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PRINCIPAL INVESTIGATOR: James G. Lyons, Ph.D.

CONTRACTING ORGANIZATION: Royal Prince Alfred Hospital
Camperdown, NSW, 2050, Australia

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Lyons, James G., Ph.D.

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Camperdown, NSW, 2050, Australia

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The aim of this project is to identify the molecular basis for the abnormal destruction of extracellular matrix that accompanies breast cancer. This is being addressed primarily by identifying the control elements within the genes of the matrix metalloproteinases (MMPs) that drive their high level of expression. It was found that the primary source of MMPs in a breast cancer cell population is the metaplastic cells, which we have demonstrated to have undergone an epithelial-mesenchymal transition. By absolute quantitation of mRNAs, it can be said that the stromelysin-1 and collagenase-3 mRNAs are major gene products of these cells. To make the MMPs, the metaplastic cells require stimulation by a factor secreted by the carcinoma cells that have retained epithelial characteristics. Using metaplastic cells stably transfected with reporter gene constructs, it was demonstrated that the 1100bp of proximal promoter of the stromelysin-1 gene is sufficient to respond to the epithelial-derived inducing factor. A P1 clone containg the gene of another MMP, collagenase-3, has been obtained and is being mapped for DNase hypersensitive sites.

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Introduction

The critical feature of breast cancer that makes it a malignant disease is its ability to spread from the breast to other parts of the body. It is these secondary breast cancer tumors whose growth and destruction of surrounding tissues can cause suffering and the death of the patient. The spread of the tumor, in the context of both invasion of tissues locally and in metastasis to distant organs, requires the destruction of the extracellular matrix (ECM). The purpose of this project is to identify the molecular and genetic basis for this abnormal ECM destruction by addressing the expression of the genes of a group of proteinases, the matrix metalloproteinases (MMPs), that are made at high levels in and around carcinomas. Specifically, the gene control regions of the MMP genes that are responsible for their high level expression in carcinoma cells will be identified. This is to be achieved by transfecting carcinoma cells with plasmids that contain reporter genes driven by wild-type or mutant MMP promoters to localize the control elements to specific regions of the MMP genes and verifying thier roles *in vivo.*

Experimental Methods, Assumptions and Procedures

Experimental Methods

The methods employed are primarily *in vitro* molecular and cellular biological methods, as described under "*Procedures*". *In vivo* tumorigenesis and metastasis assays in laboratory rats will be used in future phases of the project. The experimental model is based on the BC1 cell line, established from a mammary carcinoma that arose spontaneously in a female of the dark agouti inbred strain of *Rattus norvegicus* [1]. These cells grow continuously in serum-free culture and, when injected into immunocompetent, syngeneic host animals, produce tumors that metastasize and invade bone and muscle [2].

Assumptions

The clinical relevance of this project is based on the overall assumption that the molecular pathological basis of the rat model system being used for the experiments is similar to that of human breast cancer. This is supported by the behaviour of the tumours *in vivo*, in that they metastasize to draining lymph nodes and lungs, are able to invade bone and muscle and produce mRNAs for MMPs. The rationale for examining MMPs is based on the assumption that they are, in fact, essential for invasion and metastasis, a proposition that has not been tested scientifically for all MMPs in a rigorous, *in vivo* model of tumor invasion and metastasis. Since invasion and metastasis require destruction of the extracellular matrix, and the MMPs are able to do this efficiently and are the only known enzymes that are able to degrade interstitial collagens, it is reasonable to assume that at least some of them will play a role. Whether the MMPs used for invasion and metastasis are produced by the cancer cells, themselves, or by non-neoplastic host cells, is still a matter for conjecture. The ability of neoplastic epithelial cells that have acquired mesenchymal characteristics to produce MMPs (described in "Results") suggests that many previously published papers that concluded that non-neoplastic stromal cells are the source of MMPs in carcinomas may have done so in error, due to the misidentification of the MMP-producing cells. The current study has the potential to identify cells as being of cancer or host origin unequivocally, by virtue of the cancer cells being genetically tagged with the E. coli LacZ gene.

Procedures

The BC1 cell line and its clonal derivatives were grown under continuous serum-free conditions as described [3]. Transfections were performed using Lipofectamine (GIBCO-BRL) according to the manufacturer's instructions. Collagen-degrading activity was measured as described previously [4]. Absolute levels of MMP mRNAs were measured by competitive polymerase chain reaction (PCR) assays developed in this laboratory [5]. E-cadherin, vimentin and keratin proteins were detected in cell lysates by immunoblotting [6] using specific antibodies (Transduction Laboratories and DAKO, respectively). mRNAs for E-cadherin, fibroblast growth factor receptor-2 (FGFR2) and the β4-integrin subunit were detected by reverse transcriptase-PCR. Southern blotting was done according to standard procedures [7].

Results

Epithelial cells upregulate matrix metalloproteinases in cells within the same mammary carcinoma that have undergone an epithelial-mesenchymal transition (EMT).

The BC1 cell line that is being used as a model for breast cancer is composed of epithelial cells, that have retained their epithelial morphology, and metaplastic cells that have not. We developed a novel quantitative PCR method [5](manuscript attached) to determine absolute numbers of mRNA molecules of MMPs and their inhibitors, the tissue inhibitors of metalloproteinases (TIMPs) in cell and tumor tissue samples for the first time. This enabled us to identify the source and degree of mRNA induction in BC1 when these 2 cell types are co-cultured [2](manuscript attached). Collagenase-3, stromelysin-1 and stromelysin-2 were predominantly produced by metaplastic cells, whereas gelatinase B, TIMP-1 and TIMP-2 were produced by both cell types. Collagenase-3, stromelysin-1, gelatinase B and TIMP-2 were present in cell culture and tumor tissue samples at a level of >50 copies per cell, indicating that they are major gene products, because <2% of genes are expressed at this level [8]. The mRNA levels of collagenase-3, stromelysin-1, stromelysin-2 and gelatinase B were all substantially up-regulated in the metaplastic cells by co-culturing them with the epithelial cells.

The two cellular phenotypes have been characterized in more detail by analysis of their protein and mRNA expression profiles, to determine how they correspond to phenotypes within human neoplasms. Key differences between the two cell types are that the epithelial cells are positive for E-cadherin, keratin, β4-integrin subunit, the keratinocyte growth factor-binding splice isoform of fibroblast growth factor receptor-2 (FGFR2), whereas the metaplastic cells express the basic FGF-binding splice isoform of FGFR2 (Figure 1), are negative for the other aforementioned mRNAs and have up-regulated vimentin protein levels. These data suggest that the metaplastic cells have undergone an EMT. Preliminary experiments are underway to induce the epithelial cells to undergo an EMT *in vitro*, so that the relationship between this phenomenon and the induction of MMP genes via modulation of their promoters can be studied in more detail.

Quantitative PCR assays for measuring expression of reporter genes in cell and tissue samples.

Quantitative PCR assays based on our method for MMP mRNAs [5] have been developed for the measurement of absolute numbers of mRNAs derived from the neo and chloramphenicol acetyl transferase (CAT) genes used as reporters for wild-type and mutated promoters, respectively. These are more sensitive than activity-based assays and less likely to suffer from interference by other proteins in the sample, such as deacetylases.

Induction of MMPs is mediated by a secreted factor.

The identity of the factor produced by epithelial cells that induces MMP gene expression in the metaplastic cells is not presently known. However, experiments have determined that the effect of co-culture on the metaplastic cells can be reproduced at least partially by feeding them with medium conditioned by the epithelial cells, suggesting the secretion of a soluble MMP inducing factor by the epithelial cells (Figure 2). The factor is unlikely to be emmprin, as strong mRNA signals for it are obtained by PCR analysis of both epithelial cells and metaplastic cells.

Stromelysin-1 gene regulation.

Cells from a metaplastic clone, BC1-M3, have been stably transfected with reporter gene plasmids in which transcription of the neo gene, which confers resistance to G418, is driven by a full-length (-1100 to + 8, with respect to transcription start site) stromelysin-1 promoter, and a CAT gene driven by either a full-length stromelysin-1 promoter or a stromelysin-1 promoter truncated to its TATA box. Some of the stable transfectants were resistant to G418 in the presence or absence of epithelial cell-conditioned medium, indicating that the genes had been incorporated into the genome in a position under the influence of an endogenous constitutively active promoter or enhancer. However, the ability of several of the transfectant metaplastic cell lines to survive in medium containing the neo-selective drug G418 was dependent on feeding them with epithelial cell-conditioned medium, indicating that this region of promoter (-1100 to +8) is sufficient to confer responsiveness of the stromelysin-1 gene in the metaplastic cells to epithelial conditioned medium. The induction of the neo reporter gene by epithelial cell-conditioned medium was confirmed by PCR analysis of the neo mRNA in the absence of G418. Having established that the region of the stromelysin-1 promoter that has been cloned is sufficient to respond to the epithelial cell-conditioned medium, the sufficiency of this region of promoter to mediate stromelysin-1 gene expression *in vivo* can be determined by finer deletion and substitution mutations of this promoter can be made to drive the CAT gene.

Collagenase-3 gene regulation.

Quantitative PCR analysis indicated that collagenase-3 mRNA, like that of stromelysin-1, is upregulated in metaplastic cells by conditioned medium from epithelial cells (Figure 2).

Two bacteriophage P1 clones were obtained by an initial commercial screening of a rat genomic DNA library by Genome Systems, Inc. containing the collagenase-3 gene. After considerable time spent analysing the two clones, it was determined that neither of them contained genuine collagenase-3 sequences, and that the original PCR-based screening of the P1 library must have been flawed in some way. The company was informed and agreed to re-screen the library at no extra cost, using a new PCR primer set. A single P1 clone, verified by us to contain collagenase-3 proximal promoter sequences, was obtained. This clone gives identical bands to BC1-derived genomic DNA in Southern blots of restriction enzyme digests probed with a proximal promoter-derived probe. A region of DNA spanning ~6kbp of DNA 5' to the transcription start site is currently being examined for DNase hypersensitive sites (DHSs). If no DHSs are present in this region, the same Southern blots can be re-probed with probes specific for regions further upstream or downstream from this region.

Discussion

The use of epithelial cell-conditioned medium to induce MMP production in the metaplastic cells should facilitate the identification of gene control elements responsible for the upregulation of MMPs by simplifying protocols. Instead of co-culturing the two types of cells derived from separately maintained original cultures, then separating them for gene expression analysis, homogeneous cultures of metaplastic cells can be induced to produce MMPs by exposing them to epithelial cell-conditioned medium. The cell lines stably transfected with reporter genes may in the future form the basis of a convenient assay for the screening of candidate molecules to identify it or for monitoring its purification.

Because both epithelial cells and metaplastic cells are both exposed to the epithelial cell-derived MMP inducing factor, but only the metaplastic cells actually express their MMP genes, there must be inherent biochemical difference between the cells underlying this. This could be that an appropriate receptor for the factor is absent in the epithelial cells but present in the metaplastic cells. This may explain the different responsiveness of the collagenase-3, stromelysin-1 and stromelysin-2 genes, but would require a different factor being present for the induction of the gelatinase B gene, which is constitutively active in the epithelial cells. Alternatively, it may be that epigenetic differences between the two cell types, perhaps in the chromatin structure or methylation status of the collagenase-3 and stromelysin-1 and -2 genes, make them unresponsive in the epithelial cells. Thus, two stages are required for a high level of MMP induction in these mammary carcinoma cells: (1) the attainment of inducibility, and (2) actual induction by the inducing factor secreted by the epithelial cells.

The finding that elements of the stromelysin-1 gene responsible for the high level of its expression lie within the 1100bp 5'-proximal to the transcription start site demonstrates that the reporter plasmids that have already been constructed will be useful in resolving the elements to small stretches of the gene. The degree of labor-intensiveness and time consuming nature of generating the stably transfected cell lines has lead us to propose the use of transient transfections in future as a preliminary screening process for determining the effects of promoter mutations on MMP gene expression. The outcome of the data obtained form transiently transfected DNA will require verification with metaplastic cell lines harbouring stably transfected reporter genes, but may accelerate the rate at which the Statement of Work Tasks are achieved. Some initial experiments comparing the behavior of the reporter genes in the stably transfected cell lines that we have already generated with transiently transfected reporter genes will determine whether they are similar enough to pursue transient transfection as an initial screen.

The initial misidentification of 2 genomic DNA clones as being those of collagenase-3 wasted some time in its analysis by PCR and Southern blotting. DHS analysis is currently under way and it is hoped that DHSs induced in metaplastic cells by exposure to epithelial cell-conditioned medium will be identified. If DHSs are not detected during upregulation of the collagenase-3 gene, then an alternative method for identifying the elements responsible for high level collagenase-3 expression will be used. A reporter plasmid library can be screened for epithelial cell-conditioned medium-inducibility. This would be made by digesting the collagenase-3 P1 clone with restriction enzymes, cloning the fragments beside a minimal promoter driving a reporter gene and measuring the epithelial cell-conditioned medium-inducibility of the resulting plasmid in transiently transfected metaplastic cells.

Key Research Accomplishments

- Determination of the absolute number of MMP mRNA molecules in the mammary carcinoma cells, allowing us to determine that the mRNAs of collagenase-3, stromelysin-1 and gelatinase B are major gene products in BC1 cells and tumors, being in the top 2% of expressed genes.
- Identification of metaplastic cells as being the major source of collagenase-3, stromelysin-1 and stromelysin-2 in BC1, while gelatinase B was produced by both the epithelial cells and the metaplastic cells.
- Determining that the epithelial cell-derived MMP inducing factor is released in a soluble form.
- Determining that the collagenase-3, stromelysin-1 and stromelysin-2 genes are differentially induced in epithelial and metaplastic cells.
- Acquisition of a P1 clone containing the collagenase-3 gene.
- Demonstration that the 1100bp immediately 5'-proximal to the transcription start site are sufficient to confer inducibility to epithelial cell conditioned-medium in metaplastic cells.

Reportable Outcomes

Manuscripts:

Martorana, A. M., Zheng, G., Crowe, T. C., O'Grady, R. L. and Lyons, J. G. (1998) "Epithelial cells upregulate matrix metalloproteinases in cells within the same mammary carcinoma that have undergone an epithelial-mesenchymal transition."
Cancer Res. **58**, 4970-4979

Martorana, A. M., Zheng, G., Springall, F., Iland, H. J., O'Grady, R. L. and Lyons, J. G. (1999) "Absolute quantitation of specific mRNAs in cell and tissue samples by comparative PCR."
BioTechniques in press

Conference presentations:

Gordon Research Conference on Matrix Metalloproteinases, 1997
Martorana, A. M., Zheng, G., Springall, F., Iland, H. J., O'Grady, R. L. and Lyons, J. G.
"MMP induction in breast cancer cells via interclonal co-operativity."

Australia and New Zealand Society for Cell and Developmental Biology Annual Conference, 1998
Martorana, A. M., Zheng, G., Springall, F., Iland, H. J., O'Grady, R. L. and Lyons, J. G.
"Epithelial cells upregulate matrix metalloproteinases in cells within the same mammary carcinoma that have undergone an epithelial-mesenchymal transition."

CRC Beatson International Cancer Conference, 1999
A. M. Martorana, G. Zheng, G. Shoebridge and J. G. Lyons
"Matrix metalloproteinase production in carcinomas dependent on epithelial-mesenchymal transition."

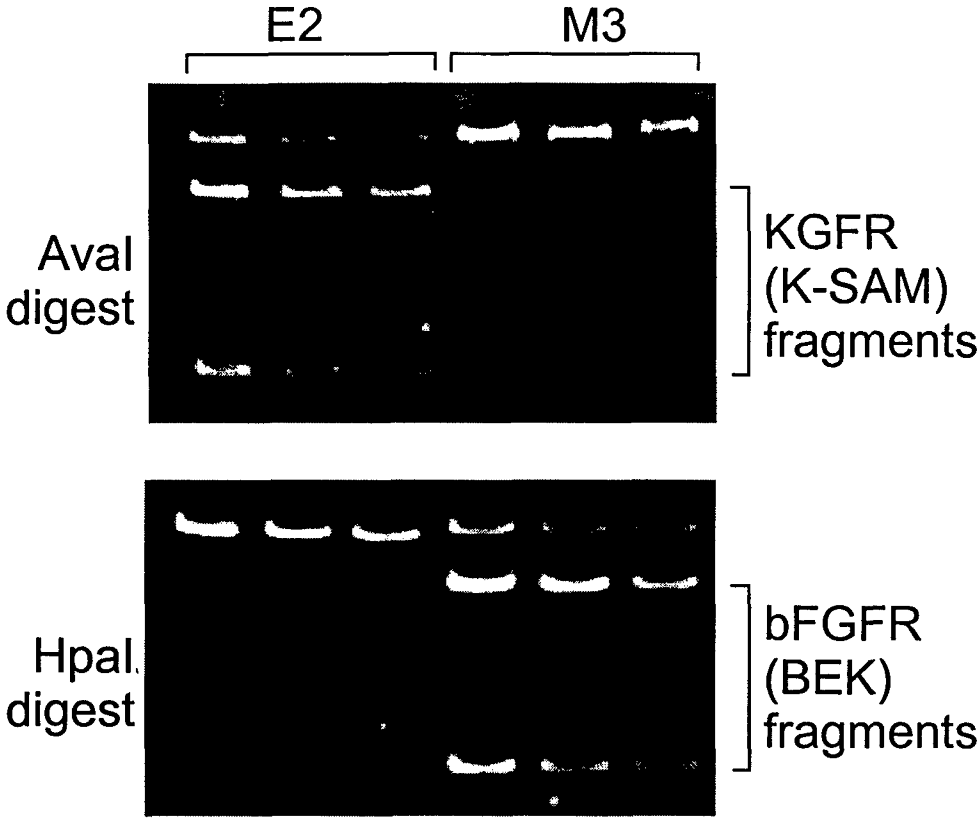

Figure 1. PCR analysis of FGFR2 mRNA splice variants. cDNA derived from 3 separate platings of clonal epithelial (E2) or metaplastic (M3) BC1 cells was subjected to PCR using primers specific for the FGFR2 cDNA. The products were then digested with AvaI, which specifically digests the keratinocyte growth factor-binding splice variant (KGFR) or with HpaI, which specifically digests the basic fibroblast growth factor-binding variant (bFGFR).

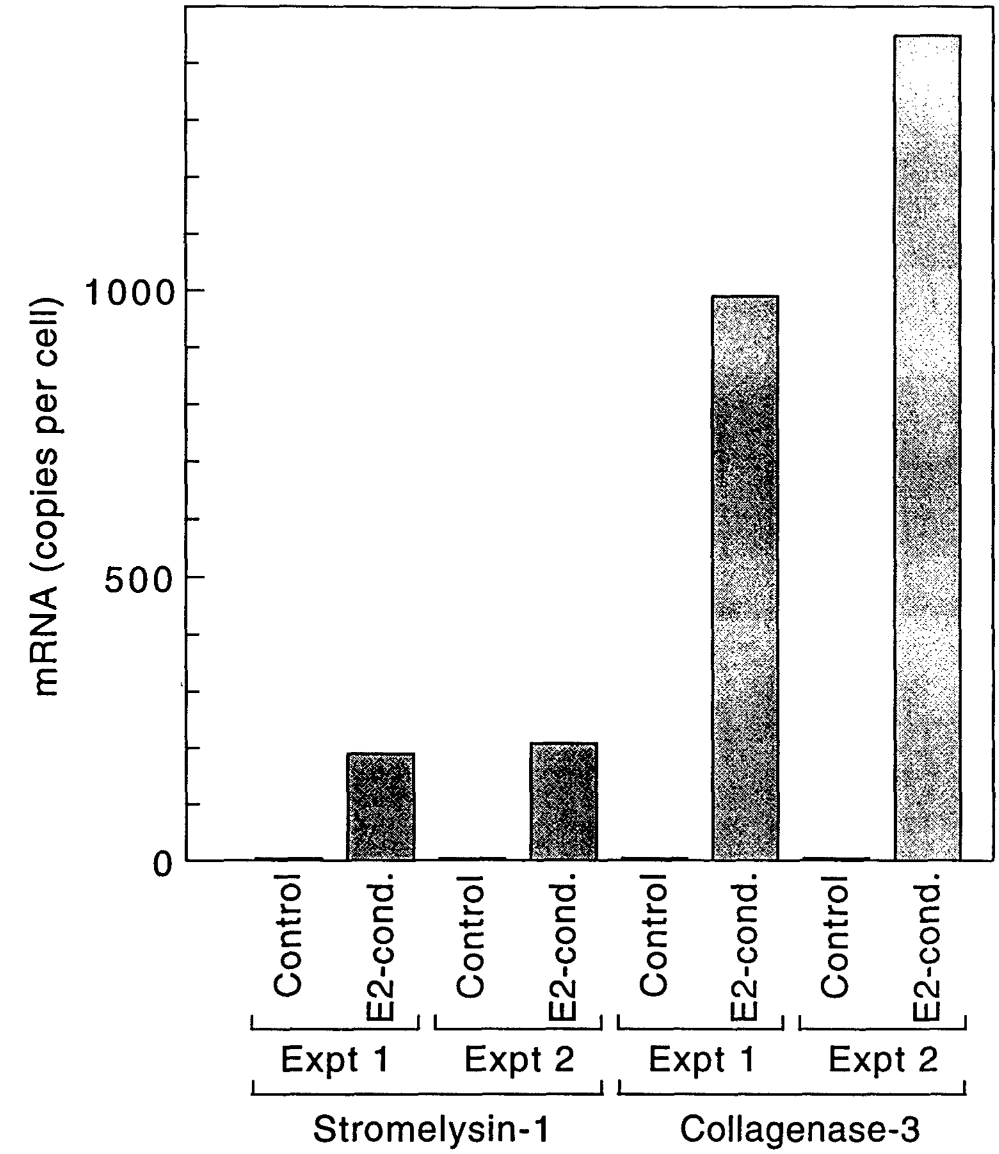

Figure 2. Induction of MMP genes in metaplastic cells by the addition of epithelial cell-conditioned medium. BC1-M3 cells that had been transfected stably with the *E. coli LacZ* gene were grown to near-confluence and exposed to fresh medium (Control) or medium that had been conditioned by epithelial cells (E2-cond.). The RNAs were isolated and the mRNAs for stromelysin-1 and collagenase-3 were quantitated by PCR. The data for two individual experiments are shown.

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Research Reports

BT 98/1825

Absolute Quantitation of Specific mRNAs in Cell and Tissue Samples by Comparative PCR

BioTechniques 26:_-_(June 1999)

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A.M. Martorana[1,2], G. Zheng[1], F. Springall[1], H.J. Iland[1], R.L. O'Grady[2] and G. Lyons[1] J.
[1]Royal Prince Alfred Hospital, Camperdown and [2]University of Technology, Sydney, NSW, Australia

ABSTRACT

A comparative PCR assay, for the absolute quantitation of specific mRNAs in cell and tissue samples, has been designed to overcome problems with previous techniques. cDNAs made from the RNAs are co-amplified with "competitor" plasmid templates under conditions in which reagents are not limiting at the equivalence point, thereby preventing competition between target and competitor templates and distinguishing the assay from competitive PCR assays. The cDNAs are serially diluted, and competitor template concentrations are kept constant, rather than vice versa as occurs in competitive PCR assays. Products from target and competitor templates are resolved by electrophoresis and measured by phosphorescent or fluorescent imagery. Both products are measured to minimize errors in the competitor:target ratio. A synthetic external standard RNA is included in the tissue lysis solution and co-purified with endogenous mRNAs, thereby being subject to identical losses of yield during subsequent procedures. The determination of the number of copies of external standard cDNA allows inefficiencies of RNA extraction and cDNA synthesis to be taken into account. Standard concentrations of plasmids containing the endogenous target sequences are also measured, so that corrections can be made for discrepancies due to unequal amplification of target and competitor sequences. These corrections, together with the use of an external standard and the PCR conditions chosen, allow for the accurate, specific and sensitive determination of the absolute number of mRNA copies in a sample.

INTRODUCTION

The amount of specific mRNAs within cells and tissues is important in the regulation of protein synthesis and is commonly used to study the expression of genes in various physiological and pathological processes. Several methods are commonly used to measure the levels of specific mRNAs, including northern blotting, primer extension, nuclease protection and reverse transcription-polymerase chain reaction (RT-PCR). As currently used, none of these methods permit absolute quantitation of mRNAs (i.e., they do not allow determination of the number of specific mRNA molecules in a particular cell culture or tissue sample). Relative levels of an mRNA are usually obtained by assaying the mRNA by one of the above-mentioned methods and normalizing the data against an internal standard RNA, usually ribosomal RNA or the mRNA of a "housekeeping" gene, such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Even these relative levels rely on the internal standard RNA being expressed at a constant level in all tissues being examined. This can be an erroneous assumption, because even the expression of housekeeping genes are subject to cell-cycle and environmental regulation (5,9). Moreover, the validity of the relative levels relies on RNA extraction yields being constant between samples, an assumption that is rarely, if ever, verified. However, it would seem unlikely that the RNA yields from tissues with physical and biochemical differences would be identical.

In this paper, we describe a method based on competitive PCR (2,6,11,14) for the absolute quantitation of specific mRNAs that overcomes these problems. We call this method, "comparative PCR", as actual competition between the target and competitor templates does not occur. It uses non-limiting amplification conditions and a synthetic external standard RNA to take into account inefficiencies in RNA yield and cDNA synthesis. This method has been used to quantitate the mRNAs of matrix metalloproteinases (MMPs) and tissue inhibitor of metalloproteinases (TIMPs) in a mammary tumor.

MATERIALS AND METHODS

Synthetic External Standard RNA

To prepare the synthetic external standard RNA for quantitative PCR assays, the APL1 plasmid (described below) was linearized with *Eco*RI and transcribed with SP6 RNA Polymerase (Promega, Madison, WI, USA), according to the manufacturer's instructions. The RNA was electrophoresed in and eluted from 6.5% polyacrylamide/8 M urea gel and ethanol-precipitated. The purified RNA was quantitated by absorbance at 260 nm, re-analyzed electrophoretically to en-

Table 1. Absolute Quantitation of mRNAs in a Rat Mammary Tumor Sample

Gene		Primer Sequence	Product Size (bp)		Correction Factor	mRNA (millions of copies per mg tissue)
APL1	F	AGAAGTGTTTCAGAAGCTTCTCCC	T	344	1.02	-
	R	AACGAGCGGCTTCACTCAGACC	C	459		
Collagenase-3	F	CTCTCTATGGTCCAGG	T	144	1.44	210
	R	TCATGGTTTCTCCTCGG	C	159		
Gelatinase B	F	CGCCAACTATGACCAGGATA	T	73	0.976	167
	R	GTTGCCCCCAGTTACAGT	C	93		
Stromelysin-1	F	GCCTGGAATGGTCTTGG	T	222	1.28	96
	R	TGGAAACGGGCCAGGTC	C	195		
Stromelysin-2	F	GGAGTGGGACAGAGCTTGGC	T	312	1.59	33
	R	GACAGAGGGCACAGGAACCAC	C	225		
TIMP-1	F	AATTTGCACATCACTGCC	T	213	0.784	4
	R	GTGATCGCTCTGGTAGC	C	179		
TIMP-2	F	CAGGCGTTTTGCAATGC	T	114	1.32	16
	R	GATCTCATATTGAATCCTC	C	90		
GAPDH	F	CCACCATGGAGAAGGCTGGGGCTC	T	239	0.336	571
	R	AGTGATGGCATGGACTGTGGTCAT	C	278		

The sequences of the forward (F) and reverse (R) primers used for comparative PCR assays and the sizes of the products that they generate from cDNA target (T) and competitor (C) templates are shown. Comparative PCR assays were performed on three independent samples of known concentration of plasmid DNA or, for GAPDH, purified PCR product, containing the wild-type target sequences. The average discrepancy between the measured value and the actual value was determined as the correction factor. These values were used to correct the measurement of absolute numbers of mRNA copies in a BC1 tumor sample, determined by comparative PCR using the synthetic external standard RNA, as described in the text.

sure integrity and stored under liquid nitrogen in aliquots until use.

Extraction of RNA

Total RNA was extracted from cultured cells and tumors according to the method of Chomczynski and Sacchi (4), with the following modifications. External standard RNA, transcribed in vitro from the APL1 plasmid, was added to the guanidinium isothiocyanate (GITC) cell lysis solution before lysis at a concentration of 5.25×10^{-17} moles of RNA per million cultured cells or per milligram of tissue. Tumors, kept frozen in dry ice, were pulverized using a mortar and pestle before the addition of cell lysis solution. The purity and quantity of total RNA extracted from samples was determined by spectrophotometric analysis at 260 and 280 nm.

Reverse Transcription

RT of mRNA was performed using 25 µg total RNA in a 100-µL reaction volume containing 100 U reverse transcriptase from the avian myeloblastosis virus (AMV) (Molecular Genetic Resources, Tampa, FL, USA), 1 mM deoxynucleotide triphosphates (Amersham Pharmacia Biotech, Castle Hill, Australia), 1.5 pg of 18-mer oligo-dT and 100 U RNasin Ribonuclease Inhibitor (Promega) in a buffer of 50 mM Tris-HCl, 8 mM $MgCl_2$, 30 mM KCl and 1 mM dithiothreitol (DTT), pH 8.5 (Boehringer Mannheim, Castle Hill, Australia). The reaction was carried out for 1 h at 42°C followed by 5 min at 98°C to inactivate the reverse transcriptase. The cDNA was subsequently stored at -70°C until assayed.

Comparative PCR

Table 1 shows the primer sequences for the quantitative PCR assays of each gene product. PCR was carried out in a 50-µL reaction volume containing 2.5 U of *Taq* DNA Polymerase (PE Biosystems, Scoresby, Australia), 0.2 mM deoxynucleotide triphosphates and 15 pmol each of forward and reverse primers in a buffer containing 10 mM Tris-HCl, 50 mM KCl, pH 8.35 (PE Biosystems). Reactions for comparative PCR in which phosphorescent imagery was used were supplemented with 0.6 µCi of [α-^{33}P]dCTP (Bre-

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satec, Adelaide, Australia) per reaction. When included, competitor plasmid was used at 0.1 amol per reaction. The standardized amplification protocol consisted of an initial denaturation step at 96°C for 4 min, followed by 25 sequential cycles of 96°C for 30 s, 54°C for 30 s and 72°C for 90 s. The optimal $MgCl_2$ concentration was determined for each primer pair to ensure specificity at the standard annealing temperature of 54°C. This was 0.8 mM for APL1, GAPDH and gelatinase B, 1.0 mM for stromelysin-2 and TIMP-1, 1.5 mM for collagenase-3 and TIMP-2 and 2.0 mM for stromelysin-1. Amplification was carried out in Omn-E or Omnigene Thermal Cyclers (Hybaid, Ashford, UK). PCR products (10 µL) were electrophoresed in 12.5% or 15% polyacrylamide gels in a Mini-Protean II Apparatus (Bio-Rad, Hercules, CA, USA). For phosphorescent imagery, the gels were dried, exposed to a phosphorescent screen overnight, imaged on a Bas1000 Phosphorescent Imager and quantitated with TINA software (both from Fuji, Tokyo, Japan). For fluorescent imagery, gels were stained for 30 min in SYBR® Green I (Molecular Probes, Eugene, OR, USA) or Vistra Green (Amersham Pharmacia Biotech), according to the manufacturers' instructions, imaged on a 312 nm UV light box with a charged-coupled device (CCD) camera and quantitated with Phoretix (both from UVP, Upland, CA, USA) or TINA software. In calculating the cDNA dilution at which target and competitor products were equivalent, differences in dC content (for phosphorescent imaging) or total length (for fluorescent imaging) were taken into account. A molar factor of two was also included, to account for the fact that cDNA is single-stranded, whereas the competitor plasmids are double-stranded and therefore offer twice as many PCR templates.

Sequencing

Polyacrylamide gel-purified PCR products were sequenced directly using the Dye Terminator Cycle Sequencing Ready Reaction kit and a Model 373A Fluorescent Sequencer (both from PE Biosystems), according to the manufacturer's instructions.

Plasmids

APL1, the wild-type plasmid used for synthesis of the external standard RNA, was made by insertion of 539 bp of multiple synthetic oligonucleotides, including the sequences for PCR amplification of BCR-ABL, between the *Sal*I and *Sac*I sites of pSP64polyA (Promega). The competitor plasmids for this and other targets were made by insertion or deletion of DNA sequences between primer sites, using restriction enzymes or by deletions introduced by PCR primers (3) (Table 1). Complete sequences of the competitor plasmids are available upon request. Plasmids were purified by centrifugation through two cesium-chloride gradients.

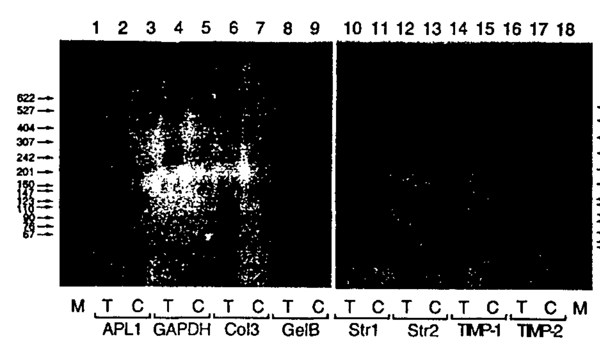
Cell Culture and Tumors

The BC1 rat mammary carcinoma cell line was cultured under continuously serum-free conditions as described (8,12). Basal media and other culture chemicals were obtained from Sigma (St. Louis, MO, USA). Tumors were induced in 7-week-old syngeneic rats by injection of 0.5×10^6 cells in 50 µL of phosphate-buffered saline (PBS) into the right footpad and allowed to grow for 40 days.

RESULTS

Design of PCR Assays for Quantitating mRNAs

As part of an ongoing investigation into the regulation of extracellular matrix (ECM) destruction by tumors, it was necessary to quantitate MMP and TIMP mRNAs in the BC1 rat mammary carcinoma cell line and in tumors derived from it. To do this, a comparative PCR assay was designed that overcomes many of the problems associated with other mRNA assays. In particular, this protocol permits absolute quantitation of mRNA species, which eliminates the need to base measurements of the levels of the mRNA of interest on comparisons with those of other mRNAs, which may themselves be subject to modulation. In designing comparative PCR assays for the rat MMPs and TIMPs, the opportunity was taken to make methodological choices and innovations that would eliminate several



Figure(s) not intended to be print quality. For position only.

Figure 1. Specificity of PCRs for target and competitor templates. Silver-stained polyacrylamide gel electrophoretograms of target and competitor PCR products for each target mRNA, amplified under optimized conditions, are shown. All target products were amplified from BC1 cDNA except for the synthetic RNA standard, which was amplified from plasmid DNA. Competitor PCR products were amplified from the respective competitor plasmids. Lanes 2, 4, 6, 8, 10, 12, 14 and 16 show the specific target (T) products for the synthetic RNA standard (APL1), GAPDH, collagenase-3 (Col3), gelatinase B (GelB), stromelysin-1 (Str1), stromelysin-2 (Str2), TIMP-1 and TIMP-2, respectively. The corresponding competitor PCR products (C) for each are shown in the adjacent lanes (i.e., lanes 3, 5, 7, 9, 11, 13, 15 and 17, respectively). A primer dimer band is evident in lane 3. Lanes 1 and 18 are size markers, with the number of base pairs indicated beside the gels.

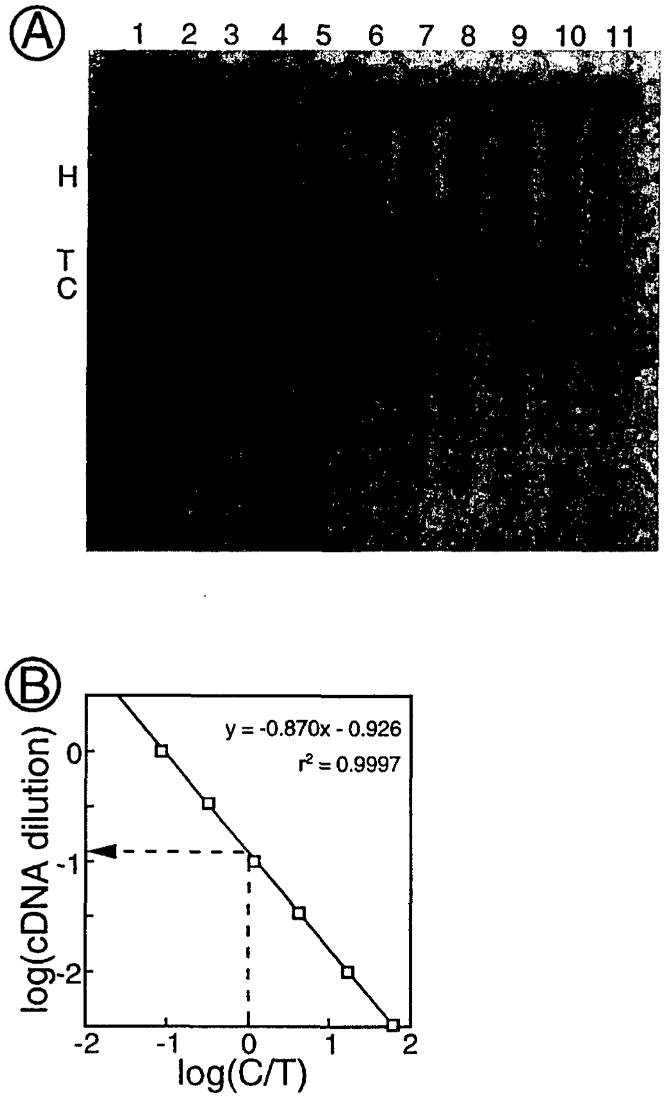


Figure 2. Comparative PCR analysis of cDNA. (A) Phosphorescent image of a polyacrylamide gel showing the PCR products in a comparative RT-PCR assay for TIMP-1. Dilutions of sample cDNA were co-amplified by PCR with 0.1 amol of competitor, in the presence of [^{33}P]dCTP. PCR products were separated by 15% polyacrylamide gel electrophoresis (PAGE) and visualized by phosphorescent imagery. Competitor (C), target (T) and heteroduplex (H) PCR products are indicated. Lanes 1, 3, 5 and 7 show the products arising from 10-fold serial dilutions of the cDNA. For each 10-fold dilution, an additional 3-fold dilution was performed, the products arising from these being shown in lanes 2, 4, 6 and 8, respectively. (B) Graphical determination of the amount of TIMP-1 cDNA. The intensities of the bands in (A) were quantitated and corrected for background intensity. The logarithm of the dilution of target was plotted against the logarithm of the ratio of competitor value to target value for each dilution (lanes 1–6). The equation describing the line of best fit, obtained by the least squares method, and the regression coefficient of the line are presented.

deficiencies in existing quantitative PCR methods. Four of these were of particular importance: (*i*) the introduction of a known amount of APL1, a synthetic external standard RNA at the beginning of RNA extraction. The quantitation of APL1 cDNA in samples permitted inefficiencies of RNA recovery and cDNA synthesis in the samples to be calculated and taken into account; (*ii*) to titrate the cDNA samples against a fixed amount of competitor, rather than vice versa. This ensured that the total amount of template DNA for a given primer set was constant for all of the samples at the dilution, at which equivalent amounts of target and competitor products were generated; (*iii*) to choose PCR conditions such that reactions at the point of equivalence were maintained in the exponential phase of amplification throughout the reaction; and (*iv*) to use a cDNA of known concentration to determine the degree to which amplification bias of target over competitor or vice versa affected the determination of the point of equivalence. The result was a sensitive method that allowed the determination of absolute numbers of copies of MMP and TIMP mRNAs in cell cultures and tissue samples. For the sake of convenience, standard thermal cycling parameters were chosen, so that assays for several mRNAs could be performed simultaneously in a single block.

Comparative PCR assays were developed for the quantitation of the mRNAs encoding rat collagenase-3, gelatinase B, stromelysin-1, stromelysin-2, TIMP-1, TIMP-2, GAPDH and the synthetic external standard, APL1. Figure 1 shows the corresponding target and competitor-derived PCR products for each of the MMPs and TIMPs investigated. The specificity of each primer pair was evaluated empirically on BC1 rat mammary carcinoma-derived cDNA and competitor templates. Under the optimized PCR conditions utilized, the primer pairs for each MMP and TIMP specifically amplified products of the expected size. Occasionally, bands corresponding to primer dimer formation were visible. However, these did not interfere with quantitation, as assays were performed under conditions in which reagents were not limiting (see Discussion). In addition to correct size, product identities were verified by direct sequencing. All of the MMP and TIMP sequences were identical to those that have been published or submitted to sequence databanks, except for stromelysin-1. GenBank® Accession No. X02601 reads C-C at bases 623 and 624, whereas BC1-derived stromelysin-1 cDNA reads A-A. This alters the predicted amino acid at codon 189 from threonine in the original sequence to isoleucine.

Figure 2A shows a phosphorescent image of a comparative PCR assay, in which the bands corresponding to target (T) and competitor (C) products were generated. In this case, the target was the low-abundance mRNA for TIMP-1. Note that, as the amount of cDNA added to the reaction increased, the intensity of the bands of the competitor product remained constant, until the amount of cDNA increased past the point of equivalence, indicating that reagents were not limiting at the point of equivalence. Figure 2A, lanes 9 and 10, show the competitor-only and cDNA-only controls, respectively, demonstrating the specificity of the reaction and absence of contamination.

In addition to the expected target and competitor products, a third band or doublet of DNA (labeled H in Figure 2A, lanes 1–4) was routinely seen in comparative PCR assays for each MMP and TIMP, in reactions at or near the equivalence point. Although the identity of the third band(s) was unknown, its appearance was not the result of a nonspecific priming event, as it consistently failed to appear in both the competitor-only and the cDNA-only PCR controls (Figure 2A, lanes 9 and 10). It was hypothesized that this third band might be the result of the formation of heteroduplexes between target and competitor products, because its occurrence was maximal near the equivalence point. To verify this, the DNA constituting the additional band was analyzed in two ways. First, gel-purified material from both homoduplex bands and the putative heteroduplex band (Figure 3A, lanes 2–4) were denatured by heating to 96°C and then allowed to renature by cooling slowly to room temperature. Under these conditions, a heteroduplex will separate at the higher temperature into single strands. Upon cooling, each target sense strand can re-anneal with antisense strands from either target or competitor products, thereby forming both homoduplexes and heteroduplexes. Similarly, the target antisense strand and both strands of the competitor product will form both homoduplexes and heteroduplexes, upon cooling. Figure 3A, lane 5, shows that bands corresponding in mobility to both heteroduplexes and homoduplexes were formed upon renaturing the putative heteroduplex product, confirming its identity as a heteroduplex. Control samples of target and competitor homoduplexes reannealed to give bands with their original mobilities (Figure 3A, lanes 6 and 7). Secondly, gel purified homoduplex and putative heteroduplex DNAs were electrophoresed under denaturing conditions. A heteroduplex will dissociate into its constituent target and competitor single strands when denatured. Figure 3B shows that the putative heteroduplex dissociates into two clusters of bands (lane 2), with mobilities corresponding to those resulting from the dissociation of the target and competitor products into single strands (Figure 3B, lanes 3 and 4).

Phosphorescent imagery and fluorescent imagery were both suitable for the quantitation of the PCR products. Phosphorescent imagery had the advantage of its response to signal intensity being linear over several orders of magnitude. The regression coefficients of the lines of best fit of data obtained by phosphorescent imagery were always greater than 0.9, and the slopes were usually between -0.85 and -1.2 (Figure 2B). A slope of -1 is predicted for this type of PCR assay (10). The small deviations from -1 might be due to variations in the measurement of background signal, because a small adjustment of the background level, by adding or subtracting a fixed amount of signal from all values, caused a substantial change in the slope, while causing just a very small change in the Y-intercept value.

The use of fluorescent imagery required that care be taken that the image be captured within the linear response range of the CCD camera, which was limited to approximately three orders of magnitude. Under these conditions, the lines of best fit were similar to those obtained by phosphorescent imagery. To ensure that measurements were taken consistently within the linear response range of the CCD camera, the signal strength of the competitor band in the competitor-only lane in each assay was adjusted by changing the aperture setting of the camera to give a fixed signal output corresponding to the middle of the linear response range. Fluorescent imagery had the advantage over phosporescent imagery of speed, in not having to dry the gels and expose them overnight to an imaging screen and avoided the expense and hazard of radioactive isotopes. Both imaging methods had equivalent sensitivity under the conditions used.

In comparative PCR, a discrepancy between the measured value and the ac-

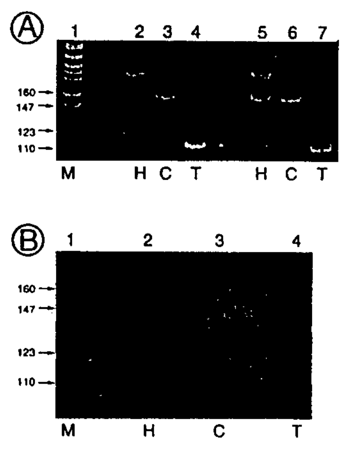


Figure 3. Heteroduplex formation in comparative PCRs. (A) Non-denaturing PAGE of renatured PCR products. Heteroduplex (H), target (T) and competitor (C) collagenase-3 PCR products were gel-purified. Each product was denatured by heating to 96°C and then cooled slowly for renaturation. The products before denaturation (lanes 2–4) and following renaturation (lanes 5, 6 and 7) were visualized by staining with ethidium bromide. The generation of target and competitor bands from the heteroduplex band is evident in lane 5. Lane 1 contains size markers, with the number of base pairs indicated to the left of the gel. (B) Gel-purified target, competitor and heteroduplex PCR products were electrophoresed in a denaturing polyacrylamide gel. Whereas the target (lane 4) and competitor (lane 3) products resolved into their respective complimentary strands, the heteroduplex (lane 2) was revealed to be composed of both target and competitor strands. Lane 1 contains size markers, with the number of base pairs indicated to the left of the gel.

tual value for an mRNA species might result when there is a difference in amplification efficiency between target and competitor templates (10). Therefore, the accuracy of each comparative PCR assay was determined by performing comparative PCR with known amounts of purified competitor and target templates, so that the discrepancies between actual and measured values could be accounted for in assays of samples. The discrepancy, or mean fold error, between the measured value and expected value for each target template, was 1–3-fold for the 8 assays (Table 1). These values were subsequently used to correct the target measurements made on cDNA samples. In this way, the values were adjusted for the amplification bias intrinsic to each assay and also for the incorporation of products into heteroduplexes.

To verify that the mRNA quantitation was independent of the amount of starting material, TIMP-2 mRNA was quantitated in the BC1-E1 clonal cell line (8), starting with 2.5×10^6, 25×10^6 and 50×10^6 cells. Six samples of each cell number were independently reverse transcribed and amplified and produced values of 102 ± 40, 122 ± 28 and 137 ± 39 copies/cell, respectively. Thus, the outcome of the assay for a given mRNA and cell type did not differ significantly, regardless of the amount of starting material over at least a 20-fold range, suggesting that any variations in the yield of RNA due to different amounts of starting material had been taken into account by the use of the external standard.

Expression of MMP and TIMP Genes in Mammary Tumors *In Vivo*

The comparative PCR assay incorporating the external standard was used to determine the absolute quantities of MMP and TIMP mRNAs in a sample of a rat mammary tumor (Table 1). High levels of mRNAs encoding the MMPs were detected, ranging from $33–210 \times 10^6$ copies per milligram tissue. Because each milligram of tissue contains approximately 10^6 cells, as determined by the density of packed cultured cells, this corresponds to approximately 33–210 copies per cell. By comparison, the TIMP mRNAs were present at lower levels, and the biologically unrelated GAPDH mRNA was present at 571×10^6 copies per mg tissue. It has been estimated that less than 2% of expressed genes are expressed at greater than 50 mRNA copies per cell (13). Thus, collagenase-3, stromelysin-1 and stromelysin-2 can be said to be expressed at high levels in this tumor.

DISCUSSION

We have described a protocol for the quantitation of mRNAs in cell and tissue samples that overcomes deficiencies in previous methods, which either do not offer absolute quantitation or do not take into account losses of yield during RNA isolation and cDNA synthesis. The current generally acceptable practice for examining RNA levels is to generate a specific signal using a technique (usually northern blotting) and compare it with the corresponding signal from the product of a housekeeping gene, such as GAPDH. This does not allow absolute quantitation of the mRNA of interest and, even for comparisons of relative mRNA levels to be made, assumes a uniform level of expression of the housekeeping gene in the different tissues or treatments of cells being compared. It is perhaps surprising, given its long history of use as a standard by which the expression of other genes are compared, that the gene for GAPDH was isolated only in 1988 (5) and that, in that paper, its expression was demonstrated to be modulated by insulin. Thus, cells that differ in their responsiveness to insulin or that grow in different concentrations of insulin or similar effectors would produce different amounts of GAPDH mRNA, thereby invalidating its use as a gene of invariant expression. Earlier work had demonstrated tissue-specific variations in the levels of GAPDH mRNA (9). It is likely that other commonly used internal standard genes undergo regulation that is dependent on environmental influences, tissue specificity or cell cycle.

Absolute quantitation of mRNA levels using the current protocol was made possible by the design of an assay that incorporated the following set of attributes: (*i*) the introduction of a synthetic external standard RNA; (*ii*) the titration of target against a constant amount of competitor, rather than vice versa; (*iii*) the use of PCR conditions in which reagents were not limiting and (*iv*) and the verification of the accuracy of the competitor assays, using known amounts of targets. No previous assay has incorporated all of these attributes, which are essential to the accurate quantitation of mRNAs in cell and tissue samples.

The inclusion of the external standard allowed inefficiencies and inconsistencies of RNA extraction and reverse transcription to be taken into account, a feature that is essential to absolute quantitation, but which has not been addressed by previous mRNA assays of cell and tissue samples, although it has been used in the semi-quantitation of in vitro transcripts (1). Without the external standard, it is not possible to take into account losses of mRNA during purification and incomplete cDNA synthesis, thereby making absolute quantitation impossible. If variability of mRNA yields occurs between tissues, even relative quantitation is impossible without knowing the extent to which the variability has occurred. The introduction of the external standard RNA to account for these errors assumes an identical behavior of the external standard and the mRNA being measured. Thus, it is important to ensure that the cell or tissue, from which the RNA is being extracted, is lysed completely and homogeneously in the GITC solution, and that reverse transcriptase, primers and nucleotides are not limiting during cDNA synthesis.

Most published protocols for competitive PCR assays determine the point of equivalence by co-amplifying a constant amount of sample against a titration of competitor (6). This is an aspect of PCR assays that has not received much attention previously, but is worthy of scrutiny because it has important consequences for the accuracy of the assay. During PCR, reagents become limiting when cycling continues after a certain amount of product has been amplified. When this occurs, the post-exponential phase of PCR is reached, and the bias in amplification efficiency between the target and competitor sequences, brought about by differences in length and sequence composition, becomes more pronounced, so that the amplification of one template is favored over the other (10). This amplification bias introduces a discrepancy between the actual and measured amounts of target that increases with cycle number. Those PCRs having a higher amount of total template DNA (target + competitor) will reach plateau phase earlier than those having a lower amount of total template DNA. Thus, the amplification bias and resultant discrepancies in measurements will be different for PCRs containing different amounts of total template DNA (10). Therefore, in PCR assays in which a constant amount of target-containing sample is co-amplified against a titration of competitor, the amount of total template DNA amplified at the point of equivalence will be different for each sample, resulting in different discrepancies between measured and actual values for each sample. Thus, in those assays, different correction factors would be needed for each different concentration of sample.

The above situation can be avoided by co-amplifying a constant amount of competitor against a titration series of sample, as is done in the present protocol. Using this protocol, the amount of total template DNA amplified at the point of equivalence is equal for all samples, regardless of their target cDNA content. Consequently, the discrepancy between actual and measured amounts of target, due to amplification bias, is constant for all samples and can be taken into account, once it has been determined empirically. Also, by restricting the PCR to 25 cycles, the reagents do not become limiting for the amount of competitor chosen (0.1 amol) (Figure 2). As a result, the bias in amplification efficiency between target and competitor sequences is minimized (10), and small amounts of nonspecific products do not interfere, because actual competition for reagents does not occur. To this extent, competitive PCR is a misnomer for assays conducted within the exponential phase of amplification. Even greater sensitivity can often be achieved by reducing the amount of competitor to 0.01 amol and increasing the cycle number to 30.

Although the use of gel electrophoresis to distinguish the products of the two templates is somewhat labor-intensive, it has the advantage of being able to measure the products of both the target and the competitor templates in each sample. Under conditions in which reagents are not limiting, such as those used in the present protocol, it is necessary to measure both products. PCR assays that detect only one product measure the product of the template that is added in a fixed amount and rely on competition between it and the template being titrated to determine the point of equivalence. Thus, those types of assays must be taken beyond the exponential phase of amplification, since competition requires that reagents become limiting, and consequently, those types of assays must be subject to the potential problems associated with entry to the post-exponential phase, such as increasing amplification biases. By separating the products electrophoretically and measuring them both to determine their ratio, the necessity for entry into plateau phase is avoided. Another advantage of measuring both products is that it diminishes the so-called tube effect, in which otherwise identical PCRs can generate different amounts of products in different tubes, due to the nonuniformity of heating blocks and tube shapes. Because the ratio of both products is being measured, and the change in amplification efficiency due to the tube effect is likely to affect both templates equally during the exponential phase, the ratio will not be affected. In contrast, if just one product is being measured, errors due to the tube effect will be incorporated into the data.

Verification of the equivalence point is important to establishing the accuracy of any competitive or comparative PCR assay, but is rarely reported. The amplification bias for each pair of target and competitor templates in the assays described here were determined empirically from a comparative PCR assay on a solution containing a known concentration of purified DNA incorporating the target sequence. The known concentration was compared with the measured concentration, and the proportional error gave a measure of the amplification bias over 25 cycles. That the discrepancy was always less than threefold confirmed that the bias for one template over another was small (<5% per cycle) under the conditions chosen. Importantly, irrespective of its size, this error factor can be taken into account in subsequent assays and is essential to ensuring accuracy of quantitation.

Absolute quantitation is important to understanding gene expression, as it will enable comparisons of mRNA levels to be made between different mRNAs within a sample, between different types of cells and tissues, between different time points and between different laboratories. Also, it enables transcription initiation rates to be determined (7). The use of the comparative PCR procedure described here avoids problems associated with previously described methods and also contributes a greater sensitivity and specificity than non-PCR-based techniques.

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REFERENCES

1. **Becker, P.B., S.K. Rabindran and C. Wu.** 1991. Heat shock-regulated transcription in vitro from a reconstituted chromatin template. Proc. Natl. Acad. Sci. USA *88*:4109-4113.
2. **Becker-André, M. and K. Hahlbrock.** 1989. Absolute mRNA quantification using the polymerase chain reaction (PCR). A novel approach by a PCR aided transcript titration assay (PATTY). Nucleic Acids Res. *17*:9437-9446.
3. **Celi, F.S., M.E. Zenilman and A.R. Shuldiner.** 1993. A rapid and versatile method to synthesize internal standards for competitive PCR. Nucleic Acids Res. *21*:1047.
4. **Chomczynski, P. and N. Sacchi.** 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. *162*:156-159.
5. **Ercolani, L., B. Florence, M. Denaro and M. Alexander.** 1988. Isolation and complete sequence of a functional human glyceralde-

hyde-3-phosphate dehydrogenase gene. J. Biol. Chem. *263*:15335-15341.

6. **Gilliland, G., S. Perrin, K. Blanchard and H.F. Bunn.** 1990. Analysis of cytokine mRNA and DNA: detection and quantitation by competitive polymerase chain reaction. Proc. Natl. Acad. Sci. USA *87*:2725-2729.
7. **Iyer, V. and K. Struhl.** 1996. Absolute mRNA levels and transcriptional initiation rates in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA *93*:5208-5212.
8. **Lyons, J.G., K. Siew and R.L. O'Grady.** 1989. Cellular interactions determining the production of collagenase by a rat mammary carcinoma cell line. Int. J. Cancer *43*:119-125.
9. **Piechaczyk, M., J.M. Blanchard, L. Marty, C. Dani, F. Panabieres, S. El Sabouty, P. Fort and P. Jeanteur.** 1984. Post-transcriptional regulation of glyceraldehyde-3-phosphate dehydrogenase gene expression in tissues. Nucleic Acids Res. *12*:6951-6963.
10. **Raeymaekers, L.** 1993. Quantitative PCR: theoretical considerations with practical implications. Anal. Biochem. *214*:582-585.
11. **Saiki, R.K., S. Scharf, F. Faloona, K.B. Mullis, G.T. Horn, H.A. Erlich and N. Arnheim.** 1985. Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science *230*:1350-1354.
12. **Stevenson, G.A., J.G. Lyons, D.A. Cameron and R.L. O'Grady.** 1985. Rat carcinoma cells in long-term, serum-free culture provide a continuing supply of collagenase. Biosci. Rep. *5*:1071-1077.
13. **Zhang, L., W. Zhou, V.E. Velculescu, S.E. Kern, R.H. Hruban, S.R. Hamilton, B. Vogelstein and K.W. Kinzler.** 1997. Gene expression profiles in normal and cancer cells. Science *276*:1268-1272.
14. **Zimmerman, K. and J.W. Mannhalter.** 1996. Technical aspects of quantitative competitive PCR. BioTechniques *21*:268-279.

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Address correspondence to:

Dr. J. Guy Lyons
Kanematsu Laboratories
Royal Prince Alfred Hospital
Missenden Rd.
Camperdown, NSW 2050, Australia
Internet:guy@kan.rpa.cs.nsw.gov.au

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Epithelial Cells Up-Regulate Matrix Metalloproteinases in Cells within the Same Mammary Carcinoma That Have Undergone an Epithelial-Mesenchymal Transition[1]

Anna M. Martorana, Guoping Zheng, Timothy C. Crowe, Robert L. O'Grady, and J. Guy Lyons[2]

Kanematsu Laboratories, Royal Prince Alfred Hospital, Camperdown, New South Wales 2050 [A. M. M., G. Z., T. C. C., J. G. L.], and Department of Cell and Molecular Biology, University of Technology, Sydney, Gore Hill, New South Wales 2065 [A.M. M., R. L. O.], Australia

ABSTRACT

A metastatic rat mammary carcinoma cell line, BC1, contains cells that have retained epithelial differentiation characteristics and metaplastic cells that have undergone an epithelial-mesenchymal transition. These two subpopulations cooperate to degrade collagen. We have used novel PCR assays to quantitate, for the first time, absolute levels of the mRNAs encoding matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) in cell and tumor samples. BC1 tumors expressed high levels of the *collagenase-3, TIMP-2, stromelysin-1,* and *gelatinase B* genes and low levels of the *stromelysin-2* and *TIMP-1* genes. This pattern of expression was repeated in cultures of BC1 and cultures containing mixed clones of epithelial cells and metaplastic cells. In both BC1 and the biclonal cultures, metaplastic cells were the main source of collagenase-3, stromelysin-1 and stromelysin-2, whereas TIMPs were equally distributed and epithelial cells were the main source of gelatinase B. High levels of all four MMP mRNAs in metaplastic cells were dependent on coculture with epithelial cells, suggesting the production of an inducing factor by the epithelial cells. In contrast, gelatinase B mRNA was produced at a high level by epithelial cells in the absence of metaplastic cells. TIMP-2 mRNA was abundant in both subpopulations grown alone and did not change substantially upon coculture. Thus, the interclonal cooperativity to degrade collagen in BC1 cells required the induction of MMPs in metaplastic cells by epithelial cells. Interclonal cooperativity may be important to the progression of neoplastic tumors, a feature of which is phenotypic heterogeneity.

INTRODUCTION

MMPs[3] are a family of enzymes that are believed to mediate much of the extracellular matrix degradation that occurs in developmental and pathological processes, including tumor invasion and metastasis (1, 2). They include the interstitial collagenases, the gelatinases, and the stromelysins. Of particular importance is the ability of the interstitial collagenases to initiate degradation of interstitial collagens, a property unique to these enzymes. After the initial cleavage by an interstitial collagenase, other proteinases can degrade the partially hydrolyzed collagen into oligopeptides; the gelatinases are particularly efficient at this, because of their high affinity for denatured collagens, and can cooperate with interstitial collagenases in the destruction of collagens.

Destruction of extracellular matrix components occurs around neoplastic tumors and is required for invasion and metastasis (3, 4). An imbalance between MMPs and their specific inhibitors, the TIMPs, in favor of the MMPs has been proposed as a mechanism for mediating this destruction and has been observed in some cases (2, 5). Both direct overproduction of MMPs by the neoplastic cells and induction of MMPs in the tumor-associated stroma have been implicated as causes of this imbalance (6-11). Expression of the *collagenase-3* gene, for example, is associated with breast cancer cells (6). Overproduction of MMPs in some cells *in vitro* can be induced by cancer-associated cytokines and by aberrant expression of oncogenic signal transduction factor and transcription factor genes (1, 12-14).

Phenotypic heterogeneity is a feature of cancer cells. Contributing to it in carcinomas is metaplastic transformation, in which the epithelial characteristics of the neoplastic cells are lost and which is associated with a more aggressive behavior (15-20). The presence of clones of different phenotypes within the neoplastic cell population gives rise to the opportunity for interactions between them, which could alter the overall behavior of the tumor. Thus, clonal populations of neoplastic cells could cooperate to exhibit a more aggressive phenotype in coexistence than when present alone. In one such instance of interclonal cooperativity, the potential of a biphenotypic mammary carcinoma cell line to degrade collagen is enhanced when both subpopulations of cells are present (21). Using a novel assay that permits absolute quantitation of specific mRNAs, we have for the first time been able to compare absolute mRNA levels of different MMPs and TIMPs with one another in tumors and cultured mammary carcinoma cells. The results demonstrate that the induction of *MMP* gene expression in cancer cells that have undergone an epithelial-mesenchymal transition by cells that have retained their epithelial differentiation characteristics underlies this cooperativity to degrade collagen.

MATERIALS AND METHODS

Cell Cultures and Tumors. BC1 and its derivative clonal cell lines, E2 and M3, were cultured under continuously serum-free conditions as described (21, 22). Basal media and other culture chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). For RNA extraction and immunoblot analysis, the cell lines were plated in medium containing 20% self-conditioned medium (v/v), at a plating ratio of 1:20 in either 75 or 150 cm^2 Corning tissue culture flasks, and grown to confluence. To obtain cocultures of E2 and M3, the cells were plated together at a ratio of 4:1, a ratio at which production of collagenolytic activity is maximal (21). Cultures were fed with fresh medium, supplemented with 20% self-conditioned medium, 2 days prior to the extraction of RNA. At the time of RNA extraction, conditioned medium was collected from each cell line, clarified by centrifugation, and stored at -20°C until assayed for collagenolytic activity by the method of Nethery *et al.* (23). Except for experiments in which epithelial and metaplastic cells were isolated by differential trypsinization, cells were lysed for RNA extraction directly in their flasks. To separate metaplastic cells from epithelial cells, 150-cm^2 flasks were rinsed gently two times with medium, followed by the addition of 5 ml of a solution of 0.1% trypsin, 0.02% EDTA in PBS. The cells were observed continuously by phase contrast microscopy to determine when the metaplastic cells were detached (~4.5 min). At this time, the metaplastic cell fraction was collected, and an equal volume of 0.1% soybean trypsin inhibitor in PBS was added concurrently to the metaplastic cell fraction and to the flask containing attached epithelial cells. In this way, both the metaplastic and epithelial cell fractions were trypsinized for the same period of time. While the metaplastic

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[2] To whom requests for reprints should be addressed, at Kanematsu Laboratories, Royal Prince Alfred Hospital, Missenden Road, Camperdown, New South Wales 2050, Australia. Phone: (61) 2 95157656; Fax: (61) 2 95156255; E-mail: guy@kan.rpa.cs.nsw.gov.au.

[3] The abbreviations used are: MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase.

cells were pelleted by centrifugation, the flask containing epithelial cells was rinsed two times with PBS to remove residual metaplastic cells, leaving behind firmly attached epithelial cells. The two cell fractions were then processed for the extraction of RNA, by the addition of cell-lysis solution to the metaplastic cell pellet, and to the epithelial cells still attached to the flask. To determine the number of cells from which RNA was extracted, a representative flask was trypsinized until all of the cells were detached and hemocytometer counts were performed on both the metaplastic and epithelial cell fractions, as appropriate.

For tumorigenesis, BC1 cells were grown to confluence, harvested by trypsinization, and washed twice with PBS. The cell pellet was resuspended in PBS at a concentration of 1×10^7 cells/ml. Cells (0.5×10^6) in 50µl were injected into the right footpad of each female Dark Agouti rat, 7 weeks of age (Animal Resources Center, Perth, Australia). Tumors were allowed to grow for a period of 40 days before harvesting. Upon harvesting, both the left and right feet were amputated and weighed. The mass of the right footpad tumor was determined to be the difference in mass between the left and right feet. The footpad tumor was excised and cut into one-third and two-third portions, which were also weighed. The one-third portion was fixed in 10% formalin for histological analysis, and the two-thirds portion was stored in liquid nitrogen for the extraction of RNA. Formalin-fixed specimens were embedded in paraffin, cut into 5-µm sections, and stained with H&E. The number of cells from which tumor RNA was extracted was calculated assuming 950×10^3 cells/mg tissue, a figure determined empirically from the packed cell volume of trypsinized cultured cells.

The right and left popliteal lymph nodes were removed by blunt dissecting along the midline of the biceps femoris muscle, which encloses the node, and detaching the exposed lymph node from its supporting connective tissue. The right and left lymph nodes were weighed, and the right lymph node was dissected into one-third and two-third portions. The one-third portion was fixed in 10% formalin for histological analysis, and the two-thirds portion was placed in Ham's F12/DME (1:1), with 100 units/ml penicillin, for the preparation of primary cultures. The left popliteal lymph node was also fixed in 10% formalin for histological analysis. The weight of the lymph node metastasis in the right lymph node was determined as the difference in mass between the right and left lymph nodes.

Cell lines were obtained from lymph node metastases by cutting the two-thirds portion of the node into small (~1 mm^3) pieces and placing them in a flask of Ham's F12/DME (1:1), supplemented with 10% FCS (Cytosystems, Castle Hill, Australia). Once cell lines were established, they were maintained under serum-free conditions as for BC1 (22).

Quantitative PCR Assays. Total RNA was extracted from cultured cells and tumors according to the method of Chomczynski and Sacchi (24), and absolute quantitation of MMP and TIMP mRNAs was done by competitive PCR[4]. Tumors, kept frozen in dry ice, were pulverized using a mortar and pestle prior to the addition of tissue lysis solution.

Immunoblot Analysis. Culture media samples were concentrated by precipitation with 60% saturated ammonium sulfate, which precipitates MMPs, but not BSA, electrophoresed according to Laemmli (25) in 10% polyacrylamide gels under nonreducing conditions and transferred to polyvinylidene difluoride membranes. Cell cultures for immunoblot analysis were washed twice with protein-free culture medium and lysed with sample buffer containing 0.1M DTT. Membranes were probed for collagenase-3 with the mouse monoclonal antibody, CoBCl-IID1 (26), for stromelysin-1 with a rabbit polyclonal antibody raised against a COOH-terminal peptide (27), for keratin with a rabbit anti-pan-keratin polyclonal antibody (Dako), for E-cadherin with a monoclonal antibody (Transduction Laboratories), and vimentin with a monoclonal antibody (Zymed), according to the suppliers' instructions. Immunoreactive bands were detected using horseradish peroxidase-conjugated second antibodies and ECL chemiluminescence (Amersham), according to the manufacturer's instructions.

RESULTS

Metastatic Behavior of BC1 Subpopulations. The cell line, BC1, was injected into the right footpads of six Dark Agouti rats. The primary tumors grew rapidly and invaded the adjacent lumbricales muscles (Table 1; Fig. 1*A*). Five of the six rats developed markedly enlarged right popliteal lymph nodes (Table 1). Histological examination showed that all six rats had lymph node metastases (Fig. 1, *B* and *C*). The enlarged lymph nodes varied with respect to the degree to which the metastasis had replaced the original architecture and lymphatic tissue. Generally, tumor cells could be seen in the subcapsular sinuses, where these were still intact, and also in the medullary sinuses. In some of the enlarged lymph nodes, entire sections of the node had been replaced by tumor cells, indicating that BC1 cells were not only capable of metastasizing via the afferent lymphatic vessel but were also able to grow within the node itself.

BC1 is composed of two morphologically distinct populations of cells: epithelial cells, which have retained epithelial characteristics, including the presence of microvilli, tight junctions, and colony-style growth; and metaplastic cells, which have not (21). Immunoblot analysis demonstrates that the production of keratin and E-cadherin, proteins characteristic of epithelial tissues, is retained by epithelial cells, but not by metaplastic cells (Fig. 2*A*, *Lanes 1-4*), suggesting that metaplastic cells are the result of an epithelial-mesenchymal transition (28). Both types of cells produce vimentin, which was more abundant in metaplastic cells than in epithelial cells (Fig. 2A, Lanes 5 and 6).

To determine whether both epithelial cells and metaplastic cells of BC1 contained subpopulations that were potentially metastatic, cell lines were established from the popliteal lymph node metastases of five of the six rats; there was insufficient material to establish a culture from the lymph node from one of the six rats. Overall, both epithelial and metaplastic cells could metastasize, as evident from their presence in at least some of these cell lines (Fig. 1, *D-H*). However, not all cell lines from metastases retained both cell types. Two cell lines, 2LN and 3LN, resembled the parental cell line in that they contained morphologically identifiable populations of both epithelial and metaplastic cells (Fig. 1, *D* and *E*). 4LN had no identifiable metaplastic cells during early passages, but metaplastic cells were noticed later among the epithelial cells, suggesting either an expansion of a small number of metaplastic cells from the primary culture or the generation of metaplastic cells from precursors within the culture (Fig. 1*F*). Neither 5LN nor 6LN contained typical spindle-shaped metaplastic cells that could be removed from the flask with less than 4 min of trypsin treatment (Fig. 1, *G* and *H*). Thus, epithelial cells metastasized to the popliteal lymph node and grew there, regardless of whether metaplastic cells were also present in the lymph node. When the 6LN cultures, which were uniformly epithelial, were subjected to a second round of footpad injection and lymph node harvest, cultures of the lymph nodes yielded cells still composed only of the epithelial morphology. Similarly, when clonal metaplastic cells (M3) were injected into the footpad, cultures of the draining lymph nodes harvested after 6 weeks showed that two of six lymph nodes had only viable M3 cells in them. No cell lines could be established from

Table 1 *Tumor and lymph node size in BC1 tumor-bearing rats*

Rat no.	Mass (g) Primary tumor	Metastasis
1	0.23	0.032
2	0.45	0.17
3	0.47	0.51
4	0.41	0.31
5	0.46	0.32
6	0.55	0.19
Mean + SD	0.43 ± 0.11	0.26 ± 0.16

For each rat, 0.5×10^6 BC1 cells were injected into the footpad and allowed to grow for 40 days, and the masses of the tumors and lymph nodes were determined on an electronic balance, as described in "Materials and Methods."

[4] A.M. Martorana, G. Zheng, F. Springall, H. J. Iland, R. L. O'Grady, and J. G. Lyons. Absolute quantitation of specific mRNAs in cell and tissue samples incorporating an external standard, submitted for publication.

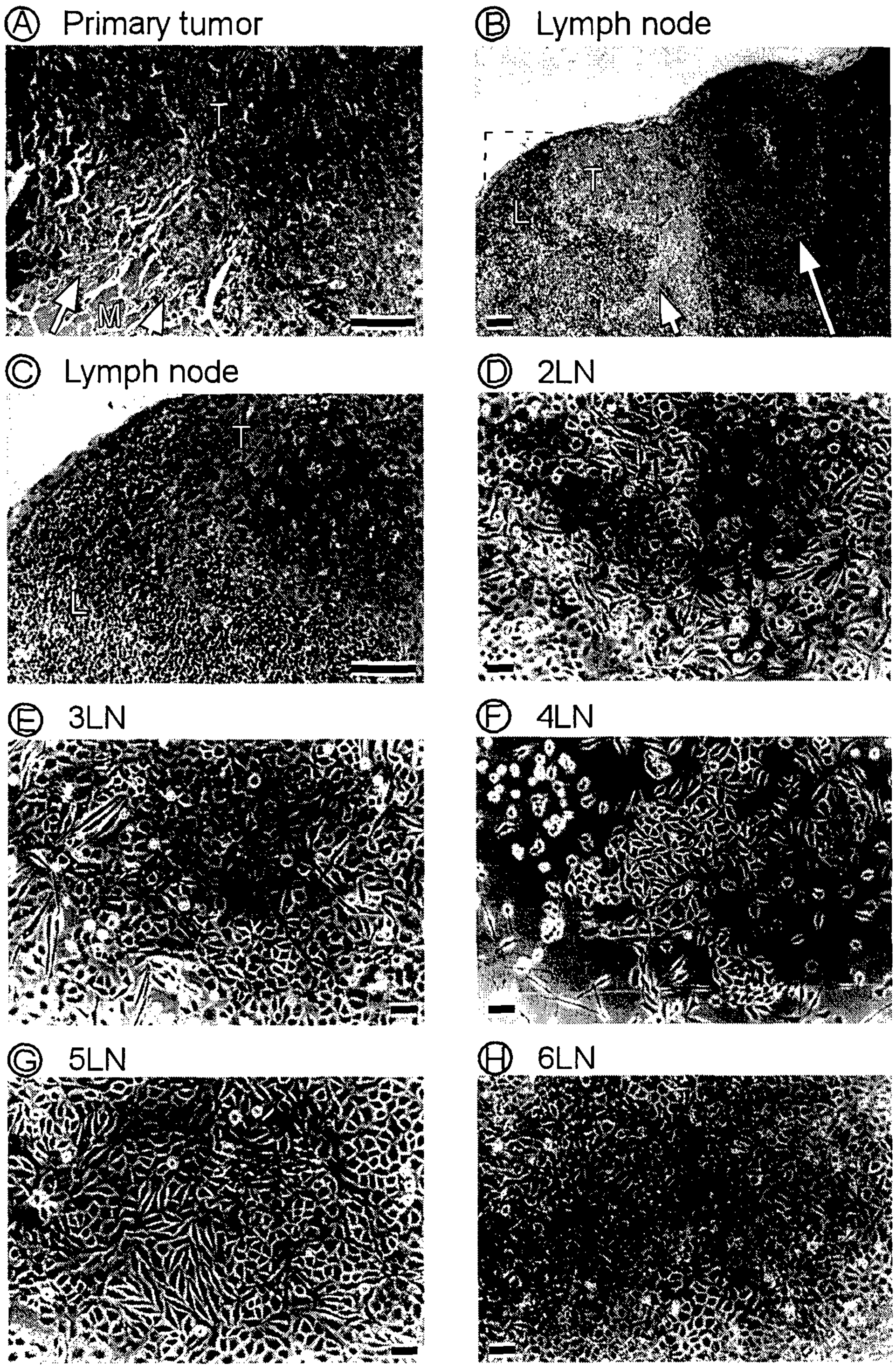

Fig. 1. Behavior of BC1 *in vivo*. Tumors were grown in the footpads of six syngeneic rats by injection with 0.5 × 10^6 BC1 cells and allowed to grow for 40 days. Samples of the primary tumor and lymph nodes were taken for histological analysis, RNA extraction, and primary culture. H&E-stained sections of primary tumor (*A*) and draining lymph node (*B* and *C*) are shown. The area *boxed* in *B* is shown at a higher magnification in *C*. Areas of tumor (*T*), muscle (*M*) and lymph node (*L*) are indicated. *Arrows*, invasive edges of tumors. The lymph nodes of rats numbers 2-6 were excised and used to establish cell lines 2LN, 3LN, 4LN, 5LN, and 6LN, depicted in phase contrast photomicrographs *D-H*, respectively. Cells exhibiting the spindle shape characteristic of the metaplastic cell phenotype are evident in panels *D-F* but not in *G* and *H*. *Bar*, 50μm

control lymph nodes under the conditions used for the BCl-derived cell lines. In both 6LN and M3, no evidence of an *in vivo* transition from metaplastic phenotype to epithelial phenotype or *vice versa* was observed. Thus, although epithelial and metaplastic cells may undergo a phenotypic transition *in vivo,* they appear to be able to reach the draining lymph nodes from the primary tumor without doing so.

Complementation of *MMP* and *TIMP* Gene Expression Is Not Sufficient to Generate Collagen-degrading Activity. Cultures of epithelial and metaplastic cells that are grown in isolation do not make significant levels of collagenolytic activity, whereas in coculture they make high levels (21). It was possible that complementation of gene expression could account for the acquisition of collagenolytic activity during coculture of epithelial and metaplastic cells, given the following scenario. The epithelial cells produce no interstitial collagenase, which is an absolute requirement for collagen degradation, but they produce an excess of MMPs (mainly gelatinase B) over TIMPs; the metaplastic cells, however, produce collagenase-3, but because their production of TIMPs exceeds their production of total MMPs, they are unable to produce a net collagen degrading activity. When the two media are mixed, the total level of MMPs exceeds the total level of TIMPs, and sufficient free collagenase-3 is available to initiate collagen degradation. This possibility was examined by mixing media

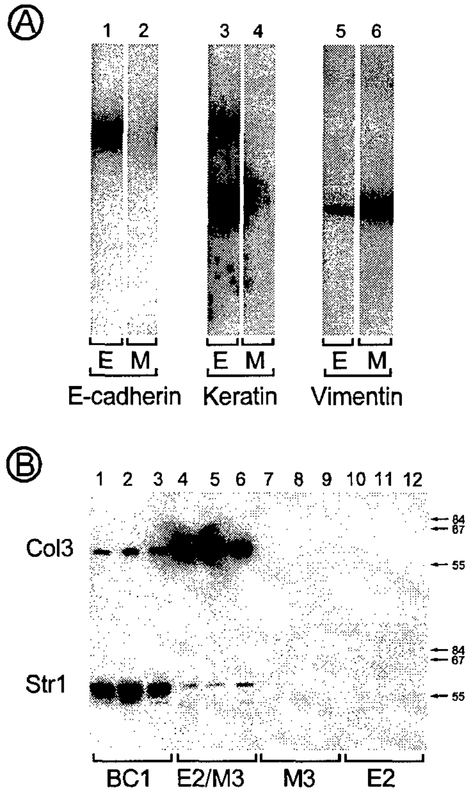

Fig. 2. Immunoblot analysis of epithelial differentiation markers and MMP production in BC1 and clonal derivatives. *A*, immunoblots of lysates of E2 or M3 cells, equivalent to 5×10^3 cells per lane, were probed with antibodies to E-cadherin *(Lanes 1* and *2)*, pan-keratin *(Lanes 3* and *4)*, and vimentin *(Lanes 5* and *6)* and detected by chemiluminescence. *B*, immunoblots of culture media from three separate platings of BC1 *(Lanes 1-3)*, cocultures of E2 and M3 *(Lanes 4-6)*, M3 grown alone *(Lanes 7-9)*, and E2 grown alone *(Lanes 10-12)* were probed for collagenase-3 and stromelysin-1 with specific antibodies. The positions of molecular weight markers (in thousands) are shown to the *right* of the membranes.

conditioned by each of the two subpopulations grown in isolation at a ratio of 4:1 (epithelial cell medium:metaplastic cell medium) and assaying it for collagenolytic activity. However, in three separate experiments, no net collagenolytic activity was generated. Thus, complementation of secreted MMPs was not normally sufficient to account for the increase in collagen degradation caused by mixing epithelial cells and metaplastic cells in culture.

Expression of *MMP* and *TIMP* Genes in BC1 Tumors *in Vivo* and Cultures *in Vitro*. To determine the levels of expression of *MMP* and *TIMP* genes *in vivo* in BC1 tumors, steady-state mRNA levels for collagenase-3, gelatinase B, stromelysin-1, stromelysin-2, TIMP-1, and TIMP-2 were quantitated by competitive PCR (Fig. 3A). Collagenase-3 and TIMP-2 mRNA were present at very high levels (>100 copies per cell), and gelatinase B and stromelysin-1 mRNAs were present at intermediate levels (20-100 copies per cell), whereas stromelysin-2 and TIMP-1 mRNAs were present at the lowest levels (<20 copies/cell).

A comparison of the levels of expression of *MMP* and *TIMP* genes in BC1 cells in culture with the levels in BC1 tumors revealed that the pattern of expression *in vivo* resembled that of BC1 cells *in vitro* (Fig. 3, A and B). Collagenase-3 and TIMP-2 mRNAs were present at very high levels in the cultured cells. TIMP-1 and stromelysin-2 mRNAs were present at very low levels *in vitro*. As in the tumors *in vivo*, gelatinase B and stromelysin-1 mRNAs also were present *in vitro*, although at relatively higher levels, comparable to those of collagenase-3 and TIMP-2 mRNAs.

E2 and M3 are clonal derivatives of BC1 that are representative of the epithelial and metaplastic phenotypes, respectively (21). To aid in the determination of which cells produce MMPs and TIMPs, E2 and M3 were cultured together, and the MMP and TIMP mRNA levels were measured by competitive PCR (Fig. 3C). The pattern of expression of *MMP* and *TIMP* genes in E2/M3 cocultures was similar to that seen in cultures of BC1. Collagenase-3 and TIMP-2 mRNA levels were high, with at least 95 copies/cell, whereas TIMP-1 and stromelysin-2 levels were consistently low, with the highest values measured at 16 and 23 copies/cell, respectively. Compared with the mRNA levels measured in BC1, gelatinase B mRNA was present in E2/M3 cocultures in somewhat higher quantities, with at least 540 copies/cell, whereas stromelysin-1 levels were somewhat lower, with the highest measured value at 60 copies/cell.

The increase in collagenolytic activity generated by mixing E2 cells with M3 cells (21) was reflected in an increase in secreted collagenase-3 and stromelysin-1 proteins, as detected by immunoblotting of

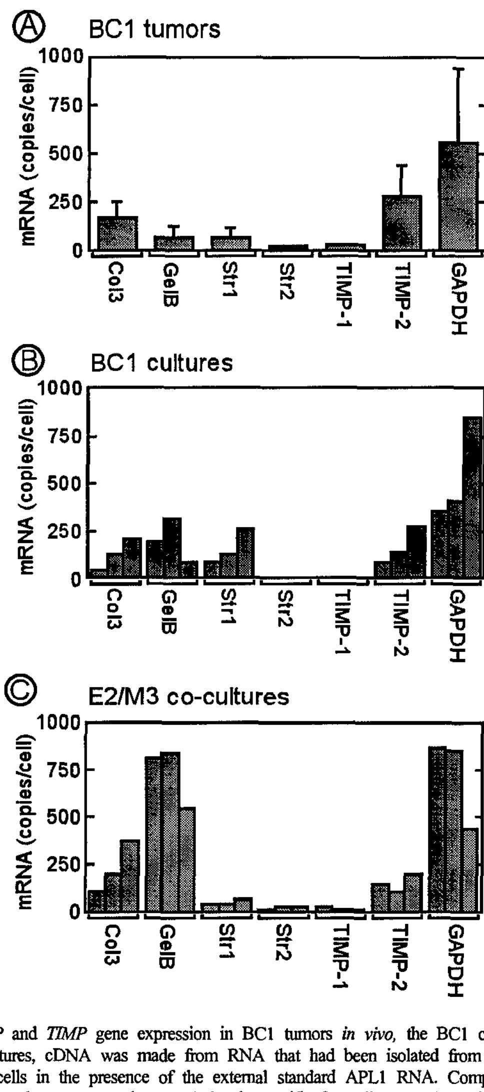

Fig. 3. *MMP* and *TIMP* gene expression in BC1 tumors *in vivo*, the BC1 cell line, and E2/M3 cocultures, cDNA was made from RNA that had been isolated from BC1-derived tumors and cells in the presence of the external standard APL1 RNA. Competitive PCR assays were used to measure the cDNA levels specific for collagenase-3 *(Col3)*, gelatinase B *(GelB)*, stromelysin-1 *(Str1)*, stromelysin-2 *(Str2)*, TIMP-1, TIMP-2, and glyceraldehyde-3-phosphate dehydrogenase *(GAPDH)* in each of the total cDNA samples, and the values were corrected using the APL1 value and the standard discrepancies between expected and measured values, as described[4]. The corrected mRNA values, expressed as the number of copies per cell, are shown for BC1 tumors (*A*), BC1 cultures (*B*), and E2/M3 cocultures (*C*). The tumor values are the means of six individual tumors; *bars*, SD. Each *column* in the BC1 and E2/M3 graphs represents one of three individual experiments.

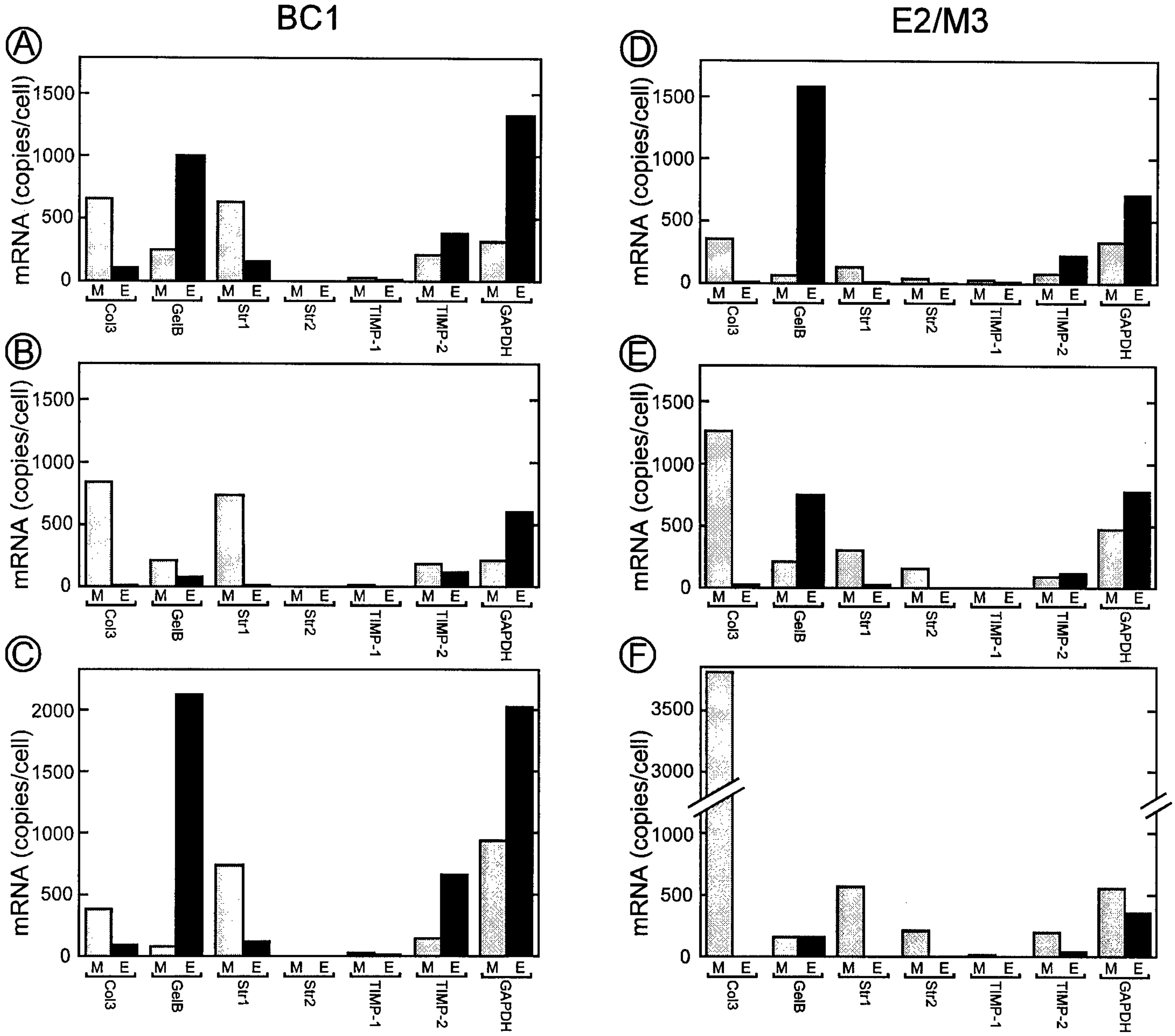

Fig. 4. Cellular origin of *MMP* and *TIMP* gene expression in BC1 and E2/M3 cocultures. Cultures of BC1 and E2/M3 cocultures were grown to confluence, and epithelial and metaplastic cell fractions were prepared from each by differential trypsinization. Competitive PCR assays were used to measure the mRNA levels for collagenase-3 *(Col3)*, gelatinase B *(GelB)*, stromelysin-1 *(Str1)*, stromelysin-2 *(Str2)*, TIMP-1, TIMP-2, and glyceraldehyde-3-phosphate dehydrogenase *(GAPDH)* in each metaplastic cell fraction (*M*) and epithelial cell fraction (*E*). *A-C*, values of three individual experiments for BC1; *D-F*, values for three independent experiments for E2/M3.

culture supernatants. Neither E2 nor M3 cultured alone consistently produced detectable collagenase-3 and stromelysin-1, whereas E2/M3 cocultures did so (Fig. 2*B*, compare *Lanes 4-6* with *Lanes 7-12)*, as did BC1 *(Lanes 1-3)*. Visualization of nonspecific bands by staining the membranes in Fig. 2*B* with Ponceau S prior to immunodetection demonstrated equivalent protein loadings (data not shown).

Cellular Origin of MMPs and TIMPs in BC1. To identify which population of cells in BC1 produces the MMPs and TIMPs, separate fractions of epithelial and metaplastic cells were prepared from BC1 by differential trypsinization. Epithelial and metaplastic cells differ with respect to their susceptibility to detachment from culture vessel surfaces by trypsin, to the extent that separate fractions of each may be prepared during the course of trypsinization of BC1 (21). Three separate differential trypsinization experiments were performed, and the mRNA levels of the MMPs and TIMPs were measured in the epithelial and metaplastic cell fractions of each by competitive PCR (Fig. 4, *A-C*). In all three replicates, metaplastic cells were the major source of collagenase-3 in BC1, with the number of mRNA transcripts per cell always measuring >380. In comparison, the number of collagenase-3 transcripts per epithelial cell was never >100, with the lowest measurement made at 12 copies/cell. In addition to providing most of the collagenase-3 mRNA in BC1, metaplastic cells were the major source of stromelysin-1 mRNA. The measured levels were always >600 copies per metaplastic cell, whereas the highest number of transcripts per epithelial cell was 150, and the lowest value was measured at 10 copies per epithelial cell. In two of the three replicates, epithelial cells produced most of the gelatinase B, containing at least 1000 copies of mRNA per cell, consistent with immunolocalization studies of the protein (26). Although the third replicate measured just 82 copies of gelatinase B per epithelial cell, the metaplastic cells in

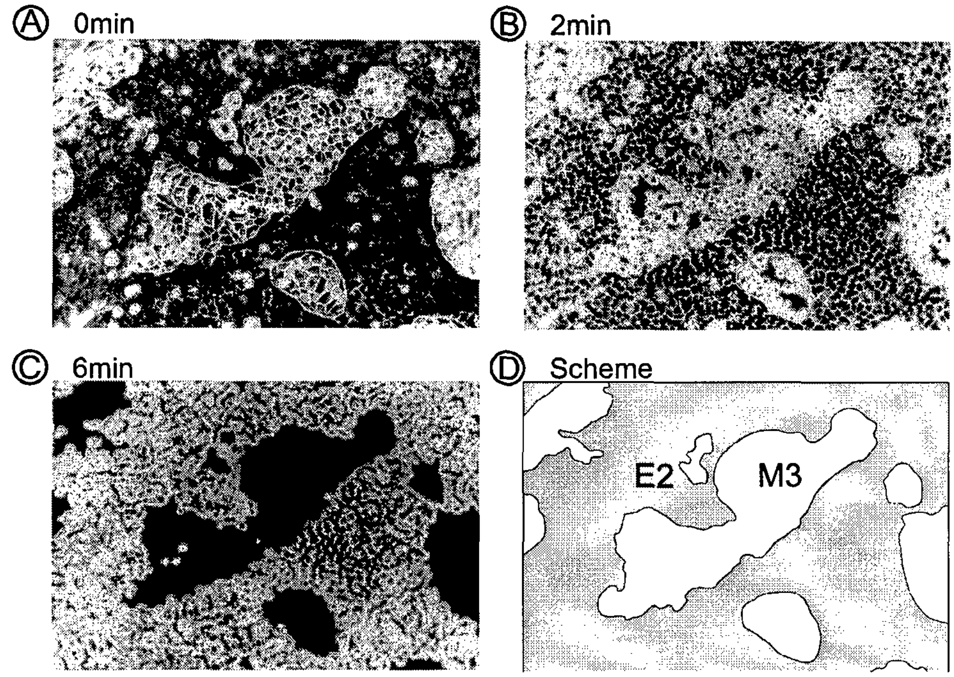

Fig. 5. E2 and M3 can be isolated from E2/M3 cocultures as separate fractions, using differential trypsinization. E2 and M3 cells were plated in the same flask at a ratio of 4:1 and allowed to grow to confluence. The coculture was rinsed once with F12/DME and treated at room temperature with a solution of 0.1% trypsin, 0.02% EDTA in PBS. Phase contrast photomicrographs of a typical field were taken. *A*, the E2/M3 coculture just prior to trypsinization. *B*, the same field of the culture after 2 min of trypsinization. *C*, the E2/M3 coculture after 6 min of trypsinization and gentle agitation to dislodge and remove detaching M3 cells. The E2 cells required an additional 5-6 min to detach. In *D*, the locations of E2 and M3 colonies are identified schematically.

BC1 never produced >240 copies/cell. TIMP-2 mRNA was found to be abundant in all epithelial and metaplastic cell fractions, with either cell fraction producing at least 120 copies of transcript per cell. TIMP-1 mRNA levels were consistently lower than TIMP-2 levels in both epithelial and metaplastic cell fractions, with the highest value measured in metaplastic cells at 18 copies/cell. The level of stromelysin-2 mRNA in whole BC1 and in any of the three epithelial cell fractions was below the limit of detection of the assay. However, it was detected in two of the three metaplastic cell fractions at 0.6 and 0.7 copies/cell, respectively, suggesting that stromelysin-2 is present in cultures of BC1, albeit at very low levels, and definitely not in all cells.

To determine whether the cellular origin of MMPs and TIMPs in E2/M3 cocultures was the same as that in BC1, separate E2 and M3 cell fractions were prepared from cocultures by differential trypsinization. Fig. 5 demonstrates that E2 and M3 can be isolated from E2/M3 cocultures in a manner analogous to that used for isolating epithelial and metaplastic cells from BC1. Three separate differential trypsinization experiments were performed, and the mRNA levels of the MMPs and TIMPs were measured in the E2 and M3 cell fractions of each by competitive PCR (Fig. 4, *D-F*). In all three replicates, the M3 cell fraction was the major source of collagenase-3 mRNA with the number of mRNA copies per cell measuring >350 and the highest value measured at 3800. In contrast, the level of mRNA transcripts for collagenase-3 in the E2 cell fraction was never >30 copies/cell. The M3 cell fraction was also the major source of stromelysin-1 and stromelysin-2 mRNA in all three replicates, with the number of transcripts per cell measuring at least 125 and 42, respectively. All three E2 cell fractions contained very low amounts of the stromelysin-1 and stromelysin-2 mRNAs, the highest values measuring at 5 and 4 copies/cell, respectively. In two of three replicates, the E2 cell fraction provided most of the gelatinase B mRNA copies, containing at least 750 copies/cell. Whereas the third E2 cell fraction contained just 165 copies per cell, the level of gelatinase mRNA in all three M3 cell fractions never exceeded 210 copies/cell, and the lowest value was measured at 65 copies/cell. TIMP-2 mRNA was present in both of the E2 and M3 cell fractions with at least 40 copies/cell in each, whereas TIMP-1 was present once more at lower levels, with the measured values in the E2 and M3 cell fractions ranging from 0.1 to 20 copies/cell.

Overall, the metaplastic cell fractions of BC1 and E2/M3 cocultures contained most of the collagenase-3, stromelysin-1, and stromelysin-2 mRNAs, whereas very high levels of gelatinase B mRNA could be produced only by the epithelial cell fractions. Both cell fractions contained TIMP-2 mRNA, at a high level, and TIMP-1 mRNA, albeit at a lower level.

Regulation of MMP and TIMP Gene Expression in Epithelial Cells and Metaplastic Cells during Coculture. Epithelial and metaplastic cells cultured in isolation do not produce collagenolytic activity in their culture medium, whereas in coculture they are able to do so (21). E2 and M3 cells in coculture are similar to the epithelial and metaplastic cells in BC1 cultures, with respect to their morphologies, their susceptibilities to detachment by trypsin, and the profile of MMPs and TIMPs that they produce. Thus, E2 and M3 were used for understanding the interactions between epithelial and metaplastic cells within BC1 that lead to a production of collagenolytic activity.

To determine whether the levels of MMP and TIMP mRNAs in epithelial cells could be regulated by metaplastic cells, the MMP and TIMP mRNA levels in E2 cells were measured and compared with those of the E2 cell fraction from cocultures of E2 and M3 (E2/M3). The collagenolytic activity in the culture medium of the E2 cells cultured alone was less than 8×10^{-3} units/ml, whereas that in the medium of the E2/M3 cocultures ranged from 40×10^{-3} to 100×10^{-3} units/ml. From Fig. *6A*, it can be seen that, other than gelatinase B mRNA, E2 cells cultured alone do not produce MMP mRNAs (collagenase-3, stromelysin-1, and stromelysin-2) at detectable levels. It can also be seen that this profile of gene expression does not change in the E2 cell fraction from E2/M3 cocultures. Thus, metaplastic cells were not required for the production of high levels of gelatinase B mRNA by E2 cells, although they may provide some degree of stimulation. Additionally, the mRNA levels of TIMP-1 and TIMP-2 did not differ between E2 cells and cocultured E2 cell fractions.

To determine whether the levels of MMP and TIMP mRNAs in metaplastic cells could be regulated by epithelial cells, the MMP and TIMP mRNA levels in M3 were measured and compared with those

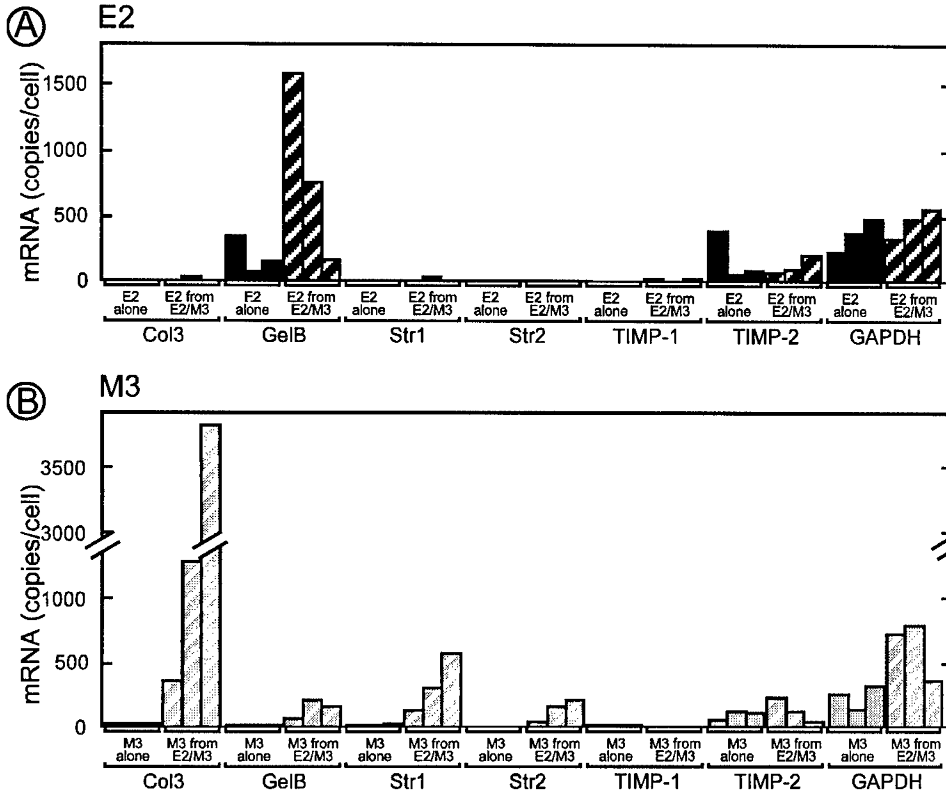

Fig. 6. Up-regulation of MMPs in metaplastic cells upon coculture with epithelial cells, mRNA levels for MMPs and TIMPs were determined by quantitative PCR analysis in E2 cells (*A*) and M3 cells (*B*) that had been grown in isolation (■) and in coculture (▨). The E2 and M3 cells from cocultures were isolated by differential trypsinization, as in Fig. 5. *Columns,* data from each of three individual experiments. *Col3,* collagenase-3; *GelB,* gelatinase B; *Str1,* stromelysin-1; *Str2,* stromelysin-2; *GAPDH,* glyceraldehyde-3-phosphate dehydrogenase.

of the M3 cell fraction of E2/M3. M3 cells cultured in the absence of epithelial cells produced less than 8×10^{-3} units/ml collagenolytic activity in their medium. From this comparison (Fig. 6*B*), it can be seen that coculturing M3 with E2 effects a marked increase in the mRNA levels of collagenase-3, gelatinase B, stromelysin-1, and stromelysin-2 in M3 cells. Collagenase-3 mRNA levels in M3 cultures were never higher than 30 copies/cell, whereas the lowest value measured for collagenase-3 mRNA in the M3 cell fraction of E2/M3 cocultures was 350 copies/cell, and the highest value was 3800 copies/cell. Gelatinase B mRNA levels were elevated in the M3 cell fractions of E2/M3 cocultures, containing at least 65 copies/cell, when compared with the mRNA level in M3 cells cultured alone, which was never >7 copies/cell. Stromelysin-1 mRNA in M3 cells cultured alone was never >30 copies/cell, whereas in the M3 cell fraction of E2/M3 cocultures, the lowest and highest mRNA values measured were 128 and 570 copies/cell, respectively. Similarly, M3 cells cultured alone never produced more than two copies of stromelysin-2 mRNA/cell, whereas the M3 cell fractions of E2/M3 cocultures contained at least 40 copies/cell and as many as 200 copies/cell. TIMP-1 mRNA levels were slightly diminished upon coculture, were low in both cases, and TIMP-2 mRNA levels were high in both M3 cultured alone and the M3 cell fraction of E2/M3 cocultures.

DISCUSSION

We have shown that phenotypically distinct clones comprising mammary tumors can produce a high level of collagen degrading activity through interclonal induction of *MMP* gene expression. In BC1, cells that retained epithelial differentiation characteristics are required for induction of MMPs in cells from the same tumor that have undergone an epithelial-mesenchymal transition. Absolute quantitation of MMP and TIMP mRNAs has not been performed before, and has made it possible, for the first time, to compare accurately the mRNA levels of different MMPs and TIMPs within a cell or tissue sample and of the same mRNA between different samples. It has been determined by others that the mRNAs of <0.2% of genes expressed in mammalian cells are present at >500 copies/cell and that mRNAs of <2% are present at >50 copies/cell (29). Thus, the mRNAs of collagenase-3, gelatinase B, stromelysin-1, and TIMP-2 truly are major gene products of BC1 cells and tumors.

The genes for collagenase-3, gelatinase B, stromelysin-1, stromelysin-2, TIMP-1, and TIMP-2 were all expressed in the BC1 tumors, suggesting that each of them could have positive or negative roles in invasion and metastasis by BC1 *in vivo.* Previous reports in the literature regarding the likelihood that mammary carcinoma cells actively express these genes *in vivo* have varied and have only been qualitative in nature. The pattern of *MMP* and *TIMP* gene expression in BC1 cells *in vitro* was found to resemble closely that seen in BC1 tumors *in vivo* (Fig. 3). By histological analysis, it was determined that most cells in the tumors were neoplastic. Therefore, it is likely that most of the abundant mRNAs measured in the BC1 tumors were contributed by the BC1 cells themselves. Low level mRNAs (stromelysin-2 and TIMP-1) could be either abundant products of a low number of cells, such as macrophages and endothelial cells, or low level products of the BC1 cells. MMPs are produced as latent proenzymes that require activation by removal of specific propeptide sequences induced by exposure to other proteinases or chemicals (30). It has been demonstrated previously that BC1 cells secrete a plasminogen activator that initiates MMP activation and collagen degradation in the presence of plasminogen (31).

The *collagenase-3, stromelysin-1,* and *stromelysin-2* genes showed similar patterns of regulation in the mammary carcinoma cells in that metaplastic cells were the major source of these mRNAs and were induced by epithelial cells to up-regulate expression of the genes. The *collagenase-3* gene was expressed at a very high level in BC1 tumors *in vivo* and in cultures of BC1 *in vitro.* This is reflected in the large amounts of collagenase-3 protein produced by BC1 cells *in vitro* (32). The production of collagenase-3 by neoplastic cells of epithelial origin is consistent with the direct localization of collagenase-3 to isolated mammary carcinoma cells by *in situ* hybridization, where the hybridization signal was found to be focal and intense (9). It is also consistent with the immunolocalization of collagenase-3 protein to the neoplastic cells of breast tumors (6), although another study by the same group identified stromal cells as the source of collagenase-3 by *in situ* hybridization of three tumor specimens (33).

The *stromelysin-1* gene was expressed at a high level in BC1 tumors *in vivo* and at a very high level in BC1 cells *in vitro.* Although some studies of stromelysin-1 in tumors have identified its origin as the stromal cells surrounding the tumor (9, 34, 35), others have observed the overexpression of the *stromelysin-1* gene by the neoplastic cells themselves, the stage of tumor progression being important in determining expression. In a study by Matrisian *et al.* (36), the *stromelysin-1* gene was shown to be expressed at higher levels in malignant than benign epidermal tumors. Further work investigating the transformation of squamous cell carcinomas to highly aggressive spindle cell carcinomas clearly demonstrated the expression of the *stromelysin-1* gene in the neoplastic cells of spindle cell carcinomas by *in situ* hybridization and immunolocalization, along with expression of the gene in the adjacent stroma (8). The squamous cell carcinomas, which represent the earlier stage of tumor progression in this model of multistage carcinogenesis, did not show expression of the gene within neoplastic cells, although stromal signals were abundant. Similarly, *in vitro* transformation of nonmetastatic squamous cell carcinoma cells by transfection results in spindle cells that are metastatic, express high levels of *stromelysin-1* gene, and cease expression of *keratin* genes (37, 38). Thus, in the epidermal carcinomas, *stromelysin-1* gene expression correlates directly with an epithelial-mesenchymal transition. Additionally, a direct association between stromelysin-1 mRNA production in mammary tissue and extracellular matrix remodeling has been suggested. An elevated level of stromelysin-1 mRNA was detected during involution of the mammary gland, a normal, physiological process that is well recognized as involving extensive matrix degradation (39). Myoepithelial cells, which represent the product of a normal developmental process involving a partial epithelial-mesenchymal transition, were identified as the source of stromelysin-1 in the involuting mammary gland, as well as in preneoplastic foci of *Ha-ras*-induced tumors (39).

Unlike the other MMPs, stromelysin-2 mRNA was present at low levels in BC1 tumors. This result is not surprising, considering the paucity of examples in the literature localizing expression of stromelysin-2 in neoplastic mammary tissue *in vivo.* However, stromelysin-2 has been identified previously in other tumors (40-42), and it appears to be selectively expressed in tissues of epithelial origin (43). Although it is not typically expressed at a high level in invasive neoplasms of the mammary gland (9), the detection of stromelysin-2 in BC1 tumors may be the result of the high level of sensitivity afforded by reverse transcription-PCR or by the stage of tumor progression.

BC1 tumors were found to contain substantial quantities of gelatinase B mRNA, although at levels that were consistently lower than collagenase-3 mRNA. In contrast to the other MMPs, epithelial cells were the main source of gelatinase B mRNA. Strong evidence for the involvement of gelatinase B in tumor metastasis is provided by a study where rat embryo cells, transformed with the *Ha-ras* and *E1A* genes and capable of forming nonmetastatic tumors, were converted to a metastatic phenotype by transient transfection with a gelatinase B expression plasmid (44). Several studies have implicated the involvement of gelatinase B in mammary carcinomas, specifically. It has been identified in human and rat breast cancer cell lines by reverse transcription-PCR or zymography (26, 45, 46) and has been immunolocalized to human breast carcinoma cells in tumor specimens (47-49). Although conflicting reports regarding the ability of mammary carcinoma cells to produce gelatinase B may be found in the literature (9, 49), the detection and quantitation of gelatinase B mRNA in BC1 cells and tumors provide further evidence that, at some stage of tumor progression, carcinoma cells can express the *gelatinase B* gene.

The level of TIMP-1 mRNA in BC1 cells and tumors was consistently low, whereas the level of TIMP-2 mRNA was very high. The absence of copurifying TIMP-1 in gelatinase B protein preparations isolated from E2 cultures on gelatin-Sepharose (26) is explained by the absence of TIMP-1 mRNA in E2 cells. The TIMP levels in BC1 are unlike those studies reporting either overexpression of the *TIMP-1* gene or similar levels of TIMP-1 and TIMP-2, where both have been investigated. TIMP-1 and TIMP-2 mRNA levels were shown to be elevated in a variety of tumor types including colorectal, pulmonary, and breast carcinoma (5, 39, 50) and were localized to both the stromal and tumor cells (9, 48). Down-regulation of *TIMP* genes can apparently confer a neoplastic phenotype upon immortalized cells (51). However, the invasiveness of BC1 cells coexists with a high level of *TIMP-2* gene expression. Clearly, then, production of TIMP-2 is not sufficient to prohibit metastasis. It may even contribute to extracellular matrix degradation by catalyzing the activation of MMPs at the cell surface (52, 53).

The behavior of tumors derived from the BC1 cell line was of interest with respect to its use as a model for investigating the expression of the genes of MMPs and TIMPs in the processes of invasion and metastasis, where they are believed to play important roles. The ability of tumors derived from BC1 cells to metastasize in an immunologically authentic host makes the model a particularly useful one. Previous studies have established the ability of BC1 tumors to invade bone, by means of inducing osteoclast-mediated resorption, and to metastasize to the draining lymph node (54, 55). In the present study, histological examination revealed their ability to invade the lumbricales muscles of the footpad and to metastasize spontaneously to the draining popliteal lymph node and to the lungs (Fig. 1). In these respects, the behavior of BC1 resembles that of human breast cancers, which usually metastasize via draining axillary lymph nodes to distant sites, such as bone, where invasion of tissues can occur. The presence of both epithelial and metaplastic cells within cell lines derived from lymph node metastases demonstrated that both cell types were capable of metastasizing. However, the absence of metaplastic cells from two of these cell lines is consistent with at least a subpopulation of BC1 epithelial cells being able to produce a lymph node metastasis independently of the presence of metaplastic cells in that lymph node.

Phenotypic heterogeneity is a common feature of neoplasms, despite their apparently clonal origin. It can arise through somatic changes in the genome (56, 57) or through normal differentiation processes (58-60). The BC1 cell line contains two readily discernible phenotypes. The epithelial cells have retained epithelial differentiation characteristics, including microvilli and tight junction formation (21), as well as expression of *E-cadherin* and *keratin* genes, whereas the metaplastic cells resemble an epithelial-mesenchymal transformation into the spindle cell or pseudosarcomatous component of carcinosarcoma-type breast carcinomas (15, 16, 28, 61). Metaplastic transformation of carcinoma cells, although well documented, is probably underdiagnosed, particularly in cases where the metaplastic cells have lost epithelial differentiation markers and do not form the majority of the tumor tissue. Loss of epithelial markers and acquisition of stromal markers in breast cancer are associated with hormone independence and a more aggressive behavior (17-20, 62), as well as a profile of *MMP* gene expression tending toward that of stromal cells (63, 64). In those cases, the neoplastic cells cannot readily be distinguished from tumor-associated stromal cells, particularly in human specimens, where it is not usually possible to isolate cells of stromal appearance and test them for tumorigenicity, as was done for the metaplastic cells of BC1 (21). Ironically, the absence of epithelial markers and presence of stromal markers are often the same criteria used to identify MMP-producing cells in carcinomas as being stromal in origin. Thus, it is possible that some reports of *MMP* gene expression in carcinomas being confined to the nonneoplastic, tumor-associated stroma have actually detected expression in metaplastic carcinoma cells that have invaded the stroma. More definitive diagnostic tools for distinguishing between tumor-associated stromal and metaplastic cells in histological specimens would clarify this situation.

The present study shows for the first time that phenotypically distinct clones of cancer cells originating from the same tumor can cooperate through the modulation of gene expression of one cell type by the other, as exemplified by the up-regulation of the four *MMP* genes in metaplastic cells by epithelial cells. This ability of epithelial cells to induce the expression of the *MMP* genes in metaplastic cells suggests the existence of a signal originating with epithelial cells to which all four promoters are responsive in metaplastic cells. This could be either a soluble factor(s) or the direct result of cell-to-cell contact between metaplastic cells and epithelial cells. Transforming growth factor-α and emmprin are both examples of factors that have been associated with breast cancer cells and can induce production of MMPs in nonneoplastic cells (14, 65). Evidently, the genes of collagenase-3, stromelysin-1, and stromelysin-2 are not responsive to this signal in epithelial cells, where they are not expressed. Gelatinase B is unique among the MMPs examined in being produced at a constitutively high level by one of the cell types (the epithelial cells) in the absence of the other cell type. In contrast, the expression of its gene in metaplastic cells was dependent on induction by epithelial cells and was at a lower level, suggesting the possibility of two distinct modes of induction of this gene in cancer. Although other studies have shown that cancer cells can induce expression of MMP genes in nonneoplastic stromal cells *in vitro* (33, 34, 65), no previous study has demonstrated the ability of a subpopulation of neoplastic tumor cells to up-regulate expression of MMP genes in another subpopulation from the same tumor.

A second mechanism of interclonal cooperativity that was examined in BC1 is complementation of gene expression. In this case, the gene products of one or more clones do not exhibit an activity in isolation, but do so when mixed. This would have been evident by the culture media conditioned by epithelial cells and metaplastic cells that had been grown separately having no intrinsic collagenolytic activity, but acquiring it when mixed, because of a shift in balance between MMPs and TIMPs in favor of collagen degradation. However, this did not occur, demonstrating that this mechanism is insufficient to account for the acquisition of collagenolytic activity in mixed epithelial/metaplastic cell cultures. It remains a possibility that complementation of gene expression such as this may contribute to or even be necessary for interclonal cooperativity. However, it is insufficient in the absence of induction of MMP gene expression in metaplastic cells.

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REFERENCES

1. Birkedal-Hansen, H., Moore, W. G. I., Bodden, M. K., Windsor, L. J., Birkedal-Hansen, B., DeCarlo, A., and Engler, J. A. Matrix metalloproteinases: a review. Crit. Rev. Oral Biol. Med., *4:* 197-250, 1993.
2. Stetler-Stevenson, W. G., Liotta, L.A., and Kleiner, D. E. Extracellular matrix 6: role of matrix metalloproteinases in tumor invasion and metastasis. FASEB J., *7:* 1434-1441, 1993.
3. Grabowska, M. Collagen content of normal connective tissue, of tissue surrounding a tumour and of growing sarcoma. Nature, *183:* 1186-1187, 1959.
4. Stetler-Stevenson, W. G., Aznavoorian, S., and Liotta, L. A. Tumor cell interactions with the extracellular matrix during invasion and metastasis. Ann. Rev. Cell Biol., *9:* 541-573, 1993.
5. Kossakowska, A. E., Huchcroft, S. A., Urbanski, S. J., and Edwards, D. R. Comparative analysis of the expression patterns of metalloproteinases and their inhibitors in breast neoplasia, sporadic colorectal neoplasia, pulmonary carcinomas and malignant non-Hodgkin's lymphomas in humans. Br. J. Cancer, *73:* 1401-1408, 1996.
6. Freije, J. M., Diez-Itza, I., Balbin, M., Sanchez, L. M., Blasco, R., Tolivia, J., and Lopez-Otin, C. Molecular cloning and expression of collagenase-3, a novel human matrix metalloproteinase produced by breast carcinomas. J. Biol. Chem., *269:* 16766-16773, 1994.
7. Basset, P., Bellocq, J. P., Wolf, C., Stoll, I., Hutin, P., Limacher, J. M., Podhajcer, O. L., Chenard, M. P., Rio, M. C., and Chambon, P. A novel metalloproteinase gene specifically expressed in stromal cells of breast carcinomas. Nature, *348:* 699-704, 1990.
8. Wright, J. H., McDonnell, S., Portella, G., Bowden, G. T., Balmain, A., and Matrisian, L. M. A switch from stromal to tumor cell expression of stromelysin-1 mRNA associated with the conversion of squamous to spindle carcinomas during mouse skin tumor progession. Mol. Carcinog., *10:* 207-215, 1994.
9. Heppner, K. J., Matrisian, L. M., Jensen, R. A., and Rodgers, W. H. Expression of most matrix metalloproteinase family members in breast cancer represents a tumour-induced host response. Am. J. Pathol., *149:* 273-282, 1996.
10. Witty, J. P., Lempka, T., Coffey, R. J., and Matrisian, L. M. Decreased tumor formation in 7,12-dimethylbenzanthracene-treated stromelysin-1 transgenic mice is associated with alterations in mammary epithelial cell apoptosis. Cancer Res., *55:* 1401-1406, 1995.
11. Sympson, C. J., Talhouk, R. S., Alexander, C. M., Chin, J. R., Clift, S. M., Bissell, M. J., and Werb, Z. Targeted expression of stromelysin-1 in mammary gland provides evidence for a role of proteinases in branching morphogenesis and the requirement for an intact basement membrane for tissue-specific gene expression. J. Cell Biol., *125:* 681-693, 1994.
12. Matrisian, L. M., Glaichenhaus, N., Gesnel, M. C., and Breathnach, R. Epidermal growth factor and oncogenes induce transcription of the same cellular mRNA in rat fibroblasts. EMBO J., *4:* 1435-1440, 1985.
13. Wasylyk, C., Gutman, A., Nicholson, R., and Wasylyk, B. The c-Ets oncoprotein activates the stromelysin promoter through the same elements as several non-nuclear oncoproteins. EMBO J., *10:* 1127-1134, 1991.
14. Lyons, J. G., Birkedal-Hansen, B., Pierson, M. C., Whitelock, J. M., and Birkedal-Hansen, H. Interleukin-1β and transforming growth factor-α/epidermal growth factor induce expression of M_r 95,000 type IV collagenase/gelatinase and interstitial fibroblast-type collagenase by rat mucosal keratinocytes. J. Biol. Chem., *268:* 19143-19151, 1993.
15. Wargotz, E. S., Deos, P. H., and Norris, H. J. Metaplastic carcinomas of the breast. II. Spindle cell carcinoma. Hum. Pathol., *20:* 732-740, 1989.
16. Wargotz, E. S. and Norris, H. J. Metaplastic carcinomas of the breast. III. Carcinosarcoma. Cancer, *64:* 1490-1499, 1989.
17. Lee, C., Lapin, V., Oyasu, R., and Battifora, H. Effect of ovariectomy on serially transplanted rat mammary tumors induced by 7,12-dimethylbenz[*a*]anthracene. Eur. J. Cancer Clin. Oncol., *17:* 801-808, 1981.
18. Sommers, C. L., Walker-Jones, D., Heckford, S. E., Worland, P., Valverius, E., Clark, R., McCormick, F., Stampfer, M., Abularach, S., and Gelmann, E. P. Vimentin rather than keratin expression in some hormone-independent breast cancer cell lines and in oncogene-transformed mammary epithelial cells. Cancer Res., *49:* 4258-4263, 1989.
19. Sommers, C. L., Heckford, S. E., Skerter, J. M., Worland, P., Torri, J. A., Thompson, E. W., Byers, S. W., and Gelmann, E. P. Loss of epithelial markers and acquisition of vimentin expression in adriamycin- and vinblastine-resistant human breast cancer cell lines. Cancer Res., *52:* 5190-5197, 1992.
20. Nakanishi, H., Taylor, R. M., Chrest, F. J., Masui, T., Utsumi, K., Tatamatsu, M., and Passaniti, A. Progression of hormone-dependent adenocarcinoma cells to hormone-independent spindle carcinoma cells *in vitro* in a clonal spontaneous rat mammary tumor cell line. Cancer Res., *55:* 399-407, 1995.
21. Lyons, J. G., Siew, K., and O'Grady, R. L. Cellular interactions determining the production of collagenase by a rat mammary carcinoma cell line. Int. J. Cancer, *43:* 119-125, 1989.
22. Stevenson, G. A., Lyons, J. G., Cameron, D. A., and O'Grady, R. L. Rat carcinoma cells in long-term, serum-free culture provide a continuing supply of collagenase. Biosci. Rep., *5:* 1071-1077, 1985.
23. Nethery, A., Lyons, J. G., and O'Grady, R. L. A spectrophotometric collagenase assay. Anal. Biochem., *159:* 390-395, 1986.
24. Chomczynski, P. and Sacchi, N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem., *162:* 156-159, 1987.
25. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, *227:* 680-685, 1970.
26. Lyons, J. G., Birkedal-Hansen, B., Moore, W. G. I., O'Grady, R. L., and Birkedal-Hansen, H. Characteristics of a 95kDa matrix metalloproteinase produced by mammary carcinoma cells. Biochemistry, *30:* 1449-1456, 1991.
27. Sanchez-Lopez, R., Nicholson, R., Gesnel, M.-C., Matrisian, L. M., and Breathnach, R. Structure-function relationships in the collagenase family member transin. J. Biol. Chem., *263:* 11892-11899, 1988.
28. Birchmeier, C., Birchmeier, W., and Brand-Saberi, B. Epithelial-mesenchymal transitions in cancer progression. Acta Anat., *156:* 217-226, 1996.

29. Zhang, L., Zhou, W., Velculescu, V. E., Kern, S. E., Hruban, R. H., Hamilton, S. R., Vogelstein, B., and Kinzler, K. W. Gene expression profiles in normal and cancer cells. Science, *276:* 1268-1272, 1997.
30. Nagase, H., Enghild, J. J., Suzuki, K., and Salvesen, G. Stepwise activation mechanisms of the precursor of matrix metalloproteinase 3 (stromelysin) by proteinases and (4-aminophenyl)mercuric acetate. Biochemistry, *29:* 5783-5789, 1990.
31. O'Grady, R. L., Upfold, L. I., and Stephens, R. W. Rat mammary carcinoma cells secrete active collagenase and activate latent enzyme in the stroma via plasminogen activator. Int. J. Cancer, *28:* 509-515, 1981.
32. Lyons, J. G., Nethery, A., Harrop, P. J., and O'Grady, R. L. The collagenase produced by neoplastic rat epithelial cells: modulation of secretion, molecular weight characteristics and purification. Matrix, *9:* 7-16, 1989.
33. Uría, J. A., Stahle-Bäckdahl, M., Seiki, M., Fueyo, A., and López-Otín, C. Regulation of *collagenase-3* expression in human breast carcinomas is mediated by stromal-epithelial cell interactions. Cancer Res., *57:* 4882-4888, 1997.
34. Ito, A., Nakajima, S., Sasaguri, Y., Nagase, H., and Mori, Y. Co-culture of human breast adenocarcinoma MCF-7 cells and human dermal fibroblasts enhances the production of matrix metalloproteinases 1, 2 and 3 in fibroblasts. Br. J. Cancer, *71:* 1039-1045, 1995.
35. Newell, K. J., Witty, J. P., Rodgers, W. H., and Matrisian, L. M. Expression and localization of matrix-degrading metalloproteinases during colorectal tumorigenesis. Mol. Carcinog., *10:* 199-206, 1994.
36. Matrisian, L. M., Bowden, G. T., Krieg, P., Furstenberger, G., Briand, J. P., LeRoy, P., and Breathnach, R. The mRNA coding for the secreted protease transin is expressed more abundantly in malignant than benign tumours. Proc. Natl Acad. Sci. USA, *83:* 9413-9417, 1986.
37. Paine, M. L., Gibbins, J. R., Chew, K. E., Demetriou, A., and Kefford, R. F. Loss of keratin expression in anaplastic carcinoma cells due to posttranscriptional down-regulation acting in trans. Cancer Res., *52:* 6603-6611, 1992.
38. Paine, M. L., Gibbins, J. R., Whitelock, J. M., O'Grady, R. L., and Kefford, R. F. Unregulated and independent expression of collagenase and transin related to tumor progression. J. Natl Cancer Inst., *85:* 1425-1427, 1993.
39. Li, F., Strange, R., Friis, R. R., Djonov, V., Altermatt, H. J., Saurer, S., Niemann, H., and Andres, A. C. Expression of stromelysin-1 and TIMP-1 in the involuting mammary gland and in early invasive tumors of the mouse. Int. J. Cancer, *59:* 560-568, 1994.
40. Nicholson, R. C., Murphy, G., and Breathnach, R. Human and rat malignant tumour-associated mRNAs encode stromelysin-like metalloproteinases. Biochemistry, *28:* 5195-5203, 1989.
41. Muller, D., Quantin, B., Gesnel, M.-C., Millom-Collard, R., Abecassis, J., and Breathnach, R. The collagenase gene family in humans consists of at least four members. Biochem. J., *253:* 187-192, 1988.
42. Breathnach, R., Matrisian, L. M., Gesnel, M. C., Staub, A., and Leroy, P. Sequences coding for part of oncogene-induced transin are highly conserved in a related rat gene. Nucl. Acids Res., *15:* 1139-1151, 1987.
43. Windsor, L. J., Grenett, H., Birkedal-Hansen, B., Bodden, M. K., Engler, J. A., and Birkedal-Hansen, H. Cell type-specific regulation of SL-1 and SL-2 genes. J. Biol. Chem., *268:* 17341-17347, 1993.
44. Bernhard, E. J., Gruber, S. B., and Muschel, R. J. Direct evidence linking expression of matrix metalloproteinase 9 (92-kDa gelatinase/collagenase) to the metastatic phenotype in transformed rat embryo cells. Proc. Natl Acad. Sci. USA, *91:* 4293-4297, 1994.
45. Alessandro, R., Minafra, S., Pucci-Minafra, I., Onisto, M., Garbisa, S., Melchiori, A., Tetlow, L., and Woolley, D. E. Metalloproteinase and TIMP expression by the human breast carcinoma cell line 8701-BC. Int. J. Cancer, *55:* 250-255, 1993.
46. Nakajima, M., Lotan, D., Baig, M. M., Carralero, R. M., Wood, W. R., Hendrix, M. J., and Lotan, R. Inhibition by retinoic acid of type IV collagenolysis and invasion through reconstituted basement membrane by metastatic rat mammary adenocarcinoma cells. Cancer Res., *49:* 1698-1706, 1989.
47. Iwata, H., Kobayashi, S., Iwase, H., Masaoka, A., Fujimoto, N., and Okada, Y. Production of matrix metalloproteinases and tissue inhibitors of metalloproteinases in human breast carcinomas. Jap. J. Cancer Res., *87:* 602-611, 1996.
48. Visscher, D. W., Hoyhtya, M., Ottosen, S. K., Liang, C. M., Sarkar, F. H., Crissman, J. D., and Fridman, R. Enhanced expression of tissue inhibitor of metalloproteinase-2 (TIMP-2) in the stroma of breast carcinomas correlates with tumor recurrence. Int. J. Cancer, *59:* 339-344, 1994.
49. Soini, Y., Hurskainen, T., Hoyhtya, M., Oikarinen, A., and Autio-Harmainen, H. 72KD and 92KD type IV collagenase, type IV collagen, and laminin mRNAs in breast cancer: a study by in situ hybridization. J. Histochem. Cytochem., *42:* 945-951, 1994.
50. Stetler-Stevenson, W. G., Brown, P.D., Onisto, M., Levy, A.T. and Liotta, L.A. Tissue inhibitor of metalloproteinases-2 (TIMP-2) mRNA expression in tumor cell lines and human tumor tissues. J. Biol. Chem., *265:* 13933-13938, 1990.
51. Khokha, R., Waterhouse, P., Yagel, S., Lala, P. K., Overall, C. M., Norton, G., and Denhardt, D. T. Antisense RNA-induced reduction in murine TIMP levels confers oncogenicity on Swiss 3T3 cells. Science, *243:* 947-950, 1989.
52. Strongin, A. Y., Marmer, B. L., Grant, G. A., and Goldberg, G. I. Plasma membrane-dependent activation of the 72-kDa type IV collagenase is prevented by complex formation with TIMP-2. J. Biol. Chem., *268:* 14033-14039, 1993.
53. Murphy, G., Willenbrock, F., Ward, R. V., Cockett, M. I., Eaton, D., and Docherty, A. J. The C-terminal domain of 72 kDa gelatinase A is not required for catalysis, but is essential for membrane activation and modulates interactions with tissue inhibitors of metalloproteinases. Biochem. J., *283:* 637-641, 1992.
54. O'Grady, R. L. and Cameron, D. A. Osteoclasts and the resorption of bone by transformed mammary carcinoma in rats. Br. J. Cancer, *51:* 767-774, 1985.
55. Whitelock, J. M., O'Grady, R. L., and Gibbins, J. R. Interstitial collagenase (matrix metalloproteinase 1) associated with the plasma membrane of both neoplastic and nonneoplastic cells. Invasion Metast., *11:* 139-148, 1991.
56. Vogelstein, B. and Kinzler, K. W. The multistep nature of cancer. Trends Genet., *9:* 138-141, 1993.
57. Sonnenberg, A., van Balen, P., Hilgers, J., Schuuring, E., and Nusse, R. Oncogene expression during progression of mouse mammary tumor cells; activity of a proviral enhancer and the resulting expression of *int-2* is influenced by the state of differentiation. EMBO J., *6:* 121-125, 1987.
58. Bennett, D. C., Peachey, L. A., Durbin, H., and Rudland, P. S. A possible mammary stem cell line. Cell, *15:* 283-298, 1978.
59. Whitehead, R. H., Bertoncello, I., Webber, L. M., and Pederson, J. S. A new human breast carcinoma cell line (PMC42) with stem cell characteristics. I. Morphologic characterization. J. Natl Cancer Inst., *70:* 649-661, 1983.
60. Heppner, G. H., Miller, B. E., and Miller, F. R. Tumor subpopulation interactions in neoplasms. Biochim. Biophys. Acta, *695:* 215-226, 1983.
61. Pitts, W. C., Rojas, V. A., Gaffey, M. J., Rouse, R. V., Esteban, J., Frierson, H. F., Kempson, R. L., and Weiss, L. M. Carcinomas with metaplasia and sarcomas of the breast. Am. J. Clin. Pathol., *95:* 623-632, 1991.
62. Sonnenberg, A., Daams, H., Calafat, J., and Hilgers, J. *In vitro* differentiation and progression of mouse mammary tumor cells. Cancer Res., *46:* 5913-5922, 1986.
63. Gilles, C., Polette, M., Seiki, M., Birembaut, P., and Thompson, E. W. Implication of collagen type I-induced membrane-type 1-matrix metalloproteinase expression and matrix metalloproteinase-2 activation in the metastatic progression of breast carcinoma. Lab. Invest., *76:* 651-660, 1997.
64. Gilles, C., Polette, M., Piette, J., Birembaut, P., and Foidart, J. M. Epithelial-to-mesenchymal transition in HPV-33-transfected cervical keratinocytes is associated with increased invasiveness and expression of gelatinase A. Int. J. Cancer, *59:* 661-666, 1994.
65. Kataoka, H., DeCastro, R., Zucker, S., and Biswas, C. Tumor cell-derived collagenase-stimulatory factor increases expression of interstitial collagenase, stromelysin, and 72-kDa gelatinase. Cancer Res., *53:* 3154-3158, 1993.