

AD \_\_\_\_\_

AWARD NUMBER DAMD17-94-J-4303

TITLE: Mechanisms of Integrin-Mediated Growth Control in Normal, Transformed, and Neoplastic Breast Cells

PRINCIPAL INVESTIGATOR: Elizabeth Wayner, Ph.D.  
Richard Tamura, Ph.D.

CONTRACTING ORGANIZATION: Seattle Biomedical Research Institute  
Seattle, Washington 98109-1651

REPORT DATE: October 1998

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, 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.

19990820047

# 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 <i>(Leave blank)</i>	2. REPORT DATE October 1998	3. REPORT TYPE AND DATES COVERED Annual (30 Sep 97 - 29 Sep 98)	
4. TITLE AND SUBTITLE Mechanisms of Integrin-Mediated Growth Control in Normal, Transformed, and Neoplastic Breast Cells		5. FUNDING NUMBERS DAMD17-94-J-4303	
6. AUTHOR(S) Elizabeth Wayner, Ph.D.  Richard Tamura, Ph.D.			
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Seattle Biomedical Research Institute Seattle, Washington 98109-1651		8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012		10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES		19990820 047	
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited		12b. DISTRIBUTION CODE	
13. ABSTRACT <i>(Maximum 200 words)</i>  We are interested in studying the role of integrins in the regulation of cell proliferation in normal and transformed mammary epithelial cells. During this past year, we have observed a possible difference in the abilities of integrins to stimulate cell proliferation in normal mammary epithelial cells but not in the highly metastatic carcinoma cell line MDA-MB-435. Differences in the strength of this stimulation among integrins may represent integrin-specific effects or possibly the level of cell surface expression. The mechanisms for stimulation of cell cycle progression through G1 by integrins remain unclear. To date, we have not observed significant impacts of integrin clustering on the time course of cyclin Ds expression, Rb phosphorylation, nor the association of growth factor receptors with the integrin complexes. Significant differences between normal and tumor mammary epithelial cells were observed for the expression of cyclin D2 and in the isoforms of the integrin a6, a6A, and a6B. During this next year, we hope to clarify the reason(s) for the inability of integrins to stimulate cell proliferation of MDA-MB-435 cells and the significances of the loss of cyclin D2 expression and changes in integrin a6 isoform expression.			
14. SUBJECT TERMS Breast Cancer  adhesion, ligand-receptor interactions, integrin, transformation, growth, signalling, humans		15. NUMBER OF PAGES 25	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 U.S. 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).

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

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

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

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

Richard N. Tamm 10/21/98  
PI - Signature Date

---

**RESEARCH GRANT DAMD 17-94-J-4303**  
**PRINCIPAL INVESTIGATORS: RICHARD N. TAMURA**  
**AND ELIZABETH A. WAYNER**

**YEAR 4 PROGRESS REPORT: OCTOBER 29, 1998**

**TABLE OF CONTENTS**

	<u>PAGE NUMBERS</u>
Front Cover.....	1
SF 298 Report Documentation Page.....	2
Foreword.....	3
Table of Contents.....	4
I. Introduction.....	5-7
II. Body.....	7-17
A. Methods.....	7-10
B. Results and Discussion.....	10-18
III. Conclusions.....	18,19
IV References.....	20-22
V. Appendix.....	23-25
A. Bibliography of publications and meeting abstracts.	
B. List of personnel.	

## I. INTRODUCTION

### A. Nature of the Problem

Breast cancer is among the leading causes of cancer-related death among women in this country and the incidence of breast cancer among young women is rising. Early detection offers the greatest hope for survival and, in recent years, the mortality rates have started to decline. The past few years have also seen several discoveries that may lead to the development of novel therapeutics in the near future. Continued development of new strategies to improve breast cancer survival will depend upon continued study of the pathogenesis of breast cancer and the events which lead to neoplastic transformation of breast epithelial cells.

Cancer cells differ from normal cells in that they undergo uncontrolled cell growth and acquire the abilities to invade adjacent tissues, enter the circulatory and lymphatic systems, and "home" to sites distal from the primary tumor (metastasis). An important aspect of tumor invasion and metastasis involves the adhesive interactions of tumor cells with other cells and the extracellular matrix. The integrin family of cell adhesion receptors (reviewed by (16)) mediates many of these adhesive interactions. Integrins provide not only a structural means of cell anchorage but also a means of transmitting signals regulating gene expression and protein function (8). Several groups have reported a role of integrins in regulating the progression of cells through the cell cycle (25, 27, 32). It is unclear if significant differences exist between normal and transformed cells. **Our efforts are focused to understand how integrins participate in the regulation of cell division in normal breast cells and to determine how breast cancer cells escape these regulatory pathways.**

### B. Background of Previous Work

**1. Integrins.** Integrins are transmembrane glycoproteins comprised of two non-covalently associated subunits ( $\alpha$  and  $\beta$ ) that mediate both cell-cell and cell-substrate adhesion. Integrin receptors bind extracellular matrix (ECM) and plasma proteins, non-integrin adhesion receptors and other integrins (16). In addition to their role as primary mediators of cell adhesion, it is now become clear that integrins are also capable of transducing signals to the cell interior (8, 12, 16, 18, 19). Tyrosine phosphorylation appears to be a key aspect of integrin mediated signal transduction and a tyrosine kinase (p125 FAK) has been identified which localizes to focal adhesions when cells adhere to an ECM protein-coated surface (30). In addition, evidence is accumulating which suggests that integrin-mediated signaling events can induce gene expression (42) and affect transit through the cell cycle (25, 27, 32). Many of the ECM proteins that serve as ligands for integrins have been identified and include: fibronectin (FN), vitronectin (VN), laminins, and collagens. Some integrins can interact with more than one ligand (e.g.  $\alpha 4 \beta 1$ ,  $\alpha v \beta 3$ ,  $\alpha 3 \beta 1$ ) while several ECM proteins are recognized by more than one integrin. For example, FN has been shown to interact with multiple integrin receptors ( $\alpha 5 \beta 1$ ,  $\alpha 4 \beta 1$ ,  $\alpha v \beta 3$ ,  $\alpha v \beta 6$ ,  $\alpha v \beta 1$ ,  $\alpha 3 \beta 1$ )

**2. Cell cycle Regulation.** The initiation of cell division and transitions between different stages of the cell cycle involves signals that activate the association of specific protein complexes (cell cycle dependent kinases (cdk) with the regulatory cyclins). At each checkpoint certain cyclins and CDKs form active complexes which phosphorylate and thereby activate specific proteins necessary for DNA replication (G1/S), mitosis, and cytokinesis (G2/M). Recently, it has been shown that the activity of cyclin/CDK complexes is regulated by a family of CDK inhibitor proteins or CDIs (p16, p21, p27) that bind to and inactivate the CDKs (15).

The original purpose of this project was to determine how signals transduced via integrin receptors, especially  $\alpha 5 \beta 1$ , regulated the formation of cdk/cyclin complexes in neoplastic and normal breast cells. This tenet was based on observations that suggested that binding of the fibronectin peptide ligand, GRGDS, to the integrin  $\alpha 5 \beta 1$  regulated cell proliferation through modulation of cdc2/cyclinA complexes in partially transformed but not fully transformed epithelial cells (33). However, when we screened a number of mammary carcinoma cell lines, we did not observe a consistent effect GRGDS peptides on either cdc2 kinase activity or cyclinA associated kinase activities which correlated with either the relative degree of transformation or the pattern of expression of RGD-binding integrins. In addition, immunohistochemical analyses suggested that the integrin  $\alpha 5 \beta 1$  was not expressed by either normal mammary epithelial cells or on tumor cells. The stimulation of cdc2 kinase and cyclin A associated kinase activity by RGD peptides along with the stimulation of Rb phosphorylation by integrin clustering in synchronized cultures of normal human mammary epithelial cells suggests that multiple integrins are capable of regulating the cell cycle machinery.

Characterization of normal human mammary epithelial cells (HMEC) either isolated directly from breast reduction tissue or obtained commercially from Clonetics (San Diego, CA) and the mammary epithelial cell line MCF12A suggested that HMEC cultures resembled basal mammary epithelial cells while MCF12A cultures resembled luminal mammary epithelial cells. Thus, the MCF12A cell line may serve as an appropriate model for studying growth regulation of normal luminal epithelial cells, which is the source of most breast carcinomas.

### **3. The Statement of Work and Specific Aims are:**

#### **Statement of Work:**

**The purpose of this project is to determine the roles of integrins in regulating the proliferation of normal and neoplastic mammary epithelial cells.**

#### **Specific Aims:**

**Aim 1: Determine whether all integrins or a subset of integrins are involved in regulating normal mammary epithelial cell proliferation.**

**Aim 2: Determine the mechanisms of integrin-mediated cell cycle regulation.**

**Aim 3. Examine carcinoma cells for possible defects in these mechanisms.****C. Purpose of the Present Work**

Most of the work for the past year has been to expand our studies to include the MCF12A and MDA-MB-435 cell lines. We have also attempted to identify possible mechanisms of integrin regulation of the cell cycle by looking at effects on RB phosphorylation and the expression of cell cycle cyclins (cyclin D1, D2, D3 and cyclin A, primarily).

**II. BODY****A. Methods****1. Patient Samples**

Tissue specimens were obtained from lumpectomy and mastectomy tissues provided by Dr. Ron Tickman who is a staff pathologist in the Laboratory of Pathology, Swedish Hospital Medical Center, Seattle, WA and used for immunohistochemistry and the isolation of breast cells (BC). These samples were removed from patients undergoing surgery at Swedish Hospital solely for diagnostic or therapeutic purposes and would otherwise be discarded. Samples were coded to permit access to information concerning the patient's age and sex, tumor histology, estrogen and progesterone receptor status, and the presence and location of metastases. This will enable me to correlate tumor type, malignancy, integrin expression and response to integrin ligation. Although no patient samples will be excluded on the basis of race, age, sex, religion, or ethnic background, the low incidence of male breast cancer will preclude collection of a large number of samples from men. I have received IRB approval from both Swedish hospital and SBRI for the use of these tissues (See Appendix).

**2. Monoclonal and Polyclonal Antibodies**

**Anti-integrin Monoclonal Antibodies.** Several monoclonal antibodies (Mabs) directed to a variety of integrin receptors expressed by normal and neoplastic epithelial cells have been produced, previously (6, 7, 38-40). Many of these Mabs perturb integrin function and will be used to determine how normal and neoplastic breast cells interact with FN or GRGDS peptide. These Mabs include the two used by Dr. Symington to ligate  $\alpha 5$  or  $\beta 1$  (P1D6 (anti-  $\alpha 5$ ) and P4C10 (anti-  $\beta 1$ )) as well as inhibitory antibodies P1H5 (anti- $\alpha 2$ ), P1B5 ( $\alpha 3$ ), P4C2 ( $\alpha 4$ ), P5H9 ( $\alpha v \beta 5$ ), LM609 ( $\alpha v \beta 3$ , courtesy of Dr. David Cheresh, The Scripps Research Institute, La Jolla, CA), and GoH3 (anti- $\alpha 6$ , Pharmingen, San Diego, CA). Other anti-integrin antibodies include AA3 (anti- $\beta 4$ , courtesy of Dr. Vito Quaranta, The Scripps Research Institute, La Jolla, CA), 3E1 (anti- $\beta 4$ , Chemicon Inc., Temecula, CA), and L230 (anti- $\alpha V$ , American Type Culture Collection, Rockville, MD).

**Antibodies Directed to Human Cell Cycle Proteins.** Monoclonal or polyclonal antibodies directed to human CDKs (cdc2, cdk2-cdk5), cyclins (A, B1, D1, D2, D3, E), CDIs (p16, p21, p27), and tumor suppressor proteins (Rb and p53) were obtained from Pharmingen (San Diego, CA).

### **3. Breast Cell Isolation and Culture**

Normal human mammary epithelial cells (HMEC) were obtained from Clonetics Corp (San Diego, CA, cat. # CC-0228) or from reduction mammoplasty tissue supplied by Dr. Ron Tickman. Breast epithelial cells were isolated according to published protocols (2, 4, 36). Briefly, the samples were minced and enzymatically digested to isolate mammary glands and single cells. After an initial plating in serum-containing medium, cells were cultured in serum-free mammary epithelial growth medium (MEGM) from Clonetics (cat. #CC-3051) or in DFCI-1 medium (1). Dr. Vito Quaranta, (The Scripps Research Institute, La Jolla) generously provided the breast carcinoma cell line, MDA-MB-435.

### **4. Flow Cytometry Analysis**

Flow cytometry analysis was used to identify the integrin receptors expressed by cultured normal and transformed BC lines. Cells were incubated with anti-integrin Mabs (10 µg/ml or culture supernatant) for 30 min in suspension in FACS buffer (HBSS supplemented with 1% goat serum and 0.02% sodium azide). Cells were then washed and incubated with affinity purified FITC-conjugated goat anti-mouse, 2 mg/ml (Southern Biotechnology, Birmingham, AL). The appropriate isotype matched controls were included with each sample. Cells were analyzed by forward light scatter (linear) versus green fluorescence (log). Flow cytometric analysis of stained cells were performed on either a Coulter EPICS C System or a Becton Dickinson FACScan equipped with an argon laser. At least 5,000 events were analyzed for each anti-integrin antibody and compared to a matched isotype control.

### **5. Integrin clustering**

Cells were first synchronized by culturing in the absence of any serum or exogenously added growth factors for 36 to 48 h. Monoclonal antibodies to specific integrin subunits were bound to the cells for 30 minutes at 4°C in growth factor-free media. Unbound primary antibodies were washed from the cells. The bound monoclonals were then cross-linked with the appropriate polyclonal secondary (rabbit anti-mouse or rabbit anti-rat) in media containing reduced levels of growth factors (1/10 to 1/100 of the normal media) and the cells were placed back at 37° C. Maximal clustering occurred approximately 30 minutes after addition of the cross-linking secondary antibodies and could often be detected within 15 minutes by immunofluorescence. Cells were lysed by scraping the cells into lysis buffer (1% NP-40 in 50 mM Tris-HCl pH 7.5, 250 mM NaCl, 2 mM EDTA, 50 mM sodium fluoride, 0.1 mM sodium orthovanadate, 1 mM PMSF, 10 µg/ml leupeptin, and 10 µg/ml aprotinin).

## 6. SDS-PAGE/ Western blots.

The protein concentration of each lysate was determined using the BCA protein assay kit (Pierce, Rockford, IL). Fifty or 100 micrograms of total protein were precipitated (41), resuspended in reducing sample buffer, and separated by SDS-PAGE. For immunoprecipitations, monoclonal antibodies were bound to goat anti-mouse Sepharose (Zymed, South San Francisco, CA) and incubated with 100  $\mu$ g of total cell lysate overnight at 4°C. Immunoprecipitations were washed extensively with lysis buffer, eluted in reducing sample buffer, and separated by SDS-PAGE. Proteins were then transferred to PVDF membranes (Biorad, Hercules, CA) and probed with antibodies to the molecule of interest (retinoblastoma protein, cyclins, growth factor receptors). Bound antibodies were detected using either the colorimetric system of alkaline phosphatase conjugated to secondary antibodies along with NBT and BCIP as substrates (Promega, Madison, WI) or the ECL system of peroxidase conjugated secondary antibodies along with the ECL+Plus Western blotting detection system (Amersham, Arlington Heights, IL).

## 7. 96 well antibody-binding cell proliferation assays.

96 well microtiter plates, which were pre-coated with goat anti-mouse antibodies, were obtained from Pierce (Rockford IL). Plates were blocked with 0.5% heat-denatured bovine serum albumin (BSA) in phosphate buffered saline (PBS). Monoclonal antibodies diluted in blocking buffer were added at 2  $\mu$ g/well in triplicate and incubated overnight at 4° C. Wells were washed three times with PBS and 3000 cells were added per well in media containing 1/100 dilution of serum or growth factors. Prior to addition to the wells, the cells were synchronized by serum deprivation for either 24 h (MDA-MB-435) or 48 h (MCF12A). The time of synchronization was based on growth curves under varying serum concentrations. Cultures were allowed to grow for 2-5 days at which time cell growth was assessed using the Cell Titer 96<sup>®</sup> Aqueous One Solution Reagent (Promega, Madison WI). This assay is based upon the ability of viable cells to reduce the tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] (3, 10). Absorbance of each well was read at 490 nm in a plate reader.

## 8. Integrin $\alpha$ 6 RT-PCR

Cells (HMEC, MCF12A, MDA-MB-435) were grown to near confluence. Frozen tissue sections of normal breast, in situ/invasive carcinoma, invasive carcinoma, and lymph node metastasis were obtained from the Department of Pathology,

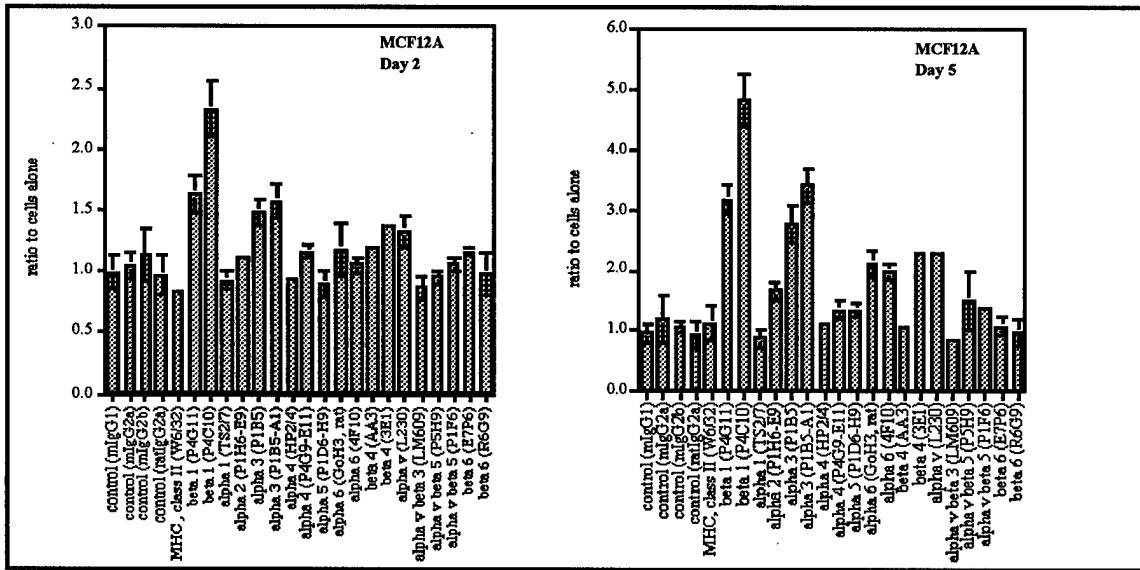
University of Michigan. Tissue sections were scraped into 1.5 ml eppendorf tubes. Total RNA was isolated from either cultured cells or frozen tissue sections by solubilization directly into TRIZOL<sup>®</sup> Reagent (Life Technologies, Gaithersburg, MD) as described by manufacturer's protocol. First strand cDNA was synthesized using the SuperSCRIPT<sup>™</sup> Pre-amplification Kit (Life Technologies, Gaithersburg, MD). PCR reactions for the integrin  $\alpha$ 6 alternatively spliced isoforms utilized nested oligonucleotides

(1156/1157-outer pair; 1681/2002-inner pair) as described previously(35). PCR products were resolved on TBE-agarose gels and visualized by ethidium bromide staining. Representative pcr products were subcloned using the TA Cloning® Kit (Invitrogen, San Diego, CA) and sequenced on an ABI Sequencer to verify that the products corresponded to either  $\alpha 6A$  or  $\alpha 6B$ .

## B. Results and Discussion

Last year we reported that multiple integrins were capable of regulating cell cycle progression in normal human mammary epithelial cells (HMEC). That is, cell proliferation was stimulated in HMEC cultures synchronized by growth factor deprivation when cells were plated on ECM proteins (collagen I or laminin5) or when integrins were clustered using monoclonal antibodies cross-linked by polyclonal antibodies.

During this past year, we expanded our studies to the normal mammary epithelial cell line MCF12A and to the metastatic breast carcinoma cell line MDA-MB-435. Cells synchronized by serum deprivation were plated onto wells coated with monoclonal antibodies to integrins in media containing 0.1% fetal bovine serum. The number of cells present in each well was assayed 2-5 days after plating. MCF12A cells were stimulated to proliferate by antibodies to integrin subunits  $\beta 1$ ,  $\alpha 3$ ,  $\beta 4$ ,  $\alpha 6$ , and  $\alpha V$  (Fig 1).



**Fig. 1. Stimulation of MCF12A cell proliferation by anti-integrin antibodies in 0.1% FBS.** Synchronized cultures of MCF12A cells were plated onto 96 well microtiter plates coated with anti-integrin antibodies in medium containing 0.1% FBS. Cell proliferation was assayed 2 and 5 days later as described in Methods. The data are expressed as a ratio of O.D. 490 nm values relative to cells plated on wells containing just the capturing goat anti-mouse antibodies.

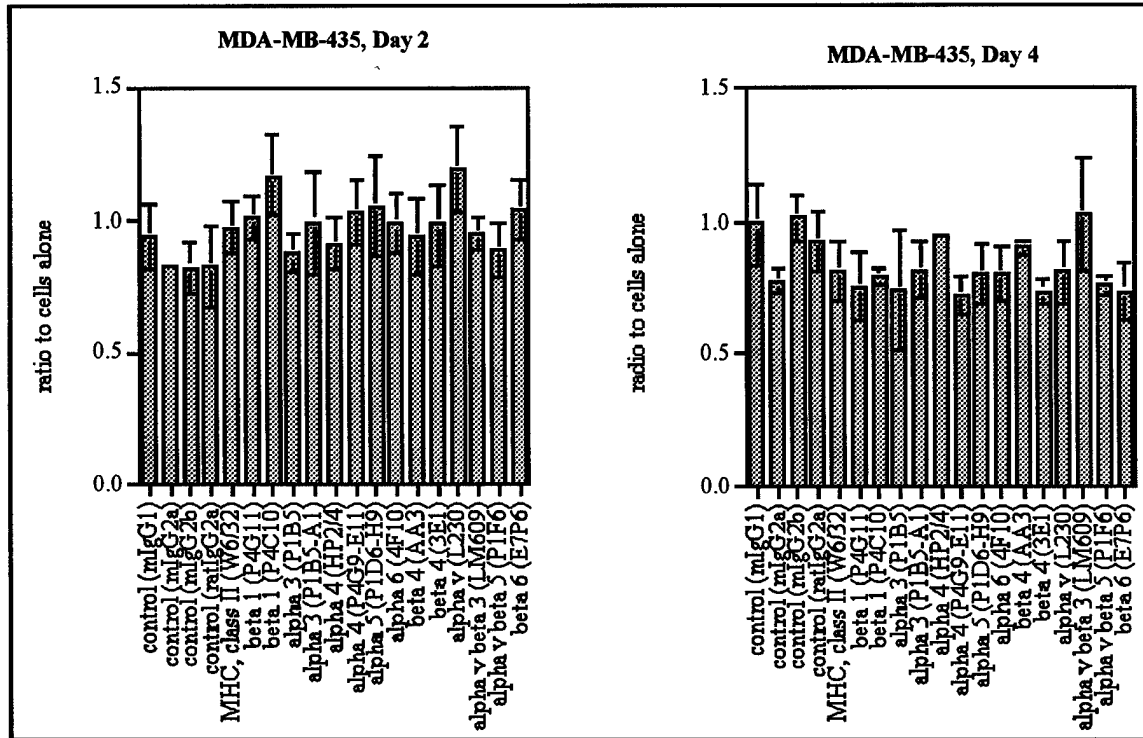
Antigen (MAb clone)	MCF12A (MFI)	cell proliferation ratio, day5
control (anti-mIgG-FITC)	7.9	1.0 (cells alone)
control (mIgG1)	7.8	0.94
control (mIgG2a)	7.7	1.18
control (mIgG2b)	7.7	1.05
control (anti-ratIgG-FITC)	8.2	ND
control (rIgG)	63.8	0.93
$\beta$ 1 (P4C10-D4)	158.9	4.82
$\alpha$ 2 (P1H6-E9)	39.3	1.65
$\alpha$ 3 (P1B5)	173.9	2.75 and 3.42
$\alpha$ 4 (P4G9-E11)	16	1.33
$\alpha$ 5 (P1D6)	12.3	1.34
$\alpha$ 6 (GoH3, rat)	520.4	2.12
$\beta$ 4 (3E1)	212.9	2.29
$\alpha$ V (L230)	42.8	2.28
$\alpha$ V $\beta$ 3 (LM609)	7.9	0.82
$\alpha$ V $\beta$ 5 (P5H9-E3)	19.8	1.49

**TABLE I Comparison of MCF12A FACS and cell proliferation data.** The mean fluorescence index (MFI) obtained by FACS analysis of an asynchronous culture of MCF12A cells is compared with cell proliferation ratio plotted in Fig 1.

Antibodies to integrin subunits  $\alpha$ 2,  $\alpha$ 4,  $\alpha$ 5, and integrin  $\alpha$ v $\beta$ 5 had minimal if any effect. Antibodies to MHC class I complex (W6/32) did not stimulate cell proliferation. These results may reflect differences among integrins to regulate cell proliferation or may reflect the relative level of integrin expression on the cell surface (Table I). That is, there may be a "threshold" along the integrin signaling pathways that must be reached before the signal to initiate cell proliferation is transmitted. We have not yet determined whether or not serum deprivation alters integrin expression.

By contrast, plating MDA-MB-435 cells onto anti-integrin antibody coated wells produced effect either 2 days or 4 days after plating (Fig 2). These results suggest that there may be defects in the integrin regulation of cell proliferation in some breast carcinomas. Alternatively, the assay may need to be optimized for MDA-MB-435 cells. A reduction of media components by 50% resulted in a greatly reduced stimulation of cell proliferation in MCF12A cells (data not shown). Studies are underway to look at the effects of media conditions on this assay. Since MDA-MB-435 cells are highly metastatic, it is also possible that they produce levels of extracellular proteases that degrade the anti-integrin antibodies to disrupt the assay. However, extracellular

collagenase activity (zymogram gels) was barely detected in the media of cells cultured in 0.1% FBS (data not shown).

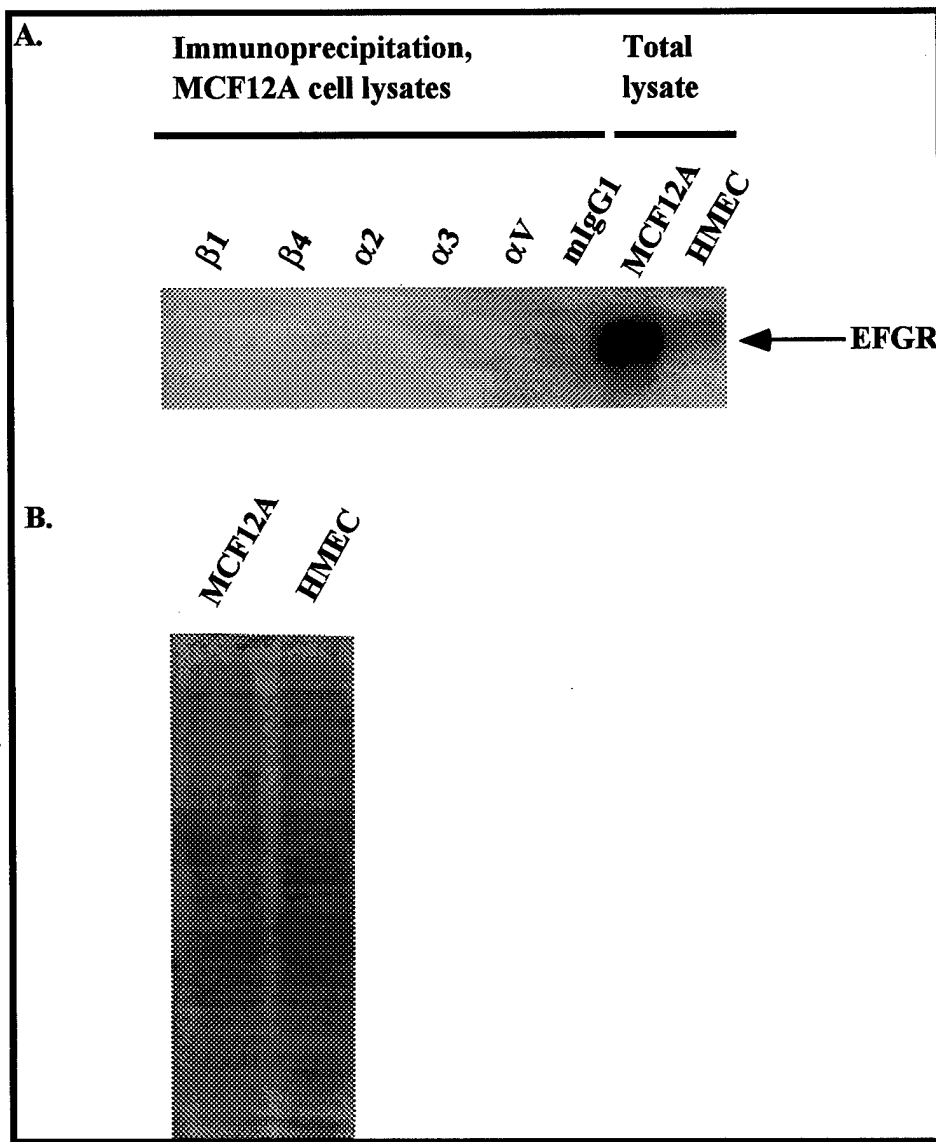


**Fig. 2. Cell proliferation of MDA-MB-435 carcinoma cells is not stimulated by anti-integrin antibodies in 0.1% FBS.** Synchronized cultures of MDA-MB-435 cells were plated onto 96 well microtiter plates coated with anti-integrin antibodies in medium containing 0.1% FBS. Cell proliferation was assayed 2 and 5 days later as described in Methods. The data are expressed as a ratio of O.D. 490 nm values relative to cells plated on wells containing just the capturing goat anti-mouse antibodies.

One possible mechanism of integrin regulation of cell proliferation is through the association and activation of growth factor receptors with activated integrin clusters as suggested by Plopper et al (28) and Miyamoto et al. (26). To date, we have not observed an association of growth factor receptors with integrins. Western blot analyses of integrins immunoprecipitated from MCF12A whole cell lysates did not detect an association with EGF receptor (EGFR) although EGFR was readily detected in the whole cell lysate (Fig 2A). The level of EGFR in MCF12A cells is much higher than in normal HMEC. Fig 2B shows that equivalent amounts of protein were analyzed in the MCF12A and HMEC cell lysates.

An initial experiment suggested that the EGF-like growth factor receptor erbB2 may be associated with  $\beta 1$  integrins in MCF12A cells. However, we have been unable to demonstrate this unequivocally and we have also been unable to detect erbB2 in whole

cell lysates of either MCF12 A or HMEC cells. We also have not detected FGFR or PDGFR in co-immunoprecipitation experiments with integrins. Although the integrin  $\alpha$

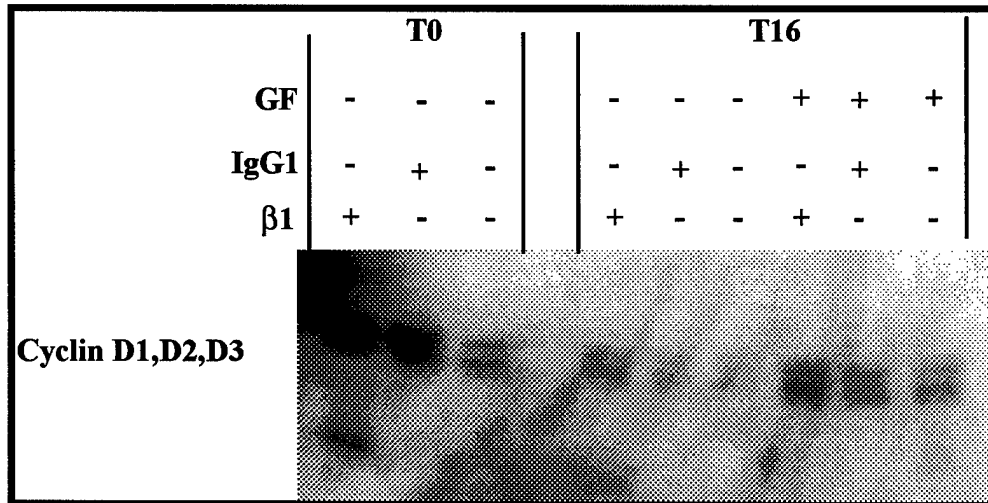


**Fig. 2 EGFR does not appear to be associated with integrins, but is expressed at much higher levels in MCF12A cells than in HMEC.** A) Western blot for EGFR in integrin immunoprecipitations of MCF12A cells or total cell lysates of MCF12A or HMEC. B) Coomassie-blue staining of total cell lysate lanes of blot membrane used for Western blot in A.

and  $\beta$  subunits remain associated during immunoprecipitation, it is possible that our lysis or immunoprecipitation buffers are too harsh and are disrupting weaker protein-protein binding interactions. To address this possible problem with the co-immunoprecipitation experiments, we attempted to look for co-localization of growth factor receptors with integrin clusters by immunofluorescence of acetone-fixed cells. Integrin clusters were

readily detected between 15 to 30 minutes after cross-linking with polyclonal secondary antibodies. Unfortunately, we have been unable to obtain a clear understanding of what is happening to the distribution of growth factor receptors (EGFR, erbB2, FGFR, or PDGFR) during the formation of these integrin clusters.

Cell synchronization by the removal of growth factors or serum blocks cell growth in the G1 phase of the cell cycle. Although cyclin D1 has been reported to be quite labile upon removal of growth factors (22-24), growth factor deprivation for 26 h did not eliminate cyclin Ds completely from HMEC cultures (Fig 3, T0 lanes). The appearance of a third band migrating slightly faster than the other two bands in the last 3 lanes of this blot suggests that cyclin D3 expression is induced by the re-addition of growth factors (Fig 3, T16, GF+ lanes). Clustering of  $\beta 1$  integrins at the time release did not appear to have a significant impact on the levels of cyclin D(s).

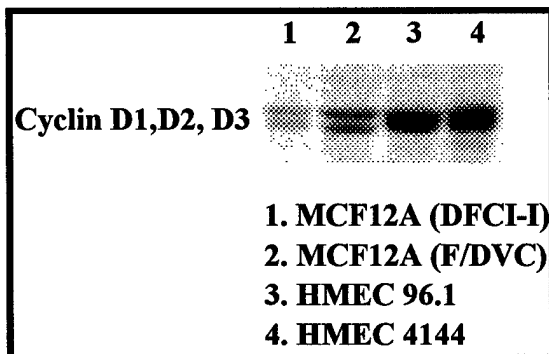


**Fig. 3. Clustering of  $\beta 1$  integrins does not appear to alter the expression of cyclin D(s).** Beta 1 integrins on HMEC cells were clustered using monoclonal antibodies in the absence (-) or presence (+) of growth factors. IgG1 samples are cells incubated with nonspecific IgG1 instead of the anti- $\beta 1$  monoclonal antibody, P4C10. Twenty five micrograms of total cell lysate protein was separated by SDS-PAGE, transferred to Immobilon membrane, and probed with monoclonal antibodies which recognize cyclins D1, D2, and D3. The lower molecular weight band present in the T0,  $\beta 1$  (+) lane is the light chain from P4C10 which is recognized by the secondary antibody-enzyme conjugate used in the Western blot procedure.

We need to utilize monoclonal antibodies specific to cyclin D1, D2, or D3 which are not cross reactive to determine which cyclin Ds are being observed in this experiment (see Fig 5). Also, since Rb phosphorylation began around 8 hours after integrin clustering (1997 report, Fig 3), these studies should be repeated with time points between 0 and 8 hours after integrin clustering. We have also looked at the time course of cyclin A expression upon release from the serum-deprivation block. Cyclin A proteins were first detected 16 h after release of MCF12A cells into media containing 0.1% FBS or 1.0% FBS and

increased in amount up to 24 h (data not shown). However, we do not know if integrin clustering alters this time course of expression.

Analyses of detergent lysates of asynchronous cultures of HMEC, MCF12A, and MDA-MB-435 cells revealed that cyclin D2 was expressed by normal HMEC cells but not by either MCF12A or MDA-MB-435 cell lines. Initial comparison between MCF12A and normal HMEC cells with monoclonal antibodies cross-reactive with cyclins D1, D2, and D3 suggested that MCF12A expressed cyclins D1 and D3, but not D2 and that normal HMEC cells expressed cyclins D1 and D2, but little or none of cyclin D3 (Fig 4). Two different cultures of normal HMEC were analyzed: HMEC96.1 was isolated in this laboratory from a breast reduction specimen and HMEC 4144 was purchased from Clonetics (San Diego, CA). Two different cultures of MCF12A cells were also analyzed: cells cultured in the presence of 10% FBS (F/DV-C) or cells adapted to growth in the HMEC-defined growth medium, DFCI-1.

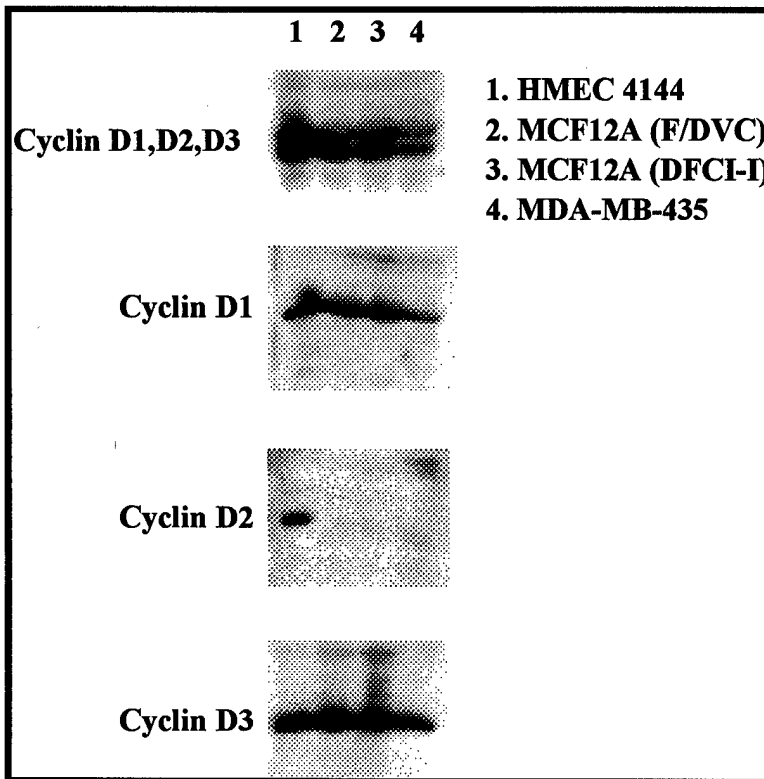


**Fig. 4. Expression of cyclins D1, D2 and D3 in MCF12A and normal HMEC cells.** One hundred micrograms of total cell lysate protein from asynchronous cultures were separated by SDS-PAGE, transferred to Immobilon membrane, and probed with monoclonal antibodies cross-reactive with cyclins D1, D2, and D3. The migration of bands suggest that MCF12A express cyclins D1 and D3 but not D2 while HMEC express cyclins D1 and D2, but not D3.

These results were confirmed using monoclonal antibodies specific for cyclin D1, cyclin D2, or cyclin D3. Fig 5 demonstrates that cyclin D1 and cyclin D3 are expressed by HMEC, MCF12A, and MDA-MB-435 cells while cyclin D2 is expressed only by HMEC cells. The predominant cell type in the normal HMEC cultures correspond to basal mammary epithelial cells. However, there are luminal cells present and these cultures have a limited life span before undergoing terminal differentiation. Studies are currently underway to determine if the expression of cyclin D2 is restricted to a particular cell population within the HMEC cultures.

Numerous attempts were made to determine if integrin clustering stimulated Rb phosphorylation in MCF12A and MDA-MB-435 cells. Unfortunately, we encountered a myriad of technical problems that have prevented us from determining the impact of integrin signaling on G1 cell cycle progression in these cell lines. Some of these problems

involved insufficient resolution of phosphorylated forms of Rb possibly due to the use of different protein precipitation techniques (acetone or trichloroacetic acid instead of our standard use of chloroform/methanol), use of an incorrect acrylamide percentage during SDS-PAGE, and high backgrounds during the Western blot procedure. There are also a number of parameters, such as the length of time for cell synchronization and the concentration of serum or growth factors, which may require optimization for each cell type or cell line. For example, Fig 3 (T16, Gf (-) lanes) demonstrates that levels of cyclin D1 and cyclin D2 decreased further when HMEC cultures were maintained for an additional 16 h in growth factor-free media. Also, the addition of growth factors/serum at 1/100 dilution of the standard media concentration is sufficient to stimulate some cell growth in cells synchronized in growth factor/serum-free media.

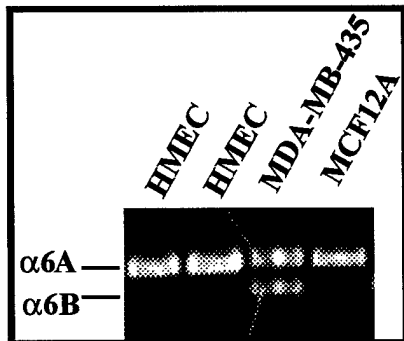


**Fig. 5. Cyclin D2 is expressed by HMEC cells but not by MCF12A or MDA-MB-435.** Western blot analyses of 50 mg of total cell lysate protein from asynchronous cultures of HMEC, MCF12A, or MDA-MB-435 cells for the expression of cyclins D1, D2, and D3. Monoclonal antibodies were either cross-reactive with all three cyclin D(s) or were specific to only one cyclin D.

The integrin  $\alpha 6$  exists as two alternatively spliced forms ( $\alpha 6A$  and  $\alpha 6B$ ) that differ in their cytoplasmic domains (9, 14, 35). In general, the  $\alpha 6A$  isoform is expressed by normal epithelial cells, such as epidermal keratinocytes while  $\alpha 6B$  is expressed by most carcinomas and tends to be the predominant form expressed by teratocarcinoma cell lines and totipotent or pluripotent cells such as mouse embryonic stem cells. The expression

of  $\alpha 6B$  may have a direct role in epidermal tumorigenesis (37). We were therefore interested in characterizing the pattern of  $\alpha 6$  isoform expression in normal HMEC and mammary carcinomas. Analyses by RT-PCR revealed that normal HMEC and the normal mammary epithelial cell line MCF12A express only the  $\alpha 6A$  isoform while the highly metastatic mammary carcinoma cell line MDA-MB-435 expresses both  $\alpha 6A$  and  $\alpha 6B$  (Fig 6).

In order to determine if these observations are relevant *in vivo*, we attempted to isolate RNA from frozen sections of normal breast, *in situ*/invasive ductal carcinoma, invasive

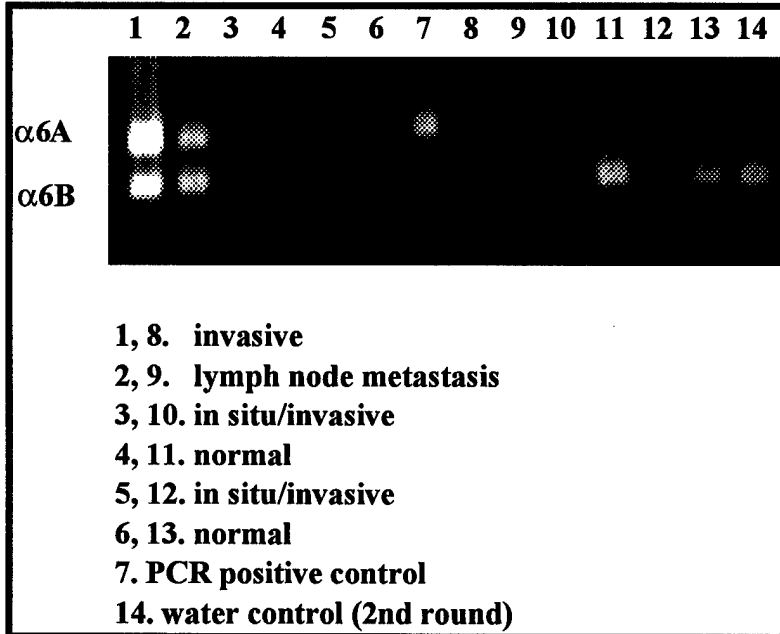


**Fig. 6. The integrin  $\alpha 6B$  isoform is expressed by MDA-MB-435 cells but not by HMEC nor MCF12A cells.** Total RNA was isolated from HMEC, MDA-MB-435, or MCF12 and analyzed for  $\alpha 6A$  and  $\alpha 6B$  expression by RT-PCR as described in Methods.

carcinoma, and a lymph node metastasis for  $\alpha 6$  RT-PCR. The invasive carcinoma and the lymph node metastasis appear to express both isoforms of the integrin  $\alpha 6$  (Fig 7). We have been unable to obtain reproducible PCR products from normal or *in situ*/invasive tissue sections. Hematoxylin and Eosin stained sections from these tissues show that the invasive carcinoma or lymph node metastasis samples have much higher cellular content than the normal or *in situ*/invasive samples which probably accounts for the differences in obtaining  $\alpha 6$  PCR products. These results do not demonstrate that the breast cancer cells express both  $\alpha 6$  isoforms. These assays have also been plagued by contamination in the negative control reactions which have produced an apparent  $\alpha 6B$  band in samples not reacted with reverse transcriptase and sometimes in the water only control (Fig 7, lanes 11, 13, and 14). The presence of an apparent  $\alpha 6B$  PCR product in the water control for the nested oligo pair suggests that either these oligos are contaminated with  $\alpha 6B$  or that the oligos are generating an artifactual product the same size as  $\alpha 6B$ . We are in the process of cloning and sequencing this PCR product to get a better understanding of how to correct this problem.

We will continue our studies of  $\alpha 6A$  and  $\alpha 6B$  expression during breast cancer progression. We will attempt to isolate RNA from blocks of tissue frozen in O.C.T.(17)

which would obviate the problem of amounts of starting material encountered when starting from tissue sections. If there appears to be a pattern of  $\alpha 6$  isoform expression that correlates with the progression of breast cancer, we will attempt to identify the cell type(s) expressing each isoform by oligonucleotide hybridization of tissue sections (21).



**Fig 7. The  $\alpha 6B$  integrin isoform is expressed in invasive breast carcinoma and breast lymph node metastatic tissue.** Total RNA was isolated from frozen tissue sections and analyzed for  $\alpha 6A$  and  $\alpha 6B$  by RT-PCR as described in Methods. Lanes 8-13 were RT-PCR reactions in which RT was omitted from the first strand cDNA synthesis reaction. Lane 14 is a water control using only the nested set of oligos (2nd round).

### III. CONCLUSIONS

To date, the cell proliferation assay using antibody coated plates has provided the most consistent and compelling evidence for integrin-mediated regulation of cell proliferation. Initial results suggest that integrin regulation occurs in normal (MCF12A) but perhaps not in metastatic (MDA-MB-435) mammary epithelial cells. However, these results require a more extensive examination of assay conditions (e.g. serum concentrations, media compositions) and a more extensive examination of cell response (cell attachment, spreading, death) during the course of the assay. Differences in the strength of stimulation may reflect functional differences among the integrins or the level of integrin cell surface expression.

The mechanism(s) of integrin regulation of cell proliferation are still unclear. We have not observed a significant impact of integrin clustering on either the level or time course of

cyclin D(s) expression or Rb phosphorylation. We also have not observed an association of growth factor receptors (EGFR, c-erbB2, PDGF, FGFR) with integrins.

We will utilize the antibody cell proliferation assay to optimize our cell synchronization experiments for length of cell synchronization and growth factors/serum concentrations. We will then use these parameters to determine if integrin clustering alters the levels or time course of expression of various cell cycle components (cyclin D(s), cyclin A, cyclin E, and Rb phosphorylation). We will also expand our analyses to the expression of a number of cyclin dependent kinase inhibitors: p15, p16, p18, p19, p21, p27, and p57.

We have observed that the G1 cyclin, cyclin D2, is expressed in cultures of normal HMEC but not by either the normal mammary epithelial cell line, MCF12A, or the metastatic carcinoma cell line, MDA-MB-435. Several other human breast carcinoma cell lines (MCF-7, ZR-75, T-47D) and other breast cell lines (HBL-100, MCF-10A) have been reported to lack cyclin D2(31, 43). Buckley et al (5) also reported that the expression of cyclin D2 was lower in breast cancer cell lines than in cultures of normal breast epithelial cells. Thus, it appears that the establishment of a cell, normal or tumorigenic, may be correlated with a loss of cyclin D2 expression. The significance of this loss in expression is unclear. Cyclin D1, D2 and D3 are a family of related proteins involved in regulation of progression through the G1 phase of the cell cycle. However, several differences have been reported among the cyclin D(s) for their binding to specific cdk(s), the formation of active or inactive kinase complexes, and binding to Rb protein (31,11, 13, 20, 34). In the study of mouse mammary tumorigenesis, Said et al (29) suggested that cyclin D2 binding to cdk4 in hyperplasias may be an attempt to maintain or induce differentiation of the mammary epithelial cells. Further investigation into the functions of cyclin D2 in normal human mammary epithelial cells appears warranted. We will examine the expression of cyclin D2 and its associations with cdk2 and cdk4 during progression through the cell cycle.

Preliminary results also suggest that the expression of the integrin  $\alpha 6B$  isoform may correlate with tumorigenesis. We will continue our efforts to determine if such a correlation exists and examine how  $\alpha 6B$  may contribute to the progression of breast cancer.

---

**IV REFERENCES**

1. Band, V., and R. Sager. 1989. Distinctive traits of normal and tumor-derived human mammary epithelial cells expressed in a medium that supports long-term growth of both cell types. *Proc Natl Acad Sci U S A.* 86:1249-53.
2. Bergstraesser, L. M., and S. A. Weitzman. 1993. Culture of normal and malignant primary human mammary epithelial cells in a physiological manner simulates in vivo growth patterns and allows discrimination of cell type. *Cancer Res.* 53:2644-54.
3. Berridge, M. V., and A. S. Tan. 1993. Characterization of the cellular reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT): subcellular localization, substrate dependence, and involvement of mitochondrial electron transport in MTT reduction. *Arch Biochem Biophys.* 303:474-82.
4. Blaschke, R. J., A. R. Howlett, P. Y. Desprez, O. W. Petersen, and M. J. Bissell. 1994. Cell differentiation by extracellular matrix components. *Methods Enzymol.* 245:535-56.
5. Buckley, M. F., K. J. Sweeney, J. A. Hamilton, R. L. Sini, D. L. Manning, R. I. Nicholson, A. deFazio, C. K. Watts, E. A. Musgrove, and R. L. Sutherland. 1993. Expression and amplification of cyclin genes in human breast cancer. *Oncogene.* 8:2127-33.
6. Carter, W. G., P. Kaur, S. G. Gil, P. J. Gahr, and E. A. Wayner. 1990. Distinct functions for integrins alpha 3 beta 1 in focal adhesions and alpha 6 beta 4/bullous pemphigoid antigen in a new stable anchoring contact (SAC) of keratinocytes: relation to hemidesmosomes. *J Cell Biol.* 111:3141-54.
7. Carter, W. G., E. A. Wayner, T. S. Bouchard, and P. Kaur. 1990. The role of integrins alpha 2 beta 1 and alpha 3 beta 1 in cell-cell and cell-substrate adhesion of human epidermal cells. *J Cell Biol.* 110:1387-404.
8. Clark, E. A., and J. S. Brugge. 1995. Integrins and signal transduction pathways: the road taken. *Science.* 268:233-9.
9. Cooper, H. M., R. N. Tamura, and V. Quaranta. 1991. The major laminin receptor of mouse embryonic stem cells is a novel isoform of the alpha 6 beta 1 integrin. *J Cell Biol.* 115:843-50.
10. Cory, A. H., T. C. Owen, J. A. Barltrop, and J. G. Cory. 1991. Use of an aqueous soluble tetrazolium/formazan assay for cell growth assays in culture. *Cancer Commun.* 3:207-12.
11. Ewen, M. E., H. K. Sluss, C. J. Sherr, H. Matsushime, J. Kato, and D. M. Livingston. 1993. Functional interactions of the retinoblastoma protein with mammalian D-type cyclins. *Cell.* 73:487-97.
12. Ginsberg, M. H., X. Du, and E. F. Plow. 1992. Inside-out integrin signalling. *Curr Opin Cell Biol.* 4:766-71.
13. Higashi, H., I. Suzuki-Takahashi, S. Saitoh, K. Segawa, Y. Taya, A. Okuyama, S. Nishimura, and M. Kitagawa. 1996. Cyclin-dependent kinase-2 (Cdk2) forms an inactive complex with cyclin D1 since Cdk2 associated with cyclin D1 is not phosphorylated by Cdk7- cyclin-H. *Eur J Biochem.* 237:460-7.
14. Hogervorst, F., I. Kuikman, A. G. van Kessel, and A. Sonnenberg. 1991. Molecular cloning of the human alpha 6 integrin subunit. Alternative splicing of alpha 6 mRNA and chromosomal localization of the alpha 6 and beta 4 genes. *Eur J Biochem.* 199:425-33.
15. Hunter, T., and J. Pines. 1994. Cyclins and cancer. II: Cyclin D and CDK inhibitors come of age [see comments]. *Cell.* 79:573-82.
16. Hynes, R. O. 1992. Integrins: versatility, modulation, and signaling in cell adhesion. *Cell.* 69:11-25.
17. Irving, J. A., G. Cain, A. Parr, M. Howard, B. Angus, and A. R. Cattan. 1996. OCT embedded sections of pathological specimens as a source of high quality RNA for reverse transcriptase/polymerase chain reaction. *J Clin Pathol.* 49:258-9.

18. Juliano, R. 1994. Signal transduction by integrins and its role in the regulation of tumor growth. *Cancer Metastasis Rev.* 13:25-30.
19. Juliano, R. L., and J. A. Varner. 1993. Adhesion molecules in cancer: the role of integrins. *Curr Opin Cell Biol.* 5:812-8.
20. Kato, J., H. Matsushime, S. W. Hiebert, M. E. Ewen, and C. J. Sherr. 1993. Direct binding of cyclin D to the retinoblastoma gene product (pRb) and pRb phosphorylation by the cyclin D-dependent kinase CDK4. *Genes Dev.* 7:331-42.
21. Larsson, L. I. 1989. In situ hybridization using biotin-labeled oligonucleotides: probe labeling and procedures for mRNA detection. *Arch Histol Cytol.* 52 Suppl:55-62.
22. Matsushime, H., M. E. Ewen, D. K. Strom, J. Y. Kato, S. K. Hanks, M. F. Roussel, and C. J. Sherr. 1992. Identification and properties of an atypical catalytic subunit (p34PSK- J3/cdk4) for mammalian D type G1 cyclins. *Cell.* 71:323-34.
23. Matsushime, H., M. F. Roussel, R. A. Ashmun, and C. J. Sherr. 1991. Colony-stimulating factor 1 regulates novel cyclins during the G1 phase of the cell cycle. *Cell.* 65:701-13.
24. Matsushime, H., M. F. Roussel, and C. J. Sherr. 1991. Novel mammalian cyclins (CYL genes) expressed during G1. *Cold Spring Harb Symp Quant Biol.* 56:69-74.
25. Matsuyama, T., A. Yamada, J. Kay, K. M. Yamada, S. K. Akiyama, S. F. Schlossman, and C. Morimoto. 1989. Activation of CD4 cells by fibronectin and anti-CD3 antibody. A synergistic effect mediated by the VLA-5 fibronectin receptor complex. *J Exp Med.* 170:1133-48.
26. Miyamoto, S., H. Teramoto, J. S. Gutkind, and K. M. Yamada. 1996. Integrins can collaborate with growth factors for phosphorylation of receptor tyrosine kinases and MAP kinase activation: roles of integrin aggregation and occupancy of receptors. *J Cell Biol.* 135:1633-42.
27. Mortarini, R., A. Gismondi, A. Santoni, G. Parmiani, and A. Anichini. 1992. Role of the alpha 5 beta 1 integrin receptor in the proliferative response of quiescent human melanoma cells to fibronectin. *Cancer Res.* 52:4499-506.
28. Plopper, G. E., H. P. McNamee, L. E. Dike, K. Bojanowski, and D. E. Ingber. 1995. Convergence of integrin and growth factor receptor signaling pathways within the focal adhesion complex. *Mol Biol Cell.* 6:1349-65.
29. Said, T. K., L. Luo, and D. Medina. 1995. Mouse mammary hyperplasias and neoplasias exhibit different patterns of cyclins D1 and D2 binding to cdk4. *Carcinogenesis.* 16:2507-13.
30. Schaller, M. D., C. A. Borgman, B. S. Cobb, R. R. Vines, A. B. Reynolds, and J. T. Parsons. 1992. pp125FAK a structurally distinctive protein-tyrosine kinase associated with focal adhesions. *Proc Natl Acad Sci U S A.* 89:5192-6.
31. Sweeney, K. J., B. Sarcevic, R. L. Sutherland, and E. A. Musgrove. 1997. Cyclin D2 activates Cdk2 in preference to Cdk4 in human breast epithelial cells. *Oncogene.* 14:1329-40.
32. Symington, B. E. 1992. Fibronectin receptor modulates cyclin-dependent kinase activity. *J Biol Chem.* 267:25744-7.
33. Symington, B. E. 1995. Growth signalling through the alpha 5 beta 1 fibronectin receptor. *Biochem Biophys Res Commun.* 208:126-34.
34. Tam, S. W., A. M. Theodoras, J. W. Shay, G. F. Draetta, and M. Pagano. 1994. Differential expression and regulation of Cyclin D1 protein in normal and tumor human cells: association with Cdk4 is required for Cyclin D1 function in G1 progression. *Oncogene.* 9:2663-74.
35. Tamura, R. N., H. M. Cooper, G. Collo, and V. Quaranta. 1991. Cell type-specific integrin variants with alternative alpha chain cytoplasmic domains. *Proc Natl Acad Sci U S A.* 88:10183-7.
36. Taylor-Papadimitriou, J., and M. R. Stampfer. 1992. Culture of Human Mammary Epithelial Cells. In *Culture of Epithelial Cells*. R. I. Freshney, editor. Wiley-Liss, Inc., New York, NY. 107-133.

- 
37. Tennenbaum, T., A. J. Belanger, A. B. Glick, R. Tamura, V. Quaranta, and S. H. Yuspa. 1995. A splice variant of alpha 6 integrin is associated with malignant conversion in mouse skin tumorigenesis. *Proc Natl Acad Sci U S A.* 92:7041-5.
  38. Wayner, E. A., and W. G. Carter. 1987. Identification of multiple cell adhesion receptors for collagen and fibronectin in human fibrosarcoma cells possessing unique alpha and common beta subunits. *J Cell Biol.* 105:1873-84.
  39. Wayner, E. A., W. G. Carter, R. S. Piotrowicz, and T. J. Kunicki. 1988. The function of multiple extracellular matrix receptors in mediating cell adhesion to extracellular matrix: preparation of monoclonal antibodies to the fibronectin receptor that specifically inhibit cell adhesion to fibronectin and react with platelet glycoproteins Ic-IIa. *J Cell Biol.* 107:1881-91.
  40. Wayner, E. A., R. A. Orlando, and D. A. Cheresh. 1991. Integrins alpha v beta 3 and alpha v beta 5 contribute to cell attachment to vitronectin but differentially distribute on the cell surface. *J Cell Biol.* 113:919-29.
  41. Wessel, D., and U. I. Flugge. 1984. A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. *Anal Biochem.* 138:141-3.
  42. Yurochko, A. D., D. Y. Liu, D. Eierman, and S. Haskill. 1992. Integrins as a primary signal transduction molecule regulating monocyte immediate-early gene induction. *Proc Natl Acad Sci U S A.* 89:9034-8.
  43. Zhou, Q., M. Stetler-Stevenson, and P. S. Steeg. 1997. Inhibition of cyclin D expression in human breast carcinoma cells by retinoids in vitro. *Oncogene.* 15:107-15.

## V. Appendix

### A. Bibliography of publication and meeting abstracts.

Publications: None

Abstracts:

**Tamura, R.N.**, Pabich, W., Symington, B., and Wayner E. (1997) Role of integrins in regulating cell proliferation of normal and neoplastic mammary epithelial cells. Era of Hope, The Department of Defense Breast Cancer Research Program Meeting, October 31, November 4, 1997.

### B. List of Personnel:

1. Richard Tamura, Ph.D., Principal Investigator
2. Elizabeth Wayner, Ph.D., Principal Investigator
3. Wendy Pabich, Research Technician I

Abstract for: Era of Hope, The Department of Defense Breast Cancer Research Program Meeting, October 31 to November 4, 1997, Washington D.C.

**ROLE OF INTEGRINS IN REGULATING CELL PROLIFERATION OF NORMAL AND NEOPLASTIC MAMMARY EPITHELIAL CELLS.**

**Richard N. Tamura, Wendy Pabich, Banu Symington, and Elizabeth Wayner**

Seattle Biomedical Research Institute  
Seattle, WA 98109-1651

The extracellular matrix (ECM) is a milieu of proteins which provide structural integrity to tissues and organs as well as cues regulating several cell functions such as proliferation, migration, differentiation, and gene expression. These cell-ECM interactions are mediated, in part through the integrin family of cell adhesion receptors.

We have been investigating the role of integrins in the regulation of cell cycle progression. Initial experiments suggested that peptides containing the cell adhesion motif of Arg-Gly-Asp (RGD) bound to the integrin  $\alpha 5 \beta 1$  and regulated cdc2 kinase/cyclin A associations in normal human epidermal keratinocytes and the non-tumorigenic breast cancer cell line HBL 100 but not in the tumorigenic cell line BT20 or virally-transformed keratinocytes. These results suggested a potential defect in the  $\alpha 5 \beta 1$  signaling pathway regulating cell cycle progression in transformed cells. We extended these studies to normal mammary epithelial cells (HMEC) and to a number of additional breast cancer cell lines (T47D, DU4475, MDA-MB-453, MDA-MB-157, BT 483, Hs578t, MDA-MB-361, MCF7, BT474, and MDA-MB-134-IV) which vary from being partially transformed (agar growth/non-tumorigenic) to fully transformed (tumorigenic/metastatic). In order to understand the interaction of the RGD peptides with these various cell lines and HMEC, we characterized the repertoire of integrin expression by FACS. Of the three integrins which bind soluble RGD peptides with the highest avidity,  $\alpha \nu \beta 5$  was expressed by nearly all of the cell lines, 40% expressed  $\alpha \nu \beta 3$ , and 60% expressed  $\alpha 5 \beta 1$ . HMEC expressed intermediate levels of  $\alpha 5$  and  $\alpha 4$ , low levels of  $\alpha 6$  and  $\alpha \nu \beta 5$ , and were negative for  $\alpha \nu \beta 3$ . The most prominent integrin expressed by HMEC and by all of the cell lines was  $\alpha 3 \beta 1$ , which is primarily a receptor for laminins but also mediates adhesion to fibronectin and collagens.

Incubation with RGD peptides resulted in increases in both cdc2 kinase activity and cyclin A associated kinase activity in HMEC and a number of the cell lines. However, in most cases, the stimulation of cdc2 kinase activity or cyclin A associated kinase activity appeared to be mutually exclusive suggesting a mechanism that is distinct from that of normal human epidermal keratinocytes or HBL100 cells. There was also no apparent

correlation between the stimulation of cell cycle kinases with the expression of the integrin  $\alpha 5\beta 1$ ,  $\alpha v\beta 3$ , or  $\alpha v\beta 5$ . Nor did there appear to be a correlation between the stimulation of cell cycle kinases with the tumorigenic potential of the cell line.

Immunohistochemical analysis of normal and tumor breast tissue indicated that \_5\_1 was not expressed by either normal mammary epithelial cells or tumor cells. Basal mammary epithelial cells expressed integrins  $\alpha 3$ ,  $\alpha 6$ ,  $\beta 4$ ,  $\alpha 2$ ,  $\beta 1$ ,  $\alpha v\beta 5$ , and possibly  $\alpha v\beta 6$ , luminal cells and cancer cells (in situ and invasive ductal) expressed predominately  $\alpha 3\beta 1$ . A few samples were positive for  $\alpha v\beta 3$  in the basal cells. The integrin  $\alpha 4\beta 1$  was not expressed by either normal epithelial cells or cancer cells. These results suggest that  $\alpha 5\beta 1$  is not of primary importance in mediating cell adhesive signaling events in either normal mammary epithelial cell or during tumorigenesis.

Recently, we have initiated studies to examine cell proliferation regulation mediated through specific integrins by clustering with monoclonal antibodies on cells synchronized to Go by serum/growth factor deprivation. Preliminary results suggest that a number of integrins,  $\beta 1$ ,  $\alpha v$ ,  $\alpha 3$ , and  $\beta 4$ , may promote transit of normal HMEC through the cell cycle under reduced serum conditions as evaluated by the time course of retinoblastoma (Rb) phosphorylation. Similar results have been demonstrated by others for  $\beta 1$  integrins in fibroblast cells and is thought to involve promotion of growth factor receptor clustering and activation of mitogen activated protein kinase (MAPK). We are looking to see if similar mechanisms are being used by normal mammary epithelial cells, both basal and luminal, and breast cancer cells. Understanding the role of integrins in the regulation of cell proliferation may lead to the development of novel therapeutics for the treatment of breast cancer.