoschuetzky, H., H. Aberle, and R. Kemler. (1994). -catenin mediates the interaction of the cadherin-catenin complex with epidermal growth factor. *J Cell Biol* **127**: p. 1375-1380.

32. Shibamoto, S., M. Hayakawa, K. Takeuchi, T. Hori, N. Oku, K. Miyazawa, N. Kitamura, M. Takeichi, and F. Ito. (1994). Tyrosine phosphorylation of beta-catenin and plakoglobin enhanced by hepatocyte growth factor and epidermal growth factor in human carcinoma cells. *Cell Adhesion & Communication* **1**(4): p. 295-305.

33. Hartmann, G., K.M. Weidner, H. Schwarz, and W. Birchmeier. (1994). The motility signal of scatter factor/hepatocyte growth factor mediated through the receptor tyrosine kinase met requires intracellular action of Ras. *Journal of Biological Chemistry* **269**(35): p. 21936-9.

34. Hsieh, J.J., T. Henkel, P. Salmon, E. Robey, M.G. Peterson, and S.D. Hayward. (1996). Truncated mammalian Notch1 activates CBF1/RBPJk-repressed genes by a mechanism resembling that of Epstein-Barr virus EBNA2. *Molecular & Cellular Biology* **16**(3): p. 952-9.

35. Fung, Y.K., G.M. Shackleford, A.M. Brown, G.S. Sanders, and H.E. Varmus. (1985). Nucleotide sequence and expression in vitro of cDNA derived from mRNA of *int*-1, a provirally activated mouse mammary oncogene. *Mol. Cell. Biol.* **5**: p. 3337-3344.

36. Miller, A.D. and G.J. Rosman. (1989). Improved retroviral vectors for gene transfer and expression. *Bio. Techniques* 7: p. 980-990.

37. Pear, W.S., G.P. Nolan, M.L. Scott, and D. Baltimore. (1993). Production of high-titer helper-free retroviruses by transient transfection. *Proc. Natl. Acad. Sci. USA* **90**: p. 8392-8396.

Figure Legends

Figure 1

Immunoblot analysis on TAC-2 cell lysates using anti-HA antibodies. TAC-2 cells programmed to express either LacZ, Wnt-1HA or int-3HA were grown in the presence or absence of Sodium Butyrate. Wnt-1 and int-3 proteins of appropriate molecular weight can be detected in the respective TAC-2 cell lines, and the level of protein expression can be increased by Sodium Butyrate treatment.

Figure 2

TAC-2 cell ductal morphogenesis assay. TAC-2 cells programmed to express LacZ (A,B,C), Wnt-1HA (D,E,F), or int-3HA (G,H,I) were grown in collagen gels either in the absence of exogenous growth factor (A,D,G), in the presence of HGF (B,E,H), or in the presence of TGFß2 (C,F,I). HGF and TGFß2 induce branching morphogenesis of TAC-2 LacZ cells (compare A to B or C). Wnt-1 expression in TAC-2 cells induces modest branching morphogenesis of TAC-2 cells in the absence of either HGF or TGFß2 (compare D to A), and robust branching is observed when TAC-2 Wnt-1 cells are grown in the presence of either HGF or TGFß2 (compare E to B, and to C). TAC-2 cells programmed to express int-3 fail to undergo branching morphogenesis when grown in the presence of either HGF or TGFß2 (compare G to H or I, compare H to B, compare I to C).

Figure 3

TAC-2 cell growth assay on collagen coated plates. TAC-2 cells programmed to express either LacZ (white bars), Wnt-1HA (gray bars) or int-3HA (black bars) were seeded at different cell densities in the presence or absence of HGF (20 ng/ml). Viable cell numbers were determined two days (A) and six days (B) after plating. No significant differences in growth rate between the different TAC-2 cells, in the presence or absence of HGF, was observed.

Figure 4

Schematic representation of int-3 deletion mutants (A) and immunoblot analysis on TAC-2 cell lysates using anti-HA antibodies (B). TAC-2 cells programmed to express either ΔNT, ΔCDC, ΔCT, and ΔNTΔCT were grown in the presence or absence of Sodium Butyrate. The int-3

deletion proteins are epitope tags and deletion proteins of appropriate molecular weight can be detected in the respective TAC-2 cell lines, and the level of protein expression can be increased by Sodium Butyrate treatment.

Figure 5

TAC-2 cell ductal morphogenesis assay. TAC-2 cells programmed to express ΔNT (A-C), ΔCDC (D-F), ΔCT (G-I), and ΔNTΔCT were grown in collagen gels either in the absence of exogenous growth factor (A,D,G,J), in the presence of HGF (B,E,H,K), or in the presence of TGFß2 (C,F,I,L). HGF and TGFß2 induce branching morphogenesis of TAC-2 ΔNT cells (compare A to B or C), TAC-2 ΔCDC cells (compare D to E or F) and TAC-2 ΔNTΔCT cells (compare J to K or L) TAC-2 ΔCT cells fail to undergo branching morphogenesis when grown in the presence of either HGF or TGFß2 (compare G to H or I).

Figure 6

Immunoblot analysis on TAC-2 cell lysates using anti-HA antibodies (A) or anti-Wnt-1 antibodies (B). TAC-2 cells programmed to express either LacZ/Wnt-1, int-3HA, or int-3HA/Wnt-1 were grown in the presence or absence of Sodium Butyrate. Wnt-1 and int-3HA proteins of appropriate molecular weight can be detected in the respective TAC-2 cell lines, and the level of protein expression can be increased by Sodium Butyrate treatment.

Figure 7

TAC-2 cell ductal morphogenesis assay. TAC-2 cells programmed to express LacZ/X (A,B,C), LacZ/Wnt-1HA (D,E,F), int-3HA/X (G,H,I), or int-3HA/Wnt-1 (J,K,L) were grown in collagen gels either in the absence of exogenous growth factor (A,D,G,J), in the presence of HGF (20 ng/ml)(B,E,H,K), or in the presence of TGFß2 (50 pg/ml)(C,F,I,L). HGF and TGFß2 induce branching morphogenesis of TAC-2 LacZ/X cells (compare A to B or C). Wnt-1 expression in TAC-2 cells induces modest branching morphogenesis of TAC-2 LacZ/Wnt-1 cells in the absence of either HGF or TGFß2 (compare D to A), and robust branching is observed when TAC-2 LacZ/Wnt-1 cells are grown in the presence of either HGF or TGFß2 (compare E to B, and to C). TAC-2 cells programmed to express int-3/X fail to undergo branching morphogenesis when grown in the presence of either HGF or TGFß2 (compare G to H or I, compare H to B, compare I to C). TAC-2 cells programmed to express both int-3HA and Wnt-1 undergo

branching morphogenesis in the absence of exogenous growth factor (J), and form a robust branching network when grown in the presence of HGF or TGFß2 (K,L) in a similar manner when compared to TAC-2 cells programmed to express Wnt-1 solely (D,E,F)

Figure 1.

Uyttendaele *et.al.*

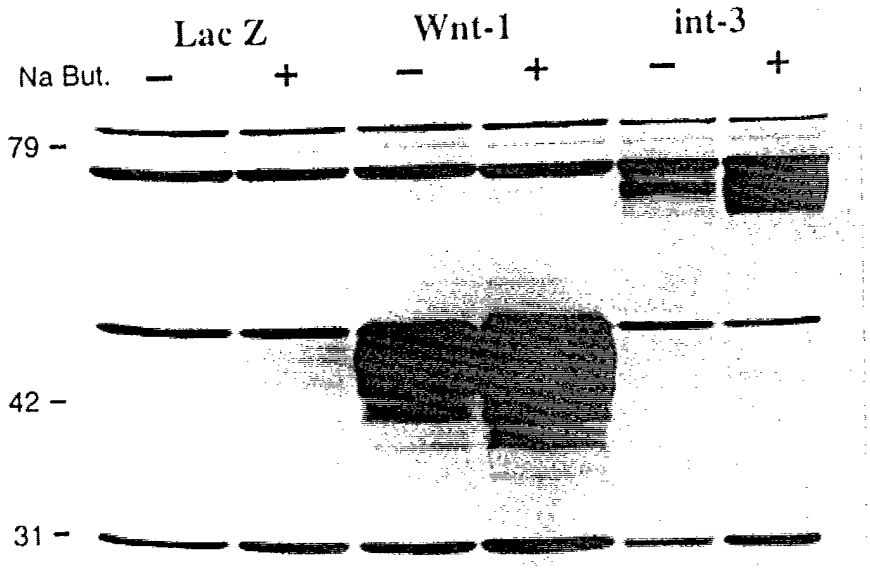


Figure 2.

Uyttendaele *et.al.*

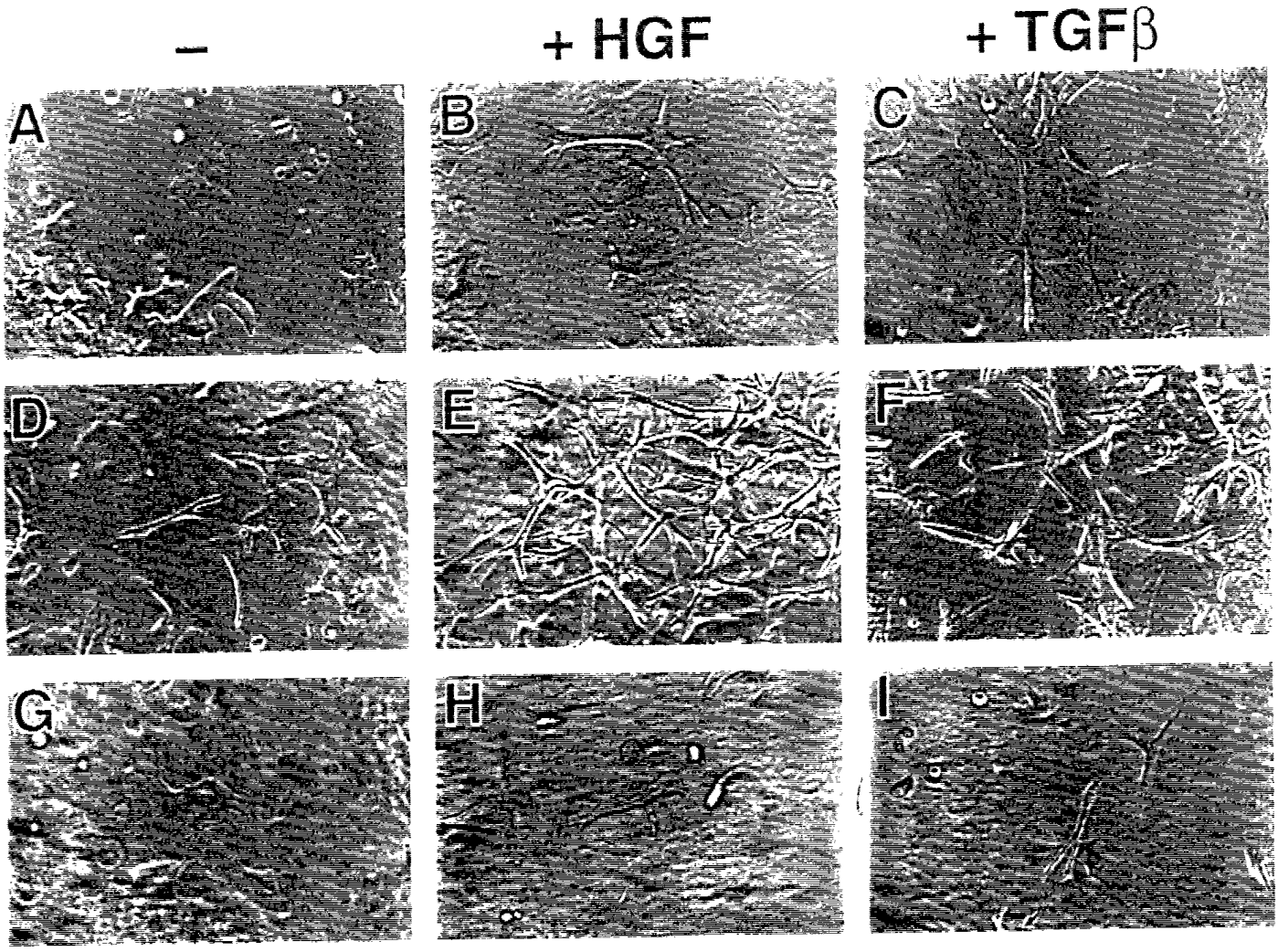
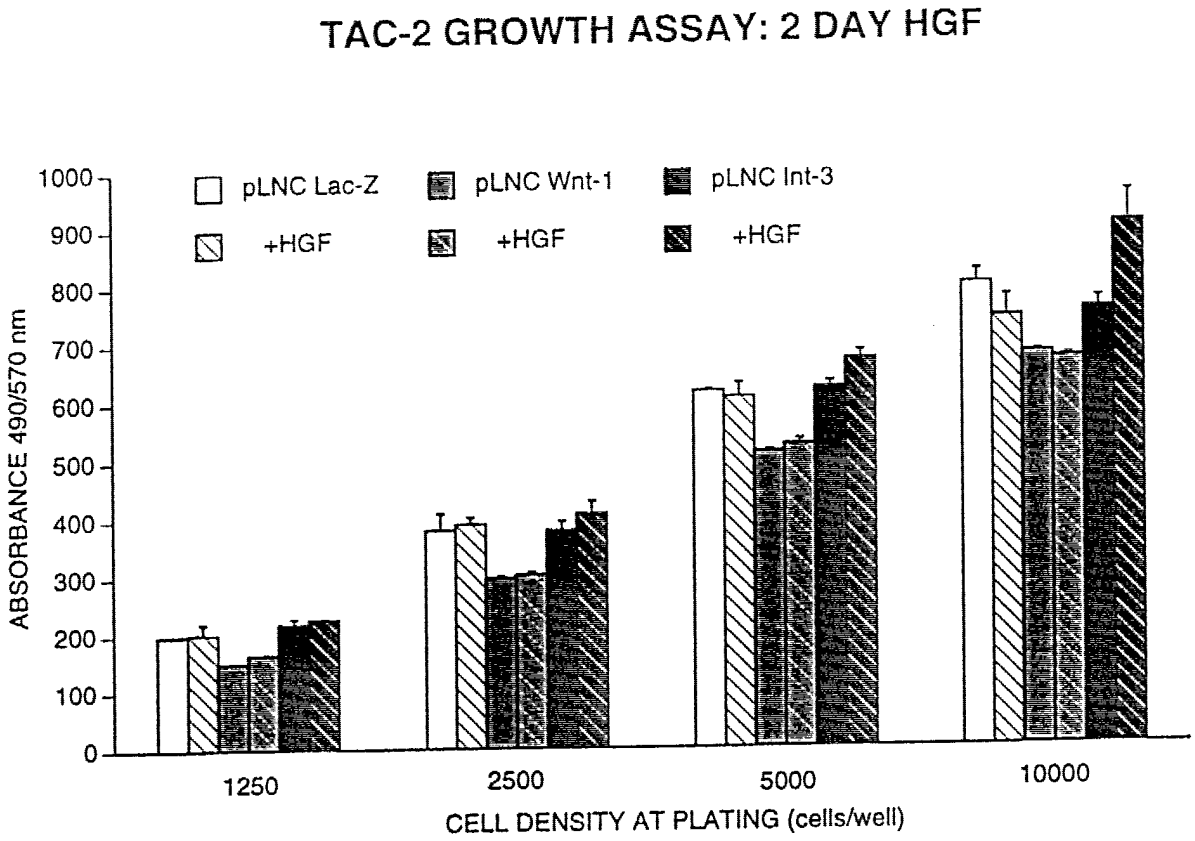


Figure 3.

Uyttendaele *et.al.*



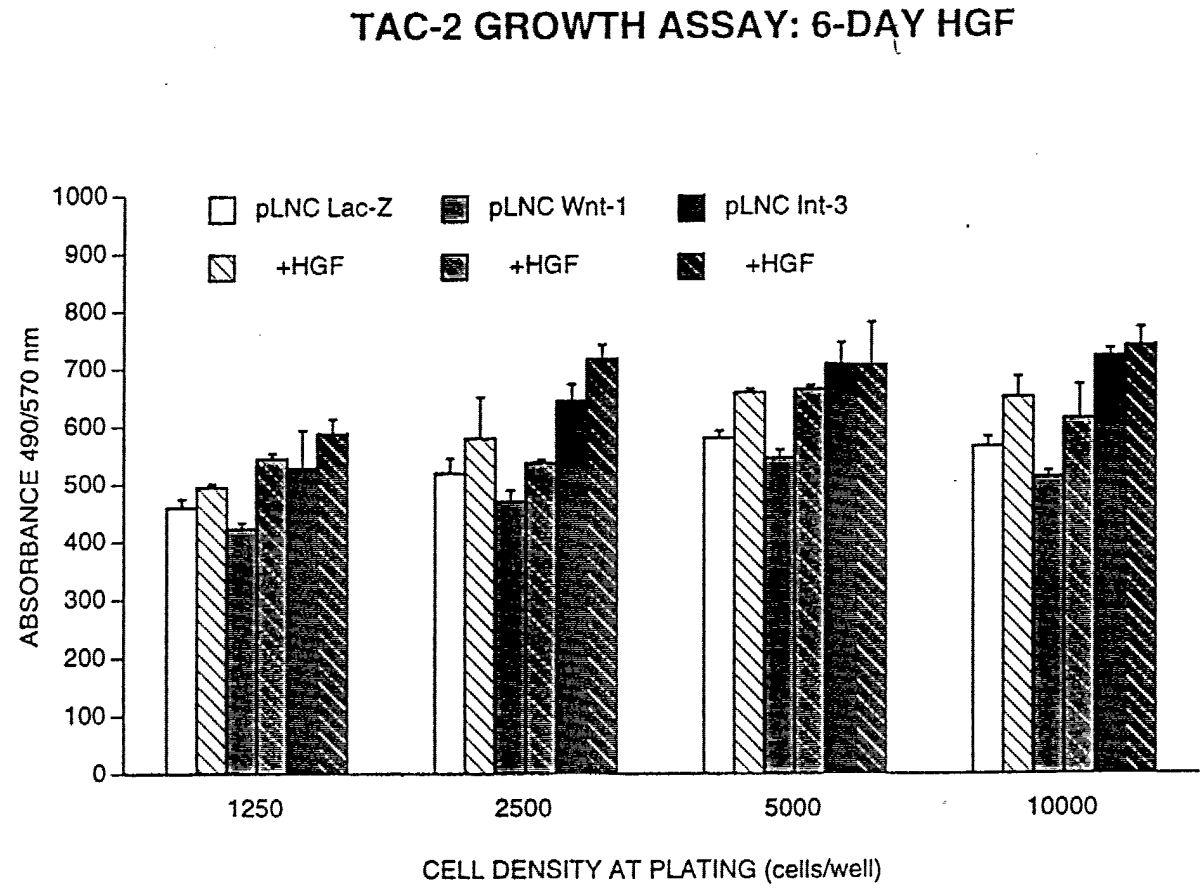


Figure 4.

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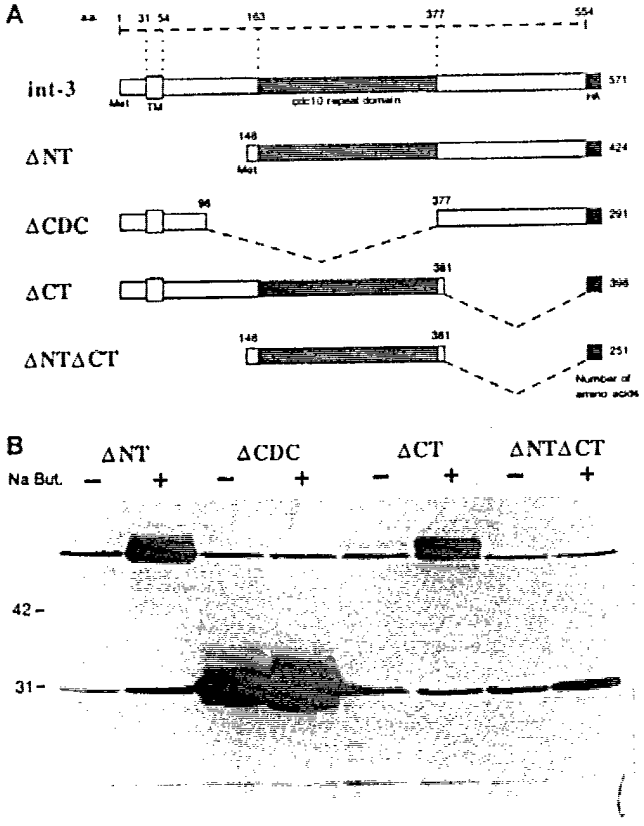

Figure 5.

Uyttendaele *et.al.*

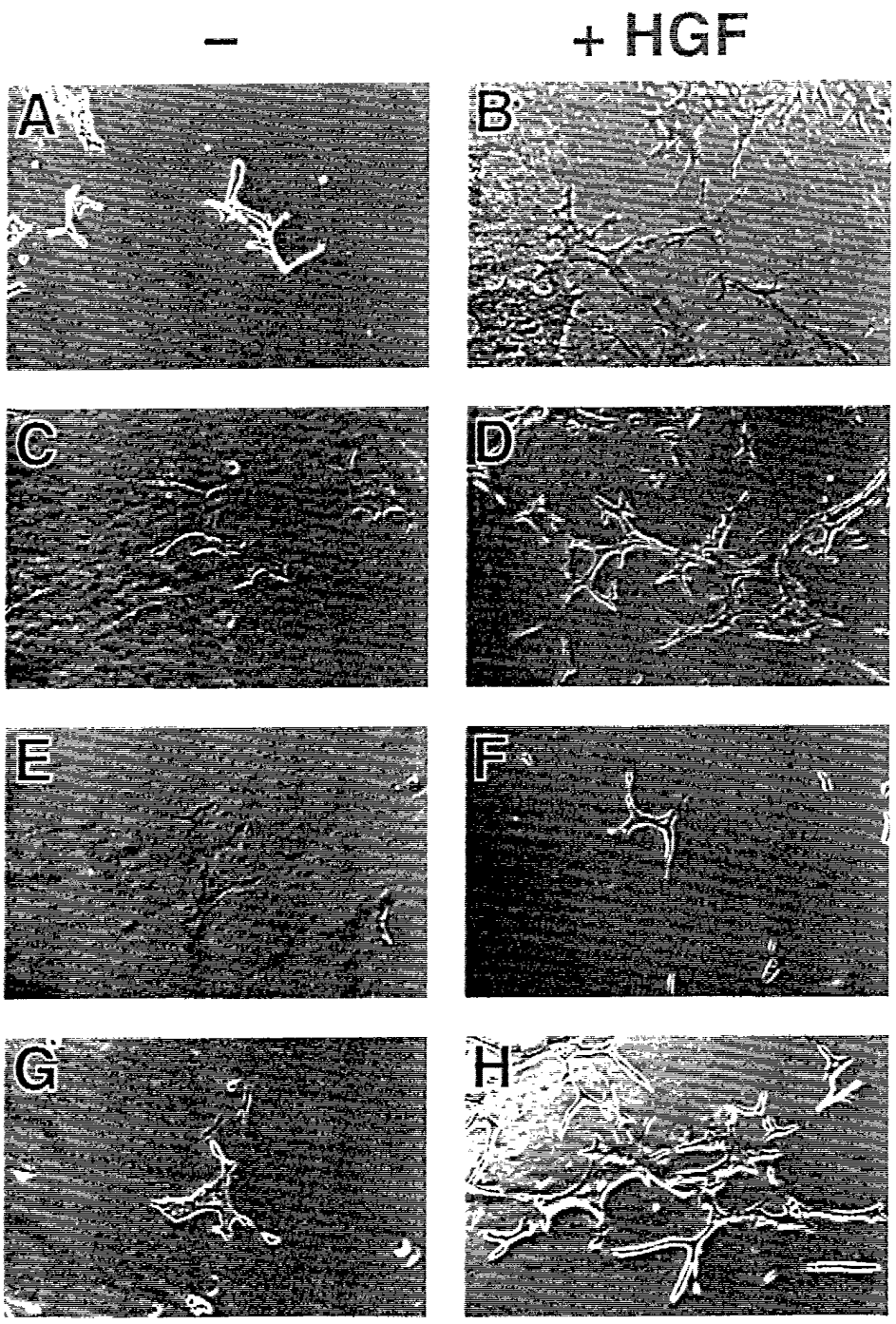

Figure 6.

Uyttendaele *et.al.*

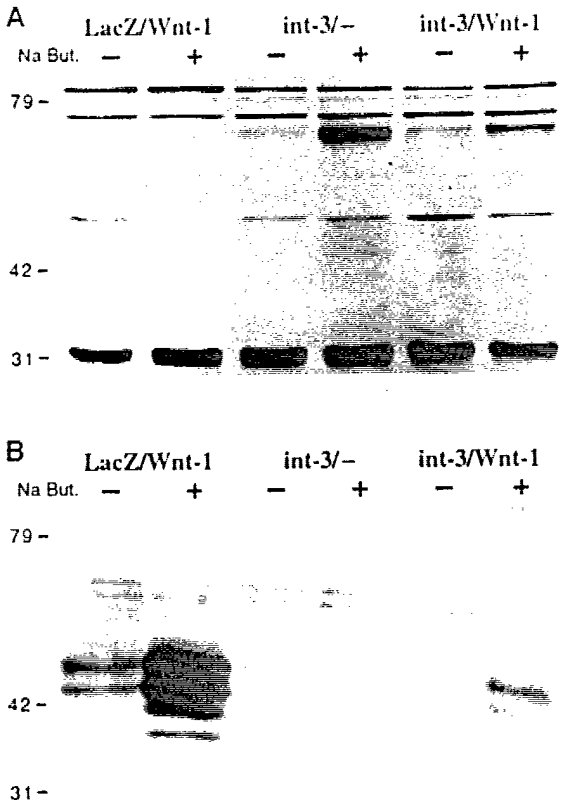

Figure 7.

Uyttendaele *et.al.*