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Award Number: W81XWH-04-1-0670

TITLE: Early Diagnosis of Breast Cancer by Identifying Malignant Cells Within Neoplasias Histologically Classified as Benign

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REPORT DATE: July 2005

TYPE OF REPORT: Annual

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

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
01-07-2005	Annual	07/01/04-06/30/05

4. TITLE AND SUBTITLE
Early Diagnosis of Breast Cancer by Identifying Malignant Cells Within Neoplasias Histologically Classified as Benign

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-04-1-0670

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Purdue University
West Lafayette, IN 47907-2040

8. PERFORMING ORGANIZATION REPORT NUMBER

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

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT: Abstract on next page.

15. SUBJECT TERMS
Nuclear Organization, NuMA, H4K20m, Three Dimensional Coculture, Imaging Analysis

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	23	19b. TELEPHONE NUMBER *(include area code)* 301-619-7325

Abstract

Current diagnostic tools permit the classification of breast neoplasias into categories that represent different relative risks of developing cancer, but they do not indicate which particular lesion of an individual will lead to cancer. The lack of precise classification of breast lesions results in patients being under- or overtreated. In three-dimensional (3D) cell cultures that mimic different breast phenotypes, we can correlate specific distributions of certain nuclear proteins to a particular cell behavior and/or degree of malignancy using an automated image analysis, referred to as local bright feature (LBF) analysis. We have proposed the novel concept that in true precancerous diseases, a small fraction of malignant cells might be detectable admixed within a cell population globally classified as benign. Using 3D cell culture, we show that the LBF analysis of proteins NuMA and H4K20m distinguishes premalignant from normal and malignant cells and that subpopulations of cells with different behaviors can be identified within a premalignant cell population. We will now use the LBF method to identify cell subpopulations within premalignant and preinvasive lesions on archival biopsy sections. The ability to recognize malignant cells in these lesions should bring critical improvement for the identification of lesions of adverse and good prognoses.

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INTRODUCTION

Early detection and prognosis of breast malignancy is a tremendous challenge (Wright and McGechan, 2003). Current diagnostic tools permit the classification of benign or premalignant breast neoplasias into categories that represent different relative risks of developing cancer, but they do not indicate which particular lesion of an individual will lead to cancer. Current classification mainly relies on gross cellular features like nucleus size, shape, and texture (Hunt et al., 1990; Mombello et al., 2001) that do not allow pathologists to identify subtle alterations, characteristic of benign or malignant behavior, at the single cell level. The lack of precise classification of a breast lesion results in patients being under- or overtreated. We have developed a novel automated imaging analysis tool, referred to as local bright feature (LBF) analysis (Knowles et al., submitted) that enables us to quantitatively measure the distribution of nuclear proteins. With this method, using three-dimensional (3D) cell culture that mimic different breast phenotypes, we can correlate a specific distribution of a defined nuclear protein to a particular cell behavior and/or degree of malignancy. We have proposed the novel concept that in **true precancerous diseases, a small fraction of malignant cells might be detectable admixed within a cell population globally classified as benign.** Our project aims at assessing whether the LBF analysis can identify cells with malignant signatures in biopsies of premalignant lesions and evaluating the prognostic value of the detection of these signatures. To do so we have proposed two aims. ***In Aim 1, we will establish the criteria for distribution analysis of chromatin-associated proteins in heterogeneous cell populations in 3D culture. In Aim 2, we will investigate, using the LBF analysis, the presence of malignant cells within neoplasms histologically classified as benign, with increasing breast cancer relative risk, including typical and atypical hyperplasias, ductal carcinoma in situ (DCIS), and lobular carcinoma in situ (LCIS).***

BODY

Progress for Aim 1

We have two nuclear makers currently utilized in this project. Heterochromatin protein histone 4-methylated on lysine 20 (H4K20m) and the nuclear mitotic apparatus protein NuMA. We had to abandon the use of histone 3-methylated on lysine 9 (H3K9m) since the best marker was the branched form (Plachot and Lelièvre, 2004) for which the antibody is still not commercially available. Therefore we have replaced the use of H3K9m with that of H4K20m. Heterochromatin marker HP1 is still part of our list of protein to investigate and will be tested. However, for the sake of time for this project we have focused on NuMA instead of HP1. We have already well defined the criteria for the LBF analysis of NuMA.

Task 1. ***Development of the LBF analysis for H4K20m***. In Collaboration with Dr. Knowles with whom we have developed the local bright feature (LBF) analysis for NuMA (Knowles et al., submitted), we have worked on the development of the criteria to measure changes in the distribution of H4K20m. The basic principle of isolation of bright features of the immunostaining is the same for all nuclear proteins studied; however, the analysis of the distribution of the bright features varies with the type of staining pattern. The isolation of bright feature was developed to mimic the way the human visual cortex perceives local bright features in a scene even when features are subtle and not well defined. Z series of confocal images are recorded from fluorescently labeled proteins. The LBF analysis uses an automated nuclear segmentation and analysis of three-dimensional images that corrects for biases that could results from nuclear shape, staining background, and recording of images with the confocal microscope (Knowles et al., submitted). Briefly, previously we have developed a model-based automatic nuclear segmentation method to isolate individual nuclei in a DAPI-stained image based on the assumption that nuclei of epithelial cells are of simple geometry in that they comprise a single

spherical core. An adaptive threshold is first applied to the DAPI-stained image to produce a binary mask of the nuclei. The technique uses a difference-of-Gaussians filter (Marr, 1982), followed by a morphological closing filter and a flood-fill algorithm to produce the binary segmentation mask (Soille, 2003). Following immunostaining, image acquisition by confocal microscopy and DAPI staining-based segmentation, bright staining features/foci are isolated by the LBF analysis technique. This technique was originally developed with NuMA staining. Pixel brightness in the raw NuMA images is normalized by the local average brightness using an extension of the difference-of-Gaussians technique (Marr, 1982). First the raw NuMA image is masked by the binarized segmentation result derived from the DAPI image as described above. Then, image brightness within each nucleus is rescaled by dividing the brightness at each point by the local average brightness in a region surrounding that point. The local average brightness is calculated over a region with a dimension half that of the dimension of the nuclear core. This is important because the LBF technique sensitively resolves light or dark features that are smaller than the region but ignores features that are larger. Thus, this technique allows the bright features and dark regions of interest within the nucleus to be resolved and normalized for the low-frequency brightness variations due to the geometry of the nucleus and the axial resolution of the microscope. In the resulting LBF images, bright image features have values above unity while dark image features have values below unity. (Figure 1; Knowles et al., submitted)

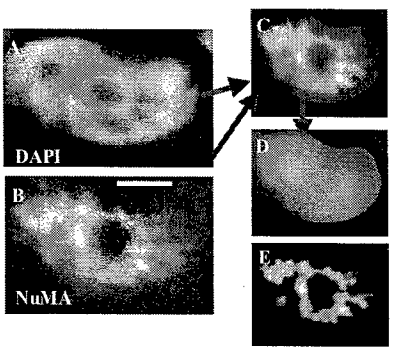

Figure 1. **Isolation of bright features of NuMA staining using the LBF algorithm**. (A-E) one nucleus analyzed from cell culture. (A) DNA counterstaining (DAPI) as a basis for the segmentation mask. (B) Immunostaining for NuMA. (C) Narrow Gaussian filtering of the NuMA image overlayed with the segmentation mask, to remove shot or detector noise. (D) Determination of the local average brightness using a 30X30 pixel kernel. (E) LBF image showing the bright features of the immunostaining.

Due to the widespread distribution of the bright features of NuMA, we had found previously that the analysis of changes in distribution is best produced by measuring changes in density of these bright features within the nuclear volume using a radial-LBF analysis. Briefly, the bright features isolated following the LBF analysis are overlayed on the segmentation mask. This technique permits a visualization of the localized accumulation of NuMA foci throughout the nuclei. In order to calculate the radial distribution of the local bright features, a distance transform (Soille, 2003) is applied to the nuclear segmentation mask. The transform calculates the shortest distance of each point within a nucleus to the nuclear boundary and in doing so, divides each nucleus into a set of concentric terraces of equal thickness (Knowles et al., submitted). The LBF image is then used in conjunction with the nuclear segmentation mask and the distance transform to compute the density of local bright features in each terrace of each nucleus. In each terrace, the density is calculated as the number of pixels in local bright features divided by the total number of pixels. To reveal the relative distribution of the density of bright features within each nucleus, the density per terrace is normalized so that the average density of bright features is unity for each nucleus. The distances defined by the distance transform are also normalized so that the distance at the nuclear perimeter is 0 and the distance at the center of the nucleus is 1.0. This normalization is done to account for variations in the number of terraces per nucleus due to variations in nucleus size and shape. Finally, the normalized density of bright features is plotted against normalized distance from the perimeter of the nucleus to its center (Figure 2; see figure 6 for resulting plot).

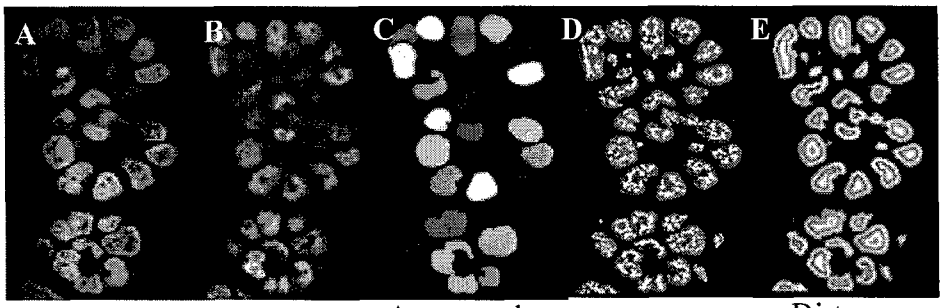

Figure 2. **Distribution density of the bright features of NuMA in acinar cells.** HMT-3522 S1 nonneoplastic cells were cultured in 3D to induce acinar morphogenesis. Each panel corresponds to the application of the different steps of distribution analysis starting from the same original image. **A.** Fluorescence micrograph of DAPI-stained nuclei from a single optical section containing three acini. **B.** Fluorescence micrograph of Texas-red immunolabeled NuMA from the optical section corresponding to the DAPI image shown in A. **C.** Segmentation mask derived from the DAPI-stained image showing a single slice of individually enumerated nuclei. **D.** Composite view of the local bright features (light gray) extracted by the LBF analysis overlayed with the segmentation mask (dark gray). **E.** Concentric terraces resulting from the application of the distance transform on the segmentation mask.

Due to the very distinct formation of H4K20m foci of large size and the paucity of such foci, the analysis of changes in H4K20m distribution cannot use the radial analysis usually applied to NuMA staining pattern. Especially, our observation is that nonneoplastic acinar cells seem to possess larger H4K20m staining foci compared to malignant cells. We first established the analysis using very different phenotypes, i.e., the differentiated luminal mammary epithelial cells and malignant cells. In order to produce these phenotypes, nonneoplastic S1 and malignant T4-2 breast epithelial cells were cultured in the presence of extracellular matrix enriched in laminin (Matrigel™) (Petersen, 1992; Lelièvre and Bissell, 2005), a process referred to as three-dimensional (3D) culture. Under these conditions nonneoplastic cells form glandular structures resembling terminal ductal lobular units in normal breast, and malignant cells from invasive tumor nodules. The analysis of H4K20m immunostaining was set up as the following: Bright features for H4K20m staining were isolated according to the LBF algorithm. For the second step of the analysis, the bright features were given a score based on their relative brightness and relative size. In order to assess the size of bright foci, the LBF image was blurred in three dimensions by convolving it with a normalized Gaussian function having a standard deviation of 1 pixel. This manipulation reduced the foci brightness as an inverse function of their size. Thus, the local brightness maximum in the blurred-LBF image is a combined measure of the foci brightness and size. The histogram of foci "score" for all nuclei within an image revealed a large number of small foci but also larger foci at much lower frequency. This result was in agreement with visual analysis of the images. Large foci were defined as having scores larger than two standard deviations away from the histogram mean. To refine the analysis of bright feature distribution, the number of larger foci in each nucleus was counted (Figure 3). For a total of 167 nuclei of S1 cells, a total of 311 larger foci were counted. Interestingly, 40% of the S1 nuclei were devoid of large foci. In the nuclei that did have large foci there were on average 3.1 large foci per nucleus. For 77 nuclei of T4-2 cells, 35 large foci were counted. 75% of T4 nuclei were devoid of large foci. In the remaining nuclei there were an average of 1.8 large foci per nucleus. The *p* value of the two probability distributions (S1 and T4-2 cells) obtained from the H4K20 analysis was calculated using a Z-test, which compares the significance of the difference between the population means (in this case it is the mean number of foci per nucleus). For the total number of nuclei the mean number of foci was 1.86+/-2 for S1 cells and 0.45+/-0.8 for T4

(Z>7 and $p<0.001$). Another way to assess that the two distributions are significantly different is to consider the significance of finding a single nucleus with three foci within the same cell population. The *p*-value for n=3 in the T4 distribution was $p=0.05$ (for a single nucleus with 3 or more foci), whereas the *p*-value for n=3 in the S1 distribution was p=0.4 (not significant), as should be the case. The analysis was on per nucleus basis and nuclei with and without large foci could be easily individually isolated.

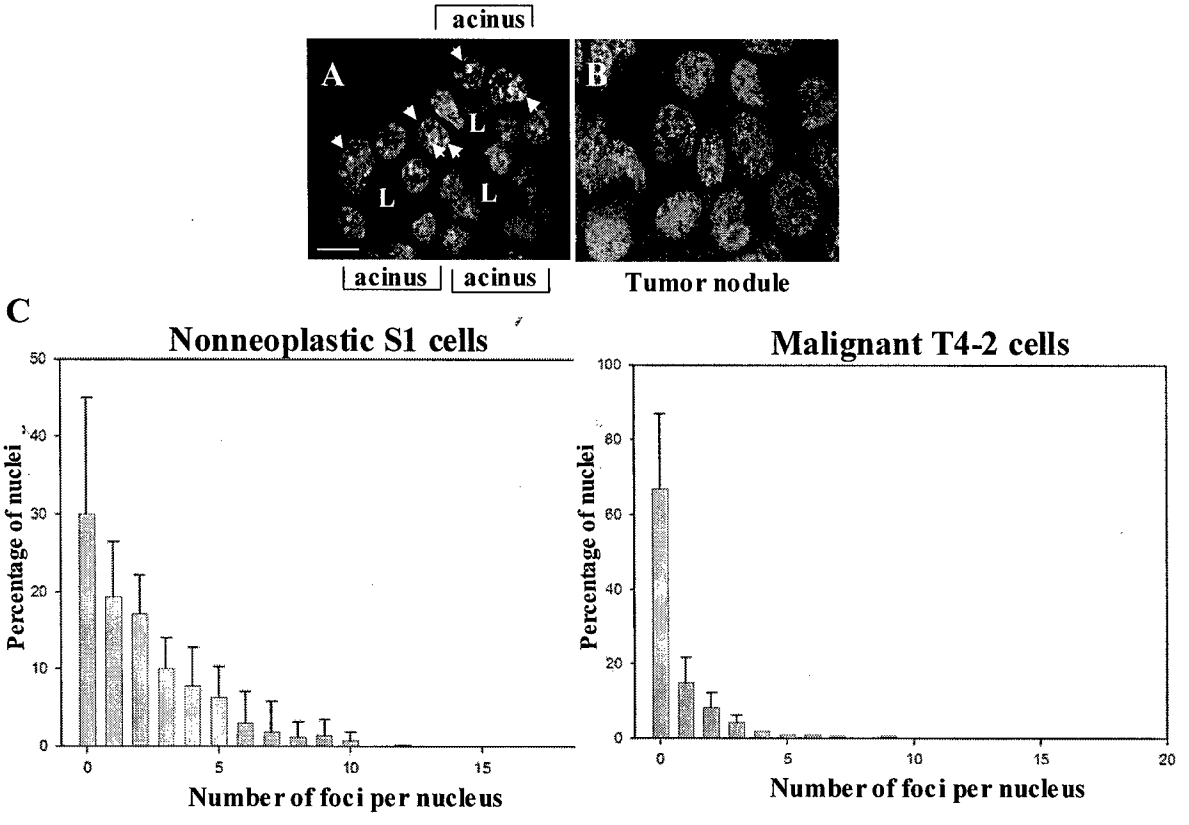

Figure 3. **H4K20m distribution depends on breast phenotype.** (A-B) Fluorescence immunostaining for heterochromatin marker H4K20m in acini (A) formed in 3D culture (3 acini are shown) and a portion of a tumor nodule (B). Arrowheads indicate nuclei with large foci of H4K20m. Size bar= 10 microns. (C) Histogram of the number of H4K20m foci per nucleus in the S1 acinar cell population and T4-2 malignant cell population. S1 and T4-2 cells were cultured in 3D for 10 days and immunostained for H4K20m and bright features were isolated using the LBF analysis. A foci-counting algorithm was developed based on the relative brightness and the relative size of the foci. The final score was generated from the LBF image convolved with a narrow Gaussian function which reduced the foci brightness as an inverse function of the foci size. X axis is the percentage of cells and Y axis is the number of large foci per nucleus.

Task 4.

Analysis of H4K20m and NuMA distribution in heterogenous premalignant S2 cell population.

In order to assess whether the LBF analysis can identify subpolulations of cells with different behaviors that are mixed in the same culture, we used premalignant HMT3522 S2 cells. These cells were derived from nonneoplastic S1 cells upon culture without adding epidermal growth factor in the culture medium and gave rise to T4-2 malignant cells after 238 passages (Briand et

al., 1987 and 2001). The S2 cell population is heterogeneous. This is exemplified by the formation of multicellular spheroids of different sizes and behavior (Figure 4) when these cells are cultured in 3D. Three subpopulations have been characterized based on the size of spheroids (less than 35 microns in diameter; 35-70 microns in diameter; above 70 microns in diameter). None of the spheroids display phenotypically normal organization (Figure 5) and the large spheroids show increase in basal apoptosis rate. The LBF analysis of NuMA and H4K20m was applied on these cells cultured in 3D. Results were then grouped into categories corresponding to spheroids of different sizes. Results show that as a whole the S2 cell population displays distribution of NuMA and H4K20 different from that seen in phenotypically normal and malignant cells (Figure 6). Most interestingly, LBF analysis of H4K20m in S2 spheroids of different sizes shows at least two subpopulations of cells within the S2 population (Figure 7). Thus, the LBF analysis enables us to distinguish between cells with different behaviors in the same microenvironment. Similar studies are currently being performed with NuMA distribution analysis.

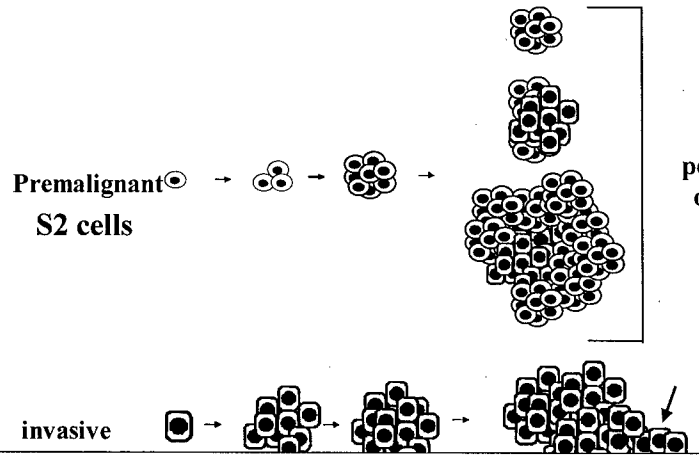

Figure 4. **Drawing of tumor nodule formation in 3D culture.** For 3D culture, premalignant, S2 cells and S2-derived, invasive carcinoma-type T4-2 cells are plated in the presence of laminin-rich Matrigel as single cells. Within 10 days, tumor nodules reach sizes ranging from 35 to several hundred microns. The heterogeneity of the S2 population is easily detected in 3D culture by the great variation is size of the tumor nodules. 3D culture also permits the visualization of invasive potentials of malignant cells (see arrow).

Establishment of coculture conditions with premalignant and malignant cells.

In order to test whether the LBF analysis of NuMA and H4K20m can identify malignant cells admixed premalignant cells, we had to set up a coculture system in which GFP-actin expressing-malignant cells are at low density compared to premalignant cells. A ratio of one malignant cell to eight premalignant cells led to the formation of small groups of malignant cells within premalignant cells (Figure 8). Images of immunostaining (red) for NuMA or H4K20m and GFP staining were recorded and are currently being analyzed. If malignant characteristics of NuMA or H4K20m distribution are identified in some of the cells, these cells will be checked for GFP staining to assess whether they correspond to malignant cells. On the images recorded for LBF analysis it is already possible to visualize differences in the distribution of NuMA in malignant (GFP stained) and premalignant cells (Figure 9). We are currently working with Dr. Knowles to set up an automated analysis that isolates single cells with protein distribution characteristics different from surrounding cells.

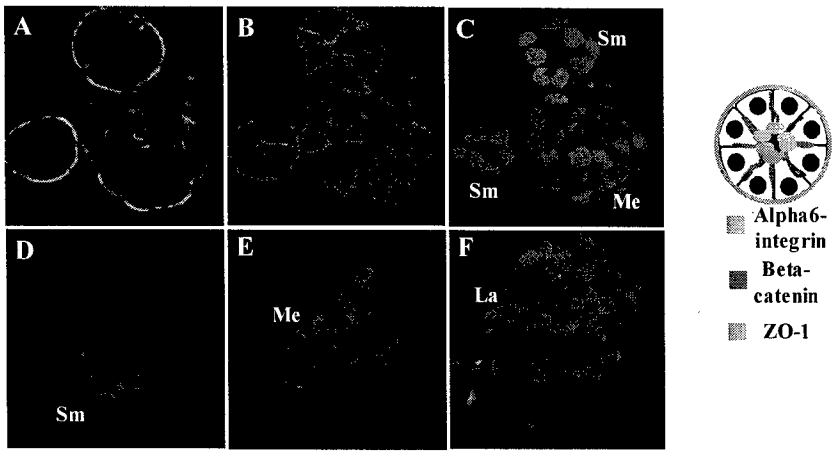

Figure 5. **3D culture of premalignant S2 cells reveals an heterogeneous phenotype**. S2 cells were cultured in the presence of Matrigel for 10 days and fixed prior to immunostaining for markers of tissue architecture, including cell-extracellular matrix adhesion molecule alpha 6 integrin (green, A), cell-cell adhesion complex component beta-catenin (red, B) and tight junction protein ZO-1 (green, D-F). Nuclei are counterstained with DAPI (blue; C is the counterstained image for A and B). Different cell types amidst the S2 population are visualized by the formation of nodules of different sizes (small- Sm; medium- Me; large- La). Markers show alterations compared to the normal situation (see drawing of an acinus to the right of the images) but they do not show dramatic or easily quantifiable differences between the nodules of different sizes. The hypothesis is that the different cell types could be distinguished at the cellular level based on the distribution of specific nuclear proteins.

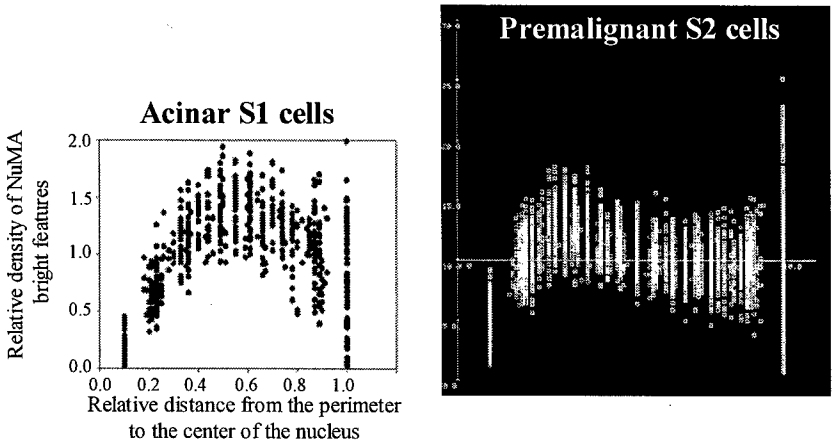

Figure 6. **Relative density of local bright features of NuMA in nuclei of nonneoplastic and premalignant cells.** Nonneoplastic S1 and premalignant S2 cells were cultured in 3D to induce acinar morphogenesis and tumor nodule formation, respectively. Multi-overlay plots represent the relative density of NuMA bright features extracted by LBF analysis as a function of the relative distance from the perimeter to the center of each nucleus. A bell shape with high density of bright features in mid-nucleus area is a characteristic of acinar cells (Knowles et al., submitted). Whereas the peak of density of bright features seems localized towards the periphery of the nucleus in premalignant cells. In malignant cells the distribution appears flat (Knowles et al., submitted, not shown). The plots correspond to measures performed in 77 acinar cells and 546 premalignant cells.

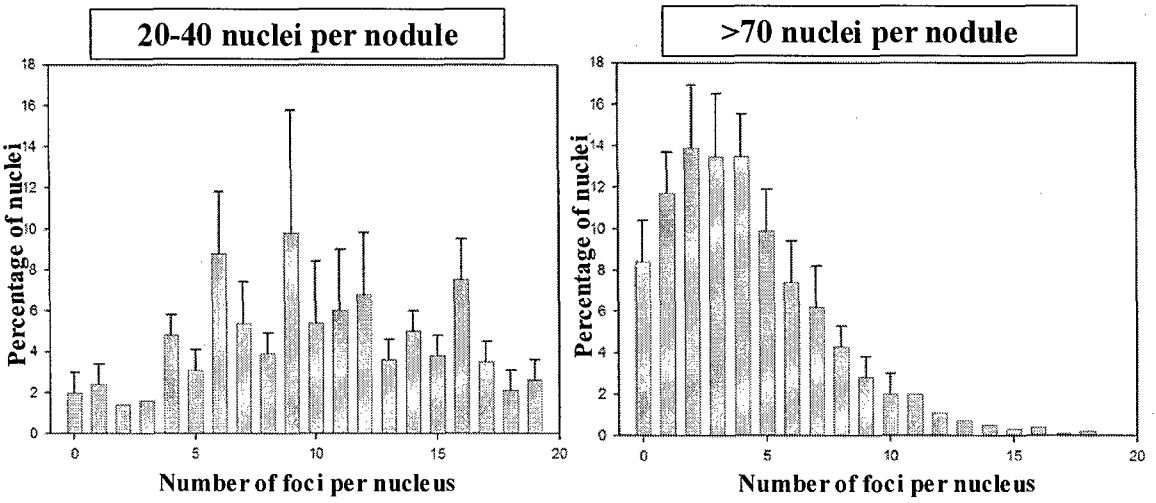

Figure 7. **Histogram of the number of H4K20m foci per nucleus demonstrates the heterogeneity of S2 cell population.** S2 cells were cultured in 3D for 10 days and immunostained for H4K20m. Bright features or foci were isolated using the LBF analysis. A foci-counting algorithm was developed based on the relative brightness and the relative size of the foci. The final score was generated from the LBF image convolved with a narrow Gaussian function which reduced the foci brightness as an inverse function of the foci size. Although the group with more than 70 nuclei per nodule seems to be fairly homogenous with regards to H4K20m distribution, the group with 20-40 nuclei per nodule displays a wide variation in H4K20m distributions, with peaks different from the group with more than 70 nuclei per nodule. The heterogeneity of the group of smaller nodules suggests that this group may itself contain additional subpopulations.

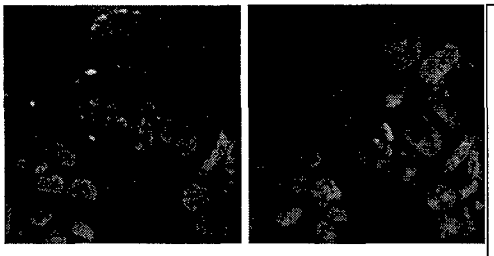

Figure 8. **Coculture of preimalignant and invasive carcinoma-type cells.** Premalignant S2 cells and S2-derived, invasive carcinoma-type (malignant) T4-2 cells expressing GFP-actin were plated in 3D at ratio 1/8 and cultured for 10 days. Cells were immunostained for the nuclear protein NuMA (red; left image) and nuclei were counterstained with DAPI (blue; right image). Upon 10 days of culture small groups of T4-2 cells (see GFP fluorescence) can be detected amidst S2 cells. These culture settings are being used to assess whether the LBF method applied to NuMA and H4K20m can detect cells with different degrees of malignancy within mixed cell populations.

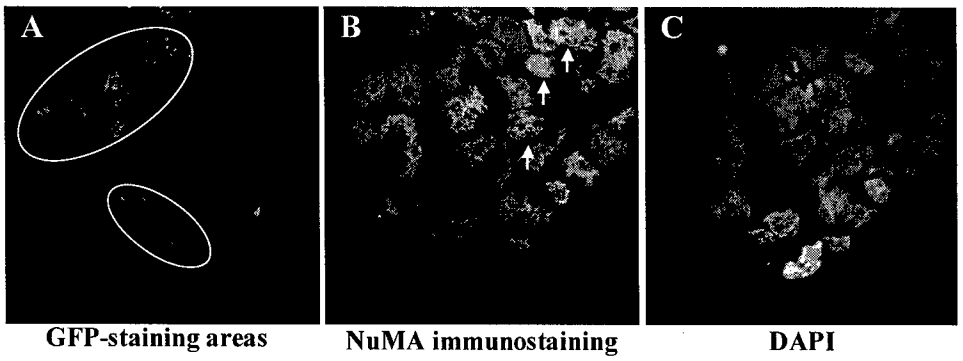

Figure 9. **Coculture of preimalignant and invasive carcinoma-type cells.** Premalignant S2 cells and S2-derived, invasive carcinoma-type (malignant) T4-2 cells expressing GFP-actin were plated in 3D at ratio 1/8 and cultured for 10 days. Cells were immunostained for the nuclear protein NuMA (central image) and nuclei were counterstained with DAPI (right image). GFP helps identify malignant cells (left image; see circled areas). Nuclei with NuMA staining patterns already visually different from nuclei located within circled areas (left image) are indicated by an arrow on the NuMA immunostaining image (central image).

Progress for Aim 2.

Task 2. ***Fluorescent immunostaining for NuMA and H4K20m on paraffin sections of breast tissue biopsies.***

In order to investigate nuclear protein distribution on tissue biopsy sections, we need to perform fluorescent immunostaining on paraffinated samples. Both NuMA and H4K20m fluorescent staining worked on sections of archival paraffinated samples of normal breast tissue (Figure 10). The best staining was obtained with the following procedure: Deparaffination and rehydration of tissue samples was achieved by washing thrice for 5 min at room temperature with xylene (Mallinckrodt, Hazelwood, MO), thrice for 2 min with 100% ethanol (Mallinckrodt), once for 2 min with 95% ethanol, once for 2 min with 70% ethanol, and twice for 5 min with Tris-Buffered Saline (TBS) [10 mM Tris base; 150 mM NaCl], pH 8.0. Samples were permeabilized for 12 min at room temperature with 0.5% Triton X-100 in cytoskeletal buffer containing proteases and phosphatases inhibitors (CSK-PI) (Plachot and Lelièvre, 2004). After washing twice for 2

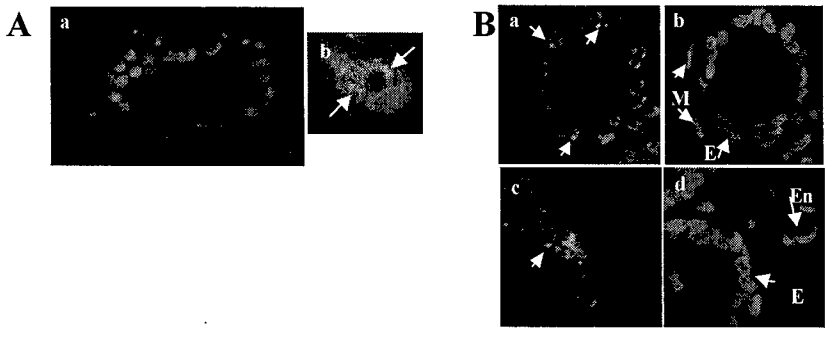

Figure 10. **Fluorescence staining for NuMA and H4K20m on paraffin section of normal breast tissue.** Samples of normal breast tissue were immunostained for NuMA and H4K20m using streptavidin-biotin-tiramide method. **A.** Low resolution images of NuMA immunostaining (a and d). Arrows indicate the concentration of NuMA bright staining features in mid nucleus (b). **B.** Low resolution images of H4K20m immunostaining (a and c) and DNA counterstain (b and d). Arrows indicate large H4K20m foci in a and c. E= luminal epithelium; M= myopepithelial cells; En= endothelial cells.

min with CSK-PI, cells were incubated for 10 min at 100°C followed by 20 min at room temperature with a 1:9 dilution of Target Retrieval Solution (DakoCytomation) in water. Samples were washed twice for 5 min with TBS. Samples were then incubated for 15 min with avidin blocking solution (Vector), washed once for 2 min with TBS, incubated for 15 min with biotin blocking solution (Vector), washed twice for 2 min with TBS, and once for 5 min with TBS containing 0.05% (v/v) Tween 20 (Sigma-Aldrich) (TBST). Samples were incubated for 30 min at room temperature with blocking reagent (PerkinElmer Life Sciences, Boston, MA), followed by incubation overnight at 4°C with NuMA or H4K20m antibody in blocking reagent. Samples were incubated twice for 10 min in TBS, once for 10 min in TBST, followed by incubation for 1 h with 15 µg/ml biotin-conjugated horse anti-mouse IgG (for NuMA) or anti-rabbit (for H4K20m) antibodies (Vector). After incubation twice for 5 min with TBS and once for 10 min with TBST, samples were then incubated for 30 min with 8 µg/ml fluorescein (DTAF)-conjugated streptavidin (Jackson Immunoresearch) in blocking reagent. After incubation twice for 5 min with TBS, once for 10 min with TBS, and once for 10 min with TBST, samples were incubated for 15 min with 1:49 dilution of biotinyl tiramide in amplification diluent (PerkinElmer Life Sciences). After incubation twice for 5 min with TBS and once for 5 min with TBST, samples were incubated for 30 min with 8 µg/ml fluorescein (DTAF)-conjugated streptavidin (Jackson Immunoresearch) in blocking reagent. Samples were then washed once for 5 min with TBS and once for 5 min with TBST. DNA was labeled for 10 min with 0.5 µg/ml of DAPI (Sigma-Aldrich) in PBS. After removal of excess DAPI, samples were mounted with the ProLong® antifade kit (Molecular Probes). The distribution of NuMA and H4K20m appeared similar in normal tissue and 3D culture of nonneoplastic cells, confirming that 3D cultures reproduce physiologically relevant distributions of nuclear proteins.

Task 3. ***LBF analysis on paraffin sections of breast tissue biopsies***
Bright features can be extracted from fluorescent staining on paraffin sections. Extraction of bright features of NuMA was first performed on normal breast tissue using LBF methods similar to those developed in 3D cell culture (Figure 11).

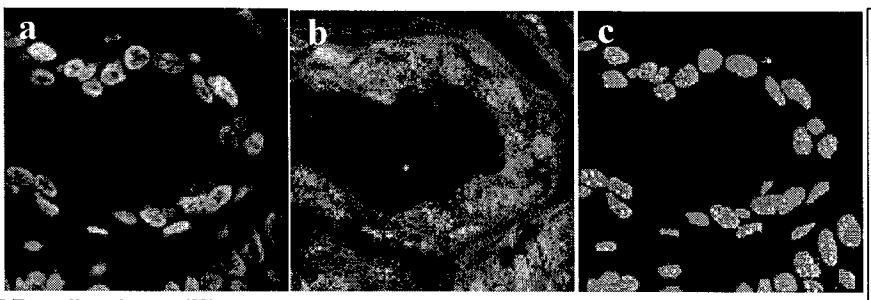

Figure 11. **Isolation of bright features on immunostained tissue samples using the LBF method.** DAPI image (a) corresponding to NuMA staining (b). Overlay of the LBF image of NuMA and the segmentation mask (for a and b) is shown in (c).

Tasks 5-9. Fluorescence immunostaining of tissue samples for NuMA and H4K20m in ongoing. These samples will be used for LBF-based extraction of bright features followed by application of the radial analysis (for NuMA distribution) or the foci-counting analysis (for H4K20m distribution).

TIMETABLE

Task 1	completed
Task 2	completed

Task 3 50% completed

Task 4 70% completed

Tasks 5-9 To be completed in the coming year

KEY RESEARCH ACCOMPLISHMENTS

- Criteria for distribution analysis have been set up for the nuclear protein H4K20m
- The LBF image analysis distinguishes premalignant from differentiated cells and from malignant cells based on NuMA and H4K20m distributions.
- The LBF analysis can identify different subpopulations of cells within the premalignant population.
- Distinct distributions of nuclear proteins in premalignant and malignant cells can be observed in these two types of cells in 3D coculture.
- Fluorescent immunostaining for H4K20m and NuMA can be performed on paraffin sections of breast tissue biopsies.
- Extraction of bright features by the LBF algorithm can be performed successfully on fluorescently labeled paraffin sections of breast tissue biopsies

REPORTABLE OUTCOMES

1) Development of H4K20m distribution analysis in cell culture
2) Establishment of a 3D coculture system to study the interaction between cell types in the context of neoplasia
3) Parts of the results obtained in this project were used in the preparation of two different grant proposals. An R33 proposal was submitted to NIH to expand the LBF analysis to establish distribution maps characterizing different tissue phenotypes (PI, David Knowles; Co-Investigator, S. Lelièvre) and an IDEA project was submitted to DOD/BCRP (PI, David Knowles; Co-Investigator, S. Lelièvre) to start implementing the use of the automated LBF analysis for assessing protein distribution in cell culture and tissue samples in individual biology research laboratories using Matlab platform.

CONCLUSIONS

In the first part of the project, we have tested the hypothesis that the LBF image analysis developed in collaboration with biophysicist David Knowles can identify subpopulations of cells with different behaviors within the same 3D culture sample. The LBF analysis of H4K20m distribution identified at least two subpopulations within premalignant cells. The ultimate goal is to be able to recognize malignant cells within premalignant or preinvasive lesions. Thus, our work with 3D culture of cells that mimic physiologically relevant phenotypes enabled us to demonstrate that in principle the LBF analysis can be used to recognize mixtures of cell subpopulations. Coculture of premalignant and malignant cells have been successfully set up and show distinct nuclear protein distributions (e.g., for NuMA) in malignant and premalignant cells when malignant cells are mixed with a large amount of premalignant cells. The image analysis is currently being modified to isolate rare events (i.e., one or a few cells with malignant characteristics as shown by the distribution of nuclear proteins, within a population of premalignant cells). We have set up the immunostaining procedure for analysis of the distribution of NuMA and H4K20m on tissue samples and are eager to start the analysis of these samples. However, such an analysis could not be started until all the parameters of the

method were first finalized in 3D culture. We are hopeful that within the next year the LBF method will help identify cell subpopulations within premalignant and preinvasive lesions. Eventually, the ability to recognize malignant cells in these lesions would bring critical improvement for the identification of lesions of adverse and good prognosis.

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Briand, P., O.W. Petersen, and B. Van Deurs. 1987. A new diploid nontumorigenic human breast epithelial cell line isolated and propagated in chemically defined medium. *In Vitro Cell Dev Biol.* 23:181-8.

Hunt CM, Ellis IO, Elston CW, Locker A, Pearson D, Blamey RW. 1990. Cytological grading of breast carcinoma--a feasible proposition? *Cytopathology.* 1: 287-95

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Lelièvre SA and Bissell MJ. Three dimensional cell culture: The importance of context in regulation of function. *Encyclopedia of Molecular Cell Biology and Molecular Medicine (EMCBMM).* (In press, 2005)

Marr, D. 1982. *Vision* (W.H.Freeman & Company)

Mombello A, Mariuzzi L, Morelli L, Granchelli G, Rucco V, Tarocco E, da Silva VD, Thompson D, Bartels HG, Bartels PH, Mariuzzi G. 2001. Quantitative study of ductal breast cancer progression: nuclear signatures for evaluation of progression grade. *Adv Clin Path.* 5: 59-70

Petersen, O.W., Ronnov-Jessen L, Howlett AR, and Bissell MJ. 1992. Interaction with basement membrane serves to rapidly distinguish growth and differentiation pattern of normal and malignant human breast epithelial cells. *Proc Natl Acad Sci U S A.* 89:9064-8.

Plachot, C., and Lelievre, S.A. 2004. DNA methylation control of tissue polarity and cellular differentiation in the mammary epithelium. *Exp Cell Res* 298: 122-132.

Soille, P. 2003. *Morphological Image Analysis* 2nd ed. (Springer)

Wright T. and McGechan A. 2003. Breast cancer: new technologies for risk assessment and diagnosis. *Mol Diagn.* 7: 49-55.

CURRICULUM VITAE

NAME	POSITION TITLE
Sophie A. Lelièvre	Walther Assistant Professor of Basic Medical Sciences

EDUCATION/TRAINING

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
University of Louvain, Belgium	Engineer	1984-1987	Veterinary Sciences
University of Liège, Belgium	Medical Degree	1987-1990	Veterinary Medicine
University of Paris VI, France	Master's	1990-1991	Molecular and Cell Pharmacology
University of Paris VI, France	Ph.D.	1991-1994	Molecular and Cell Pharmacology
Lawrence Berkeley Natl Lab, Berkeley, USA	postdoc	1995-1999	Mammary Cell Biology

RESEARCH AND PROFESSIONAL EXPERIENCE

1988-1990: **Trainee**
School of Veterinary Medicine (Cureghem, Bruxelles, Belgium)
Topics: Canine mammary cancer pathology and chemotherapy

1991-1995: **Veterinary Surgeon**
Pets Emergency Room, Paris District, France

1991-1994: **Predoctoral Fellow**
Gustave Roussy Cancer Institute (Villejuif, France)
Topics: Topoisomerases, anti-cancer pharmacology

1991-1994: **Teaching assistant**
University of Paris; topics: Embryology and Histology

1995: **Postdoctoral Fellow**
Gustave Roussy Cancer Institute (Villejuif, France), and a 3-month training in Dr. Kohwi-Shigematsu's laboratory (LJCRF, La Jolla, CA).
Topics: Resistance to topoisomerase inhibitors, metastatic phenotype, M.A.R., nuclear matrix

1995-1997: **Postdoctoral Fellow**
1997-1999: **Postdoctoral Scientist**
Lawrence Berkeley National Laboratory; Dr. Mina Bissell's laboratory (Berkeley, CA)
Topics: Extracellular matrix-nuclear structure interrelationship, regulation of gene expression in breast morphogenesis and tumorigenesis

1999-2000: **Research Scientist**
Cell and Molecular Biology Dept., Lawrence Berkeley National Lab. (Berkeley, CA)
Topics: Nuclear organization and gene expression, nuclear signaling

10/2000-ongoing: **Assistant Professor**
Basic Medical Sciences, Purdue University, West Lafayette, IN
Topics: Nuclear organization in breast differentiation and cancer, nuclear signaling, nuclear structure and genomic instability

2003-ongoing: **IU Medical School adjunct Assistant Professor** Cancer Pharmacology, Indiana University School of Medicine, Indianapolis, IN

HONORS

University of Louvain (Belgium), lifetime tuition exemption for outstanding student, 1985

National Prize for Fundamental Cancer Research/young investigator, 1995 (French Society of Cancer and National Federation of Cancer Institutes, France)- *One Prize awarded annually; paper-based competition in which trainees present ongoing and future research goals- It was awarded to me because of data showing for the first time the importance of three-dimensional organization of cells in the development of tumors showing levels of resistance comparable to clinical situations and in the acquisition of increased metastatic potential, and also for the demonstration that resistance to anti-cancer drug topoisomerase II inhibitors was accompanied by changes in the nuclear compartmentalization of topoisomerase II beta. The future goals emphasized the importance of studying the role of changes in nuclear structure and in the compartmentalization of nuclear proteins in cancer cell behavior.*

National Alexandre Joel Prize for young investigator, 1995 (Association for Cancer Research, ARC, France)- *One Prize awarded annually only if a suitable postgraduate candidate is identified. Award is made upon nomination by established scientists and reference letters that describe the accomplishments of the young investigator in the domain of cancer research.*

Lawrence Berkeley National Laboratory Outstanding Performance Award, 1998 (Lawrence Berkeley National Laboratory, Berkeley, CA)- *Award received for significant contribution to the development of the use of soft X-ray microscopy to study cellular (including nuclear) structure.*

Integrated Science Partnership Program Appreciation Award, 1999 (Lawrence Berkeley National Laboratory, Berkeley, CA)

Walther Support for New Investigator, 2000-2004 (Walther Cancer Institute, Indianapolis, IN)- *Selected candidates who are recruited as assistant professors at Purdue University receive support from Walther Cancer Institute for three years, extendable to a fourth year upon satisfactory performance. Support was received for four years.*

Teaching Fellowship: **"Moniteur" position, University of Paris**, 1991-1994- *Fellowship only awarded to top-ranked students (ranked upon completion of their Master's), accepted in a Ph.D. program and already selected for a graduate fellowship from the French Ministry of Education and Research. This program includes regular teaching assignments and workshops on teaching methods, pedagogy, and history of Science.*

Research Fellowships:
French Ministry of Education and Research (France), graduate fellowship, 1991-1994
International Agency for Research on Cancer (IARC-WHO), postdoctoral fellowship, 1995-1996
Association for Cancer Research (ARC), complementary fellowship, 1996
Department Of Defense/USA-Breast Cancer Research Program, postdoctoral training grant, 1997-1999

Collaborative Research Fellowships and Travel Awards:
French Society of Cancer Travel Fellowship, 1995
Journal of Cell Science Travel Fellowship, 1997
Philippe Foundation Travel Fellowship, 1998
Purdue University International Travel Award, 2001 and 2002

International Society of Differentiation Travel Award, 2002

Session Chair at scientific meetings:

Session on "Cellular Organization, Signal Transduction and Cancer" at the "Biology and Mathematics of Cells: Physiology, Kinetics and Evolution" European Society for Mathematical and Theoretical Biology (ESMTB) meeting, Spain, 2001

Co-organizer and session co-chair on "Nuclear Compartmentalization in Differentiation and Cancer" at the International Society of Differentiation meeting, France, 2002

Panelist and reviewer for funding agencies

Invited panelist on Focused Discussion Group – Breast Cancer, Walther Cancer Institute, 2003

Ad hoc reviewer, 2004-on: Department of Defense/Breast Cancer Research Program and Prostate Cancer Research Program; Komen Foundation for Breast Cancer Research; Department of Health (Florida State)

INVITED PRESENTATIONS

Speaker at regional, national, and international meetings:

"The solid-state signaling pathway from the extracellular matrix to the nuclear matrix: the critical role of 3D architecture at the cellular level", High resolution X-ray CMT Workshop (LBNL, Berkeley, CA), August 1996;

"Internal cell architecture-A new look", Advanced Light Source Users Meeting (LBNL, Berkeley, CA), October 1997;

"Global rearrangement of nuclear matrix-associated proteins when human mammary epithelial cells are cultured in 3-D: an analysis using confocal-, electron-, and soft x-ray microscopy", Keystone Symposium on Nuclear Matrix (Copper Mountain, CO; junior investigators workshop), April 1998;

"Nuclear structure, cell proliferation, and tissue morphogenesis", American Society for Cell Biology Meeting (San Francisco, CA), December 1998;

"Tissue architecture and gene expression: study of tissue matrix in three-dimensional models of cell culture" and ***"The non-chromatin structure of the nucleus or nuclear matrix: study of its interaction with the chromatin structure and its role in the regulation of gene expression"***, Biomathematics Summer School (Termoli, Italy), Mathematics in Cell Physiology and Proliferation, June 1999;

"Nuclear-directed signaling in mammary gland acini", Gordon Conference on Biological Structure and Gene Expression (Meriden, NH; short talk), August 1999;

"Nuclear organization in normal and malignant breast: NuMA is a marker of cell phenotype and a regulator of differentiation", Era of Hope DOD Breast Cancer Research Meeting (Atlanta, GA; platform talk), June 2000;

"Cell cycle regulation in higher order cell assemblies: the role of three-dimensional tissue architecture," Third International Congress of Nonlinear Analysts (Catania, Sicily), July 2000;

"Signal transduction and feedback signaling", ***"Cellular transformation and genomic instability"***, and ***"Tumor progression: How in vitro models may help understand in vivo situations"***, ESMTB School, Biology and Mathematics of Cells: Physiology, Kinetics and Evolution, (Siguenza, Spain), June 2001;

"NuMA functionally links cell adhesion and nuclear structure to regulate cell survival in breast", 2nd International Conference on Tumor Microenvironment: Progression, Therapy and Prevention, (Baden, Austria), June 2002;

"Multiple facets of nuclear structural proteins: The Role of NuMA in the regulation of breast epithelial phenotypes", 12th International Conference of the International Society of Differentiation, (Lyon, France), September 2002;

"Link between compartmentalization and functions of nuclear proteins in phenotypically normal and neoplastic tissues", FASEB Summer Research Conference on "Nuclear Structure and Cancer", (Saxtons River, VT), June 2003;
"Proteomics with a twist-Early detection of breast cancer by looking beyond protein expression", The Amelia Project-Giving Wings to Research, (Indianapolis, IN), February 2004;
"Tissue structure and gene expression control", Research in Cell Therapy Workshop, Session on proliferation and differentiation in normal and pathological cells, (St Louis Hospital, Paris, France), March 2004

Seminars:
"The nuclear matrix is an old concept still in its infancy", Gustave Roussy Cancer Institute, Dept. of Clinical and Molecular Pharmacology, (Villejuif, France), September 1995;
"The solid-state pathway: a model for the regulation of gene expression", University of Paris XII, CRRET Laboratory, (Créteil, France), June 1996;
"From the extracellular matrix to the nuclear matrix, the dynamic cellular architecture plays a role in the regulation of cellular behavior: a study of a model of mammary tumorigenesis" Gustave Roussy Cancer Institute, Dept. of Clinical and Molecular Pharmacology, (Villejuif, France), June 1996;
"Dynamic re-organization of nuclear architecture during tumorigenesis and tumor reversion", Harvard Children's Hospital, (Boston, MA), April 1997;
"The role of cellular and tissue structure during tumorigenesis", Institute of Immunology, (Munich, Germany), June 1997;
"Dynamic reciprocity between the extracellular matrix and the organization of the cell nucleus: a study of mammary epithelial cell morphogenesis", Institute Molecular Genetics, (Paris, France), June 1998;
"Interrelationships between the distribution of nuclear matrix proteins, chromatin structure and gene expression during mammary epithelial cells morphogenesis", Center for Atomic Energy (CEA), (Fontenay aux Roses, France), June 1998;
"Communication between the extracellular matrix and the nuclear structure in breast development and malignancy", Boston University Medical School, (Boston, MA), Dept of Biochemistry, February 1999;
"The role of nuclear organization in normal and malignant breast structures", California Pacific Medical Center Research Institute (San Francisco, CA), May 1999;
"Nuclear organization in normal and malignant breast", Division of Radiation and Cancer Biology, New England Medical Center, TUFTS University, (Boston, MA), 1999;
"What is the link between nuclear architecture and the expression of malignancy?" Purdue University, Dept. of Basic Medical Sciences (West Lafayette, IN), March 2000;
"The organization of the cell nucleus in breast differentiation and tumorigenesis. A source for the development of novel anticancer strategies," Research Institute of Molecular Pathology, Vienna Biocenter, Boehringer-Ingelheim, (Vienna, Austria), April 2000;
"Structure, Instability and Plasticity in Cancer" University of Mexico, (Mexico City), September 2001;
"Subcompartmentalization of Nuclear Proteins in Differentiation and Cancer: Multi-faceted NuMA Regulates Breast Epithelial Cell Behavior." IUPUI, (Indianapolis, IN), November 2001;
"Chromatin structure and breast differentiation: The role of the supramolecular organization of nuclear proteins" Boston University, (Boston, MA), April 2003;
"Architectural proteomics –When proteins become stars", Lawrence Berkeley National Laboratory, Life Sciences Division, (Berkeley, CA), February 2004;
"Three-dimensional culture of non-neoplastic and neoplastic breast tissues to unravel higher order control of proliferation, survival and differentiation", Georgetown University, School of Medicine (Washington, DC), January 2005

PATENTS

SA Lelièvre and MJ Bissell. "Utilization of nuclear structural proteins for targeted therapy and detection of proliferative and differentiation disorders". US 6,287,790 B1, Sep.11, 2001.

PUBLICATIONS

From Graduate Work

K Bojanowski, **S Lelièvre**, J Markovits, J Couprie, A Jacquemin-Sablon and AK Larsen, "Suramin is an inhibitor of DNA topoisomerase II *in vitro* and in Chinese hamster fibrosarcoma cells". *Proc.Natl.Acad.Sci., USA*, 89:3025-3029, 1992.

S Lelièvre and AK Larsen, "Development and characterization of suramin-resistant Chinese hamster fibrosarcoma cells: drug-dependent formation of multicellular spheroids and a greatly enhanced metastatic potential." *Cancer Res.*, 54: 3993-3997, 1994.

S Lelièvre and AK Larsen, "Suramin resistance in Chinese hamster fibrosarcoma cells is accompanied with morphological alterations and metastases formation." *Bull. Cancer*, 81: 903-905, 1994.

S Lelièvre and AK Larsen, "Chronic in vitro suramin exposure leads to the development of drug resistant sublines which grow as three dimensional cultures and are highly invasive *in vivo*. Lack of growth factor involvement in the cytotoxic action of the drug." *In* "Novel approaches in anticancer drug design. Molecular modelling-New treatment strategies. *Contrib. Oncol.*, 49: 117-123, 1995, (WJ Zeller, D'Incalci M, and Newell DR, eds), Basel, Karger.

S Lelièvre, Y Benchokroun, and AK Larsen, "Altered DNA topoisomerase I and II in suramin-resistant Chinese hamster fibrosarcoma cells." *Mol. Pharmacol.*, 47: 898-906, 1995.

From Postdoctoral Work

***S Lelièvre**, VM Weaver, and MJ Bissell, "Extracellular matrix signaling from the cellular membrane skeleton to the nuclear skeleton: a model of gene regulation in mammary epithelial cells." *Recent Progress in Hormone Research*, 51:417-432, 1996.

***S Lelièvre** and MJ. Bissell. "The solid-state signaling pathway from extracellular matrix to nuclear matrix: the critical role of three-dimensional architecture for functional differentiation." Proceedings of the 1996 Workshop on High Resolution Computed Microtomography (CMT), LBNL/UC, pp 85-96, 1997.

***S Lelièvre**, VM Weaver, CA Larabell, and MJ Bissell, "Extracellular matrix and nuclear matrix interactions may regulate apoptosis and tissue-specific gene expression: a concept whose time has come." *In* Advances in Molecular and Cell Biology: Cell Structure and Signaling, (RH Getzenberg, ed), JAI Press Inc, Greenwich CT, Vol 24, pp: 1-55, 1997.

SA Lelièvre, VM Weaver, JA Nickerson, CA Larabell, A Bhaumik, OW Petersen, and MJ Bissell. "Tissue phenotype depends on reciprocal interactions between extracellular matrix and the structural organization of the nucleus" *Proc. Natl. Acad. Sci. (USA)*, 95: 14711-14716, 1998.

***SA Lelièvre** and MJ Bissell. "Communication between the cell membrane and the nucleus: the role of

protein compartmentalization" 25th Anniversary Issue of *J. Cell. Biochem,* 30/31 suppl.: 250-263, 1998.

*MJ Bissell, VM Weaver, **SA Lelièvre**, F Wang, OW Petersen, and KL Schmeichel, "Tissue structure, nuclear organization and gene expression in normal and malignant breast" *Cancer Res.*(SUPPL), 59: 1757s-1764s, 1999.

H-M Chen, KL Schmeichel, IS. Mian, **SA Lelièvre**, OW Petersen, and MJ Bissell. AZU-1: a candidate breast tumor suppressor and biomarker for tumorigenic reversion. *Mol.Biol.Cell*, 11: 1357-1367, 2000.

SA Lelièvre, MJ Bissell, and P Pujuguet. "Cell nucleus in context." *Crit. Rev. Eukar. Gene Expression*, 10: 13-20, 2000.

W Meyer-Ilse, D Hamamoto, A Nair, **SA. Lelièvre**, G Denbeaux, L Johnson, A Lucero, D Yager, and CA. Larabell. "High Resolution Protein Localization Using Soft X-ray Microscopy." *J. Microscopy,* 201: 395-403, 2001.

C Ortiz de Solorzano, R. Malladi, **SA Lelièvre**, and SJ Lockett. "Segmentation of nuclei and cells using membrane related protein markers." *J. Microscopy*, 201: 404-15, 2001.

SK Muthuswamy, D Li, **SA Lelièvre**, MJ Bissell, and J Brugge. "ErbB2, but not ErbB1, can reinitiate proliferation and induce luminal repopulation in growth-arrested epithelial acini." *Nature Cell Biology,* 3: 785-792, 2001.

VM Weaver, **SA Lelièvre**, JN Lakins, MA Chrenek, J Jones, F Giancotti, Z. Werb, and MJ Bissell. "Beta4-integrin-dependent formation of polarized three-dimensional architecture confers resistance to apoptosis in normal and malignant mammary epithelium". *Cancer Cell*, 2: 205-19, 2002.

Since independent position (October 2000)

C Plachot and **SA Lelièvre**. "New directions in Tumour biology: from basement membrane-directed polarity to DNA Methylation". *In* Mathematical Biology and Medicine Series, "Cancer modeling and simulation", Chapman & Hall/CRC, 2003.

C Plachot and **SA Lelièvre**. "DNA methylation control of tissue polarity and cellular differentiation in the mammary epithelium" *Exp. Cell Res.*, 298: 122-32, 2004.

PC Abad, IS Mian, C Plachot, A Nelpurackal, C Bator-Kelly, and **SA Lelièvre**. "The C-terminus of the nuclear protein NuMA: phylogenetic distribution and structure". *Protein Sci.*13: 2573-2577, 2004.

P Kaminker, C Plachot, S-H Kim, P Chung, D Crippen, OW Petersen, MJ Bissell, J Campisi and **SA Lelièvre**. "Higher order nuclear organization in growth arrest of human mammary epithelial cells: A novel role for telomere-associated protein TIN2". *J. Cell Sci.* 118: 1321-1330, 2005.

SA Lelièvre and MJ. Bissell. Three dimensional cell culture: The importance of context in regulation of function. *Encyclopedia of Molecular Cell Biology and Molecular Medicine (EMCBMM).* (In press, 2005)

DW Knowles, D Sudar, C Bator-Kelly, MJ Bissell, and **SA Lelièvre**. "Automated local bright feature image analysis of nuclear protein distribution identifies changes in tissue phenotype". (Submitted)

PC Abad, J Lewis, IS Mian, S Badve, J Xie, and **SA Lelièvre**. "The nuclear apparatus protein NuMA impacts higher order chromatin structure in mammary epithelial morphogenesis". (Submitted)

G Chandramouly, PC Abad, and **SA Lelièvre**. "Restoration of higher order nuclear structure characteristic of phenotypically normal differentiation is critical for the reversion of tumor phenotype." (Submitted)

Research manuscripts in preparation for submission in the Summer:

C Plachot, H Adissu, E Asem, and **SA Lelièvre.** "The use of natural and artificial matrices for physiologically relevant culture of non-neoplastic and neoplastic breast epithelial cells"

H Adissu, S Clark, M Johnson, S Devarakonda, and **SA Lelièvre**. "Phenotypically normal mammary epithelial cells promote survival and proliferation of tumor cells via paracrine influrence"

(indicates non-peer reviewed)*

PARTICIPATION IN RESEARCH TRAINING AT PURDUE UNIVERSITY

'Administrative' member in Graduate Programs: member of the graduate committee of the Department of Basic Medical Sciences (BMS); member of the admission committee of the Interdisciplinary PULSe Program
'Participatory' member in the BMS training program, the Chromatin and Regulation of Gene Expression PULSe training program and the Integrated Molecular Signaling and Cancer Biology PULSe training program

Research trainees in the laboratory
Postdoctoral trainee: Cedric Plachot (10/2001-ongoing), Purdue Cancer Center Fellowship

Graduate trainees:
Patricia Abad (Fall 2001-Summer 2003) degree obtained: M.S.
Patricia Abad (Fall 2003- ongoing) Ph.D. program; Purdue Doctoral Fellowship and Purdue Research Foundation Fellowship
Gurushankar Chandramouly (Fall 2001- expected graduation Fall 2005) Ph.D. program, Andrews Fellowship, Purdue Research Foundation Fellowship, and Bisland Fellowship
Hibret Adissu (Fall 2002-ongoing) Ph.D. program, Ross Fellowship and ATF Fellowship

Undergraduate, veterinary student, high school student trainees (research taken for credit or as part of research programs with fellowships from DOD, Howard Hughes, Merk Merial, MARC/AIM, and Purdue Cancer Center training programs):

(1) Tushendan Rasiah (Senior Undergraduate; Biological Sciences, international exchange), Spring 2001

(2) Rosemary Ruffin (Junior Undergraduate; Food Science/Biochemistry), Spring semester and Summer 2001

(3) Sara Clark (Sophomore, Veterinary School, **Merk Merial Fellow**), Summer 2001

(4) Katie Gumble (Junior Undergraduate, Biological Sciences), Fall 2001

(5) Zoltan Metlagel (Senior Undergraduate, Biochemistry), Fall 2001, Spring and Summer semesters 2002, [Carroll County Cancer Association/Indiana County **Cancer Societies Fellow**]- HONORS program

(6) Sheela Devarakonda (Sophomore, Veterinary School, **Merk Merial Fellow**), Summer 2002

(7) Katharine Turner (Pharmacy student, **DOD Fellow**), Summer 2002

(8) Jason Lewis (Junior Undergraduate, Microbiology), 2003, including summer as **DOD Fellow**

(9) Aniysha Nelpurakal (Junior Undergraduate, Biology), Spring 2003 and Summer 2003 as a **Howard Hughes Fellow**, Fall 2003 and Spring 2004- HONORS program.

(10) Monica Johnson (Sophomore, Veterinary School, **Merck Merial Fellow**), Summer 2003

(11) Dania Jaara (Junior Undergraduate, Biological Sciences), 2004, including Summer as a **DOD Fellow**

(12) Lesley Chaboub (Senior Undergraduate, Biological Sciences), international exchange, Spring 2004

(13) Ashwini Pai (Freshman Undergraduate, Biological Sciences), Spring 2004

(14) Beverly Basham (Sophomore, Veterinary School, **Merck Merial Fellow**), Summer 2004

(15) Marissa Dixon (Junior Undergraduate, **MARC/AIM Fellow**), Summer 2004

(16) Eugenia Gabrielov (High School Student, Science Project), Fall 2004-Spring 2005

(17) Kelly Metcalf (Sophomore, Veterinary School, **Merck Merial Fellow**), Summer 2005