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GRANT NUMBER DAMD17-94-J-4069

TITLE: WNT Proteins in Mammary Epithelial Cell Transformation

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REPORT DATE: July 1996

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 1996	Annual (1 Jun 95 - 31 May 96)

4. TITLE AND SUBTITLE
WNT Proteins in Mammary Epithelial Cell Transformation

5. FUNDING NUMBERS
DAMD17-94-J-4069

6. AUTHOR(S)
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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Columbia University
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, MD 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

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. The transforming potential of Wnt proteins was tested in C57MG mammary epithelial cells. Paracrine transforming capability of *Wnt* genes was tested by co-cultivating mammary epithelial cells with *Wnt*-expressing Rat fibroblasts. Direct and paracrine transforming assays indicates 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. 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. By generating chimeric Wnt proteins we have defined two regions of Wnt-1 that are critical for transformation potential. We have identified a co-factor activity produced by mammary epithelial cells that may be required for full Wnt activity. Finally, using adenovirus vectors we have documented that Wnt proteins may be involved in inducing apoptosis in some cellular systems.

14. SUBJECT TERMS	15. NUMBER OF PAGES
WNT Proteins, Growth Factor, Mammary Oncogene, Transformation, Breast Cancer	17
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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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. On the basis of their ability to affect mammary gland growth and their expression patterns, 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). The *Wnt* gene family encodes growth factors involved in cell growth and cell fate determination during embryogenesis, organogenesis, and oncogenesis. We are interested in the role of Wnt proteins in mammary gland development and oncogenesis.

***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 initially 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 has been shown to contribute 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 searching for genes homologous to *Wnt*-1 using hybridization techniques(11, 12). and the polymerase chain reaction (PCR)(13). Each of the sequenced open reading frames encodes 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 several organisms; most extensively in those tractable to genetic or biochemical analysis of early development. Studies have shown that Wnt proteins are involved in diverse developmental phenomena. The *Wnt*-1 orthologue in *Drosophila* is the segment polarity gene *wingless* (14, 15) Genetic and biochemical analyses suggests 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 (16). *Wg* has also been

shown to have organizer activities that leads to specification of spatial patterns in adult structures, such as leg or wing and it is involved in regulating neuroblast specification in the central nervous system. 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 (17, 18, 19, 20) Current models of early embryonic patterning events in the frog invoke one or several Wnt proteins as determinants of dorsal-axial position(20, 21). The murine *Wnt* genes cloned to date are expressed in spatially restricted patterns during gastrulation, neurulation, or early organogenesis. Of the *Wnt* genes analyzed, seven of the family members show restricted expression patterns in the developing brain, and several family members are expressed in the neural tube and neural plate(22). 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 (23, 24). Wnt family proteins are also implicated in limb development(22, 25), kidney development(26, 27), and uterine development(28). 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* (29). This work indicates that myotome formation *in vivo* may be directed by the combinatorial activity of Shh secreted by the floor plate and notochord and Wnt ligands secreted by the dorsal neural tube (reprints included in Appendix).

Wnt proteins and their mechanism of action

The predicted amino acid sequence of the *Wnt*-1 gene displays many of the characteristics of secreted growth factors: a hydrophobic signal peptide, a recognition site for signal peptidase, N-linked glycosylation sites, cysteine residues, and the absence of an identifiable membrane anchor domain (30). Due to the lack of cell lines expressing endogenous *Wnt*-1 genes, most of the work on the biochemical properties of Wnt proteins has been carried out with cells programmed to express exogenous *Wnt* cDNAs. In these ectopic settings, Wnt-1 proteins behave as secretory glycoproteins, undergoing entry into the endoplasmic reticulum (ER), leader cleavage, and asparagine(N)-linked glycosylation at several sites (31, 32). Despite entry into the ER, Wnt-1 proteins are very poorly secreted. Most of the Wnt-1 protein remains associated with internal membranous components of cells. Intracellular Wnt-1 is predominantly bound to BiP; a chaperonin-like protein found in the ER (33). The appearance of extracellular Wnt-1 proteins is significantly enhanced by addition of heparin sulfate (34) or suramin (35) to the media. Wnt-1 proteins thus appear to move through the secretory pathway into the extracellular environment but 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 (35) or the extracellular matrix (34). Evidence has accumulated that Wnt proteins can act in a paracrine fashion. First, entry into the secretory pathway is necessary for Wnt-1 biological activity(36). Second, cell transformation assays have been developed that depend on paracrine effects of Wnt-1(36, 37). Paracrine assays involve co-cultivation of donor cells that do not exhibit responses to *Wnt*-1 expression (fibroblast non-responsive cells) and mammary epithelial cell lines. When *Wnt*-1 responsive cells (C57MG) are mixed with, or surrounded by, Wnt-1 donor cells, they undergo morphological changes. Finally, analysis of wg protein function, the *Drosophila* homologue of *Wnt*-1, suggests that it acts in a paracrine fashion since the *wg* mutant phenotype is cell non autonomous (38). These observations have led to the model that Wnt-1 proteins are local-acting factors that function to signal cells that are 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(39). More recently several groups have reported Wnt-specific activity in the medium of mammary epithelial cells programmed to express a *Wnt*-1 cDNA ((40)and J. Kitajewski, unpublished observations). Biological activity of soluble wingless protein from cultured *Drosophila* imaginal disc cells has also been reported(41) . These studies suggest that Wnt proteins can also act as diffusible secreted growth factors; however, the levels of active proteins found in soluble forms are low. Purification of soluble Wnt proteins has not yet been accomplished despite progress in detecting soluble forms.

Wnt signal transduction events

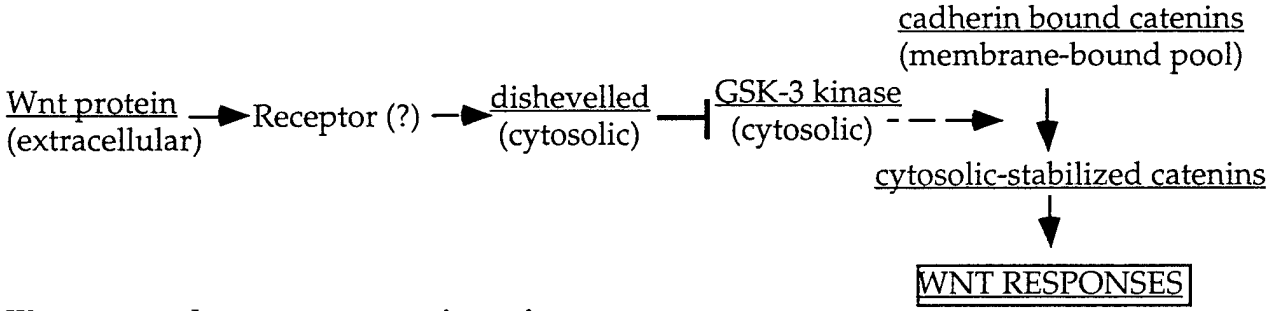
The Wnt-1 protein is now recognized as a mediator of cell-cell signaling events. Although little is known about Wnt cell surface receptors, the nature of the signaling events triggered by Wnt proteins is now becoming apparent. Genetic analysis of the *wg* signal transduction pathway in *Drosophila* embryos and biochemical analysis in cultured insect cells suggests a cascade of signaling events distinct from any previously described signal transduction pathway (16, 42, 43). A model for

the *wg*-mediated signaling pathway (schematized in Figure 1) has been proposed to involve wg binding to a cell surface receptor, yet to be identified. Within the target cell, the cytosolic dishevelled protein is the first known component in *wg*-mediated signaling, however, its function is unclear. Dishevelled has been reported to undergo hyperphosphorylation in response to wg protein (Roel Nusse, personal communication). Downstream of dishevelled is a protein kinase, zeste-white 3, whose activity must be suppressed to transmit wg signals. Suppression of the zeste-white 3 kinase leads directly, or indirectly, to the dephosphorylation and increased stability of the armadillo protein(44). Increased cytosolic armadillo is critical for the endpoint of the pathway which is transcriptional activation of the engrailed gene. The components of the *Drosophila* wg signaling pathway have been conserved in vertebrates. Several *Dishevelled* genes have been identified in the mouse(45). *Drosophila zeste-white 3* (*zw-3*) encodes a serine/threonine kinase that is the homologue of mammalian Glycogen Synthase kinase-3(46) Finally, *Drosophila* armadillo is the orthologue of vertebrate cadherin-associated molecules know as β-catenin and plakoglobin (or γ-catenin) (47). Cadherin is a cell adhesion molecule involved in homotypic associations between cells and catenins are a class of molecules that bind to the intracellular domain of cadherin ; this binding is required for cadherin function.

Conservation of this signaling pathway in vertebrates has been elegantly demonstrated by ectopically expressing Wnt signaling components in *Xenopus* embryos and the determination of their ability to induce axis duplication, as previously reported for Wnt-1(17). Ectopic expression of *Xenopus dishevelled* gives phenotypes that are similar to those conferred by *Wnt* genes(48). Dominant-negative mutants of vertebrate GSK-3 have been generated by mutating the lysines involved in ATP binding; these mutants can function as repressors of GSK-3 activity. The expression of dominant-negative GSK-3 displays activities analogous to those mediated by Wnt proteins. For instance, ectopic expression of Wnt-1 in early frog embryos results in dorsalization of the embryos or duplication of the embryonic axis. This result can be duplicated by expression of dominant-negative mammalian GSK-3(49, 50) Therefore, repression of GSK-3 activity through Wnt proteins or dominant-negative GSK-3 leads to dorsal axis induction. Finally, ectopic expression of β-catenin(51) or plakoglobin(52) mimics Wnt-like activity. Wnt signal transduction may, therefore, regulate the association of plakoglobin or β-catenin to the cadherin family of molecules. It is thought that increased cytosolic levels of catenins are critical for Wnt signaling(53). Interestingly, in *Xenopus,* high levels of cadherin expression actually inhibit β-catenin signaling activity suggesting that cadherins may counteract Wnt signaling by reducing the pools of cytosolic catenins(54).

In mammalian cells, Wnt-mediated upregulation of cadherins and catenins has been reported (55, 56) . More recently, Dr. Tony Brown's laboratory has found a dramatic and rapid upregulation of the cytosolic pool of β-catenin in cultured mammary epithelial cells (personal communication).

Figure 1. Schematic of hypothetical Wnt signal transduction pathway.



***Wnt* genes and mammary tumorigenesis**

Abnormal expression of the *Wnt*-1 gene contributes to the development of mammary tumors(57). Transgenic mice expressing the *Wnt*-1 gene in the mammary gland develop mammary tumors with high levels of *Wnt*-1 mRNA(8). Expression of the *Wnt*-1 gene in two established mammary epithelial cell lines, C57MG cells(58) or RAC311C cells(59) 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. In contrast, primary embryo cells and several established rodent fibroblast cell lines do not respond to *Wnt*-1 expression. Transgenic

experiments also suggest Wnts cooperate with Fibroblast Growth Factor (FGF) family members to induce mammary tumorigenesis(9, 60). Thus it is well established that the *Wnt* genes are potent oncogenes in mouse mammary tumorigenesis.

Several lines of evidence suggest that the proteins encoded by the *Wnt* gene family may affect mammary gland development. Mice bearing a *Wnt*-1 transgene that is expressed in the mammary gland 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 and adrenalectomy has no obvious effect on the morphology of the mammary hyperplasias in *Wnt*-1 transgenics (61). Both *Wnt*-1 and *Wnt*-3 expression can affect mammary gland growth; however, neither gene is expressed in the normal mammary gland. Since the mammary gland responds to both *Wnt*-1 and *Wnt*-3, it has been proposed that they act through Wnt specific cell surface receptors found on mammary epithelial cells and that these receptors normally respond to proteins encoded by other *Wnt* gene family members that are expressed in the mammary gland. In fact, as shown in Table 1, several *Wnt* genes are found to be expressed during post-natal development of the mammary gland(62, 63). Of the *Wnt* genes analyzed, *Wnt*-2, *Wnt*-4, *Wnt*-5a, *Wnt*-5a, *Wnt*-6, and *Wnt*-7a are expressed in the mammary gland during mammary gland growth and differentiation (in virgin and pregnant glands). In lactating glands, when the gland is no longer growing, none of the identified *Wnt* genes are expressed. The expression of several Wnt genes appears to be hormonally regulated(64). These findings suggest that regulated expression of *Wnt* gene products may play a role in the normal expansion or differentiation of the mammary epithelium before lactation. In fact, ectopic expression of Wnt-4 induces a pregnancy-like growth pattern in reconstituted mammary glands in virgin mice(65). The oncogenic effects of the *Wnt*-1 and *Wnt*-3 genes may thus interfere with the normal *Wnt*-mediated regulation of mammary gland growth.

Several human malignancies have been documented to have aberrant expression of *Wnt* genes. Human *Wnt* Genes 2, 3, 4, and 7b have been found to be overexpressed in breast cell lines and human breast tumors as compared to normal breast tissue(66). Aberrant expression of *WNT*-5a was also reported in lung, breast, and prostate carcinomas and melanomas(67). Finally, Wnt signaling may involve proteins that have been implicated in human neoplasias; including cadherin and catenin (68). These results provide a strong rationale for studies on the action of Wnt proteins as a means of understanding the normal and neoplastic development of the human mammary gland.

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

I. EXPERIMENTAL METHODS/RESULTS

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

In **specific aim 1,** we proposed 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.

In **specific aim 2,** we proposed to *determine the transforming potential of Wnt genes.* The transforming potential of the epitope tagged *Wnt* gene products have been tested by using retroviral vectors to express these genes in the murine C57MG mammary epithelial cell line (Specific aim 2). Paracrine transforming capability of *Wnt* genes has been assessed by co-cultivating mammary epithelial cells with *Wnt*-expressing Rat-B1a fibroblasts (69) (which themselves show no response to *Wnt*-1 and act as donors of Wnt proteins to adjacent C57MG cells). We have generated C57MG cells that express all ten of the HA-tagged Wnt proteins. 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, Wnt-7a 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 two sets of Rat fibroblast cell lines as donors of Wnt activity, Rat-2 and Rat-B1a fibroblasts. 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 to the expression pattern of Wnt genes in the murine mammary gland.

Table 1. Summary of Transforming Activities of Wnt Proteins

		Transforming	
	Expression in Mammary Gland	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	-	-

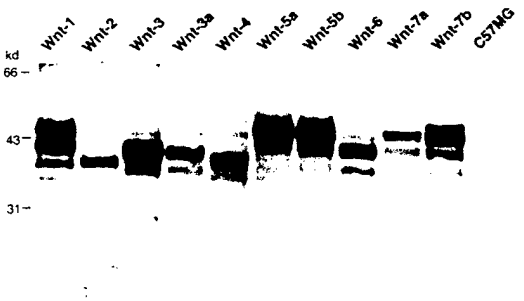


Figure 2. Immunoblot analysis of C57MG mammary epithelial cell lines programmed to express HA epitope-tagged Wnt-1, Wnt-2, Wnt-3, Wnt-3a, Wnt-4, Wnt-5a, Wnt-5b, Wnt-6, Wnt-7a, Wnt-7b.

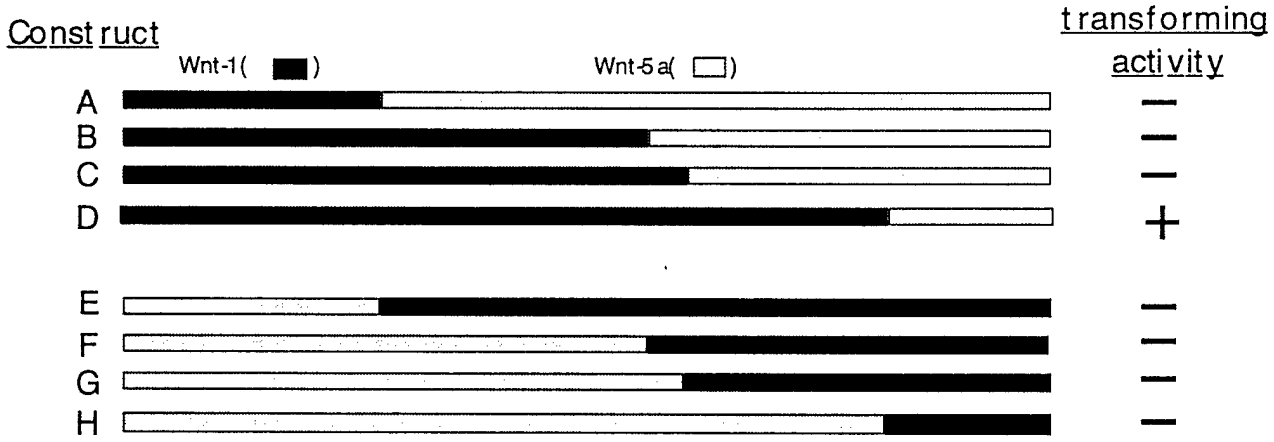
Figure 2 displays an immunoblot analysis using anti-HA antibodies to compare the levels of Wnt proteins in C57MG cells. A similar analysis was carried out with both Rat fibroblast cell lines programmed to express Wnt proteins to confirm that roughly comparable levels of proteins are produced by the cell lines (data not shown).

Our results indicate that there are two classes of Wnt proteins as evaluated by our assays in mammary epithelial cells; transforming Wnt genes and non-transforming Wnt genes. The Wnt proteins that are transforming all have the ability to transform cells in a paracrine fashion demonstrating that the *Wnt* gene family encodes paracrine-acting growth factors. Our results differ somewhat from another report on the transforming potential of Wnt genes (70), where Wnt-6 and Wnt-7a were found to have activity. However, this report used different criteria to assess transformation and did not evaluate protein levels in the cell lines. *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.

We have also utilized rat fibroblasts programmed to express Wnt proteins to address the role of Wnt proteins in the process of myogenic differentiation in the embryonic somite. In a series of experiments carried out in Dr. Andrew Lassar's laboratory (Harvard University), we have demonstrated that Wnt proteins, in combination with Sonic hedgehog (Shh), can induce myogenesis in somitic tissue *in vitro* (29). Somite myogenesis can be induced in presegmented paraxial mesoderm by co-cultivating somitic tissue with Wnt producing fibroblasts in the presence of recombinant Shh. Wnt-1, Wnt-3, and Wnt-4 proteins induce somite myogenesis, whereas, Wnt-7a and Wnt-7b do not.

In **specific aim 3,** we proposed to *map domains of Wnt proteins required for transforming potential.* We have documented that Wnt-1HA transforms mammary epithelial cells whereas Wnt-5aHA does not, despite equivalent levels of expression of the two proteins. We chose these two proteins to make chimeric proteins composed of regions of a transforming Wnt (Wnt-1) and a non-transforming Wnt (Wnt-5A). Two sets of four chimeras were constructed. These chimeras are designated as N-terminal Wnt$^{\text{amino acid \#}}$/C-terminal Wnt$^{\text{amino acid \#}}$; note that Wnt-1 consists of amino acid residues 1-359 and Wnt-5a consists of residues 1-378. One set with an increasing contribution of N-terminal Wnt-1 and decreasing amounts of C-terminal Wnt-5a; 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; Wnt-5a$^{1\text{-}111}$/1$^{100\text{-}359}$(E), Wnt-5a$^{1\text{-}197}$/1$^{187\text{-}359}$(F), Wnt-5a$^{1\text{-}218}$/1$^{200\text{-}359}$(G), and Wnt-5a$^{1\text{-}306}$/Wnt-1$^{292\text{-}359}$(H). Chimeric proteins currently generated are schematized in Figure 3. These constructs have been tested for transforming potential in mammary epithelial cell lines and the results are shown in Figure 3. We are currently characterizing the protein levels of these different chimeric Wnt proteins to control for differences in protein production. Our preliminary results suggest the transformation specific determinants of Wnt-1 proteins may lie at the most N-terminal domain and a sub C-terminal region of Wnt-1 found in chimera D but not chimera C. Further quantitation and delineation of the regions of Wnt-1 proteins required for transforming potential are described in *Specific Aim* 1 of the renewal proposal.

Figure 3. Transforming Potential of Wnt-1/Wnt-5a chimeras.



In **specific aim 4**, we proposed to *characterize Wnt proteins as ligands.* Until recently, fibroblasts programmed to express Wnt-1 proteins were thought to be unresponsive to Wnt-1. We have observed that some fibroblast lines do, indeed, undergo graded changes of morphology in two settings. Rat2 fibroblasts expressing a deregulated Wnt-1 gene, are more refractile and slightly elongated compared to their untransfected counterparts. When furnished with media that has been conditioned by normal mammary epithelial cells Wnt-1 Rat2 cells undergo further alterations that are strikingly similar to those exhibited by epithelial cells that either express or are exposed to Wnt-1. Furthermore, mammary epithelial cell conditioned medium stimulates Wnt-1 fibroblasts to grow beyond confluence to a density that is 3X greater than fibroblasts exposed to either unconditioned medium or medium conditioned by the fibroblasts themselves. Using this system, we hope to define the secreted growth factors that cooperate with Wnt-1 to induce mammary oncogenesis. This is an important observation in the sense that any assessment of the biological activity of purified Wnt-1 proteins may have to be done in the context of adding appropriate co-factors or cooperating growth factors. We have preliminary data to suggest that pre-conditioning media with cultured mammary epithelial cells to collect secreted factors followed by conditioning with fibroblasts producing Wnt-1 proteins results in an activity thought to be derived from Wnt-1 proteins. We are currently testing this hypothesis by determining if immunodepletion of Wnt proteins abrogates the conditioned media activity. This doubly conditioned media may be a source of extracellular Wnt proteins for the purification of Wnt ligands in a biologically active form.

In order to purify *Wnt* proteins, as described in *Specific Aim 4* , we will express the HA-tagged Wnt proteins using Adenovirus vectors. This system has the advantage of delivering a high copy number genome to cultured cells. In addition, Adenovirus can infect non-mitotic cells. In our hands, Adenovirus vectors can express heterologous proteins at high levels when used to infect human or rat cell lines but not mouse cells. The HA-tagged Wnt cDNAs were inserted into a Adenovirus transfer vector (kindly provided by Dr. Steven Hardy, UCSF). This vector places the gene to be expressed under the control of the CMV promoter. Using standard procedures(73) the Adenovirus transfer vector and Adenovirus genomic DNA were co-transfected into the human 293 cell line which provides E1a and E1b proteins critical for Adenovirus replication. Recombinant Adenoviruses containing the *Wnt* -1HA gene was isolated and plaque purified, we refer to this vector as Ad-Wnt-1HA. In addition, we have generated an Adenoviral Vector programmed to express a dominant negative Glycogen Synthase Kinase-3. As a control for all experiments we utilize a comparable Adenovirus vector expressing LacZ (kindly provided by Dr. Steven Hardy, UCSF), referred to as Ad-LacZ. All of these vectors lack the Adenovirus E1a and E1b genes which may affect the biology of infected target cells. These vectors do not replicate in cells lacking the E1a and E1b genes

We have used immunoblot analysis with the anti-HA antibody to examine the protein produced in infected cells. 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 (data not shown). We

are interested in assessing which of these or other cell lines infected with Ad-Wnt-1HA can provide a good source of secreted Wnt-1 proteins for subsequent purification.

Wnt-Mediated Apoptosis of neuronal PC12 cells and Breast Cancer Cells

In a bid to screen cell lines infected with Adenovirus vectors for production of secreted Wnt proteins, we infected the pheochromocytoma cell line PC12 (74). Previous reports indicated that PC12 cells programmed to express Wnt-1 exhibit Wnt-specific responses (75) that include changes in morphology and growth factor responsiveness. We now report that using Adenovirus vectors to express of Wnt-1 proteins in the PC12 cell line induces cell death. This is the first report of cell death induced by Wnt-1 proteins. Previously used methodology for expressing Wnt-1 in PC12 cells utilized retrovirual infection and selection (75) and would not have documented cell death. To characterize this phenomena, we compared survival of cells infected with control viruses (Ad-LacZ) to cells infected with Ad-Wnt-1HA. Within 1 to 2 days after infection with Ad-Wnt-1HA, dying cells containing typical "apoptotic" nuclei became visible. Cell numbers in Ad-Wnt-1HA infected cell populations decrease to roughly 25% of controls at 3-4 days post infection (Figure 4). High levels of Wnt-1HA proteins in Ad-Wnt-1HA infected cells were detected by immunoblot analysis using anti-HA antibodies (data not shown). We believe the apoptotic response is specific to Wnt-1 production since a comparable vector expressing LacZ (Ad-LacZ) does not result in apoptosis. In addition, Adenovirus vectors that express other oncoprotein products (bcl-6) do not induce apoptosis in PC12 cells (data not shown). As a positive control, we induced apoptosis in PC12 cells by serum deprivation, as previously reported (76). Serum deprived PC12 cells will not undergo apoptosis if they are pre-treated with trophic substances such as nerve growth factor (NGF) (76). Thus, NGF promotes PC12 cell survival despite serum deprivation. We have found that NGF treatment also blocks Ad-Wnt-1HA mediated apoptosis, despite the presence of high levels of Wnt-1 proteins. Finally, we believe Wnt-1 mediated apoptosis is specific to PC12 cells since we have not seen Wnt-1 mediated apoptosis in more than two dozen other cell lines, mostly mammary epithelial cells or Rat fibroblast, infected with the Ad-Wnt-1HA vector. To confirm that dying cells were apoptotic as opposed to necrotic we assessed the quality of the chromosomal DNA. Using a Southern blotting technique to detect DNA derived from PC12 cells (76) we analyzed the state of the DNA in Ad-Wnt-1HA infected PC12 cells. As shown in Figure 6, DNA fragmentation typical of apoptotic cells was detected in PC12 cells infected with Ad-Wnt-1HA vectors but not with control Ad-LacZ vectors. Serum deprivation also induced DNA fragmentation in PC12 cells (Figure 5). These points taken together indicate that Wnt-1 can induce apoptosis in the pheochromocytoma PC12 cell line.

To enlarge the scope of this survey we have acquired two dozen established human breast cancer cell lines (these cells were obtained from ATCC in collaboration with several researchers at Columbia University). These breast cancer cell lines represent a wide spectrum of hormonal-responsiveness and growth properties. The following cell lines are currently being cultured in our laboratory for this survey:
BT-20, BT-474, BT-483, BT-549, CAMA1, Du4475, HBC-100, HS 578T, MD-MBA-134VI,. MD-MBA-157, MD-MBA-175-VII, MD-MBA-231, MD-MBA-330, MD-MBA-361, MD-MBA-415, MD-MBA-435-S, MD-MBA-436, MD-MBA-453, MD-MBA-468, MCF-7, SK-BR-3, T47D, UACC 812, UACC893, Zr 75-30, ZR75-1. We have infected all of these cell lines with an Ad-LacZ vector and have demonstrated by β-galactosidase staining that Adenovirus infection and expression has occurred (data not shown). In addition, we have infected all of these cells with Ad-Wnt-1HA vectors and are testing the conditioned media for Wnt-1 protein activity. One of these cell lines, BT-549 also underwent apoptotic cell death in response to Ad-Wnt-1HA infection. Therefore, Wnt-1 may be involved in stimultating apoptotic cell death in several cell lines. We conclude that Wnt-1 may act as a mitogen under certain conditions and may act to induce cell death under other conditions.

Figure 4. Relative survival of PC12 cells infected with Adenovirus vectors expressing Wnt-1HA proteins (Ad-Wnt-1HA) as compared to control Ad-LacZ vectors. MOI-multiplicity of infection

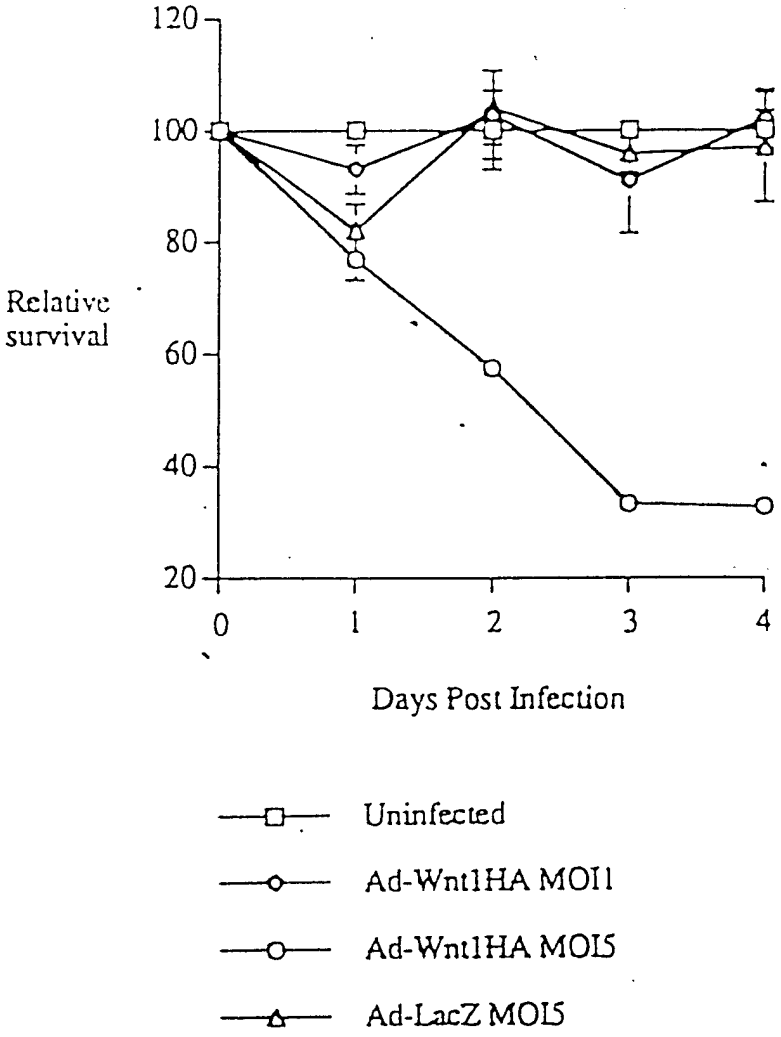


Figure 5. Southern blot analysis of DNA in serum deprived, Ad-Wnt-1HA infected (MOI=5), and Ad-LacZ (MOI=5) infected PC12 cells.

II. Goals of the Research

The data presented in this annual report represents results of experiments outlined in specific aim 1, 2, 3, 4 of the research proposal. We feel we have completed all of the major objectives in the first two aims and are well on the way toward the goals of specific aim 3 and 4.

CONCLUSIONS

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(66) 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.

By generating a panel of Wnt-1/Wnt-5a chimeric proteins, we have narrowed down a region of Wnt-1 that confers transforming ability. This regions may be made up of the most N-terminal domain of Wnt-1 and a region between amino acid 191 to amino acid 290. Future work will further define the transformation specific determinant. We have identified a potential co-factor that may be required to work in concert with Wnt-1. Finally, we have documented, for the first time a potential role for Wnt-1 in cell death or apoptosis. This apoptotic response is seen in pheochromocytoma cells and in one breast cancer cell line.

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