[CANCER RESEARCH 56, 4606-4609, October 15, 1996]

Advances in Brief

Differential Expression of Psoriasin Messenger RNA between *in Situ* and Invasive Human Breast Carcinoma[1]

Etienne Leygue,[2] Linda Snell, Tamara Hiller, Helmut Dotzlaw, Kate Hole, Leigh C. Murphy, and Peter H. Watson

Departments of Biochemistry and Molecular Biology [E. L., H. D., L. C. M.] and Pathology [L. S., T. H., K. H., P. H. W.], University of Manitoba, Faculty of Medicine, Winnipeg, Manitoba, MB R3E OW3 Canada

Abstract

The mRNA encoding the calcium-binding protein psoriasin was identified as being more highly expressed in the *in situ versus* the invasive component of the same breast tumor. Reverse transcription-PCR analysis of total RNA extracted from three independent cases of ductal carcinoma *in situ* of the comedo type and three cases of high-grade invasive ductal carcinoma revealed a detectable level of psoriasin mRNA expression in the *in situ* lesions only. Similar analysis performed on total RNA extracted from frozen sections of 32 independent breast samples, representing a continuum from normal to invasive tumor, confirmed high psoriasin expression in ductal carcinoma *in situ* of the comedo type only. The possible functional role of the psoriasin protein in breast tumor cells remains to be determined.

Introduction

The currently accepted model of breast tumorigenesis is based on epidemiological and histological observations. These have defined a series of morphological changes including epithelial hyperplasia, atypical hyperplasia, and *in situ* carcinoma that may parallel the process of evolution toward an invasive breast cancer (1). It is believed that the initial transformation event involves epithelial cells in the terminal ductal unit. Sequential additional genetic alterations accumulate which contribute to the development of phenotypic changes such as altered patterns of growth, unrestricted proliferation, and the cytological appearance of malignancy which serve as the basis for the recognition of preinvasive DCIS.[3] Further alterations within the cells in these *in situ* lesions may then lead to the acquisition of the invasive phenotype and other manifestations of tumor progression. The onset of invasive tumor cells, which are able to generate future metastasis, is perhaps the most critical step in breast tumor progression. However, little is known about the molecular mechanisms underlying this transition. Knowledge of these mechanisms is a necessary prerequisite to allow a better understanding of this critical step in tumor progression and may provide targets for new and possibly curative therapies (2). To address this issue, we have undertaken a study to identify genes which are differentially expressed between the DCIS and the invasive breast cancer components within any biopsy sample.

Received 7/26/96; accepted 8/29/96.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by grants from the Canadian Breast Cancer Research Initiative and the U.S. Army Medical Research and Material Command. P. H. W. is a Medical Research Council of Canada Clinician-Scientist, L. C. M. is a Medical Research Council Scientist, and E. L. is a recipient of a U.S. Army Medical Research and Material Command Postdoctoral Fellowship.

[2] To whom requests for reprints should be addressed. Phone: (204) 789-3812; Fax: (204) 783-0864.

[3] The abbreviations used are: DCIS, ductal carcinoma *in situ*; PDWA, proliferative disease without atypia; ADH, atypical ductal hyperplasia; ER, estrogen receptor; PR, progesterone receptor; ss, single strand; RT, reverse transcription; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Materials and Methods

Human Breast Tissues

Human breast specimens were obtained from the Manitoba Breast Tumor Bank (University of Manitoba, Winnipeg, Manitoba, Canada). All cases in the bank have been rapidly frozen at −70°C after surgical removal and subsequently processed to create formalin-fixed and paraffin-embedded tissue blocks that are matched and orientated relative to a corresponding frozen tissue block. This facilitates interpretation of frozen tissue composition by reference to adjacent high-quality histological sections to map *in situ* and invasive compartments and to distinguish specific preneoplastic histological lesions.

Microdissection Case. To select a suitable microdissection case with which to start the study, 388 primary invasive ductal carcinomas were reviewed. Of these, 32% contained an identifiable *in situ* component and 17% contained a significant (*i.e.*, >20% of the tumor component within the block) DCIS component. From this subset, a microdissection case showing DCIS associated with a small invasive carcinoma was identified. In the corresponding frozen tissue block, DCIS (solid and comedo type) was geographically distinct from an invasive ductal carcinoma component [Nottingham (3) grade 7], but both were associated with similar stromal elements and a sparse inflammatory host response.

Group 1. To confirm the differential expression between *in situ* and invasive lesions of the gene identified in the microdissection case, six additional frozen tissue samples, corresponding to six different patients, were selected. This selection of three DCIS (IS1, IS2, and IS3) and three invasive ductal carcinomas (IV1, IV2, and IV3) was achieved primarily on the basis of similarity in DCIS subtype, invasive ductal morphology, and grade to the corresponding components of the original microdissection case.

Group 2. To study the expression of the identified genes at different steps of tumorigenesis and progression, a second panel of 32 breast tissue frozen samples, corresponding to 32 different patients, was selected. This panel comprised 5 cases with normal breast tissue, 5 cases with PDWA, 3 cases with ADH, 12 cases with DCIS, and 7 cases with invasive ductal carcinoma. Histological diagnosis and confirmation of lesions in frozen sections were made by reference to the adjacent paraffin section using established criteria (4, 5). Normal breast tissues were obtained from normal tissues adjacent to tumors in mastectomy specimens. The subtypes among the DCIS cases included seven noncomedo and five comedo or mixed comedo/noncomedo types. The invasive carcinomas included three cases with good prognosis indicators (well-differentiated Nottingham grades 4 and 5, ER and PR positive, and axillary node negative) and four cases with poor prognosis indicators (poorly differentiated Nottingham grade 9, ER and PR negative, and axillary node positive).

RNA Extraction

DCIS and matched invasive components were microdissected as described previously (6) from five adjacent 20-μm frozen sections of the microdissection case. Corresponding RNA was extracted using a small-scale RNA extraction protocol (Tri-reagent; Molecular Research Center, Inc., Cincinnati, OH) as described previously (6, 7). Total RNA was similarly extracted from 20-μm frozen sections or from whole-tissue blocks for breast samples in groups 2 and 1, respectively.

Isolation and Identification of Psoriasin mRNA as Overexpressed in the in Situ Compartment Compared to the Matched Invasive One

A recently described subtractive hybridization technique (8) was used to isolate mRNA species overexpressed in the DCIS relative to the invasive breast cancer compartment of the microdissection case. Briefly, *in situ* and immediately adjacent invasive tumor cell populations were microdissected. The corresponding RNAs were extracted and mRNAs were isolated by hybridization to oligo(dT) coupled magnetic beads (Dynal, New York, NY). Single-stranded cDNAs covalently bound to the beads (sscDNA IS-beads and sscDNA IV-beads for *in situ* and invasive compartments, respectively) were synthesized according to the manufacturer's instructions. Oligo(dA) residues were then added to the 3′ ends using terminal transferase (Promega, Madison, WI), and strands complementary to sscDNA IS-beads (sscDNA IS) were synthesized using *Mbo*I-dT primer (5′-AGGGCGATCTTTTTTTTTTTTT-3′). The sscDNA IS population was then equalized (autohybridized) and subtracted three times against sscDNA IV-beads. During these three rounds of subtraction, sscDNA IS common to both *in situ* and invasive compartments were captured by hybridization to complementary sscDNA IV-beads and subsequently removed from the solution. sscDNA IS *in situ* specific, remaining in the solution, were then amplified using radioactive PCR and *Mbo*I-dT primer. Labeled PCR products were then separated on 6% polyacrylamide gels containing 7 M urea (PAGE) and visualized with autoradiography. Bands were isolated from the dried gel, subcloned, and sequenced as described previously (7).

In Situ Hybridization

Paraffin-embedded (5-μm) breast tumor sections were analyzed by *in situ* hybridization according to a previously described protocol (9). The plasmid Pso-246 that consisted of a PCR II plasmid (Invitrogen, San Diego, CA) containing a 246-bp insert of psoriasin cDNA between bases 41 and 286 (9) was used as a template to generate sense and antisense riboprobes. ^{35}S-labeled UTP riboprobes were synthetized using Riboprobe Systems (Promega) according to the manufacturer's instructions. Sections from a psoriatic skin lesion, previously shown (9) to express a high level of psoriasin mRNA, were used as positive controls.

RT-PCR Analysis

RNA (600 ng) was reverse transcribed in a final volume of 15 μl as described previously (7). One μl of the reaction mixture was taken for subsequent amplification using PCR. PCR was performed as described previously (7) using either primers corresponding to psoriasin (sense 5′-AAGAAAGATGAGCAACAC-3′ and antisense 5′-CCAGCAAGGACAGAAACT-3′) or to the ubiquitously expressed *GAPDH* gene (sense 5′-ACCCACTCCTCCACCTTTG-3′ and antisense 5′-CTCTTGTGCTCTTGCTGGG-3′). To amplify cDNA corresponding to psoriasin, 30 cycles (30 s at 94°C, 30 s at 52°C, and 30 s at 72°C) of PCR were used. For amplification of GAPDH cDNA, PCR consisted of 30 cycles (1 min at 94°C, 1 min at 52°C, and 1 min at 72°C). PCR products were separated on 2% agarose gels before staining with ethidium bromide. Identity of the 246-bp long fragment corresponding to psoriasin was confirmed by subcloning and sequencing as described previously (7).

Northern Blot Analysis

Twenty μg of total RNA from *in situ* tumor IS1 (group 1) were analyzed using Northern blotting as described previously (10). The probe used consisted of a PCR product corresponding to a 246-bp fragment of psoriasin cDNA labeled by nick translation (Amersham, Oakville, Ontario). Nitrocellulose membranes were exposed for 72 h to Kodak XAR film at −70°C.

Results

Differential Expression of Psoriasin mRNA between *in Situ* and Invasive Compartments of a Selected Human Breast Tumor. To identify genes differentially expressed between *in situ* and invasive tumor cell populations, a matched sample of DCIS associated with a small invasive carcinoma was selected. *In situ* and immediately adjacent invasive compartments were microdissected and corresponding total RNA was extracted. These RNAs provided the substrate for a recently described subtractive hybridization technique to identify differentially expressed genes (8). cDNA corresponding to the calcium-binding protein psoriasin (9) or S100A7 (11) was identified as more highly expressed in the *in situ* breast cancer compartment (data not shown). To confirm this differential expression, *in situ* hybridization was performed on sections from a matched paraffin-embedded block that was a mirror image of the original microdissected frozen tissue block (Fig. 1). A strong signal was observed in the *in situ* region of the tumor using the psoriasin antisense probe, although a very weak one was observed overall in the matched invasive breast cancer compartment (Fig. 1*A*). No signal was observed using a psoriasin sense probe (Fig. 1*B*). Assessment of the sections at a higher magnification revealed that the psoriasin expression was confined to epithelial tumor cells of the *in situ* lesion (Fig. 1*C*) and that occasional cells from the invasive compartment also expressed psoriasin mRNA (Fig. 1*D*). No signal was detected in normal ducts adjacent to the *in situ* lesion (Fig. 1*E*). A strong expression of the psoriasin mRNA was detected in the positive control that consisted of skin from a psoriatic patient (Fig. 1*F*) previously shown to contain a high level of psoriasin mRNA (9).

Differential Expression of Psoriasin mRNA between Independent *in Situ* and Invasive Human Breast Tumors. To determine whether the differential expression of psoriasin mRNA within the *in situ* compartment was limited to the specific histology of the initial sample or whether it was a more general phenomenon, we selected an initial panel (group 1) of three *in situ* (IS1, IS2, and IS3) and three invasive (IV1, IV2, and IV3) unmatched ductal carcinomas derived from six different patients and possessing similar histologies to the respective components of the initial sample. Total RNA was extracted from whole-tissue blocks, and psoriasin mRNA expression was investigated using RT-PCR (Fig. 2). The expected 246-bp PCR product, corresponding in size to psoriasin mRNA, was observed in each of the *in situ* samples. In contrast, this PCR fragment was not detected in any of the invasive tumors. The identity of the PCR product was checked by sequencing (data not shown). This differential expression was not due to differences in input cDNA, as demonstrated by the similar intensity of the 178-bp fragment corresponding to the ubiquitously expressed *GAPDH* gene. Northern blot analysis performed on total RNA from *in situ* tumor (IS1) confirmed the presence of the 0.45 kb long psoriasin mRNA (data not shown).

Psoriasin mRNA Expression Is Modified during Tumor Progression. Psoriasin mRNA expression was then investigated within a panel of histologically defined breast tissue samples chosen to represent different steps of tumorigenesis (group 2). Total RNA was extracted from frozen tissue sections and analyzed with RT-PCR. Three independent experiments were performed and gave the same results. Fig. 3 shows typical results observed. Psoriasin expression was weak or undetectable in normal breast tissues adjacent to tumor and in samples of breast PDWA. A signal corresponding to psoriasin mRNA was observed in some sections that contained ADH and in some DCIS lesions of the noncomedo type. Most of the DCIS of the comedo type expressed a comparatively high level of psoriasin mRNA as measured by this technique. An apparently lower signal was detected in those samples of invasive ductal carcinoma. The differences observed were unlikely due to differences in input cDNAs (as shown by similar intensity of GAPDH signals in each lane) or to differences in tumor cell content (assessed in immediately adjacent histological sections).

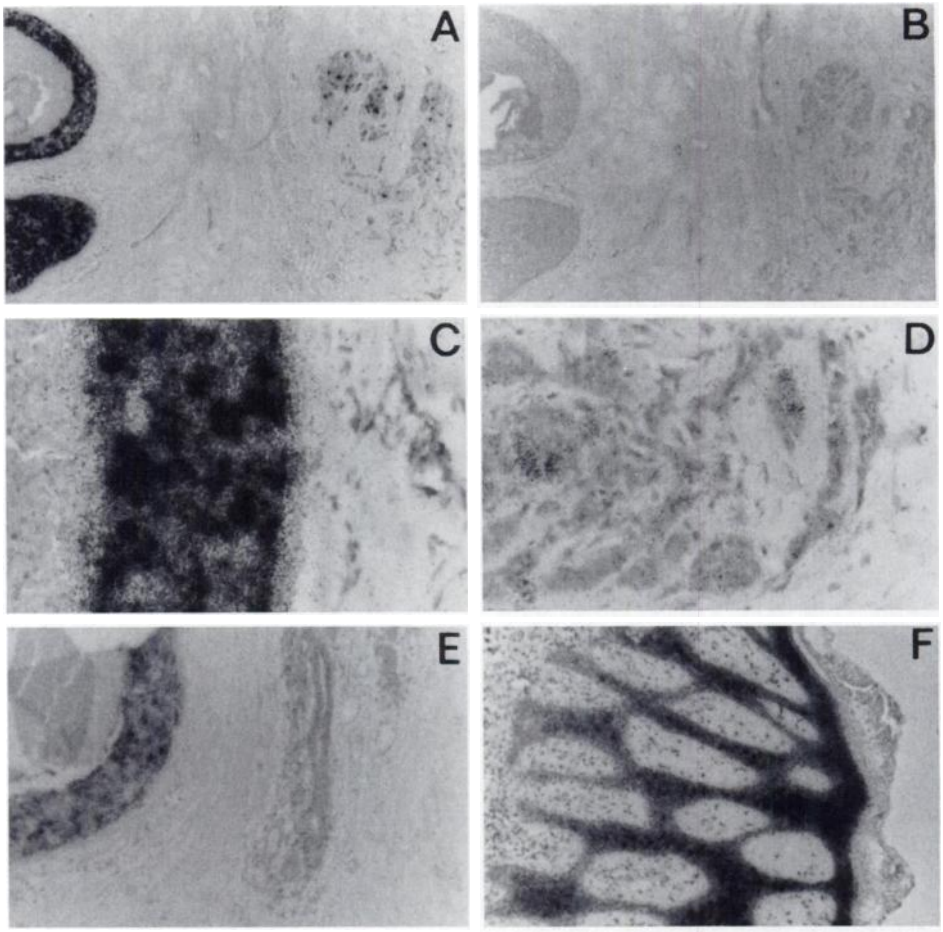

Fig. 1. Formalin-fixed and paraffin-embedded sections of the initial microdissection case were studied with *in situ* hybridization, as described in "Materials and Methods," using psoriasin antisense (*A*, *C*, *D*, and *E*) or sense (*B*) ^{35}S-labeled probes. *A* and *B*, *in situ* (*left side* of photograph) and invasive (*right side* of photograph) breast cancer in the same tissue section. *In situ* compartment (*C*), invasive compartment (*D*), and normal breast duct alongside a focus of DCIS (*E*) are shown at higher magnifications. *F*, Section of psoriatic skin from another individual studied with *in situ* hybridization using a psoriasin antisense probe and used as positive control. *A*, *B*, and *F*, ×30; *C* and *D*, ×200; and *E*, ×80.

Discussion

Psoriasin is a small calcium-binding protein of 11 kDa, belonging to the *S100* gene family, of which the exact function is unknown (9, 12). The expression of the psoriasin gene has been described previously in psoriatic skin and other skin diseases (9, 13). Psoriasin mRNA was identified here as being more highly expressed in the *in situ* compartment than in the adjacent invasive breast tumor cell population within the same human breast tumor specimen. This differential expression between *in situ* and invasive lesions was confirmed using independent breast cancer tissue samples. Interestingly, RT-PCR performed on RNA extracted from whole invasive breast tumor tissue blocks did not allow the detection of psoriasin mRNA (Fig. 2), although *in situ* hybridization revealed that a few invasive cells express this mRNA (Fig. 1*D*). This probably resulted from the high dilution of the target transcript within total RNA from whole tumor. The subsequent observation of a weak signal corresponding to psoriasin in some invasive tumor samples when total RNA from tissue sections was analyzed (Fig. 3) and where such a dilution phenomenon is decreased supported this hypothesis.

Very recently, Moog-Lutz *et al.* (14) detected psoriasin mRNA in human invasive breast cancer cells both *in vivo* and *in vitro*. In that study, psoriasin mRNA was observed using Northern blot analysis in 17% of the RNA samples from a small series of primary carcinomas. However, using *in situ* hybridization, they detected psoriasin expression only in a small fraction of the invasive cancer cells. This last result is similar to what we have also observed using the same technique in invasive carcinoma (Fig. 1*D*). The relationship with DCIS and the transition from *in situ* to invasive carcinoma was not examined by Moog-Lutz *et al.* (14). It is interesting to note that in the

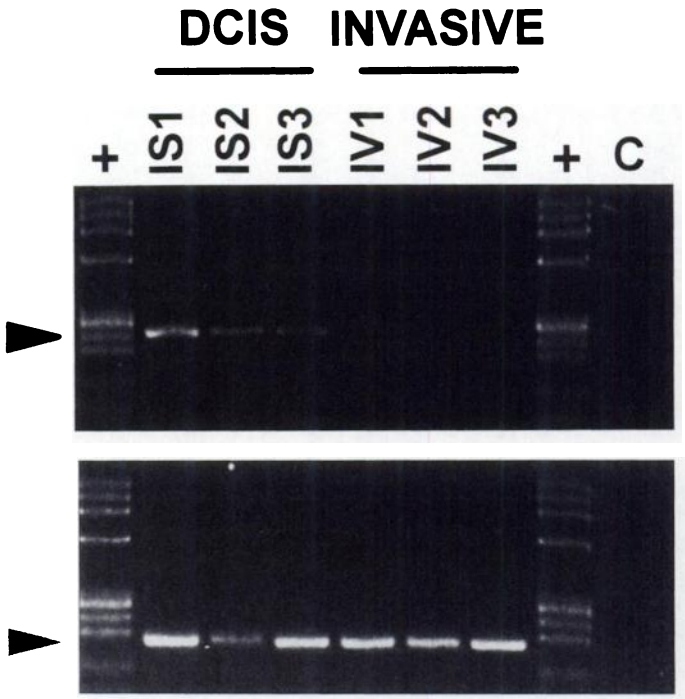

Fig. 2. RT-PCR analysis of psoriasin mRNA in three DCIS and three invasive ductal carcinomas (group 1): total RNA was extracted from three DCIS (IS1-3) and three invasive ductal carcinomas (IV1-3). RT-PCR was then performed as described in "Materials and Methods" to amplify psoriasin and GAPDH cDNAs. PCR products were separated on 1% agarose gels before staining with ethidium bromide. *Black arrowhead*, product corresponding to psoriasin; *gray arrowhead*, product corresponding to GAPDH. +, molecular weight marker (ϕX174 RF DNA/*Hae*III fragments; Life Technologies, Inc., Grand Island, NY); *C*, negative control, no cDNA added during the PCR reaction.

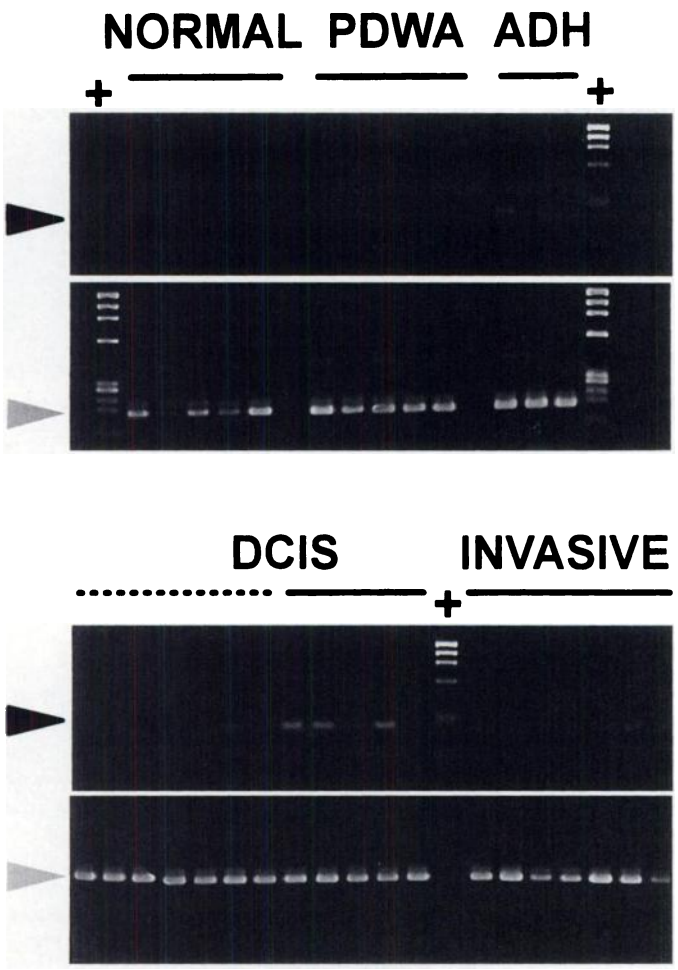

Fig. 3. RT-PCR analysis of psoriasin expression in normal breast tissue, PDWA, ADH, DCIS, and invasive ductal carcinoma from different patients (group 2): total RNA was extracted from frozen sections obtained from five normal breast tissues, five cases with PDWA, three cases with ADH, seven noncomedo-type DCIS (- - -), five comedo-type DCIS (—), and seven invasive ductal carcinomas. RT-PCR was then performed as described in "Materials and Methods" to amplify psoriasin and GAPDH cDNA. PCR products were separated on 1% agarose gels before staining with ethidium bromide. *Black arrowhead*, PCR product corresponding to psoriasin; *gray arrowhead*, PCR product corresponding to GAPDH. +, molecular weight marker.

Manitoba Human Breast Tissue Bank, DCIS constitutes a significant component (*i.e.*, >20% of the epithelial component) in approximatively 17% of the invasive ductal carcinoma samples. DCIS contamination of the whole-tumor preparations could therefore explain the percentage of tumors expressing high levels of psoriasin detected by Northern blot analysis in that previous study.

Our results, based on a selected panel of breast lesions that correspond to a sequence of potential stages of tumor progression, suggest that alteration of psoriasin gene expression occurs during tumorigenesis. This adds to the growing body of data suggesting a possible role of calcium-mediated intracellular signal transduction pathways during tumor progression. Indeed, it has been shown that alteration of those pathways may play a role in invasion (2, 15, 16) and that members of the highly conserved S100 calcium-binding protein family may be involved in breast tumorigenesis. For example, altered expression of the *Mts1* gene in murine breast cell lines can influence metastasis (17), and *CAPL* gene expression may correlate with invasiveness of breast cell lines (18). In contrast, expression of CaN19 was found to be more highly expressed in normal breast cells compared to cancer cells (19).

The mechanisms underlying the changes in psoriasin mRNA expression during tumor progression as well as the possible functional role of the encoded protein in breast tumor cells remain to be determined.

Acknowledgments

We thank Ingo Schroedter for his assistance during the set-up of the *in situ* hybridization assay. The Manitoba Breast Tumor Bank is supported by funding from the National Cancer Institute of Canada and the Terry Fox Foundation.

References

1. Allred, D. C., O'Connell, P., and Fuqua, S. A. W. Biomarkers in early breast neoplasia. J. Cell. Biochem., *17G:* 125–131, 1993.
2. Kohn, E. C., and Liotta, L. A. Molecular insights into cancer invasion: strategies for prevention and intervention. Cancer Res., *5:* 1856–1862, 1995.
3. Elston, C. W., and Ellis, I. O. Pathological prognostic factors in breast cancer. Histopathology, *19:* 403–410, 1991.
4. Page, D. L., and Dupont, W. D. Premalignant conditions and markers of elevated risk in the breast and their management. Surg. Clin. North Am., *70:* 831–851, 1990.
5. Lagios, M. D. Ductal carcinoma *in situ*. Surg. Clin. North Am., *70:* 853–871, 1990.
6. Hiller, T., Snell, L., and Watson, P. H. An approach for microdissection/RT-PCR analysis of gene expression in pathologically defined frozen tissue sections. Biotechniques, *21:* 38–42, 1996.
7. Leygue, E., Watson. P. H., and Murphy, L. C. Estrogen receptor variants in normal human mammary tissue. J. Natl. Cancer Inst., *88:* 284–290, 1996.
8. Leygue, E., Murphy, L. C., and Watson, P. H. Isolation of genes differentially expressed using minute amount of RNA. Biotechniques, in press, 1996.
9. Madsen, P., Rasmussen, H. H., Leffers, H., Honore, B., Dejgaard, K., Olsen, E., Kiil, J., Walbum, E., Andersen, A. H., Basse, B., Lauridsen, J. B., Ratz, G. P, Celis, A., Vandekerckhove, J., and Celis, J. E. Molecular cloning, occurrence, and expression of a novel partially secreted protein "psoriasin" that is highly up-regulated in psoriatic skin. J. Invest. Dermatol., *97:* 701–712, 1991.
10. Dotzlaw, H., Alkhalaf, M., and Murphy, L. C. Characterization of estrogen receptor variant mRNAs from human breast cancers. Mol. Endocrinol., *6:* 773–785, 1992.
11. Schafer, B. W., Wicki, R., Engelkamp, D., Mattei, M. G., and Heizmann, C. W. Isolation of a YAC clone covering a cluster of nine S100 genes on human chromosome 1q21: rationale for a new nomenclature of the S100 calcium-binding protein family. Genomics, *25:* 638–643, 1995.
12. Hoffmann, H. J., Olsen, E., Etzerodt, M., Madsen, P., Thogersen, H. C., Kruse, T., and Celis, J. E. Psoriasin binds calcium and is upregulated by calcium to levels that resemble those observed in normal skin. J. Invest. Dermatol., *103:* 370–375, 1994.
13. Sitzmann, J., Algermissen, B., Czarnetzki, B. M., and LeMotte, P. Expression of psoriasin mRNA in several human skin diseases. J. Invest. Dermatol., *100:* 220A, 1994.
14. Moog-Lutz, C., Bouillet, P., Regnier, C. H., Tomasetto, C., Mattei, G., Chenard, M. P., Anglard, P., Rio, M. C., and Basset, P. Comparative expression of the psoriasin (S100A7) and S100C genes in breast carcinoma and co-localization to human chromosome 1q21–q22. Int. J. Cancer, *63:* 297–303, 1995.
15. Vleminckx, K., Vakaet, L., Mareel, M., Fiers, W., and van Roy, F. Genetic manipulation of E-cadherin expression by epithelial tumor cells reveals an invasion suppressor role. Cell, *66:* 107–119, 1991.
16. Cole, K. A., and Kohn, E. C. Calcium mediated signal transduction: biology, biochemistry and therapy. Cancer Metastasis Rev., *13:* 33–41, 1994.
17. Ford, H. L., Salim, M. M., Chakravarty, R., Aluiddin, V., and Zain, S. B. Expression of *Mts1*, a metastasis-associated gene, increases motility but not invasion of a nonmetastatic mouse mammary adenocarcinoma cell line. Oncogene, *11:* 2067–2075, 1995.
18. Peddrochi, M., Schafer, B. W., Mueller, H., Eppenberger, U., and Heizmann, C. W. Expression of Ca^{2+}-binding proteins of the S100 family in malignant human breast-cancer cell lines and biopsy samples. Int. J. Cancer, *57:* 684–690, 1994.
19. Munn, K. E., Walker, R. A., and Varley, J. M. Frequent alterations of chromosome 1 in ductal carcinoma *in situ* of the breast. Oncogene, *10:* 1653–1657, 1995.