

AD_____

GRANT NUMBER DAMD17-94-J-4150

TITLE: Wnt-5a and Wnt-4 Regulates Cell Growth in C57MG Mammary
Epithelial Cells

PRINCIPAL INVESTIGATOR: Daniel J. Olson, Ph.D.

CONTRACTING ORGANIZATION: Pennsylvania State University
Hershey, Pennsylvania 17033

REPORT DATE: July 1996

TYPE OF REPORT: Annual

DTIC QUALITY INSPECTED 4

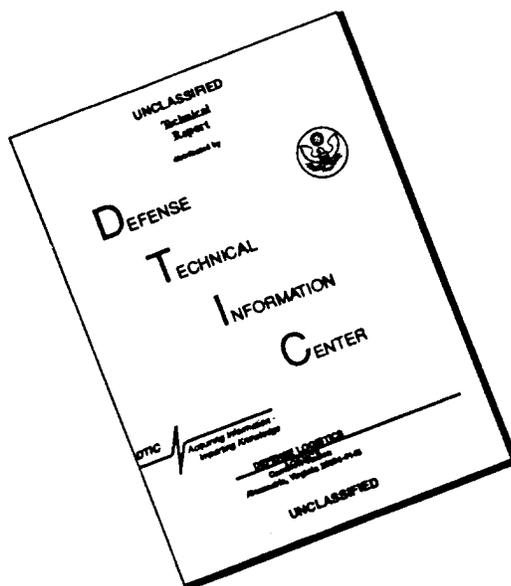
PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
distribution unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

19960930 096

DISCLAIMER NOTICE



THIS DOCUMENT IS BEST QUALITY AVAILABLE. THE COPY FURNISHED TO DTIC CONTAINED A SIGNIFICANT NUMBER OF PAGES WHICH DO NOT REPRODUCE LEGIBLY.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE July 1996	3. REPORT TYPE AND DATES COVERED Annual (1 Jul 95 - 30 Jun 96)	
4. TITLE AND SUBTITLE Wnt-5a and Wnt-4 Regulates Cell Growth in C57MG Mammary Epithelial Cells			5. FUNDING NUMBERS DAMD17-94-J-4150	
6. AUTHOR(S) Daniel J. Olson, Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Pennsylvania State University Hershey, Pennsylvania 17033			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) The implications for determining whether <i>wnt-5a</i> and <i>wnt-4</i> act as mediators of normal cell growth are relevant to the detection, diagnosis, and the treatment of breast cancer. That is, it is important ultimately to understand whether the inappropriate downregulation of certain <i>wnt</i> -genes that are spatially-temporally expressed in developing mammary glands, such as <i>wnt-5a</i> and <i>wnt-4</i> , leads to loss of growth control. C57MG mammary epithelial cells have recently been found to endogenously express <i>wnt-5a</i> and <i>wnt-4</i> whose RNA levels are reduced in the presence <i>wnt</i> -gene family members which transform this cell line. The intent of this proposal has been to develop new cell lines by transfecting C57MG cells with DNA <i>wnt-5a</i> and/or <i>wnt-4</i> constructs which express antisense or sense RNA to determinethe effect on cell transformation will occur. Results found in experiments outlined in this proposal are consistent with the importance for normal <i>wnt-5a</i> and/or <i>wnt-4</i> RNA transcripts levels in directing C57MG mammary epithelial cell phenotype, including cell proliferation and transformation. This has important ramifications for breast cancer since it is known that <i>wnt-5a</i> and <i>wnt-4</i> genes are downregulated in human breast cancer tissue and in human breast malignant cell lines.				
14. SUBJECT TERMS Breast Cancer, WNT-Genes, Transformation, Protooncogenes, Gene Expression, Gene Therapy			15. NUMBER OF PAGES 19	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the US Army.

 Where copyrighted material is quoted, permission has been obtained to use such material.

 Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

 Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

 In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

 For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

 In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

 In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

 In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.


PI - Signature

7/24/96
Date

Table of Contents

	Pages
I. Front Cover	1
II. SF 298 Report Documentation Page	2
III. Foreword	3
IV. Table of Contents	4
V. Introduction	5-7
VI. Body of Report	7-11
VII. Conclusions	12
VIII. References	13-14
IX. Appendices I-V	15-19

Introduction for 1996 interim report

Wnt-gene family

The sixteen known members of the *wnt* family of genes express growth factor-like molecules which are thought to be implicated in mammary tumorigenesis and in early developmental events. The *wnt-1* gene was first identified in mouse mammary tumor virus (MMTV) induced tumors occurring often in certain mouse strains (1). Analysis of these tumors revealed that MMTV proviral integration occurred in close proximity to either the 5' or 3' region of the *wnt-1* gene locus, presumably activating *wnt-1* through MMTV enhancer sequences (2). Since these insertions do not interrupt the protein coding sequences (3), there is expression of an intact protein. Therefore, inappropriate expression of the normal *wnt-1* protein is implicated in cellular transformation. *Wnt-2* and *Wnt-3* have also been isolated from several mouse mammary tumors with activated MMTV provirus (4).

Wnt-gene mechanism of action

The mechanism of action of *wnt* genes is unknown, although several characteristics have been reported which provide insight into how their products may be important. Analysis of genomic (3) and cDNA (5) sequences has revealed that mouse *wnt-1* encodes a cysteine rich 370 amino acid polypeptide with a leader sequence. The leader sequence, which has a potential peptidase cleavage site after amino acid 27 (6), appears to be necessary for protein function (7). Previous studies suggest that *wnt-1* protein enters the secretory pathway, is glycosylated, and may be secreted (8), but remains strongly bound to the cell surface and/or extracellular matrix (9,10), possibly to heparin (10), and is seldom found in the medium of cultured cells (9). This suggests that *wnt-1* acts locally, possibly in an autocrine or paracrine manner. This is strengthened by the finding that mouse *wnt-1* when transfected into 3T3 fibroblasts, which results in no obvious phenotype, can yet transform the mammary epithelial cell-line C57MG when co-cultured (11). This may be related to competence modification of cells by *wnt* signals to growth factors, including bFGF and activin (12). It has been speculated that this may occur at least in part through *wnt*-mediated gap junctional communication (13-15) as a result of influencing the expression of β -catenin to ultimately enhance Ca^{2+} dependent cadherin-associated cell adhesion (16).

Wnt-genes in mammary cell differentiation and in tumorigenesis

Besides the ability of the expression of certain *wnts* to determine cell competence during pattern formation in vertebrate development, an *in vivo* role for MMTV directed *wnt-1* expression in mammary tumorigenesis has been directly shown in transgenic mice (17). More recently, *wnt-3* has also been found to be transcriptionally activated by MMTV provirus in mouse mammary carcinomas (7). In addition, *wnt-2* has been implicated in mammary tumorigenesis since it is overexpressed and amplified in transplanted virally induced tumors (4). In cell culture systems, the ectopic expression of *wnt-1* or *wnt-2* is able to transform certain mammary epithelial cell lines (7), including C57MGs derived from normal mammary gland of a C57 B1/6 mouse (18). However, little is known how various members of the *wnt*-gene are capable of transforming mammary epithelial cells and are involved in mammary tumorigenesis. Some investigators believe that *wnt-1* and other transforming *wnt*-gene peptides act on endogenous *wnt*-gene signalling pathways to transform cell types.

Recent findings by Gavin and McMahon (19) have demonstrated that at least five

members of the sixteen known mouse *wnt*-genes, including *wnt-5a* and *wnt-4*, are differentially expressed during the postnatal development of the mouse mammary gland, implicating their importance in mammary development. That is, *wnt-5a* and *wnt-4* and other *wnts* are expressed in virgin glands and during early to mid-pregnancy. However, when pregnancy approaches term, these same genes become nonexpressive and other types of *wnt*-genes are concomitantly transcriptionally activated. That is, *wnt-2* has recently been detected during the ductal phase of mammary gland development and reduced at the onset of pregnancy and lactation, a profile opposite to that found for *wnt-5a* and *wnt-4* (20).

This interplay between various *wnt*-gene family members in mammary gland development may have implications for how *wnt-1* and *wnt-2* are able to transform cells. That is, the type of *wnt*-gene ultimately expressed may dictate the competence of the cell to respond to various growth factors and hormones and thus dictate the pattern of cell growth.

C57MG cells have recently been found to endogenously express *wnt-5a* and *wnt-4* (19) and that there is a reduction of endogenous *wnt-5a* and/or *wnt-4* RNA levels in the presence of *wnt-1* and *wnt-2* gene products (21). Furthermore, when C57MGs were fully transformed by the ectopic expression of activated *neu* T [c-erbB2 (22)], it was found that transcriptional expression for each endogenous gene was virtually absent.

This suggests that endogenous *wnt-5a* and *wnt-4* genes in C57MG mammary epithelial cells may play a functional role in determining cell growth and that adequate RNA expression levels of both may be important in controlling cell proliferation and perhaps transformation (21).

The implications for determining whether *wnt-5a* and *wnt-4* act as mediators of normal cell growth are relevant to the detection, diagnosis, and the treatment of breast cancer. That is, it is important ultimately to understand whether the inappropriate downregulation of certain *wnt*-genes that are spatially-temporally expressed in developing mammary glands, such as *wnt-5a* and *wnt-4*, leads to loss of growth control. This would have direct application to testing biopsied mammary tissue for loss of appropriate *wnt*-gene expression and whether the re-establishment of gene expression could result in reversal of transformation. This proposal was submitted for a **Career Development Award** to allow the opportunity to direct the laboratory toward continuing to pursue the long term goal of understanding and controlling breast cancer.

With this in mind, a series of technical objectives was formulated, as summarized below, in order to determine the importance of *wnt*-genes in mammary epithelial cell growth and differentiation. It was proposed that:

1. Fully transformed *neu*-T (c-erbB2) activated C57MG mammary epithelial cells would be transfected with sense *wnt-5a* and/or *wnt-4* DNA constructs in a manner which allows for clonal selection in hygromycin B. The same was to be done for partially transformed C57MG mammary epithelial cells constitutively expressing *wnt-1*. Anchorage independent soft agar assays were to be done to determine whether the transfectants have regained cell contact growth dependence and ³H-thymidine assays would be done to determine whether the transfectants have lost the ability to proliferate at confluence, which is a hallmark of transformation in this cell type.

2. To ascertain whether *wnt-5a* and *wnt-4* genes act as repressors of cell proliferation in C57MG mammary epithelial cells, DNA constructs were to be made to express anti-sense RNA for *wnt-5a* and *wnt-4*. These constructs would be co-transfected with plasmids containing a NEO-resistant gene into the C57MG parental cell line to allow for clonal selection in G-418. Anchorage independent soft agar assays were to be done to determine whether the transfectants have gained contact growth independence. ³H-thymidine assays were to be done to determine whether the transfectants have gained the

ability to proliferate at confluence unlike the parental cells.

3. Since there was little information concerning the expression of *wnt-5a* and *wnt-4* in mouse or human mammary cell lines or tumors when this proposal was initiated, human and mouse cell lines as well as tumors were to be probed for the expression of *wnt-5a* and *wnt-4* by Northern blot analysis.

4. Based on preliminary findings **which suggest the importance of the presence of *wnt-5a* and/or *wnt-4* RNA transcripts in directing C57MG mammary epithelial cell phenotype and cell proliferation, and may be the underlying mechanism for how *wnt-1* is able to transform cells**, it became important to extend the technical objectives by searching for genes that may be transcriptionally amended to uncover previously undescribed genes involved in mammary tumorigenesis which are regulated by *wnt*-gene family members.

This will be done using PCR mRNA differential display to identify genes which are transcriptionally regulated. Using differential display by PCR amplification of RNA should allow the recovery of genes that have are influenced by *wnt-1* signalling pathways for use as probes for RNA blot analysis and partial sequencing for data base analysis. **This systematic approach will likely open new directions in *wnt*-gene research by disclosing previously unidentified *wnt*-gene mediated transcriptional regulation.**

Body of interim report

Experimental Methods

1. Transfection of transformed C57MG mammary epithelial cells not expressing *wnt-5a* or *wnt-4* normally.

Transformed C57MG cells expressing activated V659E rat *neu* gene (provided by Oncogene Science) under the control of an MLV LTR promoter were maintained in Dulbecco's modified Eagle (DME) medium supplemented with 5% fetal calf serum and 5% bovine calf serum (HyClone), and 250 ug/ml G-418 (Gibco). The cells were transfected with the mammalian expression vector pRSV ligated to *wnt-5a* or *wnt-4* (provided by Andrew McMahon, Harvard) in the sense orientation using lipofection as described (23). The cells were co-transfected with the SV2HYG mammalian expression vector carrying the gene for hygromycin B resistance, and resistant colonies selected and expanded into cell lines. RNA was extracted (24) and cells expressing *wnt-5a* and *wnt-4* verified by Northern blot analysis using specific hybridization probes for *wnt-5a* and *wnt-4* and compared to those cells only transfected with the SV2HYG vector. The same technique was used to transfect *wnt-1* expressing C57MG cells (provided by Jackie Papkoff, Sugen, California) which are maintained normally in Dulbecco's modified Eagle medium as above also supplemented with 250 ug/ml G-418 (Gibco) with pRSV*wnt-5a* and/or pRSV*wnt-4*.

RNA isolation and Northern Blot Analysis

Total cellular RNA was isolated from multiple dishes of confluent cells (24). Twenty ug of total RNA was analyzed when probing for transfected gene expression. To ascertain effects on endogenous levels of *wnt* RNA transcripts, it was necessary to select for poly (A+) RNA using an oligo d(T) cellulose column. Two ug of each poly (A+) RNA sample were separated on a 1.2% agarose formaldehyde gel followed by transfer to a Hybond-N (Amersham) membrane. The membranes were cross-linked (Stratagene) and prehybridized for 3-6 hours at 61°C, and then hybridized at 61°C overnight with labeled riboprobes made with a Ribosystem II kit (Promega). The riboprobe vectors containing sequences for *wnt-4* or *wnt-5a* (McMahon, Harvard) have been described (19). The

hybridization/prehybridization solution consisted of 50% formamide, 4X SSPE, 0.2 mg/ml sheared salmon sperm DNA, 2.5X Denhardt's, and 1% sodium dodecyl sulfate (SDS). Membranes were washed at room temperature twice in 2X SSC, 1% SDS, followed by several washes in 0.1X SSC, 0.1% SDS at 65°C.

Cell Morphology Assays

Cells lines were maintained in G-418 and hygromycin B and grown to confluence. C57MG/*wnt-1*, C57MG/*neu T*, C57MG, C57MG/*wnt-1/wnt-5a* and/or *wnt-4*, and C57MG/*neu T/wnt-5a* and/or *wnt-4* cells were plated at a density of 5×10^5 cells per 10 cm tissue culture dish and photographed for phenotype comparison. cells per 10 cm tissue culture dish and photographed for phenotype comparison.

Soft Agar Assays

Twelve-well plates (CoStar) were used to add 0.5% agar (Difco) for a base. Cells taken from confluent plates were placed in a suspension at 10X cell density and added to the wells in a final agar concentration of 0.35%. The plates were incubated at 37°C in 5% CO₂ and observed daily for colony formation.

³H-thymidine incorporation

Cell lines above were plated into 5 cm dishes in standard growth medium and grown to confluence without a medium change (4 days). The cells were rinsed twice with phosphate buffered saline (PBS), and fed with 5 ml of serum free DME. All assays were done in triplicate. Next 4 uCi of ³H-thymidine (methyl ³H, 60-90 Ci/mmol, aqueous, ICN) were added to each dish and incubated for 2 hours at 37°C. The cells were washed twice with PBS followed by addition of 2 ml ice cold 10% trichloroacetic acid (TCA) for 30 min on ice. Cells were then washed with 10% TCA followed by the addition of 2.0 ml of 0.1 N NaOH. The dishes were incubated at 37°C for 30 min and then neutralized with 0.2 ml of 1N HCL. The dishes were carefully scraped and the extract added to 10 ml of scintillation fluid for counting.

2. Transfection of C57MG mammary epithelial cells with antisense constructs for *wnt-5a* or *wnt-4*.

The cells were initially maintained in Dulbecco's modified Eagle's medium until mid-log phase and transfected by lipofection (23) with either pRSV*wnt-5a* and/or *wnt-4* DNA mammalian expression vectors ligated in the antisense orientation. The cells were co-transfected with the mammalian expression vector SV2NEO to allow for clonal selection in G-418. Individual resistant colonies were selected and expanded into cell lines. To determine whether the antisense RNA leads to specific loss of *wnt-5a* or *wnt-4* transcripts, Northern blots were done.

RNA isolation and Northern Blot Analysis

Total cellular RNA was isolated from multiple dishes of confluent cells (24). Twenty ug of total RNA was analyzed when probing for transfected gene expression. To ascertain effects on endogenous levels of *wnt* RNA transcripts, it was necessary to select for poly (A+) RNA using an oligo d(T) cellulose column. Two ug of each poly (A+) RNA sample were separated on a 1.2% agarose formaldehyde gel followed by transfer to a Hybond-N (Amersham) membrane. The membranes were cross-linked (Stratagene) and prehybridized for 3-6 hours at 61°C, and then hybridized at 61°C overnight with labeled riboprobes made with a Ribosystem II kit (Promega). The riboprobe vectors containing sequences for *wnt-4* or *wnt-5a* (McMahon, Harvard) have been described (19). The hybridization/prehybridization solution consisted of 50% formamide, 4X SSPE, 0.2 mg/ml sheared salmon sperm DNA, 2.5X Denhardt's, and 1% sodium dodecyl sulfate (SDS).

Membranes were washed at room temperature twice in 2X SSC, 1% SDS, followed by several washes in 0.1X SSC, 0.1% SDS at 65°C.

Cell Morphology Assays

Cells lines were maintained in G-418 and hygromycin B and grown to confluence. C57MG/*wnt-1*, C57MG/*neu T*, C57MG, C57MG/*wnt-1/wnt-5a* and/or *wnt-4*, and C57MG/*neu T/wnt-5a* and/or *wnt-4* cells were plated at a density of 5×10^5 cells per 10 cm tissue culture dish and photographed for phenotype comparison.

Soft Agar Assays

Twelve-well plates (CoStar) were used to add 0.5% agar (Difco) for a base. Cells taken from confluent plates were placed in a suspension at 10X cell density and added to the wells in a final agar concentration of 0.35%. The plates were incubated at 37°C in 5% CO₂ and observed daily for colony formation.

³H-thymidine incorporation

Cell lines above were plated into 5 cm dishes in standard growth medium and grown to confluence without a medium change (4 days). The cells were rinsed twice with phosphate buffered saline (PBS), and fed with 5 ml of serum free DME. All assays were done in triplicate. Next 4 uCi of ³H-thymidine (methyl ³H, 60-90 Ci/mmol, aqueous, ICN) were added to each dish and incubated for 2 hours at 37°C. The cells were then washed twice with PBS followed by addition of 2 ml ice cold 10% trichloroacetic acid (TCA) for 30 min on ice. Cells were washed with 10% TCA followed by the addition of 2.0 ml of 0.1 N NaOH. The dishes were then incubated at 37°C for 30 min and then neutralized with 0.2 ml of 1N HCL. The dishes were carefully scraped and the extract added to 10 ml of scintillation fluid for counting.

3. To determine differential gene expression in C57MG cells which constitutively express *wnt-1* when compared to the parental cell line by PCR differential display of RNA.

C57MG and C57MG mammary epithelial cells previously transfected with *wnt-1* were maintained in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum and 5% defined bovine serum, and 250 ug/ml G-418 were grown to 70% confluence prior to their total cellular RNA extraction (24). To remove chromosomal DNA, 50 ug of total cellular RNA were incubated for 30 min at 37°C with 10 units of human placental ribonuclease inhibitor (BRL, Gaithersburg, MD), 10 units of DNase I (BRL) in 10 mM Tris-Cl, pH 8.3, 50 mM KCL, 1.5 mM MgCl₂. After extraction with phenol/chloroform, the RNA were ethanol precipitated and redissolved in RNase-free water.

mRNA differential display was done as described previously (25) with the exception that 0.2 ug total RNA was used for reverse transcription in a reaction volume of 20 uL. Oligo-dT degenerate anchored primers for reverse transcription were T12XA, T12XC, T12XG, and T12XT (already synthesized), where X is a degenerate mixture of dA, dC, and dG will be used for first strand synthesis. These primers were used in conjunction with a decamer oligonucleotide of arbitrarily defined sequence for subsequent amplification (OPA1-20 from Operon, Biotechnology, Inc., Alameda, CA). In order to minimize errors in the PCR procedure, duplicate samples with the same amount of RNA were done. The cycling parameters were as follows: 94°C for 30 seconds, 40°C for 2 minutes, were be separated on a 6% DNA sequencing gel.

4. To recover and amplify the differentially displayed cDNA bands of interest.

The DNA sequencing gel was blotted on to a piece of Whatman 3mm paper and dried without fixing. After orientation of the autoradiogram and dried gel, cDNA bands of interest were located. The gel slice along with the 3mm paper was incubated in 100 uL distilled water for 10 minutes. After rehydration of the gel, the cDNA was diffused out by boiling the gel slice for 15 minutes in a microfuge tube. After ethanol recovery, 4 uL of eluted was reamplified in a 40 uL reaction volume using the same primer set and PCR conditions as used in the mRNA display except the dNTP concentrations were at 20 uM instead of 2-4uM and no was added. 30 uL of PCR samples were run on a 1.5% agarose gel and stained with ethidium bromide. Reamplified cDNA was cloned into the pCRII vector using the TA cloning system from Invitrogen (San Diego, CA). Plasmid DNA sequencing of the cloned probes was done with either T7 or SP6 primers using the Sequenase kit from United States Biochemicals Co (Cleveland, OH). The partial sequences obtained was compared to known sequences in data banks (Blast).

To examine the pattern of gene expression in the sets of cells used for differential display and from which the cDNA bands were removed and amplified, the individual bands of cDNA after amplification were used as probes in Northern analysis. The RNA was isolated as previously described and 20 ug total RNA run on 1.2% RNA gels, blotted to nylon membranes, and probed with ³²P cDNA labeled using a Random-Prime DNA labeling kit from Boehringer Mannheim Biochemical (Indianapolis, IN).

Results and Discussion

During the first 12 months as outlined in the Statement of Work and reported in the first interim report, studies indicate that transfecting *wnt-1* expressing C57MG cells with a construct which constitutively expresses *wnt-5a* and/or *wnt-4* partially rescues the *wnt-1* mediated transformed. It was also found that C57MG cells fully transformed with *neu T* also revert to a more normal appearing phenotype by morphologic criteria particularly when the cells are transfected simultaneously with both *wnt-5a* and *wnt-4*. Furthermore, anti-sense *wnt-5a* and/or *wnt-4* dramatically alters cell phenotype when compared to the normal parental line. This change in phenotype mimics the transformed phenotype observed in the presence of the overexpression of *wnt-1*. This change in phenotype becomes even more exaggerated in those cell lines which are constitutively expressing antisense *wnt-5a* and *wnt-4* simultaneously.

These preliminary findings suggest the importance of the presence of *wnt-5a* and/or RNA transcripts in directing C57MG mammary epithelial cell phenotype and cell proliferation, and may be the underlying mechanism for how *wnt-1* is able to transform the cell phenotype. These results are consistent with the proposed hypothesis that *wnt-5a* and/or *wnt-4* control normal cell growth and provide a basis for continuing this line of research for understanding mammary cancer etiology. We have previously fulfilled the goals proposed for the first 12 months of the grant proposal by establishing all the stably transfected cell lines.

We are now characterizing these cell lines by various assays as outlined. We are currently studying these cell lines using anchorage independent assays, cell kinetics, and ³H-thymidine incorporation, as described for the **12-24 month period in Statement of Work and is considered in this second interim report.** In summary, anchorage independent assays were negative for all cell lines tested. That is, transformed C57MG cells expressing *wnt-1* which were transfected with *wnt-5a* and/or *wnt-4*, and normal C57MG cells transfected with anti-sense *wnt-5a* and/or *wnt-4* did not grow

colonies. **This is not unexpected considering *wnt-1* transformation does not allow for loss of contact growth inhibition.**

Growth kinetics reveal that when *wnt-1* C57MG cells are transfected with *wnt-4*, there is decreased cell saturation density at confluence which is similar to that for normal parental C57MG cells as shown in the Figure 1 A, Appendix I. This is further supported by the growth curves found in normal cells transfected with antisense *wnt-4* which gives a transformed phenotype and which has a similar cell saturation density at confluence to *wnt-1* transformed cells as shown in Figure 1 B, Appendix I. **This suggests that *wnt-4* is a growth regulating gene and can re-establish growth control in transformed cells.** *Wnt-1* transformed cells at confluence are known to continue proliferating as demonstrated by ³H-thymidine assays compared to the parental cell line. When *wnt-1* C57MG cells are transfected with *wnt-4*, the cells at confluence incorporate ³H-thymidine in a manner much like the parental cells as seen in Figure 1 C, Appendix II. When normal cells are transfected with antisense *wnt-4*, the cells appear transformed and incorporate ³H-thymidine similarly to *wnt-1* transformed cells.

When *wnt-1* C57MG cells are transfected with *wnt-5a*, there is decreased cell saturation density at confluence which is similar to that for normal parental C57MG cells as shown in the Figure 2 A, Appendix III. This is further supported by the growth curves found in normal cells transfected with antisense *wnt-5a* which gives a transformed phenotype and which has a similar cell saturation density at confluence to *wnt-1* transformed cells as shown in Figure 2 B, Appendix III. **This suggests that *wnt-5a* is also a growth regulating gene and can re-establish growth control in transformed cells.** When *wnt-1* C57MG cells are transfected with *wnt-5a*, the cells at confluence incorporate ³H-thymidine in a manner much like the parental cells as seen in Figure 2 C, Appendix IV. When normal cells are transfected with antisense *wnt-5a*, the cells appear transformed and incorporate ³H-thymidine in a manner similar to *wnt-1* transformed cells as seen in Figure 2 D, Appendix IV.

The data accumulated suggest that *wnt-5a* and *wnt-4* are growth regulators of C57MG mammary epithelial cells. This is particularly relevant to the manner in which *wnt-1* transforms C57MG cells. That is, *wnt-1* transformation correlates with downregulation of *wnt-4* and *wnt-5a* gene expression. **This also has important ramifications for breast cancer since it is known that *wnt-5a* and *wnt-4* genes are downregulated in human breast cancer tissue and in human breast malignant cell lines (26, 27).**

As indicated in the previous interim report, it was elected to direct our research this past year toward uncovering genes that are expressed in *wnt-1* transformed C57MG mammary epithelial cells. Using PCR based differential display, *wnt-1* transformed cells were directly compared to parental cells in an attempt to provide for a systematic approach to determine underlying genes expression which correlates with transformation by *wnt-1*, and presumably by the downregulation of *wnt-4* and *wnt-5a*. We have isolated several bands of interest from the display shown in Figure 3, Appendix V. After subcloning the cDNA fragments, ³²P labeled riboprobes were constructed and used to probe RNA from *wnt-1* transformed cells and RNA from the parental cells to confirm differential expression. In one instance we were able to show that the differentially displayed band was differentially expressed by Northern analysis. The cDNA band was then sequenced which was compared to known sequences in gene data banks using BLAST. This revealed that the isolated band is arginine-rich and some homology to protamine. This is interesting in that *wnt-1* expression in adult mice is confined to post-meiotic round spermatids at a time when protamine is being expressed. Although there is no biologic precedent for a *wnt-1* protamine connection in somatic cells, we are continuing to pursue this research direction using cells that have been transformed with antisense *wnt-4* and/*wnt-5a*.

Conclusions

The results indicate to date that *wnt-4* and *wnt-5a* gene expression is necessary for normal cell growth of C57MG mammary epithelial cells. This is an important finding since *wnt-4* and *wnt-5a* are differentially expressed in normal developing mouse mammary gland. Considering that *wnt-1* transgenic mice develop mammary gland hyperplasias and adenocarcinomas and *wnt-1* expressing C57MG cells transform, it becomes critical to understand the *wnt-1* mechanism of action. **One conclusion to draw from the results already published by this author and from the preliminary results derived from this proposal, is that it appears that *wnt-1* transforms cells at least in part by altering the normal expression of other *wnt*-gene family members, including *wnt-5a* and *wnt-4*.** It may be that whatever, *wnt*-gene family member predominates in a cell or collection of cells dictates the cell phenotype. This has important implications to determine *wnt*-gene expression in breast cancer. Interestingly, this proposal included ascertaining *wnt-5a* and *wnt-4* RNA levels in human breast cancer tissue and cell lines. Published studies have now shown that *wnt-5a* and *wnt-4* is minimally expressed in malignant breast tumors and in human breast cancer cell lines (26, 27). **The important point is that *wnt-5a* and *wnt-4* gene expression is altered depending on the state of cell growth, which is supported by this research and agrees with the original hypothesis.**

The project continues to conform with the timetable as set forth by the **Statement of Work**. That is, cell lines have been created which are stably expressing the *wnt*-genes of interest and the cell lines have been characterized. This was and has been scheduled to be completed by the end of two years. In addition, we have directed the research effort toward systematically identifying differentially expressed genes in C57MG transformed cells in an attempt to find underlying genetic mechanisms associated with *wnt*-gene function. Potentially, this would have immediate ramifications for understanding the biologic processes involved in breast cancer.

References

1. Nusse, R and Varmus, H.E. Many tumors induced by the mouse mammary tumor virus contain a provirus integrated in the same region of the host genome. *Cell* **31**:99 (1982).
2. Nusse, R. et al., Mode of proviral activation of a putative mammary oncogene (int-1) on mouse chromosomal 15. *Nature* **307**:131 (1984).
3. Van Ooyen, A. and Nusse, R. Structure and nucleotide sequence of the putative mammary oncogene int-1: proviral insertions leave the protein-coding domain intact. *Cell* **39**:233 (1984).
4. Roelink, H., Wagenaar, E., and Nusse, R. Amplification and proviral activation of several *wnt* genes during progression and clonal variation of mouse mammary tumors. *Oncogene* **7**:487 (1992).
5. Fung, Y.K., et al., Nucleotide sequence and expression in vitro of cDNA derived mRNA of *int-1*, a provirally activated mouse mammary oncogene. *Mol. Cell. Biol.* **5**, 3337-3344 (1985). (published erratum appears in *Mol. Cell. Biol.* **6**, 1362 (1986).
6. Brown, A.M.C. et al., Identification of protein products encoded by the proto-oncogene *int-1*. *Mol. Cell. Biol.* **7**, 3971-3977 (1987).
7. Nusse, R. and Varmus, H.E. *Wnt* genes. *Cell* **69**:1073 (1992).
8. Papkoff, J. Inducible overexpression and secretion of *int-1* protein. *Mol. Cell. Biol.* **9**, 3377-3384 (1989).
9. Papkoff, J. and Schryver, B. Secreted *int-1* protein is associated with the cell surface. *Mol. Cell. Biol.* **10**, 2723-2730 (1990).
10. Bradley, R.S. and Brown, A.M.C. The proto-oncogene *int-1* encodes a secreted protein associated with the extracellular matrix. *EMBO J.* **9**, 1569-1575 (1990).
11. Jue, S.F. et al., The mouse *wnt-1* gene can act via a paracrine mechanism in transformation of mammary epithelial cells. *Mol. Cell. Biol.* **12**, 321-328 (1992).
12. Christian, J.L., Olson, D.J., and Moon, R.T. *Xwnt-8* modifies the character of mesoderm induced by bFGF in isolated *Xenopus* ectoderm. *EMBO J.* **11**, 33-41 (1992).
13. Olson, D.J., Christian, J.L., and Moon, R.T. Effect of *wnt-1* and related proteins on gap junctional communication in *Xenopus* embryos. *Science* **252**, 1173-1176 (1991).
14. Olson, D.J. and Moon, R.T. Distinct effects of ectopic expression of *wnt-1*, activin B, and bFGF on gap junctional permeability in 32-cell *Xenopus* embryos. *Dev. Biol.* **151**, 204-212 (1992).
15. Moon, R.T., DeMarais, A., and Olson, D.J. Responses to *Wnt* signals in vertebrate embryos may involve changes in cell adhesion and cell movement. *J. Cell Science*, **Supplement 17**:183-188 (1993).

16. Hinck, L., Nelson, W.J., and Papkoff, J. *Wnt-1* modulates cell-cell adhesion in mammalian cells by stabilizing β -catenin binding to the cell adhesion protein cadherin. *J. Cell Biol.* **124**:729-741 (1994).
17. Tsukamoto, A.S. et al., Expression of the *int-1* gene in transgenic mice is associated with mammary gland hyperplasia and adenocarcinomas in male and female mice. *Cell* **55**, 619-625 (1988).
18. Vaidya, A.B. et al., Murine mammary tumor virus (MuMTV) infection of an epithelial cell-line established from C57BL/6 mouse mammary glands. *Viol.* **90**, 12-22 (1978).
19. Gavin, B.J. and McMahon, A.P. 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**, 2418-2423 (1992).
20. Buhler, T.A., Dale, T.C., Kieback, C., Humphreys, R.C., and Rosen, J.M. Localization and Quantification of *wnt-2* gene expression in mouse mammary development. *Dev. Biol.* **155**:87-96 (1993).
21. Olson, D.J. and Papkoff, J.P. Regulated expression of *wnt* family members during proliferation of C57MG mammary cells. *Cell Growth and Diff.* **5**:197-206 (1994).
22. Bargmann, C.I., Hung, M.-C., and Weinberg, R.A. The *neu* oncogene encodes an epidermal growth factor receptor-related protein. *Nature* **319**, 226-230 (1986).
23. Felgner, P.L. et al., Lipofection: a highly efficient lipid-mediated DNA transfection procedure. *PNAS* **84**:7413-7417 (1987).
24. Chomczynski, P. and Sacchi, N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**:156 (1987).
25. Liang, P and Pardee, A.B. Differential display of eukaryotic mRNA by means of the polymerase chain reaction. *Science* **257**:967-971.
26. Lejeune, S., et al., *Wnt-5a* cloning, expression, and up-regulation in human primary breast cancers. *Clinical Cancer Research* **1**:215-222 (1995).
27. Huguet, E.L., et al., Differential expression of human *wnt* genes 2, 3, 4, and 7B in human breast cell lines and normal and disease states of human breast tissue. *Cancer Research* **54**:2615-2621 (1994).

Appendix I

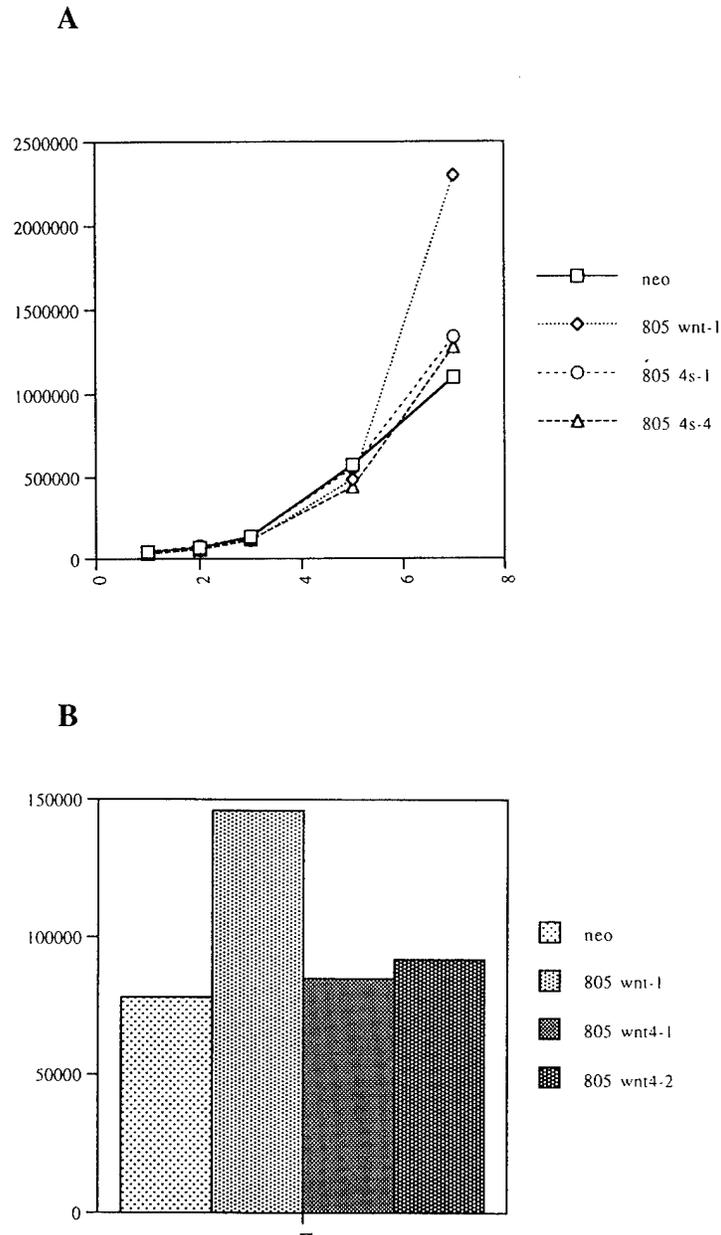
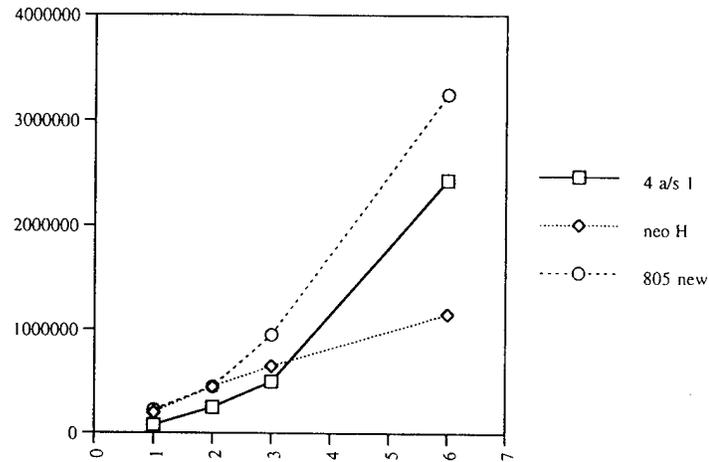


Figure 1. Growth kinetics of C57MG mammary epithelial cells transformed by ectopic wnt-1 expression which have been transfected with wnt-4. Growth curves (A) show two different clones of wnt-1 transformed cells stably re-expressing wnt-4 with similar cell saturation densities to the parental cell line. ³H-thymidine assay (B) demonstrates that wnt-1 transformed cells transfected with wnt-4 incorporate label similar to the parental cell line.

Appendix II

A



B

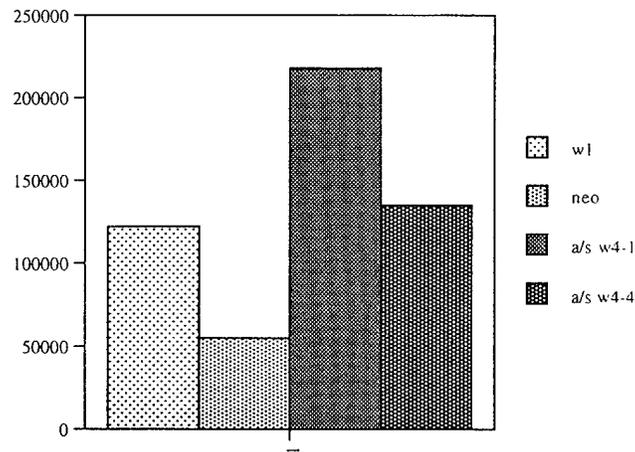
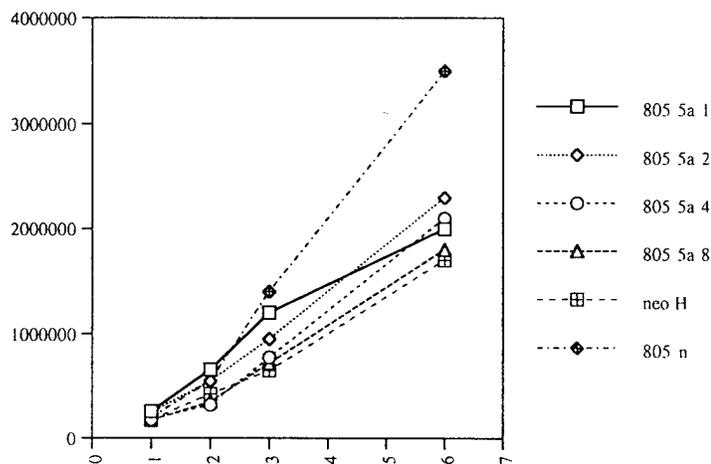


Figure 1. Growth kinetics of normal C57MG mammary epithelial which have been transfected with antisense wnt-4. Growth curves (C) shows one clone stably expressing antisense wnt-4 with a similar cell saturation density to wnt-1 transformed cells. ³H-thymidine assay (D) demonstrates that normal C57MG cells transfected with antisense wnt-4 incorporate label similar to the wnt-1 transformed cell line.

Appendix III

A



B

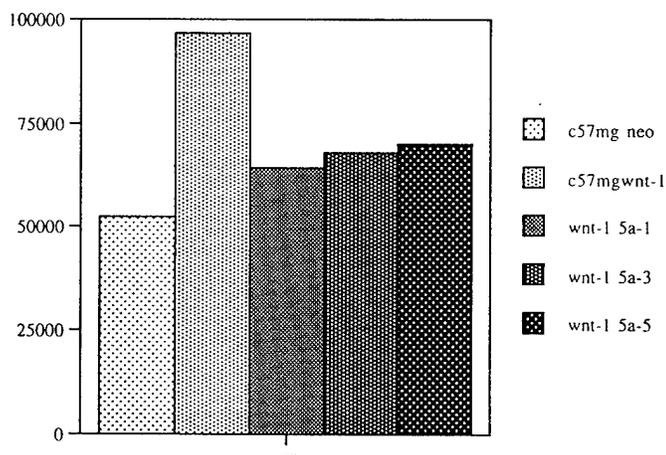
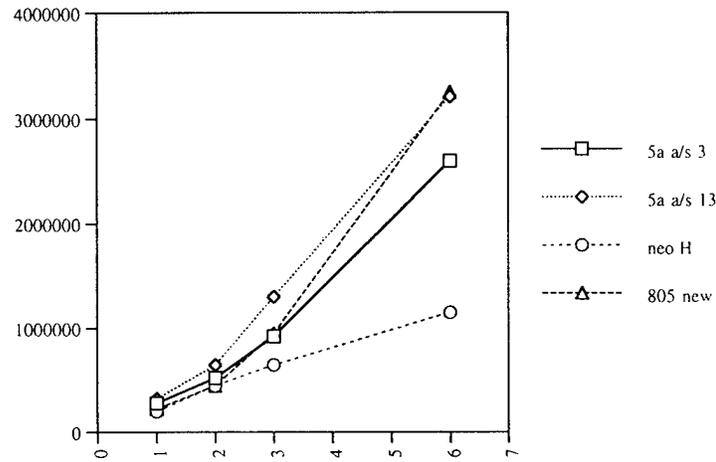


Figure 2. Growth kinetics of C57MG mammary epithelial cells transformed by ectopic wnt-1 expression which have been transfected with wnt-5a. Growth curves (A) show four different clones of wnt-1 transformed cells stably re-expressing wnt-5a with similar cell saturation densities to the parental cell line. ³H-thymidine assay (B) demonstrates three clones of wnt-1 transformed cells transfected with wnt-5a incorporate label similar to the parental cells.

Appendix IV

A



B

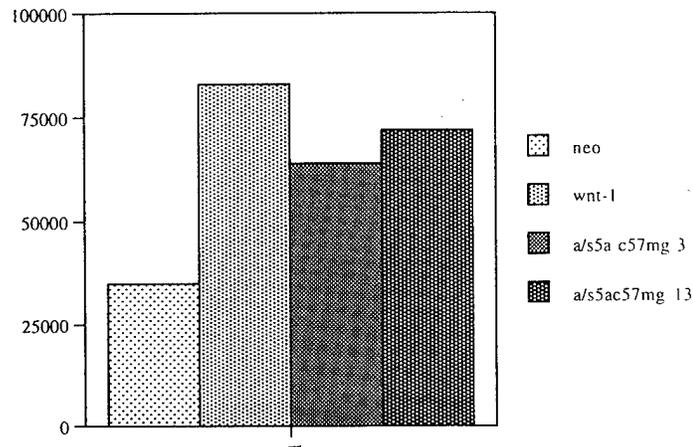


Figure 1. Growth kinetics of normal C57MG mammary epithelial which have been transfected with antisense wnt-5a. Growth curves (C) shows two clones stably expressing antisense wnt-5a with a similar cell saturation density to wnt-1 transformed cells. ³H-thymidine assay (D) demonstrates that normal C57MG cells transfected with antisense wnt-5a incorporate label similar to the wnt-1 transformed cell line.

Appendix V

#112
9/21/95
Post: 26 hrs.

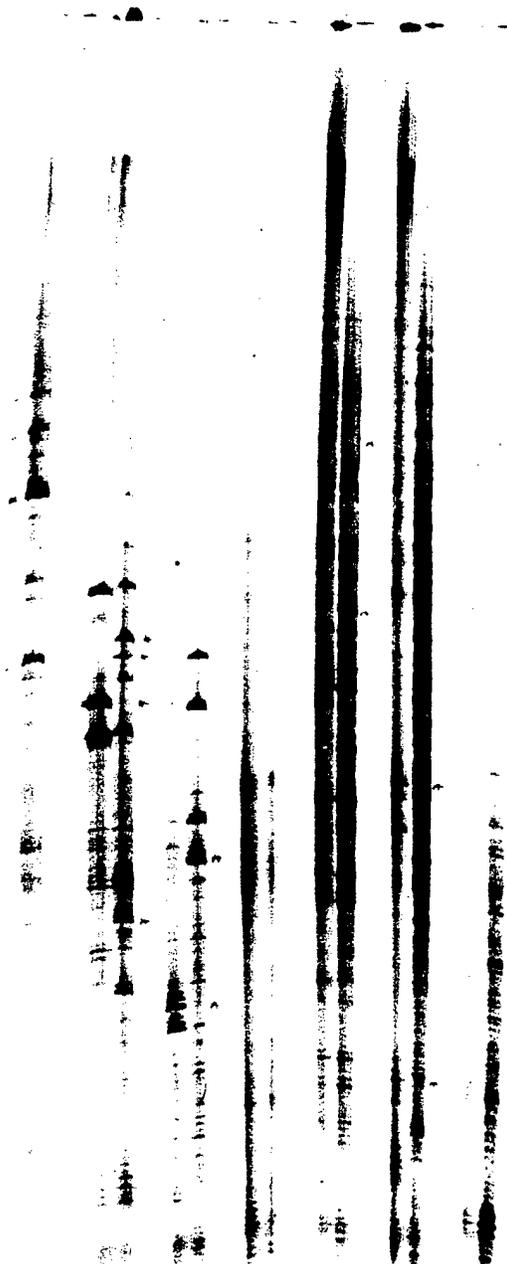


Figure 3. Using PCR based differential display, *wnt-1* transformed cells were directly compared to C57MG parental cells. Several differentially expressed bands are displayed.