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Award Number: DAMD17-03-1-0665

TITLE: Early Detection of Breast Cancer Using Molecular Beacons

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REPORT DATE: January 2008

TYPE OF REPORT: Final

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

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-01-2008	Final	1 SEP 2003 - 31 DEC 2007

4. TITLE AND SUBTITLE

Early Detection of Breast Cancer Using Molecular Beacons

5a. CONTRACT NUMBER

5b. GRANT NUMBER
DAMD17-03-1-0665

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)

Emory University
Atlanta, GA 30322

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

The objective of this research project was to develop a novel fluorescence cell imaging method for the early detection of breast cancer. We proposed to use molecular beacon technology to detect the level of expression of several biomarker genes that are highly expressed in breast cancer cells but not in normal breast epithelial cells. As the result of this DOD Idea Award, we have developed molecular beacons that detect the level of expression of survivin, cyclin D1, HIF-1 α and Her-2/Neu genes in breast cancer cells. This method can also be used to examine the level of gene expression in real time within living breast cancer cells such that changes in the expression of specific biomarker genes can be monitored. To increase the specificity and sensitivity of cancer cell detection, we further developed a multi-fluorescent cell imaging approach that detects the expression of biomarker genes and proteins simultaneously at the single cell level using combination of molecular beacon and fluorescence quantum dots conjugated to antibodies against these biomarkers. Our results show that the fluorescence cell imaging methods and tumor cell targeted nanoparticle imaging probes we have developed have potential to be used for the detection of breast cancer cells in clinical samples obtained from breast cancer patients and for in vivo non-invasive imaging of breast cancers using optical and MR imaging approaches.

15. SUBJECT TERMS
There are no subject terms provided.

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
					USAMRMC
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			**19b. TELEPHONE NUMBER** *(include area code)*
U	U	U	UU	129	

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Introduction

Breast cancer is the most common type of cancer and one of the leading causes of death among women. A crucial factor to increase survival is early diagnosis. Although early screening with mammography decreases the mortality of the disease, nearly 20% of breast cancer patients are still missed by mammography. Furthermore, of all patients with abnormal mammograms, only 10 to 20% are confirmed to be breast cancer by biopsy (1). At present, there is no reliable serum tumor marker for the diagnosis of breast cancer. Therefore, development of novel approaches for the early diagnosis of breast cancer is of critical importance for successful treatment and for increasing survival. It is well known that over 90% of breast cancers arise in the epithelial cells lining the ductal system. Most breast cancers develop over a period of 8 to 10 years before being detected by standard methods. In order to detect early stage breast cancer or ductal carcinoma in situ (DCIS), it is important to develop novel and non-invasive approaches with high sensitivity and specificity.

It is well established that cancer cells develop due to genetic alterations in oncogenes and tumor suppressor genes. These abnormalities in gene expression provide growth advantage and metastatic potential to the cells. A novel way of achieving early detection of cancer is to identify the cancer cells through detection of mRNA transcripts that are expressed in the cancer cells but at low levels or not expressed in normal cells. We have developed a molecular beacon (MB)-based approach for direct examination of gene expression in viable and fixed cells (2, 3). This objective of proposed study was to further optimize the MB-based fluorescent imaging method for detection of the expression of biomarkers for breast cancer cells, such as survivin, cyclin D1 and Her-2/neu genes, which are tumor markers found in over 50 to 80% of DCIS tissue samples (4-7). MBs are oligonucleotides with a stem-loop hairpin structure, dual-labeled with a fluorophore at one end and a quencher at the other. Delivering MBs into cells produces a fluorescent signal if the MBs hybridize to target mRNAs (8). Thus, when target mRNAs correspond to the molecular markers of a cancer, cancer cells (bright) can be distinguished from normal cells (dark) (Figure 1) (2, 3, 8).

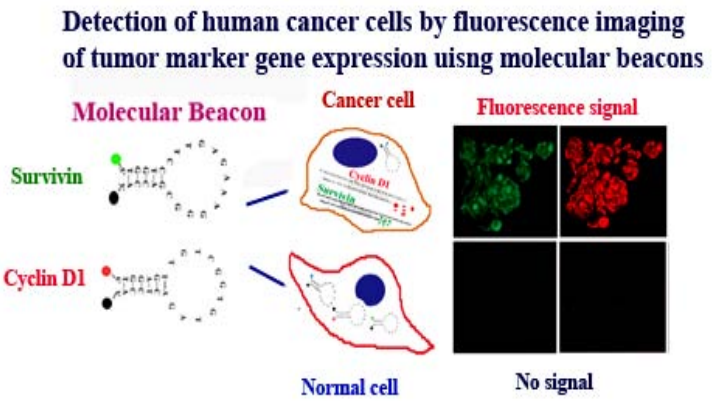


Figure 1. Schematic illustration of detecting breast cancer cells using MBs targeting to biomarker mRNAs.

Recently, a class of new fluorescent emitting nanoparticles, semiconductor quantum dots, has been developed for using as sensitive probes for biomolecular and cellular imaging (9-10). These quantum dots (QDs) are tiny, nanometer-scale light-emitting particles that have unique optical and electronic properties such as size-tunable light emission, improved signal brightness, resistance against photobleaching, and the ability

to simultaneous excite multiple fluorescence colors (9-10). These properties are most promising for improving the sensitivity of molecular imaging and quantitative cellular analysis by 1-2 orders of magnitude. Another advantage is that multicolor QD probes can be used to image and track multiple tumor markers simultaneously in single cells. It has been shown that antibodies conjugated to QDs are able to detect several protein markers in cancer cells. During the last several years, we have developed the method to conjugate antibodies or targeting peptides to QDs and have shown that these QDs specifically bind to biomarker expressing breast cancer cells and produce fluorescence imaging signal in the cells. Therefore, we would like to determine if it is feasible to detect the level of biomarkers by combining MBs for mRNA and QDs for protein level simultaneously in single cell.

Our ***Working hypothesis*** was that simultaneous detection of mRNA and protein levels of tumor markers, such as survivin, Her-2/Neu, and cyclin D1, using MBs and QDs, has a high specificity and sensitivity in identifying cancer cells. The ultimate goal is to develop a simple, sensitive and efficient clinical procedure for the early detection of breast cancers. Additionally, supported by this DOD funding, we have developed methods for detecting several other biomarkers for breast cancer cells, including urokinase plasminogen activator receptor (uPAR) and HIF-1 α. We believe that the sensitivity and specificity of cancer cell detection can be improved by using several biomarkers. In summary, we have developed novel fluorescence nanoparticle imaging probes and cell imaging approaches for the detection of breast cancer cells *in vitro* and *in vivo*.

Body of the Final Report

The development of novel molecular beacon (MB) technology for the detection of the level of expression of biomarker genes in human breast cancer cells.

Development and optimization of the MB probes for the detection of survivin, cyclin D1, Her-2/Neu and HIF-1α mRNAs.

First, we designed and tested several MB sequences for each biomarker gene. Specificity and signal strength were tested *in vitro* using a DNA oligo template that complements to each MB. The ability of detection of the level of biomarker gene expression and the specificity of MB probes were further examined in breast cancer and normal cell lines. We have selected the best MB sequences for biomarker mRNAs as shown in Table-1. Previously, we used 4-({4'-(dimethylamino)phenyl]azo)benzoic acid (Dabcyl) as a quencher molecule, which was the best available quencher. Recently, a new class of quencher molecules, called black hole quencher (BHQ), has been developed by Bioresearch Technologies. Dabcyl has inadequate absorption that overlaps very poorly with fluorescent dyes emitting above 480 nM. BHQ dyes have much larger signal-to-noise ratio, which will greatly reduce background fluorescence signals. Further, we also designed a negative control MB containing scrambled DNA sequence that does not share homology with any known gene sequences.

Table-1 Design and oligonucleotide sequence of molecular beacons.

Gene names	MB sequences
Survivin MB # 3 MB #5	 5'-Cy3-CTGAGAAAGGGCTGCCAGTCTCAG-Dabcyl-3' 5'-Cy3-TGGCTCTTTCTCTGTCCAGAGCCA-BHQ2-3'
Cyclin D1	5'-Texas-red-TGGAGTTGTCGGTGTAGACTCCA-Dabcyl-3'
Her-2/Neu MB #2	 5'-Cy5-ACAGGAGTGGGTGCAGTTG CCTGT-BHQ2-3'
HIF-1 α	5'-Texas red-TCACCAGCATCCAGAAGTGGTGA-Dabcyl-3'
Scrambled MB Control	5'-Cy3-AGACGCGCATCACATTCTC CGTCT-BHQ2-3'

Our results show that survivin and cyclin D1 MBs can specifically detect breast cancer cells by fluorescence cell imaging (Appendix I, Figure 1 and 2). To determine the specificity of survivin and Her-2/Neu MBs in detecting breast cancer cells at different stages, we employed the MCF-10A series of cell lines that represent normal (MCF-10A), pre-malignant (MCF-10AT1), DCIS (MCF-10 DCIS) and invasive breast cancer (MCF-10 CA1). Using a survivin MB, we found a high level of survivin gene expression in MCF-10 DCIS and MCF-10 CA1 cell lines but not in normal MCF-10A cells. The majority of the pre-malignant MCF-10 AT1 cells are negative for survivin gene expression except a few cells showing a low level of survivin gene expression. Her-2/neu gene is expressed at a low level in normal and pre-malignant cells. However, over-expression of Her-2/neu gene is detected by Her-2/neu MB in MCF-10 DCIS and MCF-10 CA1 cells (Figure 2).

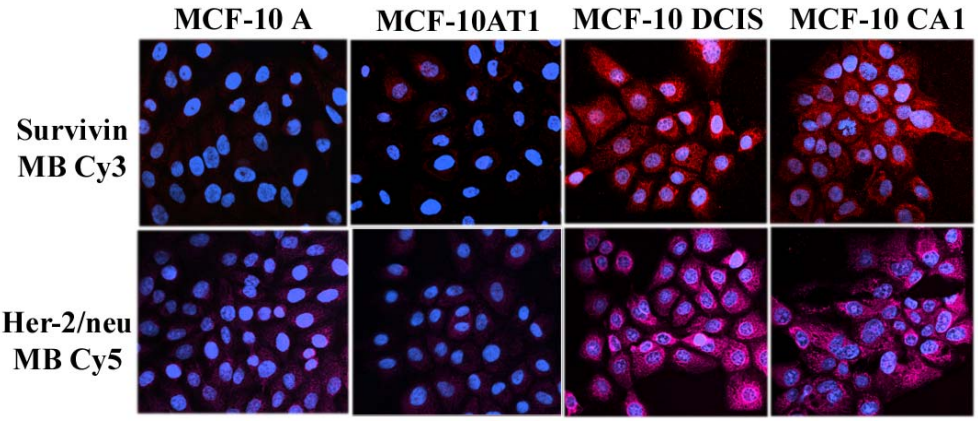


Figure 2. Detection of survivin and Her-2/neu gene expression using MBs in a series of MCF-10 cell lines representing different stages of breast cancer development.

MCF-10 A: normal immortalized human mammary epithelial cell line.
MCF-10 AT1: H-ras transformed MCF-10 A cells at pre-malignant stage.
MCF-10 DCIS: a transformed MCF-10 A cell line produces DCIS-like tumors in nude mice.
MCF-10 CA1: invasive breast cancer.
Red: Cy3 (survivin) and Cy5 (Her-2/neu).
Blue: Hoechst 33342 nuclear staining.

We further examined whether MBs targeting different tumor marker mRNAs can be labeled with different fluorophores and expression of the tumor marker genes can be determined simultaneously in single cells. Our results demonstrate that a combination of survivin and cyclin D1 MBs detects the expression of both

survivin and cyclin D1 genes simultaneously and generates fluorescent signals corresponding to either survivin (green) or cyclin D1 (red) mRNA in the cancer cells (Appendix I, Figure 2). Importantly, the fluorescent signal was very low for both survivin and cyclin D1 MBs in a normal immortalized human mammary epithelial cell line (MCF-10A), indicating that survivin and/or cyclin D1 MBs can be used as fluorescence probes for the detection of breast cancer cells (Appendix I, Figure 2). The results of examination of fluorescence intensity and the level of survivin or cyclin D1 gene expression in tumor and normal cell lines further show that the fluorescent signals detected by the MBs correlate very well with the level of survivin or cyclin D1 gene expression, both in mRNA and protein levels (Appendix I, Figure 2).

Our results demonstrate that fluorescence cell imaging using MBs is a novel approach to simultaneously detect the levels of multiple gene expressions in intact single cancer cells. At present, pathological diagnosis of breast cancer in patient samples such as fine needle aspiration, core biopsy and surgically resected tissues mainly depends on morphological classification. This method may offer a simple and fast procedure to detect biomarker gene expression in clinical samples. Our study results show that survivin MB-Cy3 was able to produce strong red fluorescent signals in breast cancer cells in frozen sections of human breast cancer tissues (Appendix I, Figure 3). Using this method, we found that survivin gene expression is an early event in the tumorigenesis of breast cancer, and is present in the ductal carcinoma in situ (DCIS) stage. A high level of survivin gene expression is also consistently detected in most breast cancer tissues of invasive ductal carcinoma and lymph node metastases but not in normal breast tissues (Appendix I, Figure 3).

In addition to its application in cancer cell detection, MBs can also be used to monitor the changes in the level of gene expression in real time within living cells. This method should allow for specific and sensitive evaluation of the effect of molecular targeted therapeutics on the level of target gene expression in cancer cells (Appendix I, Figure 4 and 5). Unlike the traditional linear probes where unbound probes also generate fluorescent signals, MBs should only produce fluorescent signals when hybridized specifically to their target mRNAs, which makes them ideal probes for detecting the level of mRNAs in viable cells.

<u>MBs can be used to monitor the level of gene expression and location of specific mRNA in living cells real time.</u>

In collaboration with Dr. Paraskevi Giannakakou, we found that MBs can be used to determine the location of mRNAs in living cells. MCF-7 breast cancer cells stably transfected with a GFP-tubulin fusion gene were transfected with Survivin MB (Cy-3) and then cultured in the presence or absence of 50 nM docetaxel, at which docetaxel concentration induces upregulation of survivin expression. The cells were examined using a confocal microscope with a live-imaging

Real-time detection of the level of location of survivin mRNA in living cells

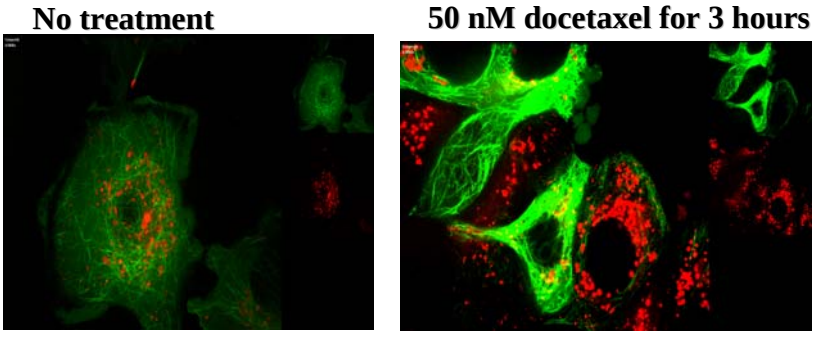


Figure 3. Detection of localization of survivin mRNAs and changes in the level of survivin gene expression in MCF-7 cells after docetaxel treatment using survivin MB.
MCF-7 breast cancer cells stably transfected with a GFP-tubulin fusion gene (green). Red: survivin MB (Cy3).

system. First, we observed that red fluorescence signals were co-localized to green microtubules, suggesting that survivin mRNAs are located around microtubules. Interestedly, docetaxel-treatment stabilizes microtubulin resulting formation of bundles of green microtubules. However, in cancer cells with a high level of survivin, as indicated as red clusters of fluorescence dots, few green microtubule bundles were found. It is possible that upregulation of survivin gene expression in those cells prevents the effect of docetaxel even before activation of the apoptotic pathway.

Identification of new biomarkers that are overexpressed in the ductal carcinoma in situ (DCIS) stage of human breast cancer.

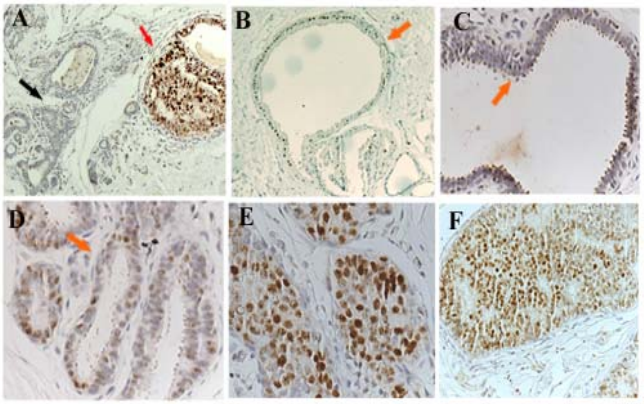
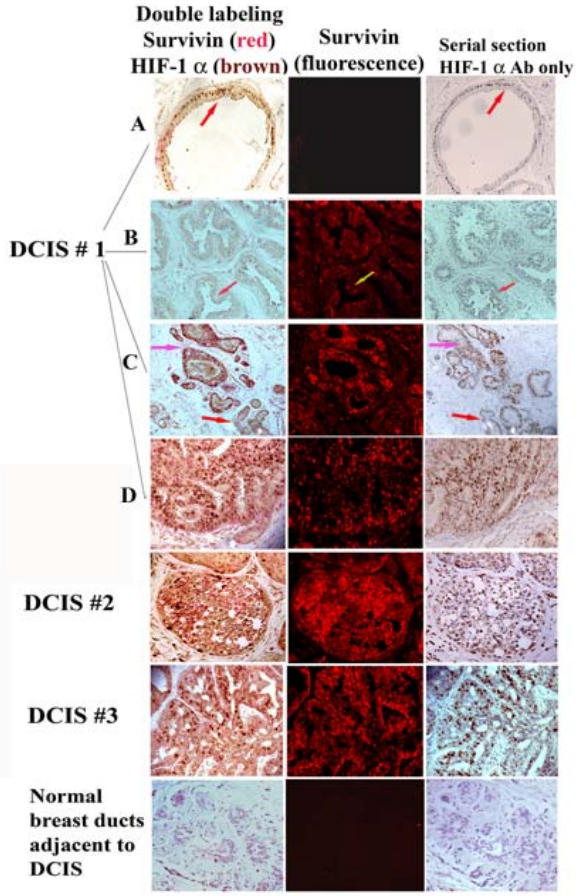


Figure 4. Examination of HIF-1α in a human DCIS tissue by immunohistochemical staining.



Since the objective of this research project is to detect breast cancers at the early stage, it is important to identify biomarkers that are upregulated in DCIS tissues. It is well known that human tumors containing a heterogeneous cell population that expresses various biomarkers. MB probes that detect several biomarkers should increase the sensitivity and specificity of the early detection of breast cancer cells. We have examined 16 human DCIS tissues and identified several new biomarkers for breast cancer cells at the DCIS stage. First, we found that the level of hypoxia-inducible factor 1 alpha (HIF-1α) is upregulated in 30% of human DCIS tissues but is undetectable in normal breast ductal epithelial cells. Interestingly, we found that this upregulation is not necessarily correlated with tumor hypoxia since many of HIF-1α positive cells were detected in DCIS lesions containing a single cell layer of ductal epithelial cells (Figure 4). We also found high levels of HIF-1α in solid type DCIS lesions where the tumor hypoxia is present. We further found that upregulation of HIF-1α in DCIS tissue occurs before survivin expression, suggesting the importance of the HIF-1α in the progression of breast cancer (Figure 5). We further examined the levels of

Figure 5. Co-localization of survivin and HIF-1α expression in DCIS tissues.
HIF-1α-Ab positive tumors were labeled with an HRP-rabbit anti-mouse secondary antibody followed by DAB substrate (brown). Survivin-Ab positive cells were detected using an alkaline phosphatase-labeled donkey anti-goat antibody followed by alkaline phosphatase substrate kit 1, which gave rise to red staining under light microscope and red fluorescence observed with fluorescence microscope.

expression of those biomarker genes in a series of MCF-10 cell lines. As shown in Figure 6, we found that Her-2/neu, EGFR, HIF-1α and survivin were highly expressed in MCF-10 DCIS cell line. Additionally, the results from immunofluorescence staining also show that uPAR is expressed in DCIS tissues (Figure 6). Therefore, in addition to three tumor markers that were originally proposed for this study, we added HIF-1α and uPAR as biomarkers for cancer cell detection.

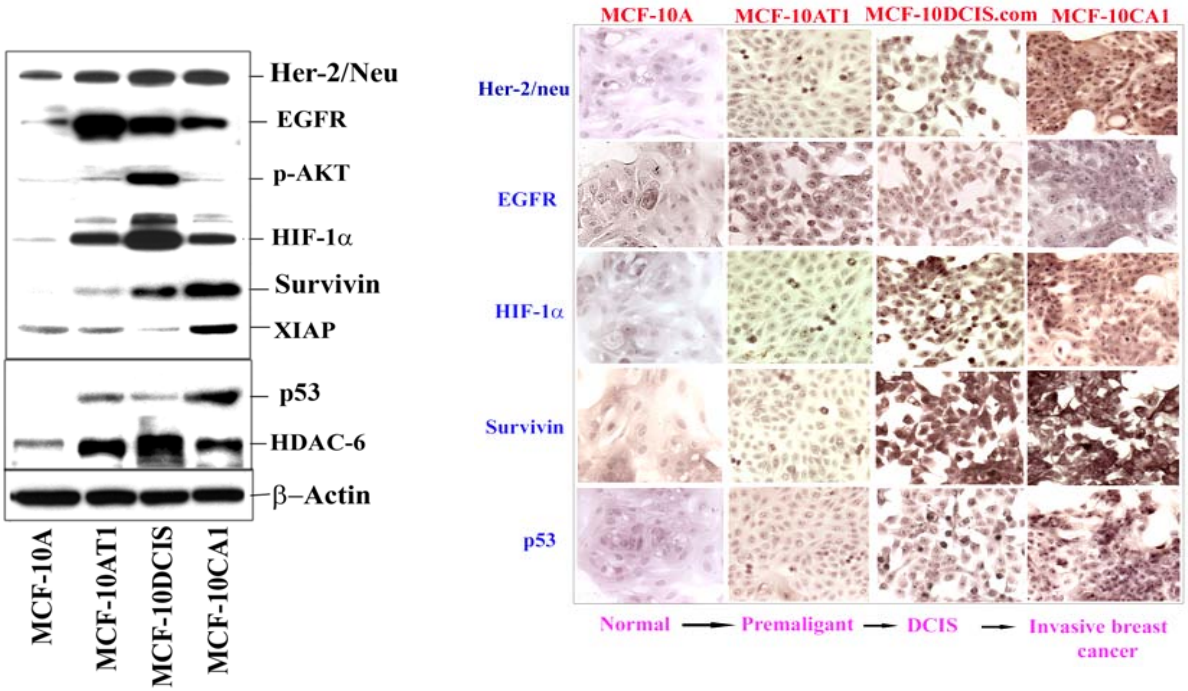


Figure 6. Detection of cellular factors regulating HIF-1α/ survivin pathway in the MCF-10 cell lines.

Left panel: Western blot analysis of the levels of Her-2/neu, EGFR, p-AKT, HIF-1α, survivin, XIAP, mutant p53 and HDAC6 proteins in MCF-10 series of cell lines cultured under normoxic conditions. **Right panel**: Immunohistochemical staining of above markers in the MCF-10 serial cell lines. DAB substrate was used and positive cells were stained as brown. Hematoxylin was used as counterstaining (blue).

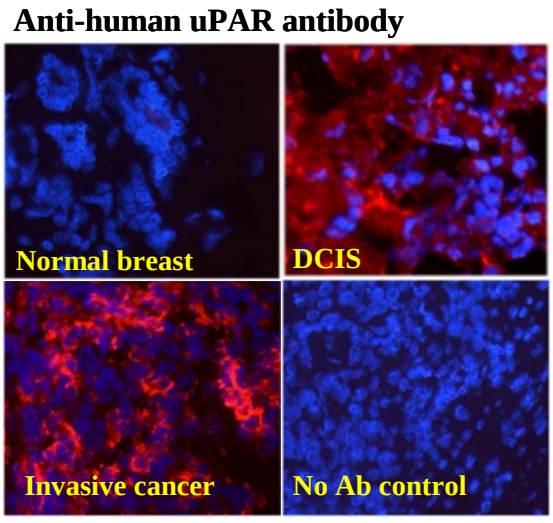


Figure 7. Detection of uPAR level in human breast cancer tissues.

Frozen tissue sections obtained from normal breast, DCIS and invasive breast cancer tissues were stained with a polyclonal anti-uPAR antibody followed by Texas-red labeled secondary antibody (Red). Blue: background staining.

Development of QD-based fluorescence cell imaging for the detection of protein levels of biomarkers in breast cancer cells

It is well known that post-translational regulation of the levels and activities of proteins is important in the development of human cancer. It is likely that novel approaches that allow for detection of both mRNA and protein levels of breast cancer biomarkers will increase the specificity of cancer cell detection. Although several research labs have developed multicolor QD probes for image and semi-quantification of multiple tumor markers in cancer cells, our group is the first to developed the combined MB and QD approach for detecting the level of mRNA and protein simultaneously in cells. Unlike most other studies using antibody conjugation kits provided by a commercial company, we have developed two novel methods for conjugation of antibodies and peptides onto the nanoparticle. The first method is developed for conjugating his tagged recombinant proteins to an amphiphilic copolymer layer that coated on the surface of QDs, which is mediated by NTA-Ni (Figure 8 A). This method is a simple, fast and efficient way to conjugate any his-tagged recombinant targeting peptide ligands and single chain antibodies to the surface of the nanoparticles at the correct binding orientation. The second method uses a protein G as a mediator to conjugate antibodies. First, protein G is conjugated directly to QDs. Antibodies were then added to the protein-G-QDs by highly affinity interaction of the Fc region of an antibody with a protein G (Figure 8 B).

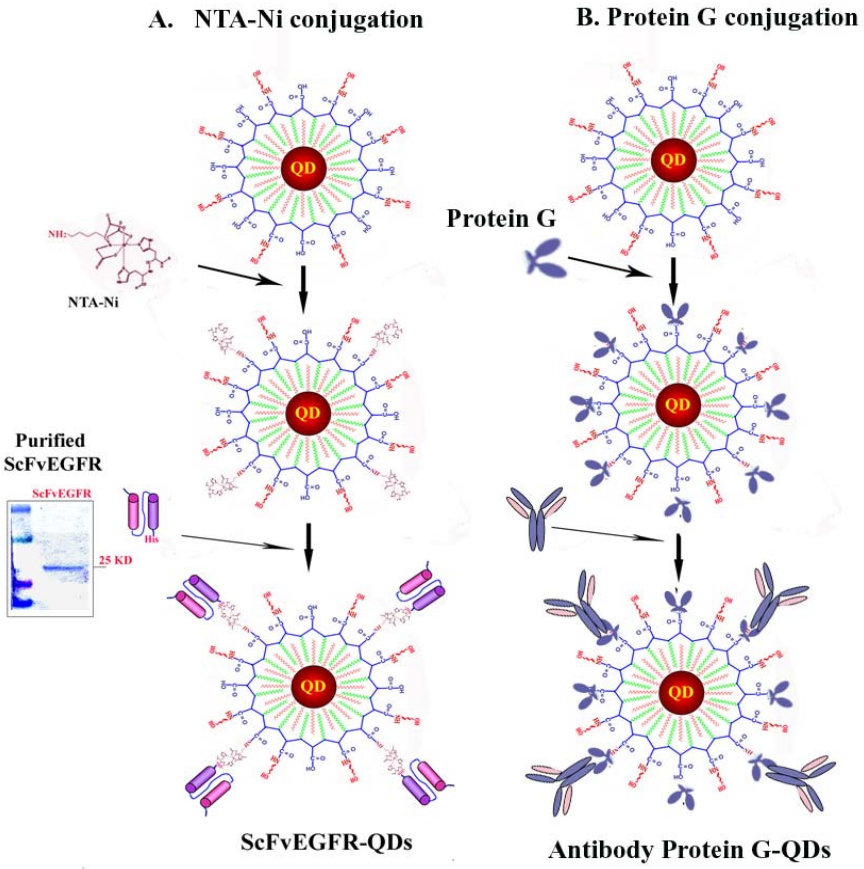


Figure 8. Schematic illustration of novel methods for conjugating biomarker targeted ligands and antibodies to QDs.

We have also produced a single chain anti-EGFR antibody (ScFvEGFR) and the receptor binding domain of the amino-terminal fragment (ATF) of uPA in bacteria-recombinant protein expressing system. As shown in Figure 8, ScFvEGFR is conjugated to QDs through Ni-NTA, which binds to 6x his tag in the C-terminal of the single chain antibody. We showed that those ScFvEGFR-QDs specifically bind to breast cancer cells expressing a high level of EGFR (Figure 9). Using a similar approach, we produced a uPAR-binding QDs by conjugating ATF peptides to QDs and demonstrated the binding specificity in breast cancer cells. For other biomarkers such as survivin, cyclin D1, Her-2/neu and HIF-1 α, we conjugated their antibodies to QDs with different emission wavelengths. The specificity of each QD probe in MCF-10 DCIS cells is shown in Figure 11.

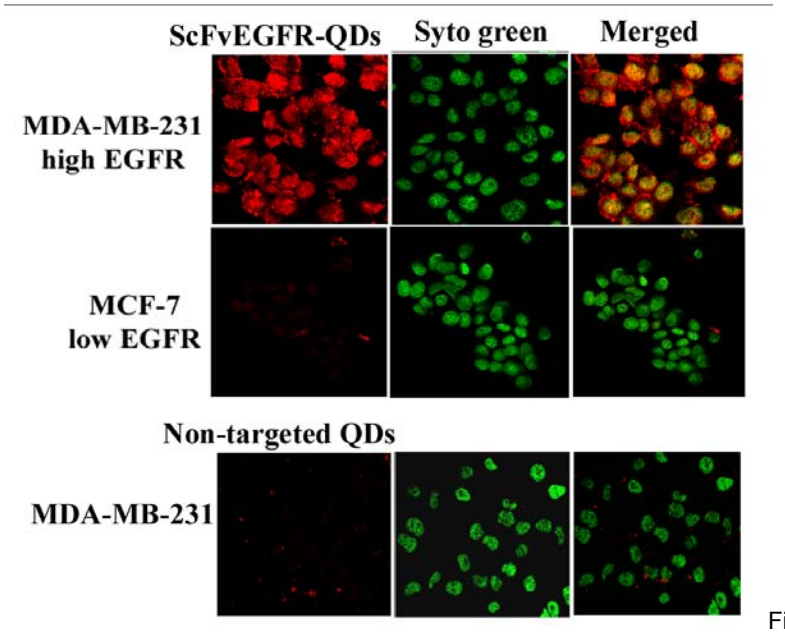


Figure 9. Examination of target specificity of ScFvEGFR-QDs in breast cancer cell lines expressing different levels of EGFR. Red: QDs, Green: Syto green nuclear counterstaining.

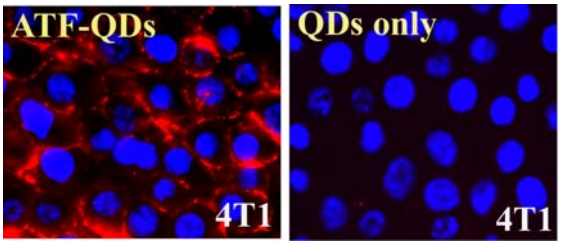


Figure 10. ATF-QDs specifically bind to uPAR expressing mouse mammary tumor 4T1 cells.

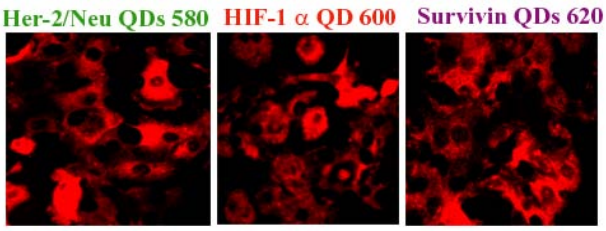


Figure 11. Anti Her-2, HIF-1α and survivin antibody conjugated QDs are able to bind to MCF-10 DCIS cells and produce fluorescence signal. QDs with different emission wavelengths were used as indicated in the figure. A pseudo color (red) was used for all QDs with emissions of 580, 600 or 620 nm.

Examination of the feasibility of simultaneous detection of the level of mRNAs and proteins of the biomarkers in breast cancer cells.

We have developed the combination approach using MBs and QDs to detect the levels of gene expression (mRNA) and proteins of biomarkers simultaneously in breast cancer cells. As shown in Figure 12, Her-2-MBs detect MCF-10 DCIS cells expressing Her-2/neu mRNAs. Double labeling with survivin QDs reveals that most Her-2/neu gene expressing cells also have a high level of survivin protein. However, some survivin positive cells lack Her-2/neu gene expression. A similar result was observed when the survivin MB was used to detect survivin gene expression and Her-2/neu QDs were used for labeling Her-2/neu protein (Figure 12). Furthermore, we found that MCF-10 DCIS cells that are highly positive for Her-2/neu also upregulate the level

of HIF-1α (Figure 12). These results demonstrate that it is feasible to detect the levels of mRNA and protein expression in cancer cells simultaneously using MBs and QDs.

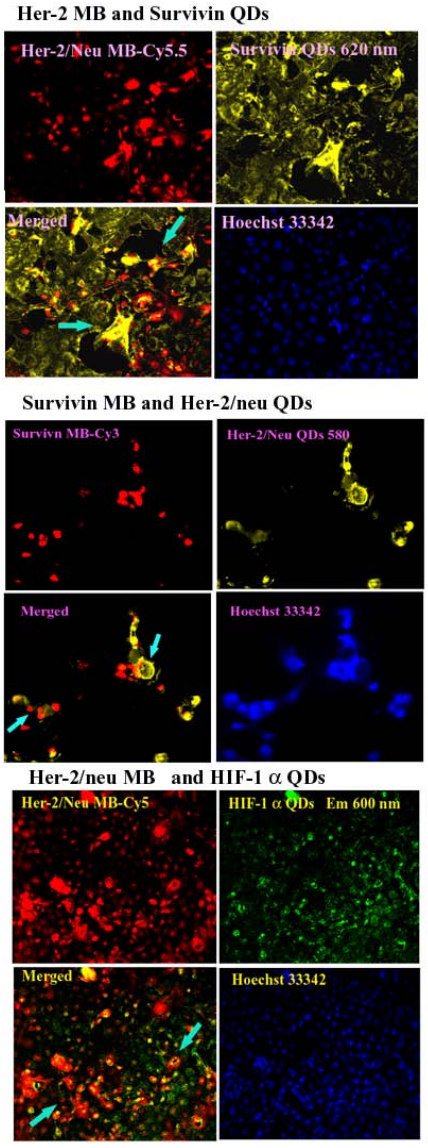


Figure 12. Simultaneous detection of mRNA and protein levels of biomarkers using MBs and antibody conjugated QDs.
Blue arrows: cells positive for both biomarkers.

New research projects for the detection of breast cancer cells by fluorescent nanoparticle imaging probes that are supported in part by this DOD award.

The development of biomarker targeted QDs also allowed us to explore the feasibility of *in vivo* tumor targeting and optical imaging of breast cancers. Since uPAR is highly expressed in breast cancer cells and tumor environment, we wanted to determine if uPAR-targeted, ATF-QDs can be used for *in vivo* tumor imaging.

It is well known that the lung is one of the most common organ sites for developing metastatic disease from breast cancer. To determine the specificity of targeting tumor lesions *in vivo* using ATF-QDs, we examined the distribution of the ATF-QDs in mice bearing 4T1 mouse mammary tumors in the lung. First, we used QDs that give rise to visible red fluorescence (emission: 580 nm), which allow us to determine the amount of QDs in the tissue sections under a fluorescence microscope. We found that systemic delivery of the ATF-QDs leads to the accumulation of the QDs in the lung metastases but not in nearby normal lung tissues (Figure 13 A). Interestingly, ATF-QDs also target tumor endothelial cells, resulting in red fluorescent vascular structures in the tumor areas. In normal tissues, unconjugated QDs are mostly detected in the liver and spleen. However, ATF-QD injected mice show much less QD accumulation in the liver and spleen (Figure 13 B). We found a few ATF-QDs in the kidney and occasionally scattered QDs in the lung.

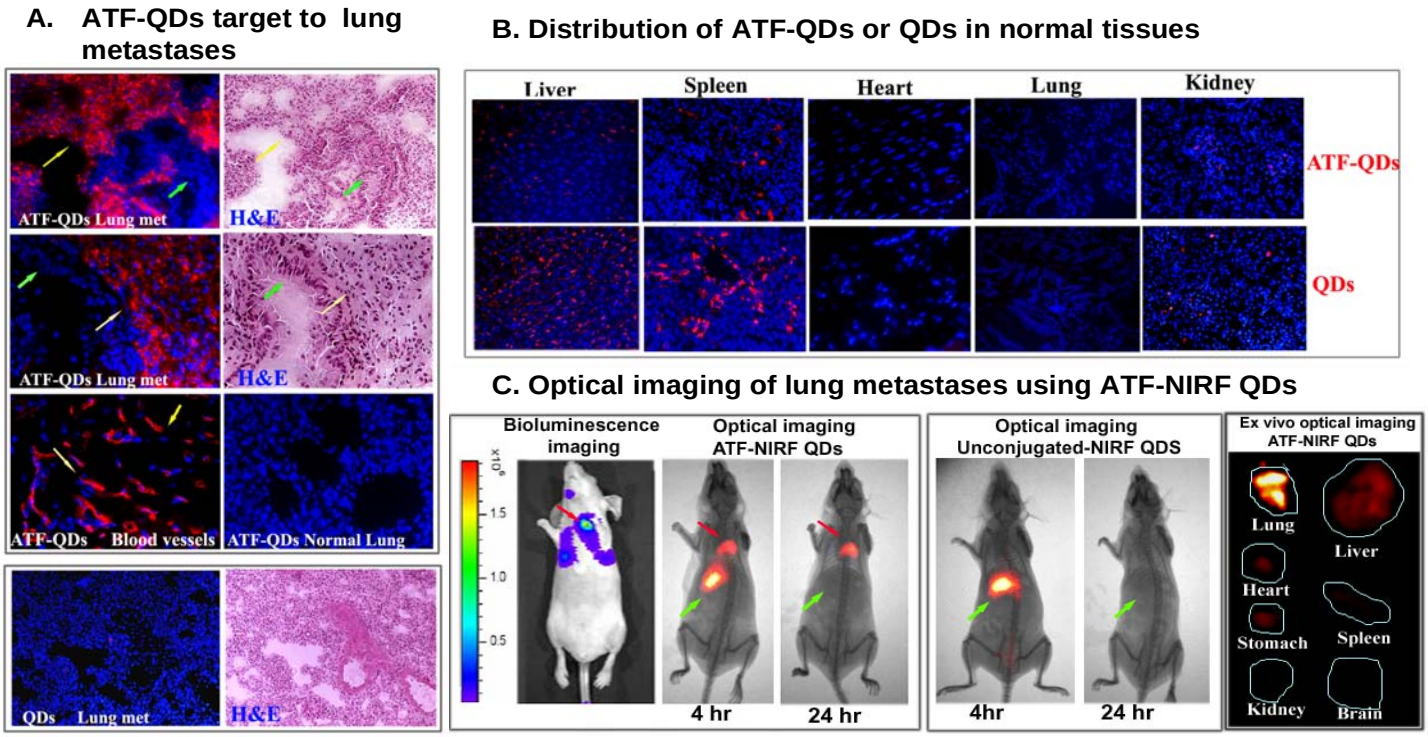


Figure 13. Examination of target specificity of ATF-QDs in the 4T1 mouse mammary tumor model.
4T1 tumor cells were injected via the tail vein to produce consistent lung metastases in a short time. **A&B**: To determine the target specificity, 580 nm QDs (Red) were used to visualize the location of ATF-targeted or non-targeted QDs. 24 hrs following the tail vein injection of the ATF-QDs, the mice were sacrificed and lung and other normal tissues were collected. Frozen tissue sections were counterstained with Hoechst 33342 (blue) and observed under a fluorescence microscope. **A**. Frozen tissue sections from the lung show that ATF-QDs specifically target the tissue areas with lung metastases (**yellow arrows**). However, nearby normal tissues do not have ATF-QDs (**green arrow**). Upper two image panels are paired images from the same areas, showing the histology of the ATF-QD targeted areas (H&E staining). The left image of the third panel shows that the ATF-QDs target vascular structures in the tumor, indicating that ATF-QDs may bind to tumor endothelial cells that express a high level of uPAR *in vivo*. On the other hand, ATF-QDs are not found in the normal lung tissues after the tail vein injection. Injection of unconjugated QDs into the mouse with lung metastases (Lung met) only shows a few QDs in the tumor areas. **B**. Systemic administration of unconjugated-QDs leads to accumulation of the QDs mostly in the liver and spleen. Small amounts of the QDs are also detected in the kidney and lung. Compared to non-targeted QDs, a marked decrease in QDs is seen in the liver and spleen obtained from the ATF-QD injected mouse. **C**. *In vivo* NIR optical imaging of lung metastases after the tail vein injection of ATF-NIR QDs. 10 days after the tail vein injection of 4T1 cells into nude mice, BLI shows the presence of lung metastases. The mice were then injected via the tail vein with either ATF-NIR QDs (emission 860 nm) or unconjugated NIR QDs and were imaged using the Kodak *in vivo* FX imaging system at different time points (the left panel). Different organs were collected and imaged with the Kodak imaging system to confirm NIRF signals in different tissues (the right panel).

To determine the ability of ATF-QDs to be used for *in vivo* tumor imaging, we developed a near infrared QD emitting at 860 nm, which has strong penetration in tissues and produces an optical imaging signal beyond the spectral range of autofluorescence in tissues, allowing for the detection of signals inside animals. ATF peptides were conjugated to NIR QDs. At 4 hrs and 24 hrs after the tail vein delivery, lung metastases were detectable using the Kodak *in vivo* FX imaging system in a mouse that received ATF-NIR-QDs (red arrow) while the mouse injected with unconjugated-NIRF QDs mainly showed NIR signal in the liver (Figure 13, green arrow). Interestingly, 24 hrs after delivery of either ATF- or unconjugated NIR QDs, the optical signal became undetectable in the liver, which may be due to oxidation process in the liver that quenches the signal of QDs. However, the NIR signal remained to be very strong in the lung metastases of the ATF-NIR QD injected mouse (Figure 13).

Biomarker targeted QDs and magnetic iron oxide nanoparticles for in vivo imaging of breast cancers using optical and MR Imaging.

We have developed a method for labeling a Cy5.5 NIR dye to ATF peptides, which provides us with a means to detect the location of the uPAR-targeted magnetic iron oxide (IO) nanoparticles and NIR optical signal in tumor lesions. ATF- or Cy5.5-ATF peptides were conjugated to the IO nanoparticles using the reaction of –COOH on IO to –NH2 of amino acids. Using Prussian blue staining and confocal microscopy, we examined the specificity of ATF- or Cy5.5-ATF-IO nanoparticles in cancer cell lines and found that the ATF-nanoparticles specifically bind to and are internalized by uPAR (+) 4T1 and a transformed mouse endothelial cell line (MS1) but not by uPAR (-) T47D breast cancer cells after incubation of the IO nanoparticles with viable cells for 2 hrs (Appendix II).

To determine the feasibility of targeting metastatic lesions, we injected ATF-IO nanoparticles into the tail vein of a mouse bearing intraperitoneal (i.p.) 4T1 tumors stably expressing a luciferase gene. The presence of tumor lesions was determined by bioluminescence imaging (BLI). We found that ATF-IO nanoparticles selectively target to two tumor lesions on the top of the right kidney (Appendix II, red-lined area).

We further demonstrated that the tail vein delivery of Cy5.5-ATF-IO nanoparticles allows for the detection of 4T1 mammary tumors by NIR optical and MR imaging (Appendix II). At 48 hrs after injection of the nanoparticles, MRI using a 3T scanner of the mice showed that s.c. mammary tumors in the mouse that received Cy5.5 ATF-IO nanoparticles, but not in the tumor of mouse injected with un-conjugated, has areas with significant T2 signal decrease. Optical imaging of the mice using Kodak *In Vivo* FX imaging system reveals the NIR signal in the tumor and the strangest signal corresponds well with the tumor area with T2 contrast decrease (Appendix II). T2 map analysis of the MR image of the tumor region suggests that distribution of the ATF-IO nanoparticles inside the tumor mass is not uniform and that the tumor regions showing the strongest T2 signal decrease (orange red) are in the peripheral areas of the tumor, which correlates very well with the location of NIR signals detected in the tumor (Appendix II). Co-localization of Cy5.5 signal with Prussian blue iron staining in tumor cells was also detected in the frozen section of tumors received Cy5.5-ATF-IO nanoparticles using Olympus fluorescence microscope and CRI imaging system.

Statement of work in proposal and revised statement of work

Statement of Work in the proposal:

Aim 1. **Synthesis and characterization of molecular beacons for the detection of expression of survivin, cyclin D 1 and Her-2/neu genes in breast cancer cells** (Month 1 to 12)

1) To design and synthesize MBs. (Month 1-3)

- Designing and synthesizing MBs for cyclin D 1 and Her-2/neu genes. (Month 1 to 2)
- Examination of specificity of the MBs *in vitro* with synthesized oligonucleotide targets (Month 3 to 4)

2) Determination of specific detection of Cyclin D 1 or Her-2/Neu gene expression by MBs in human breast tumor cell lines and normal human cell lines. (Month 3 to 8)

3) Establishment of the procedures for analysis of gene expression by multiple MBs using fluorescence microscope or FACS analysis. (Month 3 to 8)

4) Examination of sensitivity of MB detection of cancer cells by fluorescence microscope and FACS analysis. (Month 6 to 12)

We have conducted all studies proposed in Specific Aim 1. We have developed and optimized MBs for all biomarker genes for our study. We have successfully established methodologies and protocols for the detection of breast cancer using fluorescence imaging of tumor marker genes. Additionally, we have also identified new biomarkers for DCIS tissues and designed MBs for these mRNAs.

Aim 2. Evaluation of the feasibility of using MBs targeting survivin, cyclin D 1 and Her-2/neu mRANs to detect breast cancer cells in ductal lavage and aspirates of fine-needle aspiration.

(Month 6 to 36)

1) Evaluation of specificity and sensitivity of the MB detection of breast cancer cells in ductal lavage and samples of fine needle aspiration from breast cancer patients (Month 6 to 24)

2) Examination of the feasibility of detection of survivin, cyclin D 1 and Her-2/neu gene expressing cells in ductal lavage or aspirates of FNA obtained from women at high risk of breast cancer and the predicative value of identifying gene expressing cells in detection of breast cancer at DCIS stage. (Month 12 to 36)

Although we were ready to conduct human trial in breast cancer patients, our human study proposed in Specific Aim 2 was delayed due to regulatory issues on human study both at Emory IRB and the DOD. As the result, we were not able to enroll breast cancer patients for our study. In April 2007, as requested by the DOD, a revised Statement of Work was submitted and approved by the Grant office of the DOD.

Revised Specific Aim 2.

Aim 2. Simultaneous examination of the expression levels of biomarker genes and proteins in a series of human cell lines representing different stages of breast cancer development in single cell level using MBs and quantum dots.

(From April 2007 to December 31 2007)

As shown in the body of the final report, we have accomplished the study proposed for this revised Aim 2.

Key Research Accomplishments:

1). We have demonstrated the specificity of all MB probes in detection of corresponding mRNAs in breast cancer cell lines.
2). We have improved our MB design using more advanced quencher molecules and better cDNA sequence region of biomarker genes.
3). We have developed procedures for detecting biomarker gene expression in breast cancer cells using MBs and antibody-conjugated QD nanoparticle probes.
4). We have developed fluorescence QD and magnetic iron oxide nanoparticle probes for the detection of breast cancer cells *in vitro* and *in vivo*.
5). We have produced promising results on tumor targeted nanoparticles for the detection of breast cancer by non-invasive tumor imaging. Those results were used as preliminary results for NIH funding of two center grants including the Cancer Center of Nanotechnology of excellence (CCNE) in March 2005 and Emory Molecular and Translational Imaging Center grant (EMTIC, P20 was funded in September 2007 and P50 is pending for funding). Our R01 grant application on optical imaging guided surgery for determining breast cancer margin is also pending for NIH funding, which is score in the 2008 pay line of NCI.

Reportable outcome

Publications:

Peng XH, Cao ZH, Xia JT , Carlson W. G, Lewis MM, Wood, WC and **Yang L**. Real-time detection of gene expression in cancer cells using molecular beacon imaging: New strategies for cancer research. Cancer Research, 65 (5), 1909-1917, 2005.

Peng XH. Karna P. Cao Z. Jiang BH. Zhou M. **Yang L**. Cross-talk between epidermal growth factor receptor and hypoxia-inducible factor-1 alpha signal pathways increases resistance to apoptosis by up-regulating survivin gene expression. Journal of Biological Chemistry. 281(36):25903-14, 2006.

Yang L, Peng XH, Wang YA, Wang X, Cao Z, Ni C, Karna P, Zhang X, Wood WC, Gao X, Nie S and Mao H. Receptor-Targeted Nanoparticles for In Vivo Imaging of Breast Cancer, Submitted to Journal of Clinical Investigation, 2008

Yang L Peng XH, Gao X, Nie S and Wang YA. *In vivo* optical imaging of breast cancer using peptide-targeted near infrared quantum dots. To be submitted, 2008

Karna P, Cao Z, Zhong H, Li F and **Yang L**. HIF-1 alpha activated tumor specific expression of survivin gene is mediated by a novel transcriptional mechanism. To be submitted.

Presentations in national and international conferences:

Oral presentations

2005 The 2005 AACR Annual Meeting, Experimental and Molecular Therapeutics, Anaheim, CA
Crosstalk Between EGFR and HIF-1 alpha Signaling Pathways Increases Resistance to Apoptosis Under Normoxic Conditions by Upregulating Survivin in Human Cancer Cells.

2005 Era of Hope Department of Defense Breast Cancer Program Meeting, Philadephia, PA
Detection of Breast Cancer Cells by Fluorescence Imaging of Tumor Marker Gene Expression Using Molecular Beacons.

2006 The Second Annual Meeting of American Academy of Nanomedicine, Washington, DC
Tumor Targeted Nanoparticles for Molecular Imaging and Therapy of Human cancer.

2006 International Conference on Bio and Pharmaceutical Science and Technology. San Diego, CA
Multifunctional Magnetic Iron Oxide (IO) Nanoparticles for *in vivo* Imaging and Therapy of Breast and Pancreatic Cancers.

Poster presentations

2005 Peng XH, Karna P, Cao ZH, Zhong H, Jiang BH, Wood WC and **Yang L**. Cross-Talk between EGFR and HIF-1 □ signaling pathways increases resistance to apoptosis under normoxic conditions by upregulating survivin in human breast cancer cells. Proceedings of the American Association for Cancer Research, 46:4387, 2005.

2005. Peng XH, Cao ZH, Carlson, G, Lewis MM, Wood WC and **Yang L**. Detection of breast cancer cells by fluorescence imaging of tumor marker gene expression using molecular beacons. Proceedings of Era of Hope Department of Defense Breast Cancer Research Program Meeting, p-456, 2005.

2006. Peng XH, Gao XH, Wang YA, Yuan QA, Admas G, Wood WC, Nie S and **Yang L.** Engineering Multifunctional Quantum Dots for *in vivo* Imaging and Therapy of Breast Cancer. American Association for Cancer Research Annual Meeting.

2006 Peng XH, Wang AY, Wang X, Cao Z, Ni C, Wood WC, Nie S, Mao H and **Yang L**. Peptide-conjugated superparamagnetic iron oxide nanoparticles (SPIO) targeting to urokinase plasminogen receptor for MR Imaging in vivo and treatment of breast cancer. The Fifth Annual Meeting of the Society of Molecular Imaging, Sept 2006, The Big Island of Hawaii.

Patent applications

U.S. patent, 60/439,771, full patent application filed in January, 2004,
International patent filed in January, 2005.
Methods of detecting gene expression in normal and cancerous cells.

U.S. patent, full patent application (# 10/542,117) filed on July 15, 2005.
Methods and applications of molecular beacon image for cancer cell detection.

Above two patents have been licensed by Alvitae Pharmaceuticals, San Francisco, CA.

U.S. patent, full patent application (60/676,812), file on May 2, 2006

Targeted Multifunctional Nanoparticles for Cancer Imaging and Treatment.

U. S. patent, (# 60/797,613), filed on May 4, 2007
Nanostructures, methods of synthesizing Thereof, and methods of use thereof

Research funding as the result of the DOD Idea Award

Emory-GA Tech Nanotechnology Center for Personalized and Predictive Oncology

NIH NCI Center of Cancer Nanotechnology Excellence (CCNE) 1 U54 CA119338-01

Funding for total of 6 projects and 5 cores: $ 19 millions for five years

10/2005- 9/2010

(CCNE PIs: Drs. Shuming Nie and Jonathan Simons)

Project 1. Quantum Dots and Targeted Nanoparticles Probes for Tumor Imaging

(Co-PI, 20%), (CO-PIs: Dr. S Nie, **L Yang**, H. Mao)

Emory Molecular and Translational Imaging Center grant (EMTIC)

NIH, NCI P20 **(PI. C. Meltzer)**

Project 3. uPAR Targeted *in vivo* Molecular Magnetic Resonance Imaging of Breast Cancer 5%

Project Co-PIs: H Mao (MR imaging technology) and L Yang (Developing uPAR targeted magnetic iron oxide nanoparticles, conducting cellular and animal tumor model studies)
$100,000/year Project 3 direct 2007 – 2010

Pending applications:

HIF-1α/ survivin pathway in progression of breast cancer
NIH, NCI, R01, (PI. L Yang**)** $1,912,500 2007 – 2012

Targeted Nanoparticles For Intraoperative Optical Imaging of Breast Cancer Margins
NIH, NCI, R01, (PI. L Yang), 30% $250,000/year 2008 – 2013
This R01 application is scored at 9.2%, which is within the pay-line of the NCI for 2008.

Emory Molecular and Translational Imaging Center grant (EMTIC)

NIH, NCI P50 (PI. C. Meltzer)

Project 3. uPAR Targeted *in vivo* Molecular Magnetic Resonance Imaging of Breast Cancer

Mutil-PI: H Mao and L Yang $200,000/year Project 3 direct 2008 – 2013

Labile Catalytic Packaging of siRNA for Breast Cancer Therapy
NIH, NCI, R21, Co-PI (PI, H He, Rutgers University).
$ 120,000 (direct, subcontract) 2008-2010

Nanoparticle Based Magnetic Microfluidic Enrichment System (MMES)
NIH, STTR, Co-PI **(**PI, YA Wang**,** Ocean Nanotech)
$75,000 Phase 1 (direct, subcontract) 2008-2009
This proposal is scored within the payline for 2008.

Conclusions

In summary, we have developed a novel multiplex fluorescence cellular imaging approach using molecular beacon and nanoparticle imaging probes for detection of the early stage breast cancer cells *in vitro* and for *in vivo* non-invasive tumor imaging. The nanotechnology developed in our research lab has great potential for translating these novel nanoparticle imaging probes into clinical applications for the detection and treatment of breast cancer. We will continue to pursue these research projects developed as the result of the DOD Idea Award and to bring them into phase I clinical trials in the near future.

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Appendices

Appendix I

Peng XH, CZ, Xia JT, Carlson W. G, Lewis MM, Wood, WC and Yang L Real-Time Detection of Gene Expression in Cancer Cells using Molecular Beacon Imaging: New Strategies for Cancer Research. Cancer Research, 2005; 65(5).

Appendix II

Yang L, Peng XH, Wang YA, Wang X, Cao Z, Ni C, Karna P, Zhang X, Wood WC, Gao X, Nie S and Mao H. Receptor-Targeted Nanoparticles for In Vivo Imaging of Breast Cancer, Submitted to Journal of Clinical Investigation, in review, 2008.

Appendix III

Peng XH. Karna P. Cao Z. Jiang BH. Zhou M. **Yang L**. Cross-talk between epidermal growth factor receptor and hypoxia-inducible factor-1 alpha signal pathways increases resistance to apoptosis by up-regulating survivin gene expression. Journal of Biological Chemistry. 281(36):25903-14, 2006.

Appendix VI

Human study protocol
Consent form
Emory IRB approval letter

Real-time Detection of Gene Expression in Cancer Cells Using Molecular Beacon Imaging: New Strategies for Cancer Research

Xiang-Hong Peng,[1] Ze-Hong Cao,[1] Jin-Tang Xia,[3] Grant W. Carlson,[1] Melinda M. Lewis,[2] William C. Wood,[1] and Lily Yang[1]

Departments of [1]Surgery, Winship Cancer Institute and [2]Pathology, Emory University School of Medicine, Atlanta, Georgia and [3]The First People's Hospital of Guang Zhou, Guang Zhou, P.R. China

Abstract

Development of novel approaches for quantitative analysis of gene expression in intact tumor cells should provide new means for cancer detection and for studying the response of cancer cells to biological and therapeutic reagents. We developed procedures for detecting the levels of expression of multiple genes in fixed as well as viable cells using molecular beacon imaging technology. We found that simultaneous delivery of molecular beacons targeting survivin and cyclin D1 mRNAs produced strong fluorescence in breast cancer but not in normal breast cells. Importantly, fluorescence intensity correlated well with the level of gene expression in the cells detected by real-time reverse transcription–PCR or Western blot analysis. We further show that molecular beacons can detect changes of survivin gene expression in viable cancer cells following epidermal growth factor stimulation, docetaxel treatment, and overexpression of *p53* gene. Thus, molecular beacon imaging is a simple and specific method for detecting gene expression in cancer cells. It has great potential for cancer detection and drug development. (Cancer Res 2005; 65(5): 1909-17)

Introduction

Development of new approaches for detecting cancer cells and determining the responses of the cells to therapeutic reagents holds great promise to increase the survival of cancer patients. It is well known that human cancer cells develop due to abnormalities in gene expression that provide growth advantages, metastatic potential, and apoptosis resistance to the cells (1–3). Methods for specific detection of abnormal gene expression in intact single cancer cells should provide new tools for identifying cancer cells in clinical samples, studying biological effects, and evaluating the effects of therapeutic reagents on specific molecular targets in cancer cells.

In this study, we developed a molecular beacon fluorescence imaging approach to detect the levels of expression of multiple genes simultaneously in single cells. Molecular beacons are stem-loop type oligonucleotide probes dual-labeled with a fluorophore and a quencher. In the absence of the target, the stem brings the fluorophore and quencher molecules together, which prevents the production of a fluorescent signal. When the molecular beacon hybrids to its specific target sequence, the stem is forced to break apart, which enables it to generate a fluorescent signal (4–6). Because binding conditions between the loop and complementary target sequences are very stringent, only a target with perfectly matching sequences is able to hybridize to the molecular beacon (5). During the last several years, molecular beacon technology has been used in various applications to detect oligonucleotides in solution, including DNA mutation detection and real-time quantification of PCR products and protein-DNA interaction (6–8).

The ability of molecular beacon probes to detect specific target molecules without separation of unbound probes also provides an opportunity to detect intracellular mRNA molecules in intact cells. The feasibility of detecting intracellular mRNA has been examined in several laboratories (9–13). It has been shown that molecular beacons were able to visualize mRNA molecules in several human and animal cell lines after introducing into cells through microinjection or liposome delivery (9–11, 14). It has also been shown that the detection limit of preformed molecular beacon/β-actin mRNA duplexes microinjected into the cells is 10 mRNA molecules, suggesting that molecular beacon technology is a very sensitive method for detecting mRNAs in cells (9).

Although previous studies suggested that detection of intracellular mRNA using molecular beacons is a feasible approach, the question remains of how to develop this novel technology into a simple procedure that can be used broadly in basic research and clinical laboratories. To address this issue, we developed procedures that enable us to detect gene expression in fixed as well as viable cells. We designed molecular beacons targeting survivin and cyclin D1 mRNAs, which are highly expressed in breast cancer cells (15, 16). Survivin is a member of the inhibitor of apoptosis protein family that plays a crucial role in the apoptosis resistance of tumor cells (17). Increasing evidence indicates that survivin is also a promising tumor marker because it is normally expressed during fetal development but is not expressed in most normal adult tissues (18). However, high levels of survivin are detected in many human cancer types including 70% of breast cancers (16, 19). Also, cyclin D1, an important regulator of cell cycle, is overexpressed in 50% to 80% of breast cancer tissues, whereas it is low or absent in normal breast tissues (15). In this study, we examined the feasibility of detecting expression of survivin and cyclin D1 genes in human breast cancer cells using the molecular beacon–imaging technology.

Materials and Methods

Human Breast Cancer or Normal Cell Lines and Tissues

Breast cancer cell lines SKBr-3, MDA-MB-231, and MCF-7 and normal immortalized human mammary epithelial cell line MCF-10A were obtained from the American Type Culture Collection (Manassas, VA). MDA-MB-435 cell line was provided by Dr. Zhen Fan (MD Anderson Cancer Center, Houston, TX).

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Frozen human breast cancer and normal tissues were obtained according to an approved institutional review board protocol at Emory University from breast cancer patients during surgery to remove the tumors. Tissues were frozen immediately in liquid nitrogen and kept at −80°C.

Design and Synthesis of Molecular Beacons

The sequences of molecular beacons targeting survivin or cyclin D1 mRNAs were unique for each gene. These include (*a*) survivin MB-FITC: 5′-FITC-TGGTCCTTGAGAAAGGGC*GACCA*-Dabcyl-3′, (*b*) survivin MB-Cy3: 5′-Cy3-*C*TGAGAAAGGGCTGCCAG*TCTCAG*-Dabcyl-3′, and (*c*) Cyclin D1 MB-Texas Red: 5′-Texas-Red-TGGAGTTGTCGGTGTAGA*CTCCA*-Dabcyl-3′. Control molecular beacons for targeting human glyceraldehyde-3-phosphate dehydrogenase (GAPDH), GAPDH MB-Cy 3 or GAPDH MB-6-FAM, were also synthesized as the following: 5′-Cy3 or 6-FAM-*C*GAGTCCTTCCACGATAC-CC*ACTCG*-Dabcyl-3′. The underlined bases were those added to form a stem with an optimal Tm condition. All molecular beacons were synthesized by MWG-Biotech Inc. (High Point, NC).

The specificity of the molecular beacons in solution was determined using synthesized oligonucleotide targets (Sigma Genosys, Woodlands, TX). These include (*a*) survivin target: 5′-CCTGCCTGGCAGCCCTTTCTCAAG-GACCACCGCATCTCTACATTCAAGAAC-3′, (*b*) cyclin D1 target: 5′-AGAAGCTGTGCATCTACACCGACAACTCCATCCGGC-3′, (*c*) HER-2/*neu* gene target: 5′-AGTGTGCACCGGCACAGACATGAAGCTGCGGCTCCCT-3′, and (*d*) K-*ras* gene: 5′-GTAGTTGGAGCTGGTGGCGTAGGCAA-GAGTGCCTTGACGATACAGCTAATT CAG-3′. Survivin or cyclin D1 molecular beacon (200 nmol/L) was mixed with 1 μmol/L of various DNA targets in 100 μL of Opti-MEM (Invitrogen, Carlsbad, CA). After incubating at 37°C for 60 minutes, fluorescence intensity was measured by a fluorescence microplate reader (Bioteck FL600 fluorometer, Winooski, VT).

Real-time Reverse Transcription–PCR

Total RNAs were isolated and amplified with an Omniscript reverse transcription kit (Qiagen Inc, Valencia, CA). Real-time PCR was done on an ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA). The primer pairs for detecting the expression of survivin gene were survivin forward 5′-TCCACTGCCCCACTGAGAAC-3′ and survivin reverse 5′-TGGCTCCCAGCCTTCCA-3′. PCR primers for cyclin D1 were forward 5′-AGAAGCTGTGCATCTACACCGACAACTC-CATCCGGC-3′ and reverse 5′-GGTTCCACTTGAGCTTGTTCACAA-3′. The primer pairs for β-actin gene were β-actin forward, 5′-AAAGACCTGTA CGCCAACACAGTGCTGTCTGG-3′, and β-actin reverse, 5′-CGTCA-TACTCCTGCTTGCTGATCCACATCTGC-3′, and for GAPDH were forward 5′-TGAAGGTCGGAGTCAACGGATTTGGT-3′ and reverse 5′-CATGTGGGC-CATGAGGTCCACCAC-3′.

Western Blot Analyses

Cell lysates were collected after different treatments and total cellular protein was resolved on polyacrylamide SDS gels. Western blot analysis for the level of survivin protein was done according to a standard protocol as described (19). The membranes were incubated for 1 hour with goat anti-human survivin (Santa Cruz Biotechnology, Santa Cruz, CA) and mouse monoclonal antibodies to β-actin (Sigma Chemical Co, St. Louis, MO). The levels of specific proteins in each lysate were detected by enhanced chemiluminescence using ECL plus (Amersham International, Buckingham, United Kingdom) followed by autoradiography.

Detection of Gene Expression in Fixed Cells

Cells were plated on chamber slides for 24 hours and then fixed with ice-cold acetone for 5 to 10 minutes. The slides were stained with a mixture of 200 nmol/L of survivin MB-FITC and cyclin D1 MB-Texas Red in Opti-MEM at 37°C for 60 minutes and then examined using a confocal microscope (LSM 510 Meta, Carl Zeiss Microimaging, Inc., Thornwood, NY).

For detecting survivin gene expression on tissue sections, 5-μm frozen sections of breast normal and cancer tissues fixed with ice-cold acetone were incubated with 200 nmol/L survivin MB-Cy3 for 60 minutes and then counterstained with 10 μg/mL Hoechst 33342 (Molecular Probes, Inc., Eugene, OR). For immunofluorescence labeling, acetone-fixed frozen sections were incubated with a goat anti-human survivin antibody and then with FITC-conjugated anti-goat antibody. For double-labeling survivin MB and human endothelial cell marker CD31, tissue sections were incubated with survivin MB-Cy3 and then with an anti-CD31 antibody followed by a FITC-conjugated secondary anti-mouse antibody. One tissue section was double-labeled with goat anti-human survivin and mouse anti-human CD31 antibodies followed by FITC-labeled donkey anti-goat antibody or biotinylated horse anti-mouse antibody and Texas Red avidin. The tissue slides were observed under a Nikon fluorescence microscope (Nikon Eclipse E800, Nikon Instruments Inc. Melville, NY). Fluorescence images were taken using an Optronics Magnafire digital imaging system (Meyer Instruments, Houston, TX).

Quantification of the Level of Gene Expression in Viable Cells Using Molecular Beacons

FACScan Analysis. Cells were plated in six-well plates and cultured in medium containing 2% fetal bovine serum overnight. The cells were transfected with 400 nmol/L of either survivin MB-FITC or GAPDH MB-6FAM using LipofectAMINE 2000 in Opti-MEM (Invitrogen). Three hours after transfection, 100 ng of human recombinant epidermal growth factor (EGF; Invitrogen) were added to the EGF-treated group for 1 hour and the cells were collected for FACScan analysis (Becton Dickinson, Mansfield, MA).

Cells transduced with an adenoviral vector expressing a wild-type *p53* gene (Adp53, Qbiogene, Carlsbad, CA) or control adenoviral vector (Adcmv) at a multiplicity of infection of 50 plaque-forming units for 24 hours were collected and divided into two groups. One group of the cells was transfected with 400 nmol/L of survivin MB-FITC and the other was transfected with 400 nmol/L of GAPDH MB-6-FAM using LipofectAMINE 2000 in Opti-MEM (Invitrogen). Fluorescence intensity of the cells from all groups was examined using FACScan analysis.

Fluorescence Microplate Reader. Cells were plated in 96-well culture plates at 80% confluence for 24 hours. EGF-treated and nontreated groups were cultured in the medium with 2% fetal bovine serum. The cells were then transfected with a mixture of 400 nmol/L of survivin MB-FITC and internal control GAPDH MB-Cy 3. At 3 hours after transfection, 100 ng/ml of EGF was then added to the wells in the EGF-treated group and 10 or 50 nmol/L of docetaxel (Aventis Pharma, Bridgewater, NJ) were added to the docetaxel-treated group. The culture plates were immediately placed in the microplate reader and fluorescence units in each well were measured at different time points.

Results

Survivin and Cyclin D1 Molecular Beacons Specifically Bind to DNA Targets. The design of the survivin and cyclin D1 molecular beacons and illustration of the mechanism of binding molecular beacons to specific oligonucleotide targets are shown in Fig. 1*A*. We showed that survivin or cyclin D1 molecular beacon specifically bound to its DNA target and generated 5- to 8-fold higher fluorescent signal when mixed with specific DNA target compared with other targets (Fig. 1*B*).

Detection of Human Breast Cancer Cells Using Molecular Beacons Targeting Tumor Marker mRNAs. We examined whether molecular beacons targeting different tumor marker mRNAs can be labeled with different fluorophores and expression of the tumor marker genes can be determined simultaneously in single cells. We found that a combination of survivin and cyclin D1 molecular beacons detected the expression of both survivin and cyclin D1 genes simultaneously and generated fluorescent signals corresponding to either survivin (green) or cyclin D1 (red) mRNA in the cancer cells (Fig. 2*A*). Importantly, the fluorescent signal was very low for both molecular beacons in a normal immortalized human mammary epithelial cell line (MCF-10A), indicating that survivin and/or cyclin D1 molecular beacons can be used as fluorescence probes for the detection of breast cancer cells (Fig. 2*A*). The results of examination of fluorescence

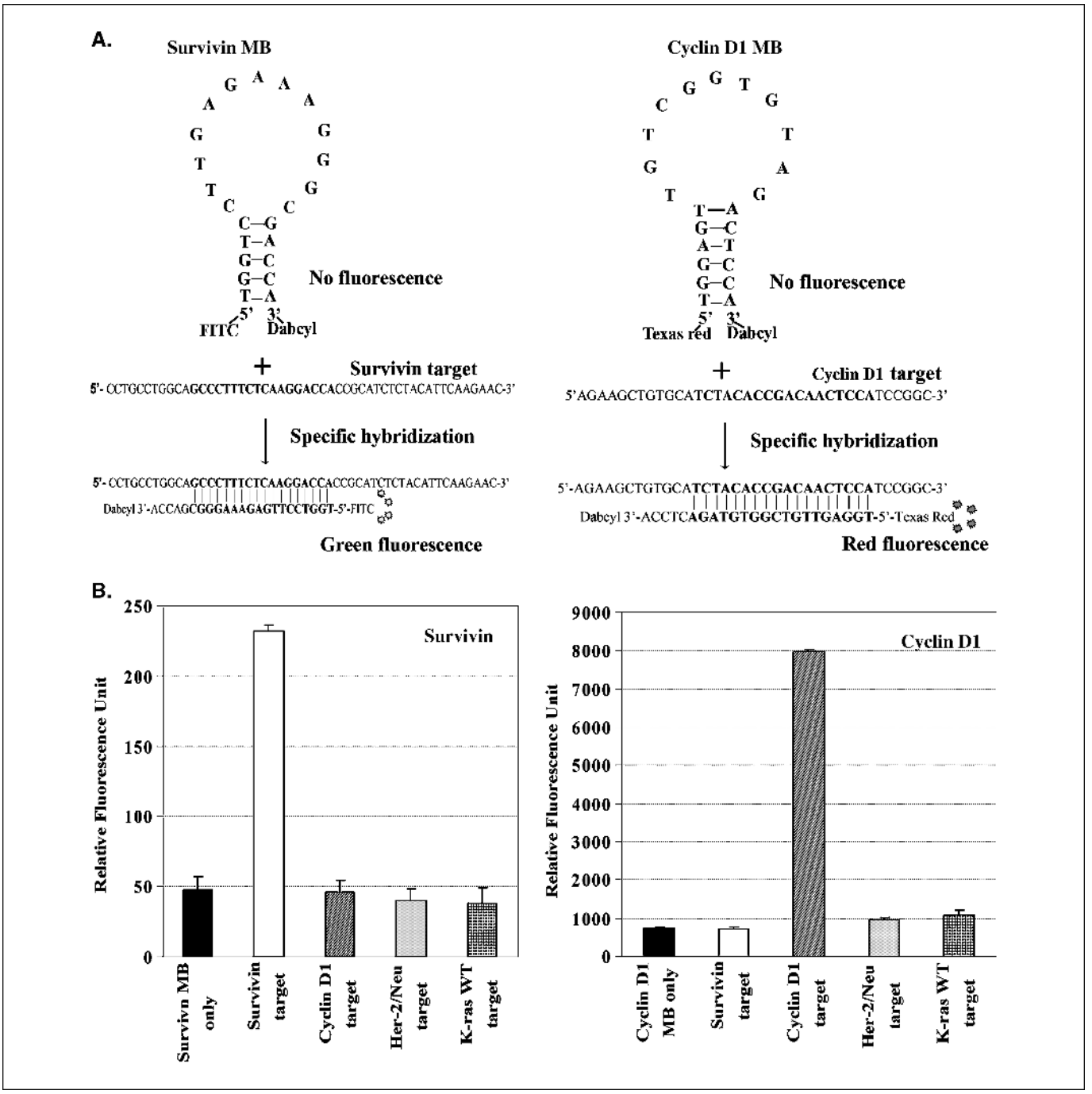

Figure 1. Schematic illustration of molecular beacon design and examination of specific binding of the molecular beacons to their oligonucleotide targets. *A,* both survivin and cyclin D1 molecular beacons have 23 nucleotides with 5′ stem and loop sequences complementary to survivin or cyclin D1 gene. The stem length for survivin molecular beacon is 5 nucleotides with the 5′ end labeled with FITC and the 3′ end labeled with a quencher (*Dabcyl*). Cyclin D1 molecular beacon has a stem containing 6 nucleotides with the 5′ end labeled with Texas Red and the 3′ end with Dabcyl. Survivin and cyclin D1 molecular beacons only generate fluorescent signals when hybridized to their specific DNA target. *B,* examination of specificity of the molecular beacons *in vitro.* Survivin or cyclin D1 molecular beacon was mixed with various synthesized DNA targets. The fluorescence units were measured using a fluorescence microplate reader. Survivin or cyclin D1 molecular beacon only bound and generated strong fluorescent signal when mixed with its specific DNA target. *WT,* wild-type.

intensity and the level of survivin or cyclin D1 gene expression in tumor and normal cell lines further showed that the fluorescent signals detected by the molecular beacons correlated very well with the levels of survivin or cyclin D1 gene expression, both in mRNA and protein levels (Fig. 2*B-D*). For example, MDA-MB-435 and SKBr-3 expressed very high levels of survivin gene, and the strongest fluorescent signal was detected in these cell lines. Conversely, these cell lines expressed low levels of cyclin D1 gene and showed a weak red fluorescence staining (Fig. 2*A-D*). MCF-7 cells expressed a moderate level of survivin gene but had a very high level of cyclin D1 gene expression. Delivery of survivin and cyclin D1 molecular beacons into this cell line produced a strong red fluorescent signal (cyclin D1) and an intermediate level of green fluorescent signal (survivin; Fig. 2*A-D*). Our results show

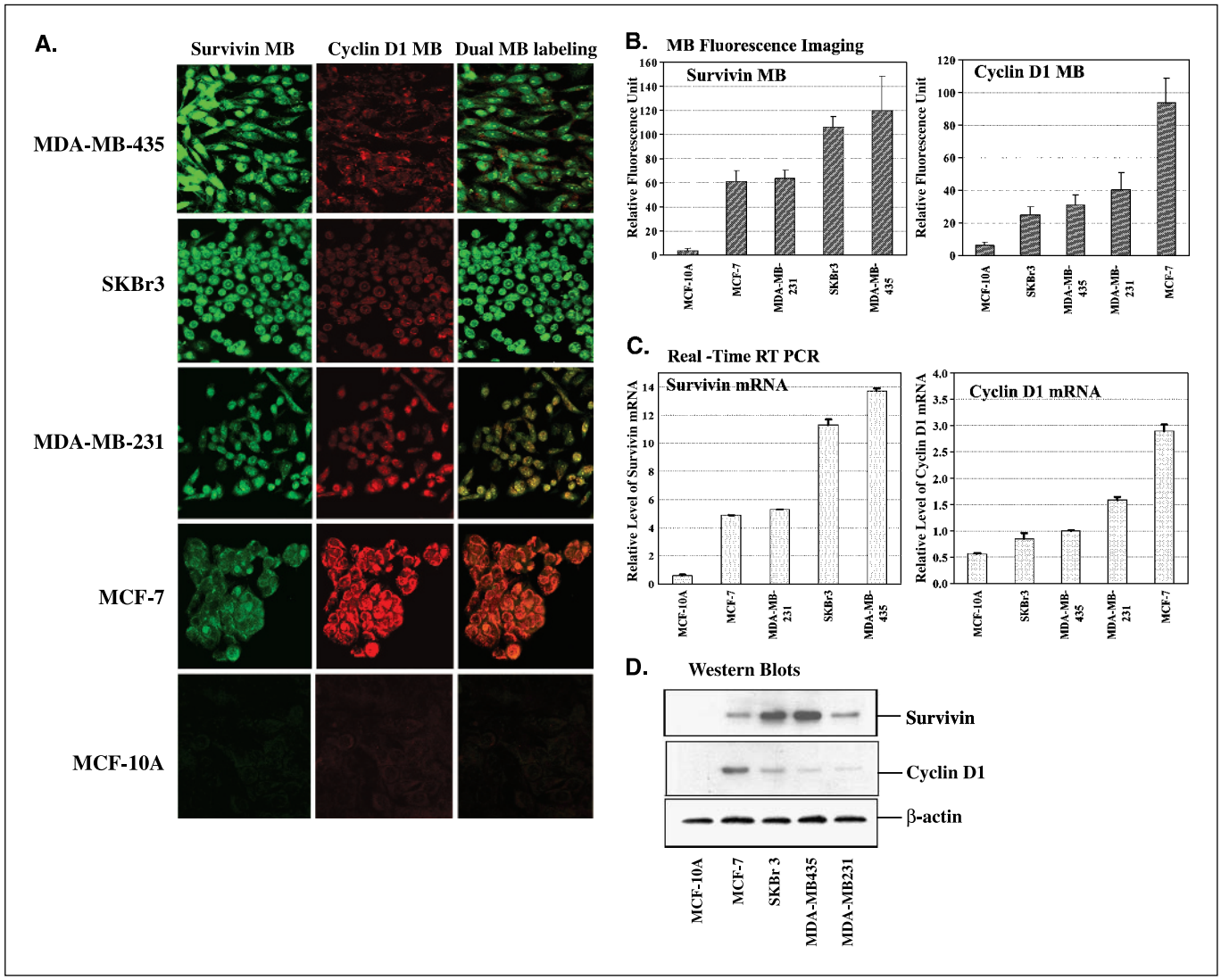

Figure 2. Simultaneous detection of the levels of survivin and cyclin D1 mRNAs in breast cancer cells. *A,* dual molecular beacon (*MB*) imaging of breast cancer cells. A mixture of survivin and cyclin D1 molecular beacons was incubated with the fixed cells and then examined under a confocal microscope. *B,* quantitative analysis of the level of fluorescence intensity produced in breast cancer and normal cells. Fluorescence intensity was determined by measuring the mean fluorescence units from four randomly selected areas for each image taken under a confocal microscope. The mean fluorescence unit from four areas of each cell line is shown in the figure. Similar results were observed in repeat experiments. *C,* detection of the levels of survivin and cyclin D1 mRNAs by real-time RT-PCR. Relative level of survivin or cyclin D1 mRNA was calculated from the quantity of survivin or cyclin D1 PCR products and the quantity of β-actin PCR products. *D,* examination of the levels of survivin protein in tumor and normal cell lines by Western blotting. The levels of survivin or cyclin D1 protein correlated well with the levels of survivin or cyclin D1 mRNA detected *in situ* in fixed tumor cells using molecular beacon detection or with the real-time RT-PCR results.

that a combination of molecular beacon technology with fluorescence imaging is a novel approach to simultaneously detect the levels of multiple gene expressions in intact single cells.

Molecular Beacons Detect Cancer Cells on Frozen Sections of Breast Cancer Tissues. We further developed a simple and fast procedure that allows us to detect survivin gene expression *in situ* on frozen tissue sections. Our previous study showed that survivin is expressed in 72% of breast cancer tissues, including 34 invasive breast ductal carcinoma and 2 lymph node metastases, using Western blot analysis of tissue lysates obtained from frozen tissue samples of the patients with cancer (19). In this study, we examined survivin gene expression on frozen tissue sections of those cancer tissues using survivin MB. We found that survivin MB-Cy3 was able to produce strong red fluorescent signals in breast cancer cells on frozen tissue sections (Fig. 3*A*). A high level of survivin gene expression was consistently detected in the breast cancer cells in nine of nine invasive ductal carcinoma tissues and one lymph node with metastastic lesions that were previously found positive for survivin protein by Western blot analysis. Two breast cancer tissues that were negative for survivin protein expression also lacked survivin MB positive cells (data not shown). Moreover, the survivin MB positive cells were not found in frozen tissue sections of all five paired normal breast tissues (Fig. 3*A* and *B*, representative results of survivin molecular beacon imaging and immunofluorescence labeling with a survivin antibody).

We have also examined the expression of survivin proteins in ductal carcinoma *in situ* (DCIS) tissues by immunohistochemical staining on frozen or paraffin sections using a polyclonal anti-survivin antibody. Eleven of 17 DCIS tissues displayed various levels of survivin protein expression (data not shown). We further

examined frozen tissue sections from two DCIS tissues and found that breast cancer cells in those DCIS tissues were positive for survivin molecular beacon, suggesting that survivin gene expression is an early event in the tumorigenesis of breast cancer (Fig. 3*A*).

In addition, we found that survivin gene–expressing cells in breast cancer tissues included cancer cells as well as cells in the vascular structures (Fig. 3*C*). When the same section was double-labeled with an antibody specific for a CD31 human endothelial cell marker (20), those survivin-expressing cells in the vascular structures were shown to be endothelial cells (Fig. 3*C*). Establishment of this molecular beacon detection method for measuring gene expression *in situ* should provide pathologists with a new tool to identify cancer cells in clinical samples.

Monitoring the Level of Real-time Gene Expression Using Survivin Molecular Beacon. We used three model systems to determine whether survivin molecular beacon was able to detect changes of survivin gene expression in viable cells, including EGF or docetaxel induced up-regulation and tumor suppressor gene *p53*–induced down-regulation of survivin gene expression (21, 22). Breast cancer cells were transfected with a mixture of survivin and GAPDH molecular beacons and observed under a fluorescence microscope after treatment with EGF for 1 hour or docetaxel for 24 hours. Our results showed that treatment of the cells with EGF or docetaxel increased the level of survivin gene expression. Under a fluorescence microscope, the green fluorescence intensity (survivin MB-FITC) was stronger in the cells treated with either EGF or docetaxel compared with untreated control whereas the fluorescent signal for GAPDH molecular beacon (Cy3, red) was relatively consistent (Fig. 4*A*). We further used FACScan analysis to determine the mean fluorescence intensity in each cell population. Consistent with our observation with the fluorescence microscopy, we detected higher levels of fluorescent signal in EGF-treated cells compared with the untreated group in breast cancer cells (Fig. 4*B*). The relative level of survivin mRNA could be quantified from the FACScan data using the fluorescence unit of *GAPDH* gene as an internal control. We found that EGF treatment induced ~1.5-fold increases in the level of survivin gene expression in breast cancer cells.

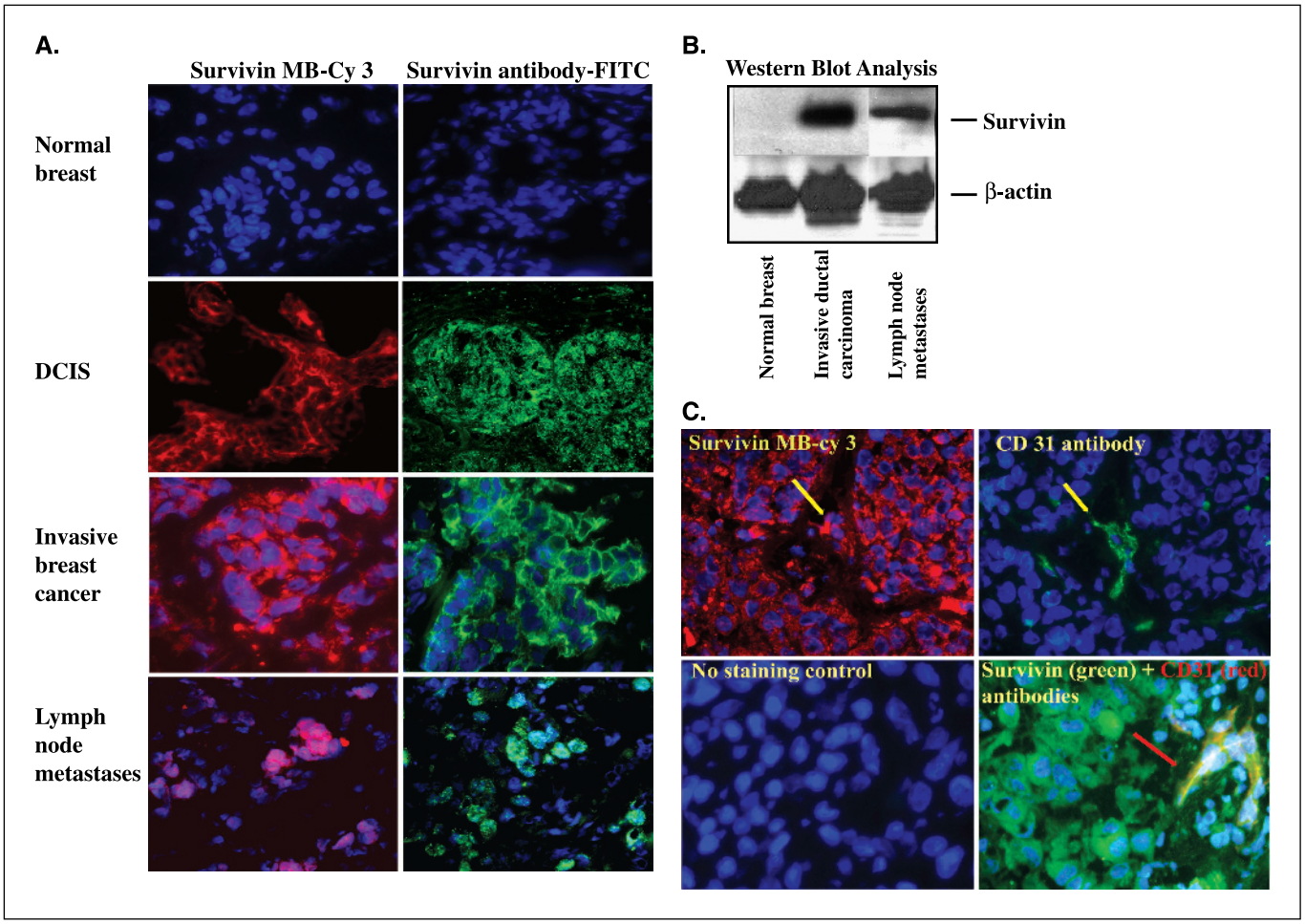

Figure 3. Detection of survivin gene expression on frozen tissue sections obtained from patients with breast cancer. Expression of survivin gene was detected in different stages of breast cancer tissues. Frozen tissue sections were fixed with acetone and incubated with survivin MB-Cy3. The sections were counterstained with Hoechst 33342 (*blue nuclei*). Survivin-expressing cells (*red*) were found in all stages of breast cancer tissues including DCIS, invasive carcinoma, and lymph node metastases, but not found in normal breast tissues. Different sections from the same tissues were also stained with a survivin antibody to confirm the presence of survivin positive cells (*green*). *B,* Western blot analysis showed a high level of survivin protein (16.5 kDa) in primary breast cancer and lymph node with metastases but not in normal breast tissues. *C,* detection of survivin gene expression in breast cancer and tumor endothelial cells in breast cancer tissues using double-labeling survivin MB-Cy3 with an antibody to CD31. Expression of survivin mRNA was labeled by survivin MB-Cy3 (*red*) and tumor endothelial cells were labeled with an anti-CD31 antibody (*green, yellow arrow*). Another tissue section was double-labeled with goat anti-human survivin (FITC, *green*) and mouse anti-human CD31 antibodies (*red*). All sections were counterstained with Hoechst 33342 (*blue*). *Red arrow,* tumor endothelial cells expressed both survivin and CD31 (*orange*).

In addition to the detection of levels of up-regulated genes, we examined the feasibility of quantifying the relative level of down-regulated gene expression. It has been shown that overexpression of *p53* gene decreases the expression of survivin gene (22). We transduced the tumor cells with Adp53 vector or control vector Adcmv for 24 hours and then delivered survivin or GAPDH molecular beacons into the transduced cells. Using FACScan analysis, we found that the relative fluorescence was decreased ~2-fold in Adp53 vector–transduced cells compared with the untreated or empty Adcmv vector control group (Fig. 4*C*). The ability of molecular beacons to detect a decreased level of gene expression suggests that the fluorescent signals detected intracellularly after molecular beacon transfection are not from nonspecific degradation of the molecular beacons because the same amount of survivin and GAPDH molecular beacons is delivered into Adp53 and control vector–transduced cells. The results from real-time reverse transcription-PCR (RT-PCR) further confirmed that EGF increased the transcription of survivin gene and overexpression of the *p53* gene decreased the level of survivin mRNA (Fig. 4*D*).

Although detection of the level of gene expression by FACScan could accurately measure the fluorescence intensity in individual cells as well as in cell populations, the procedure for FACScan is time-consuming and does not easily detect changes of gene expression in real time in the same cell population. To develop a high-throughput method for monitoring the changes of gene expression in real time in viable cells, we examined the feasibility of detecting levels of gene expression in cells cultured in 96-well plates using the molecular beacon-transfection approach. Breast cancer cells were plated in 96-well plates and transfected with a mixture of survivin and GAPDH molecular beacons for 3 hours. After adding EGF or docetaxel, the fluorescence units were

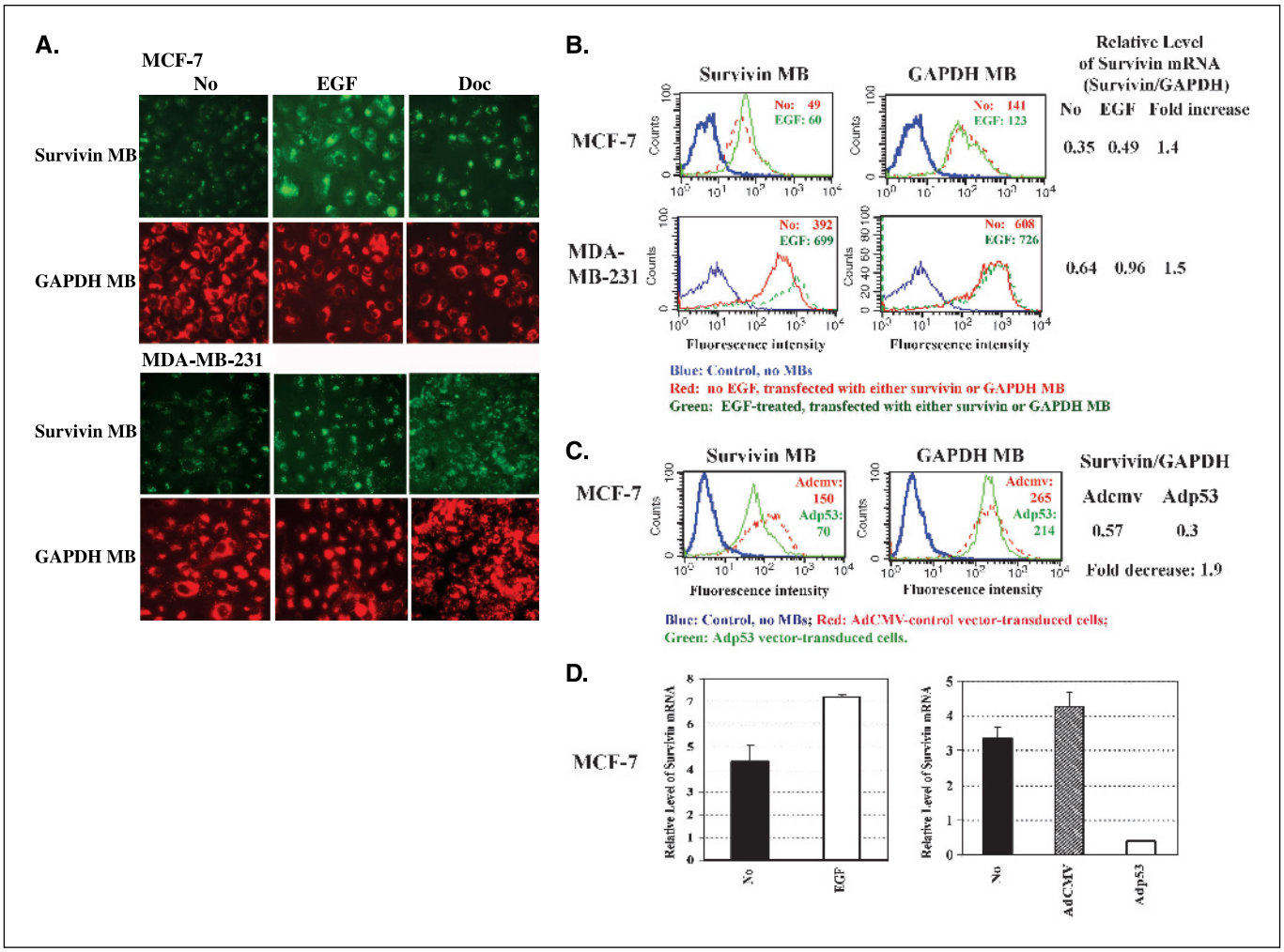

Figure 4. Detection of the levels of survivin gene expression in viable cells using survivin molecular beacon. *A,* survivin MB-FITC produced green fluorescence in cytoplasm of breast cancer cells after transfecting into viable cells. Treatment of the cancer cells with EGF for 1 hour or docetaxel (Doc) for 24 hours increased the fluorescence intensity in the cells. The fluorescence intensity generated by GAPDH MB-Cy3, which was cotransfected with the survivin molecular beacon, was relatively consistent in the cells. *B* and *C*, The level of survivin or GAPDH mRNA in molecular beacon-transfected cells could be measured by FACScan analysis to determine the mean fluorescence unit for each sample. The level of survivin mRNA was increased in EGF-stimulated cancer cell lines (survivin molecular beacon, *green line*), whereas there was no change in the fluorescence units detected in GAPDH molecular beacon–transfected cells (GAPDH molecular beacon, *green line*). On the other hand, the level of survivin mRNA decreased in Adp53 vector- (*green line*) but not in Adcmv vector–transduced cells (*red dotted line*). Numbers in the figure represent the mean fluorescence intensity for each group. Relative levels of survivin mRNA were calculated from the ratio of mean fluorescence intensities of survivin molecular beacon and GAPDH molecular beacon. Real-time RT-PCR analysis showed that the level of survivin mRNA was increased by EGF treatment but decreased after overexpression of *p53*. The numbers in the figure represent the mean numbers from three repeat samples. The relative level of survivin gene expression was calculated as a ratio of the quantity of survivin and GAPDH PCR products.

measured at different time points using a fluorescence microplate reader. We found that EGF-induced up-regulation of survivin gene expression occurred as early as 15 minutes after the treatment and lasted for >3 hours (Fig. 5*A*). There were 2.3 (MCF-7)- to 2.8 (MDA-MB-231)-fold increases in the relative levels of survivin mRNA after EGF treatment. We also examined the level of survivin protein using Western blot analysis and further confirmed that the level of survivin protein increased after EGF treatment (Fig. 5*A*).

For real-time detection of the level of gene expression in viable cells, it is important to determine how long the molecular beacon probes will stay in the cells and still be able to produce fluorescent signals that reflect the relative level of the gene expression. It has been shown that the chemotherapy drug docetaxel increases in the level of survivin gene expression as early as 4 hours after the treatment (21). We examined the level of real-time survivin gene expression in molecular beacon–transfected cells after docetaxel treatment from 0 to 48 hours. We found that the level of survivin mRNA was increased at 5 hours and reached higher levels 24 and 48 hours after treatment (Fig. 5*B*). The relative level of survivin mRNA is ~1.5-fold higher in docetaxel-treated cells than control cells and the difference detected 48 hours after docetaxel treatment is statistically significant (Student's t test, $P < 0.05$ for both MCF-7 and MDA-MB-231 cell lines). We also found a similar increase in the level of survivin mRNA detected by real-time RT-PCR compared with survivin molecular beacon detection, and the level of increase in survivin protein after docetaxel treatment (Fig. 5*B*, *inset* is real-time RT-PCR result).

One of the important issues to be addressed in developing an oligo-based approach for detecting gene expression in viable cells is whether the binding of the molecular beacon probes to their target RNA leads to degradation of the mRNA by RNase H, which may affect the level of target mRNA (23). To answer this question, we transfected breast cancer cells with either survivin molecular beacon or control GAPDH molecular beacon for 24 hours and then examined the level of survivin protein by Western blot analysis. We found that compared with cells transfected with a nonspecific GAPDH molecular beacon, the presence of the survivin molecular beacon in the cells did not reduce the level of survivin protein (Fig. 5*C*).

Discussion

We have developed a novel molecular beacon-based molecular imaging approach that allows identification of tumor cells expressing specific marker genes. Because molecular beacon is highly specific in detecting target mRNAs, and molecular beacons targeting various genes can be labeled with different fluorescent dye molecules and delivered into single cells, expression of several tumor marker genes in a single cell can be analyzed at the same time. Human cancers contain heterogeneous cell populations with various genetic changes (24). Simultaneous detection of overexpression of several tumor marker genes, especially when a single cell expresses more than one marker gene, may have a high predicative value for identifying cancer cells and therefore increase the sensitivity and specificity of cancer detection. Using molecular beacons targeting survivin and cyclin D1 mRNAs, we showed that delivery of a mixture of survivin and cyclin D1 molecular beacons into fixed cells produced fluorescent signals in breast cancer cells but not in normal breast cells. Interestingly, the fluorescence intensities in the cells correlated well with the level of the gene expression in different tumor cell lines. Previous methods for detecting gene expression *in situ* were not quantitative because the signals were amplified by either the presence of multiple fluorescent dye labeled nucleotides in an oligonucleotide probe or amplification of the signals with secondary antibodies to labeled nucleotides. Because each molecular beacon has only one fluorophore and unbound molecular beacons do not fluoresce, the fluorescence intensity generated by hybridization of the molecular beacon with a specific mRNA should reflect more accurately the level of the mRNA expressed in the cells.

At present, molecular beacon technology has been mainly used in various applications *in vitro*, which were done in solutions with defined molecular beacon-target conditions. Although previous studies showed the feasibility of detecting mRNAs and monitoring the transportation of RNAs in cells, the procedure for delivery of the molecular beacons through microinjection or by liposome delivery has made it difficult to apply this technology into broad research areas or into a routine clinical procedure (9–12). A recent study showed that it is feasible to transfect a molecular beacon into living cells to detect doxorubicin-induced activation of p21 gene expression (13).

We developed this molecular beacon-based procedure for the detection of gene expression in viable cells. We showed that transfecting survivin molecular beacon into cells produces a strong fluorescent signal in survivin-expressing tumor cells and the level of survivin gene expression can be monitored real time in cells either by FACScan or by using a fluorescence microplate reader. Using these methods, we detected an increase in the level of survivin gene expression following EGF and docetaxel treatment. Although we used GAPDH molecular beacon as an internal control for our experiments, simultaneous detection of survivin and *GAPDH* gene expression real time in viable cells indicates that it is feasible to monitor the levels of expression of several genes in the same cell population using molecular beacons labeled with different fluorophores.

Quantitative measurement of mRNA levels by molecular beacons is very important for the future use of this technology for cancer cell detection because many tumor marker genes are not unique to cancer cells and the difference between normal and cancer cells can be only the level of gene expression. Although we used two molecular beacons to detect the expression of tumor marker genes, a proof of principle from this study will lead to the use of more molecular beacons with multiple dye molecules to analyze the expression of several tumor genes. In addition, because only a small amount of abnormal cells are present in a large amount of normal cell background in clinical samples, there is a clear advantage of direct fluorescence imaging of individual cells expressing tumor marker genes for early detection of cancer cells compared with conventional RT-PCR to amplify the expression of tumor marker genes from isolated total RNA, which may be difficult to detect the differences in the level of gene expression in a few cancer cells over the normal background.

Current methods for the identification and classification of cancer cells from clinical samples rely on examining the morphology of the cells or immunostaining with antibodies for tumor-related protein markers. Although the *in situ* hybridization using labeled linear probes has been used to detect gene expression in tissue sections, it is very time-consuming and

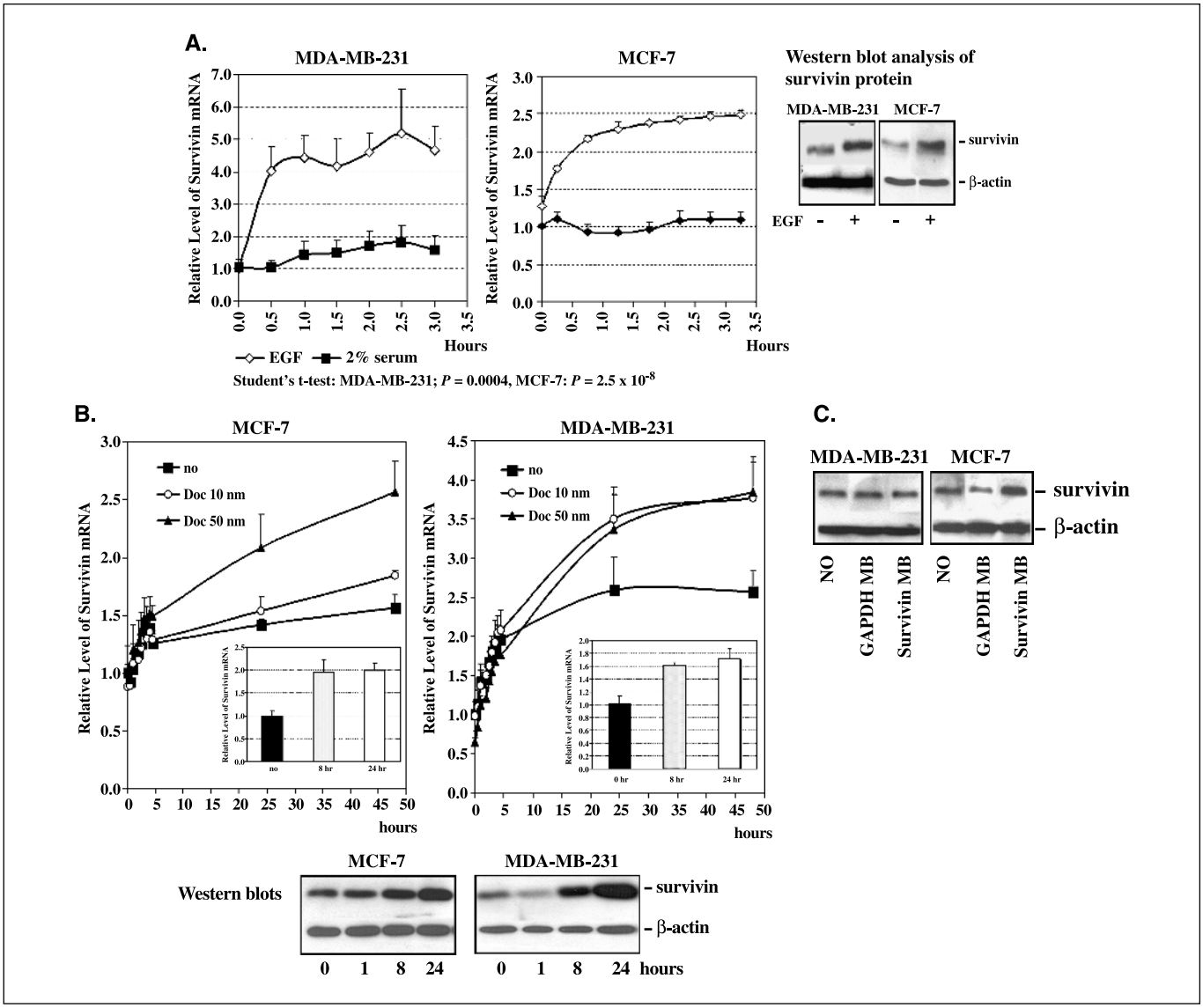

Figure 5. Real-time monitoring the level of survivin gene expression in breast cancer cells. Cells cultured in 96-well plates were transfected with a mixture of survivin MB-FITC and GAPDH MB-Cy3 and then added human EGF or docetaxel. The fluorescence intensity was measured at different time points following treatment using a fluorescence microplate reader. Each point in the curve is a ratio of the mean fluorescence unit of survivin molecular beacon (FITC, Ex/Em 480/530) and mean fluorescence unit of GAPDH molecular beacon (Cy3, Ex/Em 530/590) from four repeat samples. Similar results were obtained from three independent studies. *A,* EGF treatment significantly increased the level of survivin mRNA (Student's *t* test for all time points, $P < 0.0005$). Western blotting further showed that EGF increased the level of survivin protein. *B,* docetaxel treatment increased the level of survivin gene expression. Significant increases in the level of survivin mRNA were seen 24 to 48 hours following the treatment (Student's *t* test, $P < 0.05$). The levels of survivin gene expression after treatment were also examined by real-time RT-PCR (*inset*). Western blot analysis showed up-regulation of survivin protein by docetaxel. *C,* transfection of survivin or control GAPDH molecular beacon into viable cells did not significantly decrease the level of survivin protein as determined by Western blot analysis of cell lysates after transfected with either survivin molecular beacon or GAPDH molecular beacon for 24 hours.

usually accompanied by a high background because unbound probes also produce fluorescent signals. In our study, we found that molecular beacons could be used to detect the expression of genes on frozen tissue sections. The procedure is very simple and results can be examined within 30 to 60 minutes without the extensive staining and washing steps. Demonstration of the feasibility of combining the molecular beacon and immunofluorescence approaches to detect the expression of tumor marker genes and proteins *in situ* in the same cell population makes its potential application in pathologic diagnosis of human cancers more appealing. It is possible that the level of gene expression detected by molecular beacon-fluorescence imaging in clinical samples with intact tumor cells, such as fine-needle aspirates and exfoliated cells in body fluids is more quantitative than that detected in cancer cells on frozen tissue sections because most cells in tissue sections have been cut through and lost part of their cellular components.

One concern in the delivery of unmodified molecular beacons to viable cells is that the molecular beacons may be digested by nucleases in the cells or nonspecific interaction between molecular beacons, and cellular proteins may open up the stem of the molecular beacons, resulting in nonspecific fluorescence. However, our results showed that the fluorescence intensity detected by either FACScan or microplate reader correlated well

with the level of survivin mRNA in the tumor cells. Because a similar level of the molecular beacons was delivered into the tumor cells, it seemed that increases in the fluorescence intensity in EGF- and docetaxel-treated cells or a decrease in *p53*-expressing cells were not due to nonspecific degradation of the molecular beacons.

In this study, we showed that molecular beacon imaging of tumor cells is a simple and specific approach for the detection of breast cancer cells. This study is the first to apply state-of-the art molecular beacon–based methodology for cancer cell detection and for real-time monitoring the level of expression of tumor marker genes in viable cells. Based on this study, high-throughput assays for measuring the expression of multiple genes critical for drug response can be developed for screening cancer drugs that target specific molecules or pathways in cancer cells. To increase the specificity of molecular beacon detection, the molecular beacons can be further modified to make them resistant to nuclease or RNase H, such as by using 2′-*O*-methyl molecular beacon probes (12).

Acknowledgments

Received 9/3/2004; revised 12/8/2004; accepted 12/29/2004.

Grant support: Idea Award from the Breast Cancer Research Program of the Department of Defense (BC021952), the Avon Foundation, NIH grants CA95643 and CA80017, and the Wallace H. Coulter Foundation.

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We thank Dr. Toncred Styblo and Beth Sumpter (Department of Surgery, Emory University School of Medicine, Atlanta, GA) for providing us with breast cancer and normal tissues and Drs. Mark Behlke for his suggestion on survivin MB-Cy3 sequence and Gang Bao for helpful discussions.

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Receptor-Targeted Nanoparticles for *In Vivo* Imaging of Breast Cancer

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Running Title: Receptor-Targeted Nanoparticles for Tumor Imaging

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Foot note: This research project is supported by Emory-Georgia Tech Nanotechnology Center for Personalized and Predictive Oncology of NIH NCI Center of Cancer Nanotechnology Excellence (CCNE, U54 CA119338-01) and in part by the Idea Award of the Breast Cancer Research Program of the Department of Defense (BC021952).

Abstract

Biomarker targeted magnetic resonance imaging (MRI) contrast agents provide molecular imaging capability to improve the specificity for the detection of human cancer. The present study reports the development of a novel receptor-targeted MRI probe using a recombinant peptide containing the amino-terminal fragment (ATF) of urokinase plasminogen activator (uPA) conjugated to magnetic iron oxide (IO) nanoparticles (ATF-IO). This MRI probe targets urokinase plasminogen activator receptor (uPAR) that is overexpressed in breast cancer tissues. ATF-IO nanoparticles are able to specifically bind to and be internalized by uPA receptor-expressing tumor cells. Systemic delivery of ATF-IO nanoparticles into mice bearing subcutaneous and intraperitoneal mammary tumors leads to the accumulation of the particles in tumors, generating a strong MRI contrast detectable by a clinical field strength MRI scanner. Target specificity of ATF-IO nanoparticles demonstrated by *in vivo* MRI is further confirmed by near infrared fluorescence (NIR) imaging of the mammary tumors using Cy5.5 NIR dye-labeled ATF peptides that conjugated to IO nanoparticles. Furthermore, mice administered ATF-IO nanoparticles exhibit lower uptake of the particles in the liver and spleen compared to those receiving non-targeted IO nanoparticles. Our results suggest that uPAR-targeted ATF-IO nanoparticles have potential as molecularly-targeted, dual modality imaging agents for *in vivo* imaging of breast cancer.

Keywords: Targeted magnetic resonance imaging, near infrared optical imaging, magnetic iron oxide nanoparticle, urokinase plasminogen receptor, breast cancer

Abbreviations: uPA: urokinase plasminogen activator, uPAR: urokinase plasminogen activator receptor, ATF: amino-terminal fragment, MRI: magnetic resonance imaging, IO: magnetic iron oxide nanoparticles, NIR: near infrared fluorescence, PEG: poly(ethylene glycol), QDs: quantum dots, ROI: region of interest, SPIO: superparamagnetic iron oxide.

Introduction

Breast cancer is the most common type of cancer and second leading cause of cancer related death among women. Novel approaches for the detection of primary and metastatic breast cancers are urgently needed to increase the survival of patients. A promising strategy to improve the specificity and sensitivity of cancer imaging is to use biomarker target-specific imaging probes (1-3). Currently, targeted radionuclide probes have been used for cancer detection by positron emission tomography (PET) or single photon emission tomography (SPECT) (2, 4, 5). Although nuclear imaging modalities show a high sensitivity, they lack good resolution and anatomic localization of the tumor lesion and require complicated and expensive radiochemistry. In addition, the half-life of the radiotracer often limits the ability for dynamic and time-resolved imaging and may not be sufficient to allow the biomarker targeting agent to reach and accumulate in the tumor. Magnetic resonance imaging (MRI) offers a high spatial resolution and three-dimensional anatomic details and has been widely used in the clinical oncology imaging. Recently breast MRI was recommended by the American Cancer Society as a screening approach, adjunct to mammography, for the early detection of breast cancer in women at high risk for developing the disease (6). However, current breast cancer MRI mainly delineates morphological features of tumor, tissue and organs and therefore has limited ability to provide specific and functional and molecular information on the disease (2, 7). Conventional MRI suffers from low sensitivity, and the generically used MRI contrast enhancing media, such as Gd-DTPA, have low specificity for different cancer types.

Recently, biocompatible and functionalized nanoparticles have been shown to target the tumors and produce optical, magnetic and/or radioactive signals for enhancing sensitivity and specificity of non-invasive tumor imaging. Previous studies have shown the feasibility of

producing such imaging probes for *in vivo* MRI of cancer (8-13). The unique features of nanoparticles that make them suitable for receptor-targeted imaging include: 1) having a prolonged blood retention and circulation time, 2) providing reactive function groups for conjugation of tumor targeting ligands, and 3) providing large surface area for loading large numbers or multiple types of tumor targeting ligands. However, several issues remain to be addressed in the production of nanoparticle imaging probes for clinical application. These include the identification and availability of suitable imaging biomarkers, the delivery of sufficient probes *in vivo*, and the development of imaging probes with sufficient signal amplification and contrast enhancement (14).

Paramagnetic iron oxide (IO) nanoparticles can induce remarkably strong imaging contrast, and have a large surface area and versatile surface chemistry that allow for surface functionalization and the introduction of biomolecules (15-17). Their biological safety in human has been tested, with non-targeted IO nanoparticles currently in use to detect liver tumor lesions or lymph node metastases in patients (18, 19). Previous attempts to develop targeted IO nanoparticles used dextran or poly(ethylene glycol) (PEG)-coated IO to conjugate targeting ligands and demonstrated the feasibility and improved sensitivity and specificity of such receptor-targeted MRI nanoprobe (3, 11, 17, 20, 21). However, most of these ligands were directed to molecular targets that were expressed only in a small percentage of tumor tissues or subpopulations of tumor cells, such as Her-2/Neu, transferrin, and folate acid, which have limited sensitivity and applications for molecular-targeted cancer imaging in patients.

In this study, we used the amino-terminal fragment (ATF) of the high affinity receptor binding domain of urokinase plasminogen activator (uPA) to target its cellular receptor (uPAR), which is upregulated in a high percentage of tumor cells and tumor-associated stromal cells, such

as endothelial cells, macrophages and fibroblasts, of many human cancer types (22-25). It is well known that the interaction of uPA with its cellular receptor (uPAR) results in conversion of plasminogen to serine protease, a central regulator of the activation of other proteases including matrix metalloproteinase, which promotes tumor metastasis and angiogenesis. An elevated level of uPAR is associated with tumor aggressiveness, the presence of distant metastasis, and poor prognosis in several types of human cancers (26-30). In human breast cancer tissues, high levels of uPAR are detected in 54% of ductal carcinoma *in situ* (DCIS) and in 60 to 90% of invasive breast cancer tissues (26, 31).

To achieve optimal tumor targeting and imaging, we have developed novel paramagnetic IO nanoparticles that have uniform sizes and are functionalized through surface coating with amphiphilic polymers. This surface coating provides a stable hydrophobic protective inner layer around single IO particles with carboxylate groups in the outer layer readily available for conjugation with ATF peptides. Conjugated ATF-IO nanoparticles specifically bind to and are internalized by uPAR-expressing cells. We showed that systemic delivery of ATF-IO nanoparticles into mice bearing mammary tumors led to the accumulation of the ATF-IO nanoparticles in subcutaneous and metastatic mammary tumor lesions, inducing MRI contrast changes sufficient for *in vivo* tumor imaging. By conjugation of Cy5.5, a near infrared (NIR) dye, to the ATF peptides, we were able to achieve *in vivo* tumor imaging with both MRI and optical imaging.

Methods

Breast Cancer Cell Lines. Mouse mammary carcinoma cell line 4T1 stably expressing a firefly luciferase gene was obtained from Dr. Mark W. Dewhirst at Duke University (Durham, NC).

Human breast cancer cell line T47D was purchased from American Type Culture Collection (ATCC, Rockville, MD). 4T1 cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS). T47D cells were grown in RPMI 1640 containing 10% FBS, 100 ug/ml gentamicin, and 0.2 IU insulin/ml.

Preparation of ATF-IO Nanoparticles. A cDNA fragment encoding amino acids 1–135 of mouse uPA, isolated by PCR amplification using a PCR primer pair containing forward (5'-CACCATGGGCAGTGTACTTGGAGCTCC-3') and reverse (5'-GCTAAGAGAGCAGTCA-3') primers, was cloned into pET101 /D-TOPO expression vector (Invitrogen, Carlsbad, CA). The cDNA sequences were confirmed by DNA sequencing. Recombinant ATF peptides were expressed in E. coli BL21 (Invitrogen) and purified from bacterial extracts under native conditions using a Ni^{2+} NTA-agarose column (Qiagen, Valencia, CA). Purification efficiency was determined using electrophoresis on sodium dodecyl sulfate (SDS)-PAGE gel and greater than 95% of purified proteins were ATF peptides. Cy™5.5 maleimide (GE Healthcare Piscataway, NJ), a near infrared dye, was conjugated to ATF peptides using the manufacture's protocol. Non-targeted dye molecules were separated from the Cy5.5 dye labeled ATF peptides using Sephadex G25 column.

Paramagnetic iron oxide (IO) nanoparticles were prepared using iron oxide powder as the iron precursor, oleic acid as the ligands, and octadecene as the solvent as described (32). The core size and hydrodynamic size of the IO nanoparticles were measured using transmission electron microscopy (TEM), and light scattering scan, respectively. The particles were coated with amphiphilic polymers using a similar method as reported previously (33), which stabilizes IO nanoparticles and provides reactive carboxyl groups on the particle surface. ATF peptides were conjugated to the surface of the IO nanoparticles via cross-linking of carboxyl groups to

amino side groups on the ATF peptides as shown in Figure 1. Briefly, the polymer-coated IO nanoparticles were activated with ethyl-3-dimethyl amino propyl carbodiimide (EDAC, Pierce, Rockford, IL) and sulfo-NHS for 15 min. After purification using Nanosep 100k OMEGA (Pall Corp, Ann Arbor, MI), activated IO nanoparticles were reacted with ATF or Cy5.5-ATF peptides at a molar ratio IO:ATF of 1:20 in pH 7.0 PBS buffer and at 4 °C overnight, generating ATF-IO or Cy5.5-ATF-IO nanoparticles. The final ATF-IO conjugates were purified using Nanosep 100k column filtration and centrifuged at 9600 rpm for 20 min. After washing 3 times with PBS, ATF-IO nanoparticles were resuspended in PBS buffer (pH 7.0). Conjugation efficiency of ATF peptides to the IO nanoparticles was confirmed by the measurement of fluorescence intensity of Cy5.5-ATF-IO nanoparticles using fluorescence spectroscopy and the measurement of the ζ potential change before and after the conjugation of the ATF ligands to IO nanoparticles. The numbers of ATF peptides conjugated to each IO nanoparticle were estimated by measuring the fluorescence intensity of a diluted sample of Cy5.5-ATF-IO nanoparticles using an emission wavelength of 696 nm and then comparing the value to a linear standard curve prepared using various concentrations of Cy5.5-ATF peptides.

Western Blot Analysis. Western blot analysis was performed as described using a standard protocol in our laboratory (34). To confirm the presence of ATF peptides in SDS/PAGE gel, the protein was transferred to PVDF membranes (Bio-Rad laboratories, Hercules, CA). An anti-His tag monoclonal antibody (Novagen, Madison, WI) was used to identify the His-tagged ATF-peptides. After reacting with a HRP-labeled rat anti-mouse IgG antibody, the ATF peptide band was detected by enhanced chemiluminescence using ECL plus (GE Healthcare) followed by autoradiography. The level of uPAR was determined by an anti-uPAR polyclonal rabbit antibody (Santa Cruz Biotechnology, Santa Cruz, CA) that reacts with both mouse and human uPAR and

a HRP conjugated goat anti-rabbit IgG. The protein bands were detected using enhanced chemiluminescence.

Ni-NTA-Agarose Pull-down Assays. To determine whether the purified recombinant ATF peptides, either free peptides or conjugated to IO nanoparticles, are still able to bind to uPAR, we performed a combined pull-down and Western blot analysis. Ni^{2+}-NTA-agarose beads were incubated with appropriate concentrations of His-tagged ATF peptides or ATF-IO nanoparticles at 4 ºC for 30 min. The conjugated beads were then washed twice with the binding buffer and incubated with 500 μg of total cell lysate obtained from 4T1 or T47D cells for 2 hrs. The bound proteins were eluted from the beads using the elution buffer containing 400 mM imidazole and examined by Western Blot analysis to determine the amount of uPAR pulled down by ATF- or ATF-IO conjugated Ni-NTA agarose beads in each sample as described above.

Immunofluorescence Labeling. Frozen normal breast and cancer tissue sections were fixed with ice-cold acetone for 15 min and blocked with 1% (wt/vol) BSA for 20 min. Slides were incubated with 5 μg/ml of a polyclonal rabbit anti-uPAR antibody for 60 min and followed by biotinylated-goat anti-rabbit IgG for 30 min. The slides were then incubated with Texas-red avidin for 30 min. After washing, the slides were examined under a fluorescence microscope and images were taken using an imaging system (Zeiss Axioplan with Axiovision software, Carl Zeiss MicroImaging, Inc, Thornwood, NY). To detect the expression level of uPAR in living cancer cells, cells were dissociated from the culture dish and cell suspensions were incubated with an anti-uPAR antibody at 4 ^{o}C for 30 min. After incubation with FITC-goat anti-rabbit IgG for 30 min, cells were placed on glass slides and examined under the fluorescence microscope.

Specificity of ATF-IO Nanoparticles in Cancer Cells. Cells cultured on glass chamber slides (Nalge Nunc International, Naperville, IL) were incubated with 13.5 pmol of Cy 5.5-ATF-IO

or non-targeted IO nanoparticles at 37 °C for 3 hrs. After washing with the PBS buffer and fixing with ice-cold acetone, the slides were examined under a confocal microscope (Perkin Elmer Ultraview ERS, PerkinElmer Life and Analytical Sciences, Inc, Wellesley, MA). To localize the IO nanoparticles, the cells incubated with Cy5.5-ATF-IO or IO nanoparticles were fixed with 4% formaldehyde in the PBS buffer and Prussian blue staining (35) was used to confirm the presence of IO nanoparticles in the cells.

***In Vitro* MRI Scan**. 1x 10^7 of mouse mammary tumor 4T1 or control human breast cancer T47D cells were harvested from the cell culture and incubated in serum free medium containing 270 pmol of non-targeted IO or ATF-IO nanoparticles at 37 °C for 3 hrs. Cells were washed with PBS buffer 5 times and then embedded in 0.8% agarose in 24-well plates. Plates were then scanned in a 3T MRI scanner (Philips Medical Systems, Bothell, WA) using a T_1-weighted gradient echo sequence and a multi-echo T_2 weighted fast spin echo sequence which simultaneously collects a series of data points at different echo times (TE_i) (i = 30 and ranges from 8-200 ms, with increment of 8 or 10 ms) for T2 relaxometry measurement. T_2 values of each sample/well were calculated from multi-echo images by fitting the decay curve on a pixel-by-pixel basis using the non-linear mono-exponential algorithm of $M_i = M_0 * \exp(-TE_i/T_2)$.

Mouse Mammary Tumor Models:

Subcutaneous tumor model: Mouse mammary tumor 4T1 cells were injected subcutaneously into the back flank area of 6- to 8-week old female Balb/c or nude mice. We used nude mice for optical imaging to reduce background fluorescence.

Intraperitoneal metastatic mammary tumor model: 4T1 cells stably transfected with a firefly luciferase gene were directly injected into the upper right side of the peritoneal cavity. The tumor

growth was monitored by bioluminescence imaging using the Xenogen bioluminescence imaging system (Xenogen Corp., Hopkinton, MA).

***In Vivo* MRI of Mouse Mammary Tumors.** Tumor-bearing mice were examined using a 3T MRI scanner with a customized rodent coil to obtain pre-IO contrast MR images. Since MRI contrast effect is greatly dependent on the magnetic field strength (36), we chose to test the feasibility of detecting IO nanoparticle-induced contrast at a clinically relevant field strength (3 tesla) with consideration of the potential application of this imaging probe in patients. The imaging sequences included: T_1 and T_2 weighted spin echo or gradient echo methods; The three-dimensional fast spoiled gradient echo technique (TR/TE = 31.2 msec/8 msec; TI = 71 msec; flip angle = 30° with 512 frequency encoding steps and 256 phase encoding steps, and 40 slices at 0.5 mm slice thickness without gap). A multi-echo T_2 weighted fast spin echo sequence was used to obtain T_2 relaxometry of the whole mouse. The mice were injected with ATF-IO, Cy5.5™-ATF-IO, or IO nanoparticles suspended in PBS buffer though the tail vein at dosages ranging from 11.2 to 27 nmole/kg body weight and then scanned at different time points. Images from pre- and post-contrast administration were compared to evaluate the efficacy of contrast enhancement. Region of interest (ROI) analysis was used to evaluate and quantify the contrast agent induced changes of MRI signal or T_2 value in the tumor and other selected tissues and organs. Signal from the leg muscle was used to normalize the signals in ROIs. T_2 maps before and after contrast administration were calculated from fitting the data points at 8 different echo times ranging from 10 to 90 million seconds.

NIR Optical Imaging of Mouse Mammary Tumors. The tumor bearing mice were placed on an alfalfa-free rodent diet (Teklad Irradiated Global 18% Protein Diet #2918, Harlan Teklad, Madison, WI) to reduce background fluorescence for over two weeks. NIR images of the tumor-

bearing mice were taken using the Kodak *in vivo* FX imaging system (Carestream Molecular Imaging, New Haven, CT) before and at different time points following the injection of Cy5.5-ATF-IO or control IO nanoparticles. For each NIR image, a corresponding X-ray image was taken to provide anatomic registration of the tumor.

Histological Analysis. Prussian blue iron staining was used to confirm the presence of IO nanoparticles in the tissue sections. Tumor and normal tissues were collected from the mice at the end of *in vivo* imaging experiments. 5 μm frozen tissue sections were incubated with Prussian blue staining solution containing a 1:1 mixture of 5% potassium ferrocyanide and 5% HCl acid for 30 min at 37°C (35). The slides were then rinsed and counterstained with nuclear fast red (Vector Laboratories, Burlingame, CA, USA) for 10 min.

Results

Characterization of ATF-IO nanoparticles

The magnetic IO nanoparticles synthesized and used in this study have a 10 nm core size. We coated the nanoparticles with a monolayer layer of amphiphilic polymers grafted with carbon alkyl side chains to stabilize and functionalize their surface, resulting in a 18 nm water soluble IO nanoparticle with carboxyl side groups (Figure 1). To reduce nonspecific binding and uptake by normal tissues, short PEG chains were conjugated to a portion of the carboxyl side groups on the amphiphilic polymers. Our results showed that the amphiphilic polymer-coated 10 nm IO nanocrystal has a strong T_1 and T_2 shortening effect with R_1 (e.g., $1/T_1$) = 3.6 ± 0.3 $mM^{-1}.s^{-1}$ and R_2 ($1/T_2$) = 124 ± 7.2 $mM^{-1}.s^{-1}$ at 3T MRI, respectively (Figure 1). The resulting amphiphilic polymer-coated IO nanoparticles have the combined characteristics of a relatively small particle

size for easy *in vivo* delivery, a large surface area for conjugating biomolecules and sufficient T_2 effect for MRI contrast.

Recombinant mouse ATF peptides were examined by gel electrophoresis and Western blot analysis to determine the amount and purity of the ATF peptides. Coomassie blue staining of the SDS-PAGE gel revealed an ATF band located at approximately 17 kDa (Figure 1). The presence of His-tagged ATF peptides was confirmed by Western blot using a monoclonal anti-histidine tag antibody, showing a strong positive band in the location corresponding to the ATF-peptides identified by coomassie blue staining (Figure 1). Conjugation of ATF peptides to the negatively charged carboxyl groups on the particle surface was further confirmed by the reduction of surface potential from an average of -30 mV to -11 mV after attachment of ATF peptides, suggesting the charge neutralization of carboxyl groups after conjugation with peptides (Figure 1). From the measurement of fluorescent intensity produced from the Cy5.5 dye conjugated to the ATF-IO nanoparticles, we estimated that 8 to 10 ATF peptides were attached to each nanoparticle using our conjugation method (Figure 1).

Specific binding and internalization of ATF-IO nanoparticles to uPAR-expressing mammary tumor cells

We examined the level of uPAR expression in human breast cancer and normal tissues. A high level of surface uPAR was found in mouse mammary tumor 4T1 cells by immunofluorescence labeling of viable cells using an anti-uPAR antibody. However, human breast cancer T47D cells lacked uPAR expression (Figure 2A). We showed that uPAR is strongly expressed in invasive breast cancer tissues but is not detected in normal breast tissues, consistent with previous observations (26). To determine whether ATF peptides maintain a high binding affinity after being conjugated to the IO nanoparticles, we performed a pull-down assay

using cell lysates obtained from uPAR-positive 4T1 and -negative T47D cells. Our results showed that both free ATF peptides and ATF-IO nanoparticles bound to and precipitated uPAR proteins in the cell lysates, resulting in a positive uPAR band detected by Western blot assay in 4T1 but not in T47D cell lysates (Figure 2B). It has been shown that interaction of uPA with uPAR leads to the internalization of the ligand/receptor complex (37). To determine whether the binding of ATF to uPAR leads to receptor-mediated endocytosis, we examined 4T1 and T47D cells after incubation with Cy5.5-ATF-IO at 37 °C for 3 hr under a confocal microscope. We found that Cy5.5-ATF-IO nanoparticles were internalized by 4T1 cells but not by T47D cells (Figure 2C). The presence of intracellular IO nanoparticles in 4T1 cells was further demonstrated by Prussian blue staining (Figure 2C).

Binding and internalization of ATF-IO nanoparticles in tumor cells and MRI contrast effect

Receptor-mediated binding and internalization of imaging probes promote the accumulation of imaging probes at the tumor site, which contributes and increases contrast effect. 4T1 cells incubated with ATF-IO nanoparticles showed MRI contrast with signal drops in T_2 weighted gradient echo imaging (Figure 3B upper panel). T_2 relaxometry measurements indicated that the T_2 value of 4T1 cells bound with ATF-IO nanoparticles dropped significantly compared with those of the T47D cells incubated with ATF-IO nanoparticles and the samples treated with non-targeted IO nanoparticles (Figure 3 lower panel). Since the T_2 value is a function of the iron concentration, the T_2 relaxometry data suggest that T_2 weighted MRI contrast is induced by the specific binding of ATF-IO nanoparticles to the uPAR expressing 4T1 cells. Prussian blue staining detected a high level of IO particles in 4T1 cells incubated with

ATF-IO but not with non-targeted IO nanoparticles. uPAR-negative T47D cells showed only a very low level of nonspecific uptake (Figure 3A).

***In vivo* targeting and MR imaging of subcutaneous mammary tumors in mice**

In Balb/c mice bearing subcutaneous mouse mammary tumors derived from the 4T1 tumor cell line, T_2 weight gradient echo imaging and multiecho fast spin echo imaging showed that ATF-IO nanoparticles were selectively accumulated in tumors, as evidenced by a reduction in T_2 values and signal decrease of T2 weighted images in various areas of the tumor mass (Figure 4A). The ROI analysis of MRI signal change showed a three-fold signal reduction in animals receiving ATF-IO nanoparticles when compared with that of mice receiving non-targeted IO particles (Figure 4B). Although we observed deceases in MRI signals in the liver and spleen in ATF-IO-injected mice due to IO particle-induced T_2 effect, the reduction in MRI signal was 50% (liver) to 80% (spleen) less than that in mice that received non-targeted IO nanoparticles, suggesting that liver and spleen uptake of the nanoparticles was reduced for ATF-IO nanoparticles (Figure 4B). To further confirm the distribution of non-targeted or ATF-IO nanoparticles in normal and tumor tissues, Prussian blue staining was performed on the frozen tissue sections obtained from the mice that received control IO or ATF-IO nanoparticles. Prussian blue-stained cells were detected in the tumor sections of animals receiving ATF-IO nanoparticles but not in the sections from animals receiving non-targeted IO nanoparticles. High magnification images showed the intracellular localization of IO nanoparticles in the cells (Figure 4C). It is well known that the subcutaneous 4T1 mammary tumor model produces spontaneous lung metastases (38). We also found Prussian blue-positive cells in tissue sections of the lung metastases obtained from a mouse that received ATF-IO nanoparticles (Figure 4C). In normal tissues, we found high levels of Prussian blue-positive cells in the liver and spleen of

the mice that received non-targeted IO injections. However, liver and spleen tissue sections from the ATF-IO mouse group had fewer positive cells. We did not detect IO nanoparticles in tissue sections of the brain or heart obtained from mice injected with either non-targeted IO or ATF-IO nanoparticles (Figure 4C). For both ATF-IO and IO nanoparticle-injected groups, the lung and kidney tissues were negative in most cases (Figure 4C) and only a few scattered iron staining positive cells were detected in some of the tissue sections.

Targeted MR imaging of intraperitoneal mammary tumor lesions using ATF-IO nanoparticles

We tested the feasibility of targeting and *in vivo* imaging of metastatic lesions using an animal model bearing intraperitoneal (i.p.) 4T1 tumors. The presence and development of the 4T1 tumor was determined and followed by bioluminescence imaging (BLI) (39), since 4T1 tumors stably express a firefly luciferase gene. At 5 hrs after injection of the ATF-IO nanoparticles, MRI signals decreased in two tumor lesions on top of the right kidney (Figure 5, upper panel). MRI signal in the region recovered gradually 30 hrs after the injection of the ATF-IO nanoparticles. In contrast, we did not detect such a T_2-induced MRI signal change in a mouse bearing a tumor mass after injection of non-targeted IO (Figure 5, lower panel). The selective accumulation of the uPAR targeted ATF-IO nanoparticles in this metastatic i.p. tumor model was further confirmed by Prussian blue staining of tissue sections from the sacrificed animals. A high percentage of iron positive cells was found in the tumor lesion while the kidney beneath the tumor was negative (Figure 5, upper panel). On the other hand, we did not detect iron positive cells in tissue sections of the i.p. tumor mass obtained from the mouse that received the non-targeted IO injection, while the adjacent normal liver tissue had a high level of iron positive cells (Figure 5, lower panel).

Validation of tumor-targeted MRI in 4T1 mouse mammary tumors using NIR optical imaging

Conjugation of Cy5.5 dye-labeled ATF peptides to IO nanoparticles allows for tumor imaging using both sensitive NIR optical and high resolution MR imaging. NIR optical imaging also provides a simple and sensitive approach to monitor the distribution of ATF-IO nanoparticles in small animals. When monitoring mice bearing s.c. 4T1 tumors at different time points after injection of Cy5.5-ATF-IO nanoparticles, we observed the NIR signal in the peripheral area of the tumor mass 24 hrs after the nanoparticle administration. The signal intensity of NIR images gradually increased to a peak level between 48 to 72 hrs. ATF-IO nanoparticles also appeared to enter the areas of the tumor center at those time points (Figure 6A). The signal intensity of NIR images in the tumor started to decline at 80 hrs. We also detected an NIR signal in the kidneys and bladder, suggesting that some Cy5.5-ATF-IO nanoparticle conjugates and/or degraded components may be eliminated through the kidney. Areas with MRI contrast change were registered well with the presence of NIR signal in the tumor mass when comparing optical and MR images (Figure 6B). Examination of the tissue sections with positive Prussian blue staining revealed that Cy5.5 NIR signals were co-localized with blue iron positive cells (Figure 6C). This result also suggests that most Cy5.5-ATF-IO nanoparticles are stable *in vivo* and remain a single unit when bound and internalized by tumor cells.

Reduced nonspecific uptake of ATF-IO nanoparticles

Previous studies reported that IO nanoparticles can be non-specifically taken up by macrophages as well as the reticuloendothelial system in the liver and spleen. We examined the tumor tissue sections stained with both Prussian blue and an antibody to CD68, a marker for

macrophages (40). We found that Prussian blue-positive cells are present in both CD68-positive and -negative cell populations. A high percentage of Prussian blue-positive cells do not express CD68, suggesting that non-macrophage cell types, such as tumor cells, contain IO nanoparticles. It has been shown that active macrophages in breast cancer tissues also express a high level of uPAR (23, 41). It is possible that the presence of iron positive macrophages may be due to active targeting of uPAR-expressing macrophages rather than non-specific uptake of IO nanoparticles, since we did not find Prussian blue-positive cells in the tumors of mice that received non-targeted IO nanoparticle injections, which should have a similar level of intratumoral macrophages to that of the animals that received ATF-IO nanoparticles.

Additionally, an MRI relaxometry T_2 map of a s. c. mammary tumor showed that accumulation of ATF-IO nanoparticles is not uniformly distributed inside the tumor mass. At various levels of tumor MR images, MRI signal decreases were heterogeneous in the tumor (Figure 6C). Interestingly, areas with the greatest decline in signal were largely in the periphery regions of the tumor mass which are enriched in blood vessels, relative to the necrotic areas at the center of the tumor.

DISCUSSION

Molecular imaging probes targeting specific cancer markers have been long sought-after in applying molecular imaging for disease-specific detection and personalized therapeutics. However, the development of receptor-targeted imaging and its *in vivo* applications are particularly challenging, with current obstacles including: 1) the identification of cell surface biomarkers that are expressed sufficiently in tumor cells or tumor environments for sensitive tumor imaging; 2) the production of stable and high affinity targeting ligands in large amounts

for chemical modification, conjugation and *in vivo* studies; and 3) the development of safe and biodegradable contrast agents producing strong imaging signal or contrast.

The uPAR-targeted IO nanoparticle imaging probe reported in this study provides an example that addresses these challenges and demonstrates the feasibility of *in vivo* receptor-targeted tumor imaging. Results of our study showed that ATF-IO nanoparticles are capable of targeting uPAR-expressing tumor cells *in vitro* and *in vivo,* and enable receptor-targeted *in vivo* MR and optical imaging of the tumor. We believe that following factors of ATF-IO nanoparticles enabled uPAR-targeted imaging *in vivo*. First, we used a tumor targeting ligand from a natural high affinity receptor binding domain of uPA. uPA is composed of three independently folded domain structures: growth factor domain (GFD), Kringle domain, and serine protease domain. Enzymatic digestion of uPA by plasmin generates an amino-terminal fragment (ATF), consisting of GFD and kringle domains, and the low molecular weight fragment serine protease domain (42). uPA binds to uPAR with a high affinity through the GFD of ATF (Kd=0.28 nm) (43). Studies have shown that ATF (residues 1-135 aa) of uPA is a potent uPA binding antagonist to uPAR. Our results revealed that recombinant ATF peptide maintains its binding affinity with uPAR even after being cross-linked to the surface of the IO nanoparticles. Second, efficient internalization of the ligand/receptor complex may increase the concentration of the IO nanoparticles in tumor cells, which enhances the effect of uPAR-targeted tumor imaging (37, 44). Third, nanoparticle imaging probes provide favorable pharmacokinetics by prolonging blood circulation time that allows for sufficient amounts of the IO nanoparticle probes to reach the tumor. Furthermore, we can produce the recombinant protein in a large scale using the standard protein engineering method by cloning the gene sequence of ATF into a bacterial expression plasmid, which is essential for preclinical and eventually clinical studies. It

should be mentioned that this study was done using mouse ATF peptides and the 4T1 mouse tumor model to investigate the feasibility of targeting uPAR. Although it has been shown that the interaction of uPA to its receptor has a species specificity (45), we found that mouse ATF peptides bind efficiently to mouse tumor cells and also show cross reactivity with human uPAR expressing tumor cells. A major advantage of using mouse ATF peptides to conduct studies in a mouse tumor model is that the targeting specificity, sensitivity and biodistribution in normal tissues of this imaging probe can be studied in greater detail. For future clinical application, it is desirable to use a human ATF peptide and we have produced human ATF in our laboratory.

uPAR is abundantly expressed in a high percentage of tumor cells in many human cancer types. Extensive studies in breast cancer cells and tissues have shown that human breast cancer cells have a much higher level of uPAR compared to normal breast cells (46, 47). For example, about 130,000 to 500,000 uPAR molecules per cell were detected in breast cancer cells while primary normal human mammary epithelial cells have only 2,500 uPAR molecules per cell (47). Such a high level of receptor expression in tumor cells makes uPAR a particularly suitable target for molecular MR imaging of breast cancer. Several studies have shown that the highest level of uPAR expression is detected in the invasive edge of the tumor regions (25, 30), which are usually enriched in blood vessels, making this area particularly accessible for uPAR-targeted IO nanoparticles. Supporting this notion, the results of both NIR optical and MR imaging as well as Prussian blue staining of tumor tissue sections obtained from the mice that received ATF-IO nanoparticles showed that the IO nanoparticles preferentially accumulated in the tumor cells close to the tumor boundary.

Another important factor to consider when developing molecular imaging probes is the potential ability to quantify the level of probe *in vivo* via imaging methods. We believe that size

uniformity of nanoparticles is essential in this regard. It is likely that nanoparticles exhibit averaged magnetism of disperse size IO nanoparticles, such as Feridex, a commercially available and FDA approved superparamagnetic iron oxide (SPIO) nanoparticle formulation consisting of a mixture of 20-30 nm core size or 80-150 nm overall diameter with dextran coating (16). In addition, a portion of the larger sized particles in these products may never be able to reach the tumor site, therefore reducing the effective concentration of the probe. We prepared high quality and uniformly sized IO nanoparticles with thin amphiphilic copolymer coating (estimated at ~2 nm). Compared with conventional dextran or PEG-coated SPIO nanoparticles used in previous studies, amphiphilic copolymer-coated IO nanoparticles form a relatively small particle complex (~18 nm), which is desirable for *in vivo* delivery of the imaging probe. Our results suggest that our targeted IO nanoparticles are stable *in vivo* and in intracellular environments for over 48 hrs during our imaging experiments.

Although other small molecule imaging agents may have better intratumoral distribution compared with nanoparticle-based imaging agents, these imaging agents are usually eliminated from the blood circulation in a relatively short time (less than 30 min), which makes it unlikely that sufficient levels of the targeted contrast agents can accumulate at the tumor site (2). It has been shown that polymer-coated IO nanoparticles have over 8 hours of plasma retention time (14). This longer circulation time could be an important factor enabling targeted IO nanoparticles to reach the tumor site and to bind and be internalized by tumor cells, as demonstrated by our NIR optical imaging of Cy5.5-ATF-IO nanoparticles in animals. We observed that the intratumoral NIR signal increased over time and reached its highest level around 48-72 hrs after the administration of IO nanoparticles, suggesting that the long blood retention time may facilitate nanoparticles targeting the tumor. Although the production of dual

modality SPIO nanoparticles by direct conjugation of Cy5.5 dye to SPIO through cross-linking has been reported previously (11), we used Cy5.5 dye-labeled ATF-peptides to conjugate to IO nanoparticles so that the results of optical imaging and tissue distribution are not affected by non-specific signals generated from the possible presence of Cy5.5-IO nanoparticles without conjugated ATF. The addition of the near infrared dye Cy5.5 to ATF-peptides in a time course imaging study in live small animals provides an important validation of distribution and tumor targeting of MR imaging probe *in vivo*. Other potential applications of such dual modal imaging probes can be explored further.

It has been reported by several other groups that large portions of SPIO nanoparticles are taken up by the reticuloendothelial system in the liver and spleen, and then are subsequently utilized for iron storage or metabolism (15, 16). Our data showed that ATF-IO nanoparticles have reduced liver and spleen uptake compared to that of non-targeted IO nanoparticles. This suggests that conjugation of ATF-peptides to the IO nanoparticles attenuates the non-specific capture and retention of nanoparticles in the liver and spleen that commonly occurs after their systemic delivery.

In conclusion, we have developed a uPAR-targeted molecular imaging nanoprobe that has an uniform-sized IO nanocrystal core, a thin amphiphilic copolymer coating, and a high affinity receptor binding domain of uPA conjugated with an NIR dye. This receptor-targeted nanoprobe selectively binds to and is internalized by tumor cells and can specifically accumulate in primary and metastatic tumor lesions, facilitating *in vivo* MR and optical imaging in a mouse mammary tumor model. Such uPAR-targeted imaging nanoparticles are promising probes for the molecular imaging of breast cancer with MRI.

ACKNOWLEDGMENTS

We would like to thank Dr. Anthea Hammond for her critical editing of the manuscript and Dr. Mark W. Dewhirst for kindly providing us with luciferase gene stable 4T1 cell line. We also thank Drs. Adam Marcus and Katherine Schafer-Hales in the Cell Imaging Core of the Winship Cancer Institute for their assistance in confocal microscopy, and Dr. Hongwei Duan for his help with zeta potential and particle size analysis.

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FIGURE LEGENDS

Figure 1. Characteristics of ATF-IO nanoparticles.

Size uniformed IO nanoparticles have 10 nm core size as shown in transmission electron microscopy (TEM) and are coated with amphiphilic copolymers modified with short PEG chains. The purity of recombinant ATF peptides produced from a bacterial expression system was confirmed by Crossman blue staining on SDS-PAGE gel as well by Western blotting using an anti-His tag antibody. Cy5.5™ maleimide was conjugated to ATF peptides through a bond between maleimide esters and free thiol groups of cystidine residues of the ATF peptide. ATF peptides or Cy5.5-ATF-IO peptides were then conjugated to the carboxyl side groups mediated by EDAC. ζ potential measurement showed that conjugation of ATF ligands to the carboxyl groups on the surface of IO nanoparticles reduced the negative surface charges (from -30 to – 11 mV). ATF-IO nanoparticles have high relaxation rates of R_2 at 124 $S^{-1}.mM^{-1}$ (3T).

Figure 2. Determination of specificity of ATF peptides or ATF-IO nanoparticles.

A. Detection of uPAR expression by immunofluorescence labeling. Using an anti-uPAR antibody, a high level of uPAR (red) is detected in frozen tissue section of an invasive human breast cancer tissue but is not detected in normal breast tissue obtained from the same patient (upper panel). Blue: Hoechst 33342 background staining. Surface-labeling of viable cells using anti-uPAR antibody and FITC-labeled secondary antibody shows a high level of uPAR in mouse mammary tumor 4T1 cells but not in human breast cancer T47D cells (green, lower panel). **B**. Determination of specificity of recombinant ATF peptides and ATF-IO nanoparticles using an immunoprecipitation assay. Lane 1: Free ATF peptides pulled down uPAR protein from 4T1 cell lysates; Lane 2: ATF-IO nanoparticles are also capable of binding and pulling down uPAR

protein; Lane 3: Control total lysates of 4T1 cells; Lanes 4 & 5: The uPAR protein band is not detected after incubation of free ATF peptides (lane 4) or ATF-IO nanoparticles (lane 5) with T47D cell lysates. **C**. Specific binding and internalization of Cy5.5-ATF-IO nanoparticles in uPAR expressing tumor cells. Confocal images show intracellular localization of Cy5.5 fluorescence signal (red) in 4T1 but not in T47D cells (upper panel). Prussian blue staining further confirms the presence of blue iron staining inside the 4T1 cells (lower panel).

Figure 3. ATF-IO nanoparticle-induced MRI signal change after binding to tumor cells.

A. Prussian blue staining shows specific binding and internalization of ATF-IO nanoparticles in 4T1 tumor cells after incubation at 37°C for 3 hrs. A low level of scattered blue staining is found in 4T1 cells incubated with non-targeted IO or uPAR (-) T47D cells incubated with either IO or ATF-IO nanoparticles. **B**. uPAR specific binding resulted in the reduction of T_2 values in uPAR (+) 4T1 cells but not uPAR (-) T47D cells after incubation with ATF-IO or control IO nanoparticles. Reduction of T_2 values in 4T1 cells (upper panel) measured by MRI T_2 relaxometry indicates specific binding of ATF-IO nanoparticles. A low T_2 value (orange to red color) correlates with a high iron concentration. The fastest T_2 value drop was detected in 4T1 cells incubated with ATF-IO nanoparticles (lower panel, red curve). TE: MRI scanning echo time in million seconds.

Figure 4. *In vivo* MRI of 4T1 mammary tumor using ATF-IO nanoparticles.

A. MRI of subcutaneous (s.c.) mammary tumor**.** A marked MRI signal drop with T_2 weighted contrast was observed in s.c. tumor areas (pink dash-lined) 6 hrs after the tail vein administration of ATF-IO nanoparticles. Heterogeneous signal changes suggest that intratumoral distribution of

ATF-IO nanoparticles is not uniform in the s.c. tumor (red arrow). T_2 contrast change is also found in the liver (yellow arrows and Pink *). Selected MR image is a representative image of seven mice that received ATF-IO nanoparticles. An MR image of the mouse that received non-targeted IO nanoparticles is shown in Figure 6B. **B**. Organ-specific profiling of MRI signal change. Signal changes in the mice that received non-targeted IO or ATF-IO nanoparticles for 6 hrs were measured in the regions of tumor or various normal tissues. Relative intensity was calculated using the intensity in the leg muscle as a reference. Fold decreases in the intensity of the MR image were compared between pre- and post- ATF-IO injection and plotted in the Figure. The bar plot represents mean values of three regions. **C**. Examination of the IO nanoparticles in tumor and normal tissues 48 hrs after particle injection using Prussian blue staining. Blue iron-positive cells are found in tumor tissue sections of a mouse that received ATF-IO nanoparticle injection but not in a mouse that received non-targeted IO nanoparticles. Spontaneous lung metastasis (pink arrow) also shows a high level of iron positive cells, although MRI could not detect those small metastases at the current detection sensitivity. A markedly lower level of iron positive cells was found in the liver and spleen of the mouse injected with ATF-IO nanoparticles compared to those from the mouse that received non-targeted IO nanoparticles. Red: background staining with nuclear fast red.

Figure 5. Targeting and *in vivo* MR tumor imaging of intraperitoneal mammary tumor lesions. Upper panel: 10 days after injection of 4T1 cells stably expressing the luciferase gene, bioluminescence imaging confirmed the presence of intraperitoneal tumors on the upper right of the peritoneal cavity. Two areas located near the right kidney (red dash-lined) showed decrease of MRI signals 5 or 30 hrs after the tail vein injection of 11.2 nmole/kg of body weight of ATF-

IO nanoparticles, which corresponded to the locations detected by bioluminescence imaging. Examination of peritoneal cavity of the mouse confirmed the locations of two tumor lesions on the top of the right kidney. Specific accumulation of ATF-IO nanoparticles in the tumor tissues was confirmed by Prussian blue staining. **Lower panel:** A control mouse with a tumor lesion in the upper right of the peritoneal cavity received non-targeted IO nanoparticles. There is no MRI contrast change in the tumor area (pink dash-lined) 6 hrs after administration of IO nanoparticles, while the liver of the mouse shows a marked contrast decrease. Prussian blue staining of tissue sections revealed iron positive cells in the normal liver tissue near the tumor region but not in the tumor tissues (A red dashed line divides normal liver and tumor tissue areas).

Figure 6. Dual modality imaging of s.c. 4T1 mouse mammary tumor using Cy5.5-ATF-IO nanoparticles.

A. A time course NIR optical imaging of tumor targeting and tissue distribution of Cy5.5-ATF-IO nanoparticles after the tail vein injection. A nude mouse bearing a s.c. 4T1 tumor received the tail vein injection of 27 nmole/kg of Cy5.5-ATF-IO nanoparticles. NIR optical imaging was performed at different time points using Kodak *in vivo* FX imaging system equipped with a filter set with an excitation wavelength of 625 nm and emission wavelength of 700 nm. **B**. Simultaneous MR and optical imaging of mammary tumor. A tumor-bearing mouse that received the tail vein injection of Cy5.5-ATF-IO nanoparticles for 48 hrs showed a T_2 signal drop in the lower left half of the s.c. tumor in T_2 weighted MR image. Optical imaging reveals the NIR signal in the tumor area corresponding well with the MRI results (Lower panel, pink dash lined). Neither T_2 contrast change nor optical imaging is detected in the s.c. tumor of the mouse that received non-targeted IO nanoparticles (upper panel). MRI shown represents the results of a total

of seven control tumor bearing mice that received IO nanoparticles. **C**. Examination of the location of ATF-IO nanoparticles and Cy5.5 signal in tumor tissues. Double-labeling of Prussian blue staining and an Alexa Fluor 488-anti-CD68 antibody, a marker for macrophages, reveals that many blue iron positive cells are negative for CD68 (red arrow). Moreover, co-localization of blue iron staining and Cy5.5 signal is also detected in the tumor tissues (white arrows). **D**. T_2 map of a series of MR images taken from a s.c. mammary tumor lesion. Multi-echo T_2 weighted spin echo imaging using 3D-MRI reveals intratumoral distribution of ATF-IO nanoparticles throughout different sectional levels of a tumor mass. Upper panel shows control MR images taken before ATF-IO nanoparticle injection. Lower panel is corresponding MR images taken 48 hrs after the tail vein injection of ATF-IO nanoparticles. Orange to red colors represent tumor areas with the greatest T_2 contrast decreases. Regional of interest (ROI) analysis of T_2 mapping is used.

Figure 1

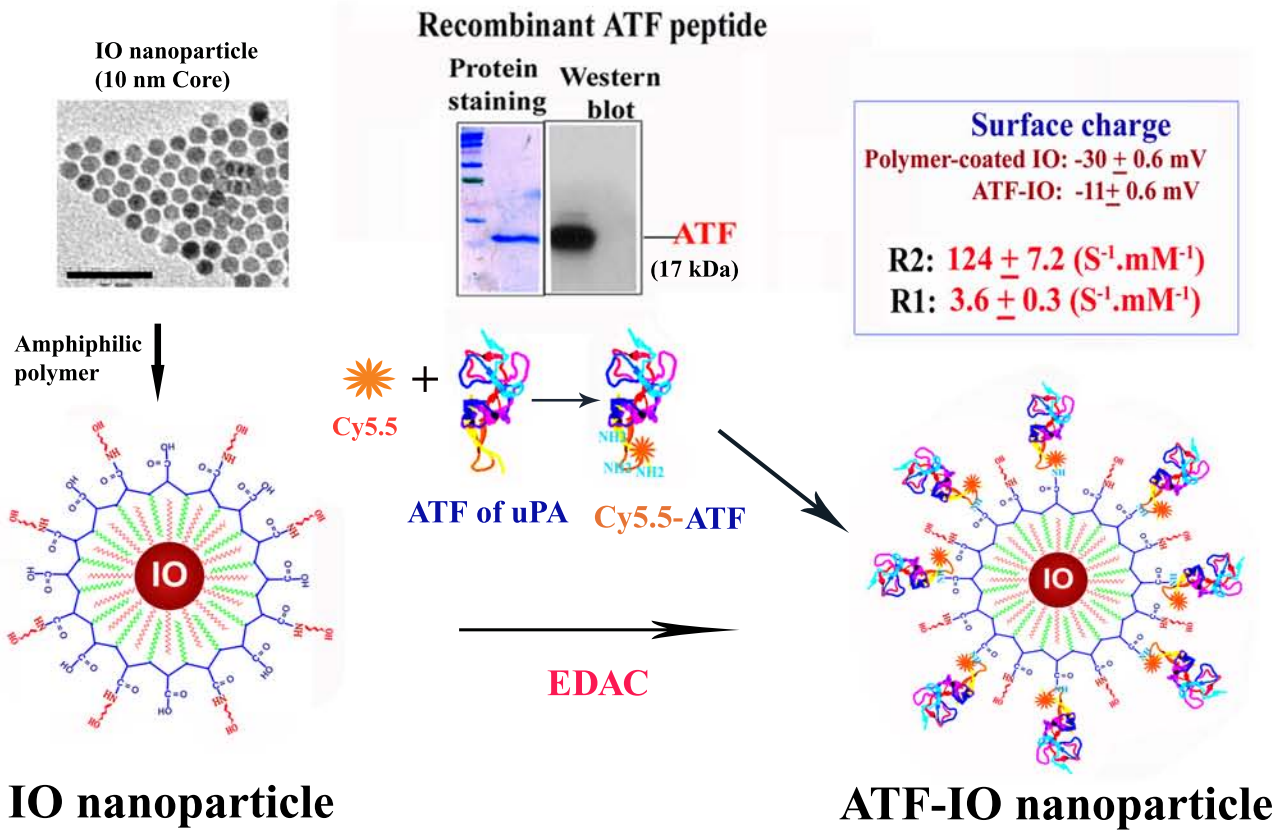
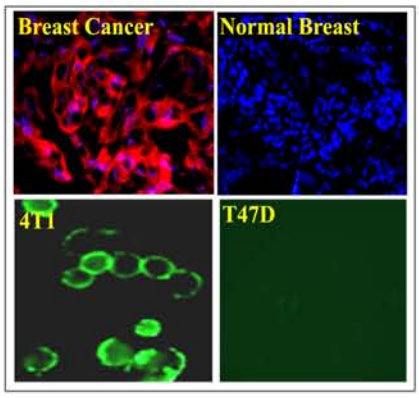
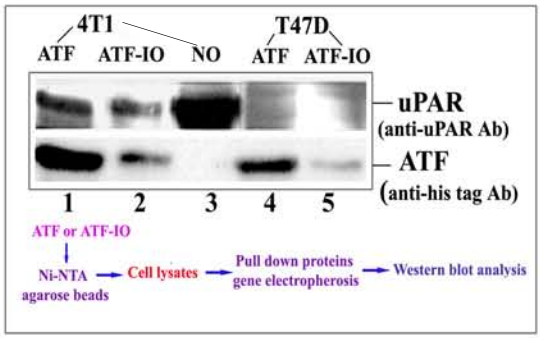

IO nanoparticle
(10 nm Core)
Amphiphilic
polymer
IO
IO nanoparticle
Recombinant ATF peptide
Protein
staining
Western
blot
ATF
(17 kDa)
Cy5.5
+
ATF of uPA
Cy5.5-ATF
EDAC
Surface charge
Polymer-coated IO: -30 ± 0.6 mV
ATF-IO: -11± 0.6 mV
R2: 124 ± 7.2 (S-1.mM-1)
R1: 3.6 ± 0.3 (S-1.mM-1)
IO
ATF-IO nanoparticle

Figure 2

A.


Breast Cancer
Normal Breast
4T1
T47D

B.


4T1
T47D
ATF
ATF-IO
NO
ATF
ATF-IO
uPAR
(anti-uPAR Ab)
ATF
(anti-his tag Ab)
1
2
3
4
5
ATF or ATF-IO
Ni-NTA
agarose beads
Cell lysates
Pull down proteins
gene electropherosis
Western blot analysis

C.

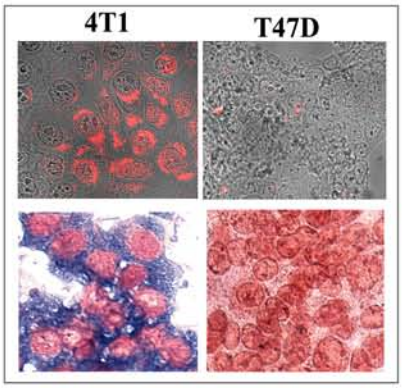
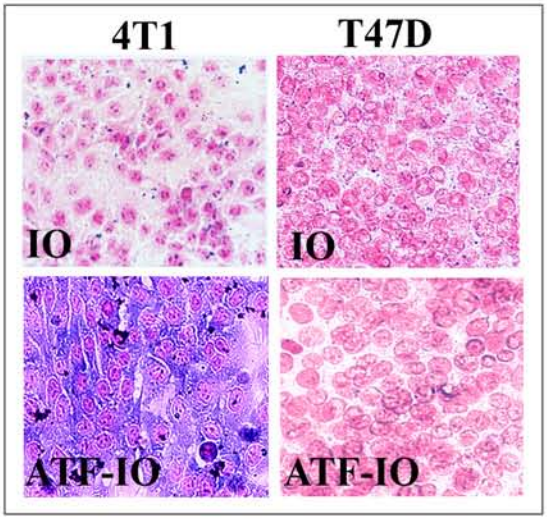

4T1
T47D

Figure 3

A.


4T1
T47D
IO
IO
ATF-IO
ATF-IO

B.

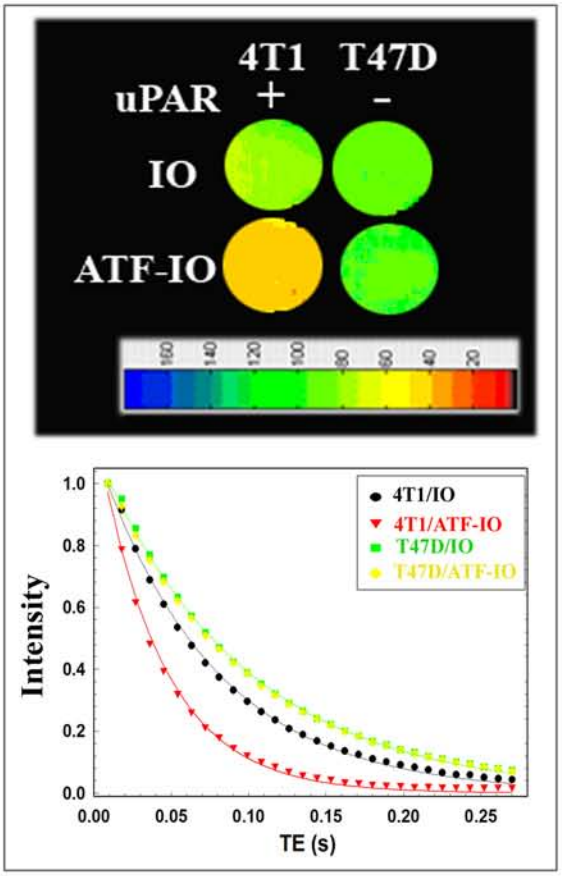

4T1
T47D
uPAR
+
-
IO
ATF-IO
160
140
120
100
80
60
40
20
4T1/IO
4T1/ATF-IO
T47D/IO
T47D/ATF-IO
Intensity
1.0
0.8
0.6
0.4
0.2
0.0
0.00
0.05
0.10
0.15
0.20
0.25
TE (s)

Figure 4

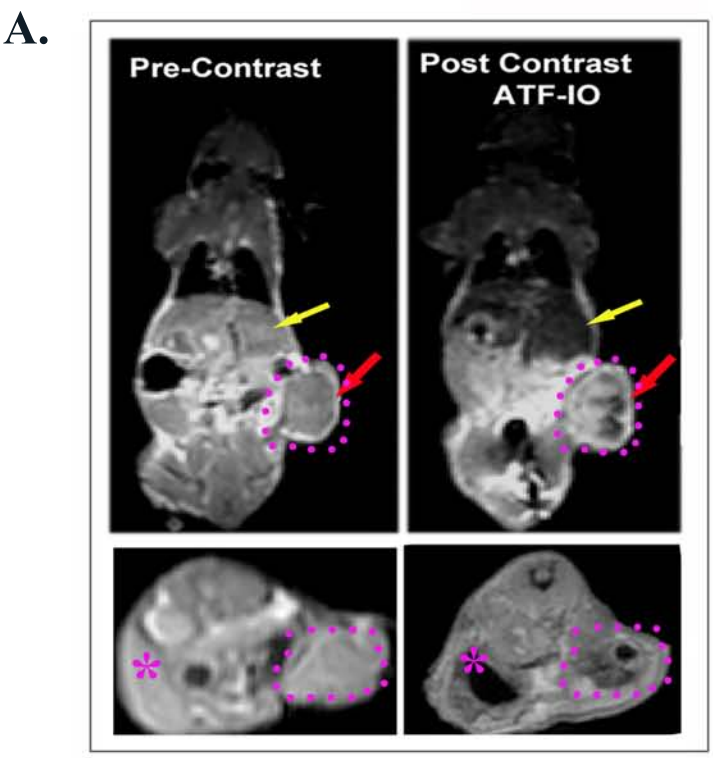
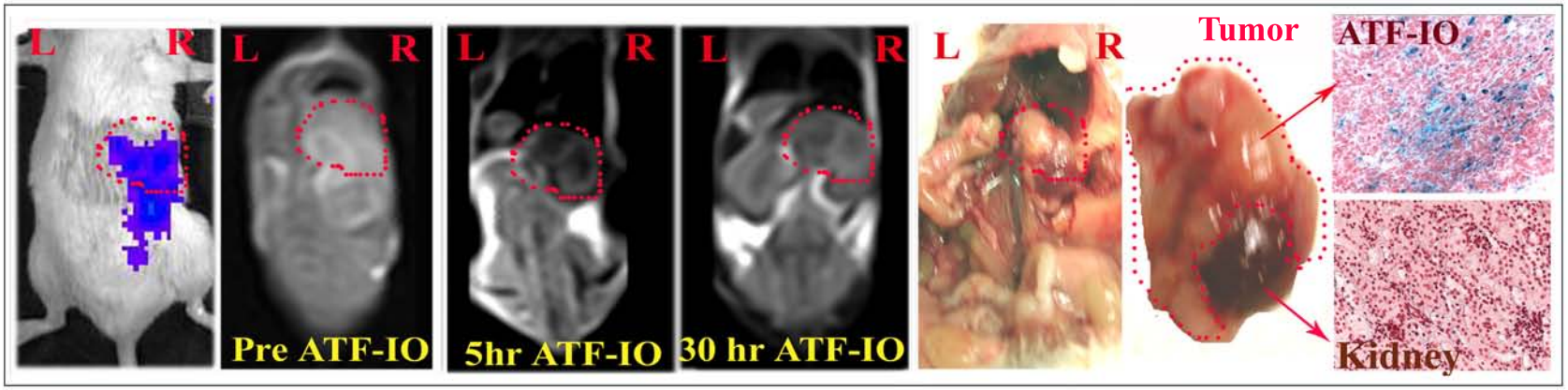
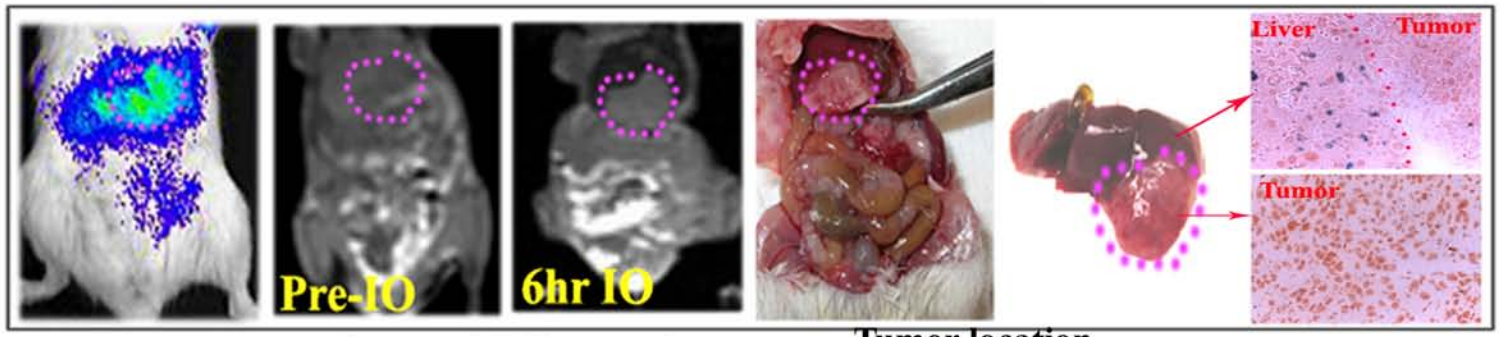

A.
Pre-Contrast
Post Contrast
ATF-IO
B.
Relative fold decrease in MRI contrast
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Non-targeted-IO
ATF-IO
Relative fold decrease in MRI contrast
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
Non-targeted-IO
ATF-IO
Brain
Kidney
Liver
Spleen
C.
S.C. tumor
IO
ATF-IO
ATF-IO
Lung metastasis
Lung
ATF-IO
ATF-IO
Normal tissues
Liver
Control
IO
ATF-IO
Spleen
Control
IO
ATF-IO
Kidney
Lung
Brain
Heart
IO
ATF-IO

Figure 5



L: Left, R: Right.



Bioluminescence

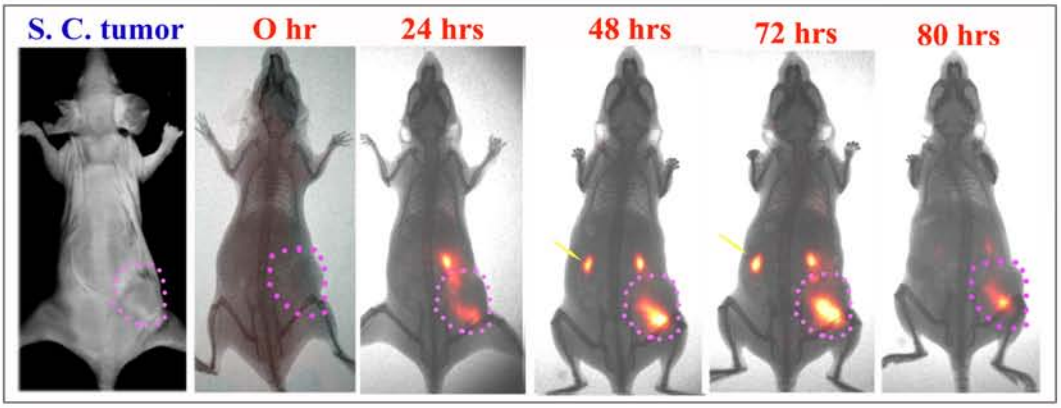
MRI

Tumor location

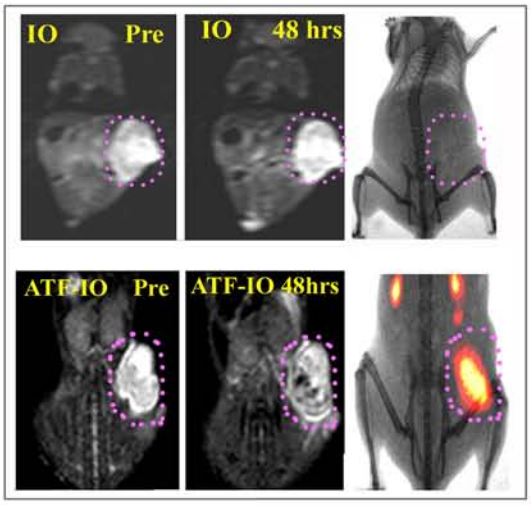
Prussian blue staining

Figure 6

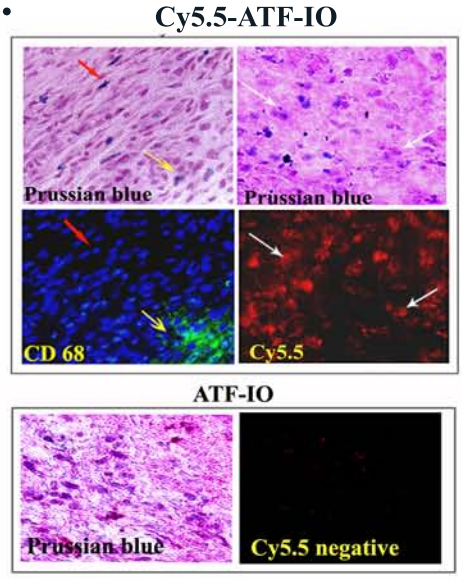
A.



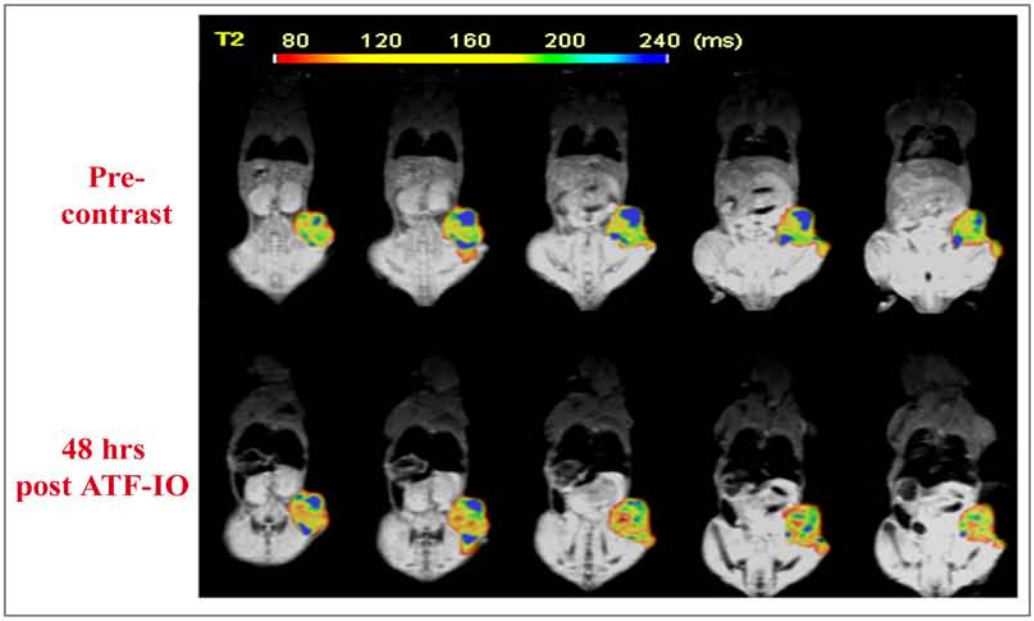
B.



C.



D.



THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 281, NO. 36, pp. 25903–25914, September 8, 2006

Cross-talk between Epidermal Growth Factor Receptor and Hypoxia-inducible Factor-1α Signal Pathways Increases Resistance to Apoptosis by Up-regulating Survivin Gene Expression*

Received for publication, April 10, 2006, and in revised form, July 12, 2006 Published, JBC Papers in Press, July 17, 2006, DOI 10.1074/jbc.M603414200

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Although increasing evidence supports a link between epidermal growth factor receptor (EGFR) signaling and resistance to apoptosis, the mechanism by which the EGFR signaling pathway inhibits apoptosis is not well understood. In this study, we found that epidermal growth factor (EGF) stimulation increased the level of expression of the inhibitor of apoptosis protein survivin in breast cancer cells but not in normal mammary epithelial cells. We further demonstrated that activation of survivin gene expression is mediated by oxygen-independent hypoxia-inducible factor (HIF)-1α up-regulation in EGF-treated cancer cells. EGFR signaling activated the phosphoinositide 3-kinase/AKT pathway, subsequently increasing the level of HIF-1α under normoxic conditions. HIF-1α then activated survivin gene transcription through direct binding to the survivin promoter. Furthermore, we found that overexpression of HIF-1α small interfering RNA blocks EGF-induced survivin gene up-regulation and increases apoptosis induced by the chemotherapy drug docetaxel. However, transfection of a plasmid expressing HIF-1α gene activates survivin gene expression and reduces the apoptotic response. Our results demonstrate a novel pathway for EGFR signaling-mediated apoptosis resistance in human cancer cells. Although the role of HIF-1α in regulating cell survival under hypoxic conditions has been studied extensively, our results show that normoxic breast cancer cells utilize cross-talk between EGFR signals and HIF-1α to up-regulate the anti-apoptotic survivin gene, providing a strong rationale for the targeting of HIF-1α as a therapeutic approach for both hypoxic and normoxic tumor cells. Understanding key molecular events in EGFR signaling-induced apoptosis resistance should provide new information for the development of novel therapeutic agents targeting EGFR, HIF-1α, and/or survivin.

The EGFR[2] signaling pathway plays a key role in the regulation of cell proliferation, survival, and differentiation (1, 2). It has been shown that the level of EGFR is up-regulated in many human tumor tissues. Activation of EGFR signaling has been associated with highly aggressive cancer types and poor responses to therapeutic agents (3–7). Prior preclinical and clinical studies have shown that blocking the EGFR signaling via monoclonal antibodies or inhibition of the EGFR tyrosine kinase with small molecules reduces the growth of breast cancers and sensitizes responses to chemotherapy (8–10).

Recently, we and others have shown that activation of the EGFR signaling pathway leads to the up-regulation of survivin, a member of the inhibitor of apoptosis (IAP) protein family (11–14). Survivin is broadly expressed in fetal tissues but is undetectable in the most normal adult tissues (15). However, a high level of survivin is found in most common tumor types, including over 70% of human breast cancer tissues at all stages of cancer development (16–18). It has been shown that in breast cancer cells, levels of survivin expression correlate with susceptibility to apoptosis (17). At present, the mechanism by which this up-regulation of survivin occurs in tumor cells having activated EGFR signaling is not fully understood. Recent studies have suggested, however, that activation of the phosphoinositide 3-kinase (PI3K)/AKT pathway by EGFR signaling causes up-regulation of survivin expression (12, 13). It is still unknown how PI3K/AKT signaling leads to survivin gene transcription.

Several studies have shown that under normoxic conditions, activation of EGFR signaling also increases the level of hypoxia-inducible factor 1α (HIF-1α) through the PI3K/AKT pathway (19–21). HIF-1α, a member of the basic helix-loop-helix-PAS protein family (22), normally becomes highly up-regulated under hypoxic conditions, mostly as a result of inhibition of protein degradation. HIF-1α can then activate transcription of many genes that are critical for continued cellular function

* This work was supported by NCI, National Institutes of Health Grants R29 CA 80017 and R01 CA95643, an Idea Award of the Department of Defense Breast Cancer Research Program, and the Avon Breast Cancer Research Foundation. 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.

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[2] The abbreviations used are: EGFR, epidermal growth factor receptor; IAP, inhibitor of apoptosis; EGF, epidermal growth factor; HIF, hypoxia-inducible factor; Doc, docetaxel; HRE, hypoxia-responsive element; 7-AAD, 7-amino-actinomycin D; PI3K, phosphoinositide 3-kinase; siRNA, small interfering RNA; FBS, fetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PE, phycoerythrin; nt, nucleotide(s); MAPK, mitogen-activated protein kinase; VEGF, vascular endothelial growth factor.

under hypoxic conditions (22). Our previous study results have shown that survivin gene transcription is increased in hypoxic tumor cells (23). Due to the combined results of these studies, we speculated that the observed EGFR signaling- induced survivin gene expression might be mediated by transcriptional activity of HIF-1α in an oxygen-independent manner.

In this study, we examined the effects of EGFR activation on the apoptotic response and survivin gene expression in human breast cancer cells. We found that EGF stimulation increases survivin gene expression specifically in breast cancer but does not in normal breast cells. Up-regulation of survivin gene expression reduces apoptosis induced by the chemotherapeutic drug docetaxel. We also discovered that in breast cancer cells, EGF up-regulates the level of HIF-1α and that by down-regulation of HIF-1α using HIF-1α siRNA, we could significantly decrease those EGF-induced levels of survivin expression. Thus, cross-talk or a feedback loop between EGFR activation and HIF-1α expression is implied. Furthermore, we demonstrated direct binding of HIF-1α to the survivin promoter, which strongly suggests that EGF-activated survivin gene expression is indeed mediated by induction of transcriptional activity of HIF-1α under normoxic conditions.

MATERIALS AND METHODS

Cell Lines—Normal immortalized human mammary epithelial cell line MCF-10A and breast cancer cell lines SK-BR-3, MDA-MB-231, and MCF-7 were obtained from the ATCC (Manassas, VA). SK-BR-3 and MDA-MB-231 cells were maintained in RPMI 1640, and MCF-7 cells were maintained in Dulbecco's modified Eagle's medium/F-12 medium (50:50; Mediatech) supplemented with 10 μg/ml of insulin. All of the above media were supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT) as well as 2 mM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin (Mediatech Herndon, VA). MCF-10A cells were cultured in Dulbecco's modified Eagle's medium/Ham's F-12 medium supplemented with 20 ng/ml EGF, 500 ng/ml hydrocortisone, 100 ng/ml cholera toxin, 10 μg/ml insulin, 2 mM L-glutamine, and 5% FBS.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) Cell Proliferation Assay—To measure the effects of EGF alone or in combination with docetaxel on breast cancer cells, 8×10^3 cells/well of MCF-7 and SK-BR-3 cells were plated in 96-well plates and cultured in the medium containing EGF and/or docetaxel (Aventis Pharma, Bridgewater, NJ) in various combinations of the drugs for 3 days. The percentage of viable cells in each well was examined by an MTT cell proliferation assay (Sigma), and the remaining viable cells were determined using Spectra Max Plus (Molecular Devices, Sunnyvale, CA).

Transfection—Plasmids expressing a full-length HIF-1α cDNA gene were provided by Dr. Hua Zhong (Emory University, Atlanta, GA). The control empty pcDNA3 or pHIF-1α plasmids were transfected into cultured tumor or normal cell lines in 6-well tissue culture plates using Lipofectamine 2000 (Invitrogen). Some groups of the plasmid-transfected cells were treated with docetaxel in the absence or presence of 100 ng/ml human EGF. Twenty-four hours after transfection, the cells were collected for Western blot analysis to determine the levels of HIF-1α and survivin proteins or FACScan analysis for the percentage of the apoptotic cells.

Apoptosis Assay—Cellular apoptosis was determined using Annexin V-phycoerythrin (PE) and 7-amino-actinomycin D (7-AAD) (BD Biosciences). SK-BR-3 and MCF-7 cells were treated for 3 days with 25 or 50 nM of docetaxel in the absence or presence of 100 ng/ml EGF. Floating and adherent cells were labeled with Annexin V-PE and 7-AAD and then analyzed by FACScan (BD Biosciences) to determine the percentage of apoptotic cells.

Real Time Reverse Transcription-PCR—Total RNAs were isolated using the RNA Bee kit (Tel-test, Friendswood, TX). Each 2-μg sample of RNA was amplified with the Omniscript RT kit using an oligo(dT) primer (Qiagen Inc., Valencia, CA) to generate 20 μl of cDNAs. A 1–2-μl sample of the cDNA was then quantified by real time PCR using primer pairs for survivin or β-actin with SYBR Green PCR Master mix. Real time PCR was performed using the ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA). The primer pairs for detection of survivin gene expression were as follows: survivin forward, 5′-TCCACTGCCCCACTGAGAAC-3′; surviving reverse, 5′-TGGCTCCCAGCCTCCA-3′. These amplify a 77-nt PCR product located at nt 130–206 of the survivin mRNA. Amplification of the β-actin gene was used as an internal control for real time reverse transcription-PCR. The primer pair for the β-actin gene was as follows: forward, 5′-AAAGACCTGTACGCCAACACAGTGCTGTCTGG-3′; reverse, 5′-CGTCATACTCCTGCTTGCTGATCCACATCTGC-3′, which generates a 219-nt PCR product from nt 870 to 1089 of the β-actin mRNA sequence. The quantity of PCR product generated from amplification of the survivin gene was standardized using the quantity of β-actin product for each sample to obtain a relative level of gene expression.

Western Blot Analysis—For the various treatments described, cells were cultured in medium containing 10% serum and then changed to medium containing 2% serum or no serum when treated with 100 ng/ml human EGF (Invitrogen) and/or docetaxel. Inhibitors for PI3K (LY294002), MAPK (PD98059), and EGFR (AG1478) were obtained from Calbiochem. At the end of the assay, cells were lysed in lysis buffer, and Western blot analysis was performed as previously described (18). The detection antibodies for phosphoserine 473 Akt, p44/p42 MAPK, XIAP, and cleaved caspase-3 were from Cell Signaling Technology Inc. (Beverly, MA). Goat anti-human survivin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and mouse monoclonal anti-β-actin antibody (Sigma) were also used.

Caspase Activity Assay—Cells were treated with or without docetaxel, in the absence or presence of 100 ng/ml human EGF, for a period of 2 days. Cells were collected, and their lysates were examined for caspase-3-like activity using a specific substrate, Ac-DEVD-7-amino-4-trifluoromethylcoumarin, which detects the activities of caspase-3, caspase-7, caspase-10, or caspase-9 activity using Ac-LEHD-7-amino-4-trifluoromethylcoumarin, according to a standard protocol (Calbiochem). The results were measured using a Spectra Max fluorescence microplate reader (Molecular Devices). For each experiment, control groups with specific caspase inhibitors, including caspase-3 inhibitor (benzyloxycarbonyl-DEVD-aldehyde; BD Biosciences) and caspase-9

inhibitor (benzyloxycarbonyl-LEHD-aldehyde; Alexis Biochemicals, San Diego, CA), were done to ensure the specificity of the assay.

Chromatin Immunoprecipitation (ChIP) Assay—To demonstrate direct binding of HIF-1α protein to the survivin promoter region in EGF-stimulated cancer cells, the SK-BR-3 cells were treated with 100 ng/ml EGF for 4 h. After cross-linking chromatin with proteins by 1% of formaldehyde, the assay was performed using a ChIP assay kit from Upstate (Charlottesville, VA), according to the company's protocol. A monoclonal anti-HIF-1α antibody (BD Biosciences) was added to precipitate the protein-chromatin complexes. A PCR primer pair for amplification of a 230-nt survivin promoter fragment is as follows: forward primer, 5′-GCGTTCTTTGAAAGCAGT-3′; reverse primer, 5′-ATCTGGCGGTTAATGGCG-3′.

Modified McKay Assay—A core survivin promoter containing 269 nt of the 5′-flanking region of the survivin gene was cut out from a survivin promoter-luciferase reporter plasmid (pluc-cyc1.2) (23). A DNA fragment containing six repeats of the hypoxia-responsive element (HRE) of vascular endothelial growth factor (VEGF) was also cut from pBI-GL V6R plasmid (23) as a positive control. The purified promoter fragments were 5′-end-labeled with [γ-^{32}P]dATP using T4 polynucleotide kinase (New England Biolabs, Ipswich, MA). The radiolabeled promoter fragments were then incubated with nuclear extracts obtained from breast cancer cells treated with or without 100 ng/ml EGF in a 1× binding buffer containing 10 mM Tris-HCl (pH 8.0), 150 mM KCl, 0.5 mM EDTA, 0.1% Triton X-100, 12.5% glycerol, and 0.2 mM dithiothreitol on ice for 1 h. Protein A-Sepharose beads conjugated with monoclonal anti-HIF-1α antibody were added to precipitate the protein-DNA complexes. After washing for three times with a 1× TE buffer containing 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA, the final pellet was resuspended in a 0.1 M $NaHCO_3$, 0.1% SDS, 1× sample buffer and heated to 65 °C for 2 min. Then the protein A-Sepharose beads were pelleted, and the supernatant was analyzed by electrophoresis on a 1.4% agarose gel followed by autoradiography to determine whether the EGF-induced HIF-1α protein had bound to the survivin promoter DNA fragments.

Luciferase Assay—The effect of EGF stimulation on survivin promoter activity was determined in breast cancer cells after transfecting pluc cyc-1.2 plasmid, a survivin promoter-luciferase reporter plasmid (23), for 24 h, followed by EGF treatment for 4 h. A pRL-SV-40 plasmid that expresses a *Renilla* luciferase gene (Promega, Madison, WI) was also cotransfected for all studies as an internal control. To determine whether direct overexpression of the HIF-1α gene activates the survivin promoter, breast cancer and normal cells were cotransfected with pluc cyc-1.2 and pHIF-1α plasmids for 24 h. The ability of direct activation of HRE by EGF-induced HIF-1α was demonstrated using a MDA-MB-231 cell line, stably transfected with pBI-GL V6R plasmid containing six copies of HRE fragments of the VEGF gene and a firefly luciferase reporter gene (24) (provided by Dr. Hyunsuk Shim at Emory University). The transfected cells or MDA-MB-231 stable cells were cultured in the absence or presence of 100 ng/ml of EGF for 45 min. After various treatments as described above, the promoter activity of the cell lysates was determined using a dual luciferase activity assay kit from Promega.

Production of Adenoviral Vectors Expressing HIF-1α siRNA—Plasmid vectors containing either HIF-1α siRNA sequence 5′-CAGTGGATTACCACAGCTGA-3′ or survivin siRNA 5′-GGCTGGCTTCATCCACTGCCC-3′ were generated by cloning the synthesized oligonucleotide into pSilencer 2.1-U6 Neo plasmid (Ambion Inc., Austin, TX). Control pSilencer 2.1-U6 Neo plasmid vector containing a scrambled siRNA sequence, 5′-ACTACCGTTGTTATAGGTGT-3′, was obtained from the company. Adenoviral vectors expressing siRNA to HIF-1α and control siRNA were produced by cloning a HindIII-EcoRI fragment from pSilencer 2.1-U6 Neo plasmid, which contains a U6-promoter-siRNA cassette (HIF-1α or control siRNA), to a pcDNA3 HindIII-EcoRI site and then transferring the NotI-HindIII fragment from the pcDNA3 to the pAdtrack plasmid (25). After performing homologous recombination with an AdEasy adenoviral DNA backbone, the viral vectors were produced by transfecting into the human embryonic kidney cell line 293 (ATCC). The AdEasy system was provided by Dr. Bert Vogelstein at The Johns Hopkins University. Additional vector amplification was also performed in the 293 cell line. High titer viral vectors were purified by centrifugation and CsCI banding.

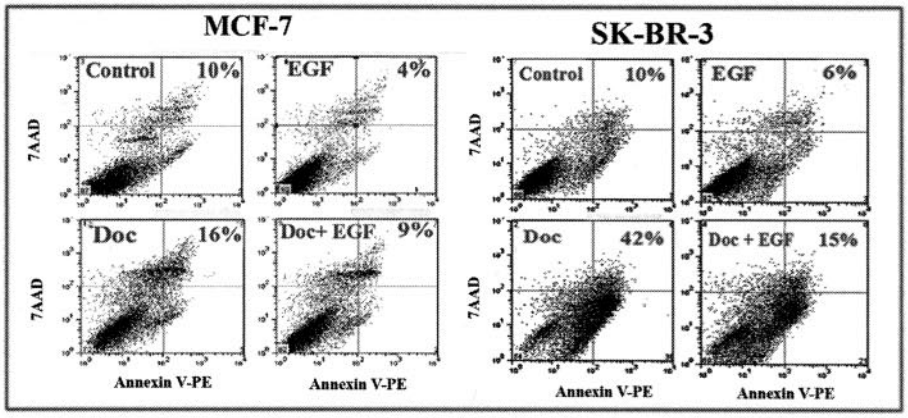
To determine the effect of down-regulation of HIF-1α on tumor cells, 2×10^5 tumor cells/well were first cultured in 6-well plates and then transduced with Ad HIF-1α siRNA or Ad Control siRNA vectors. 24 h after transduction, cells were cultured in serum-free medium overnight and treated with (or without) EGF for 45 min. Some groups received cotransfection of pSilencer HIF-1α siRNA and pHIF-1α plasmids to determine whether overexpression of HIF-1α gene could rescue HIF-1α siRNA-mediated down-regulation of survivin in EGF-treated cells. Last, collected cell lysates were examined for the levels of both HIF-1α and survivin by Western blot analysis.

To examine the effect of EGF-induced HIF-1α and survivin on apoptotic death in breast cancer cells after docetaxel treatment, 5×10^4/well of MCF-7, SK-BR-3, and MDA-MB-231 cells were plated in 12-well tissue culture plates. Cells were then transduced with AdControl siRNA or AdHIF-1α siRNA vector. Some treatment groups were transfected with psilencer 2.1 U6-survivin siRNA plasmids. Following a 2-day treatment with docetaxel, the percentage of remaining viable cells was examined using an MTT cell proliferation assay.

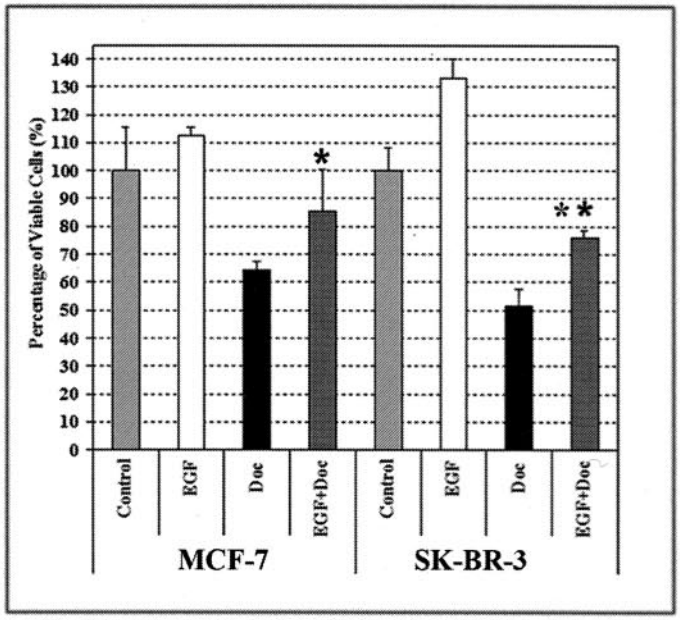
RESULTS

EGF Stimulation Decreased Docetaxel-induced Apoptotic Response in Breast Cancer Cells—In order to determine the effect of activation of the EGFR signaling pathway on the apoptotic response in human cancer cells, we treated the human breast cancer cell lines SK-BR-3 and MCF-7 with a chemotherapeutic drug, docetaxel, in the absence or presence of human recombinant EGF. Previous reports have shown that SK-BR-3 cells have high levels of EGFR and Her-2/neu expression, whereas MCF-7 cells express low levels of EGFR and Her-2/neu (26). We chose those two cell lines to determine whether the levels of the EGF receptors present affect the EGF-induced apoptotic response. After treating the cancer cells with docetaxel in

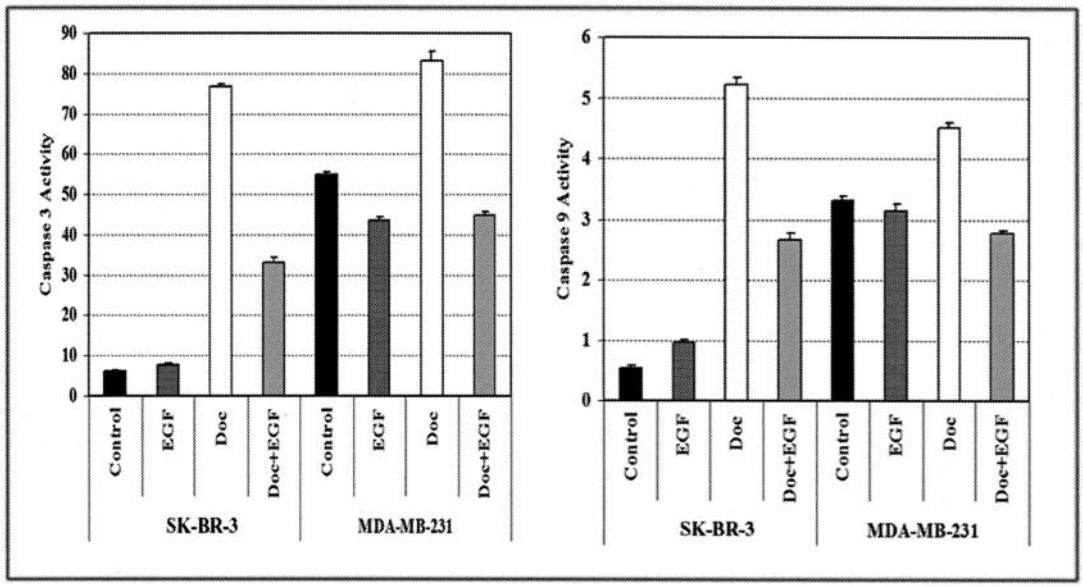
A. FACScan analysis of percentage of apoptotic cells



B. MTT cell proliferation assay



C. Caspase activity assay



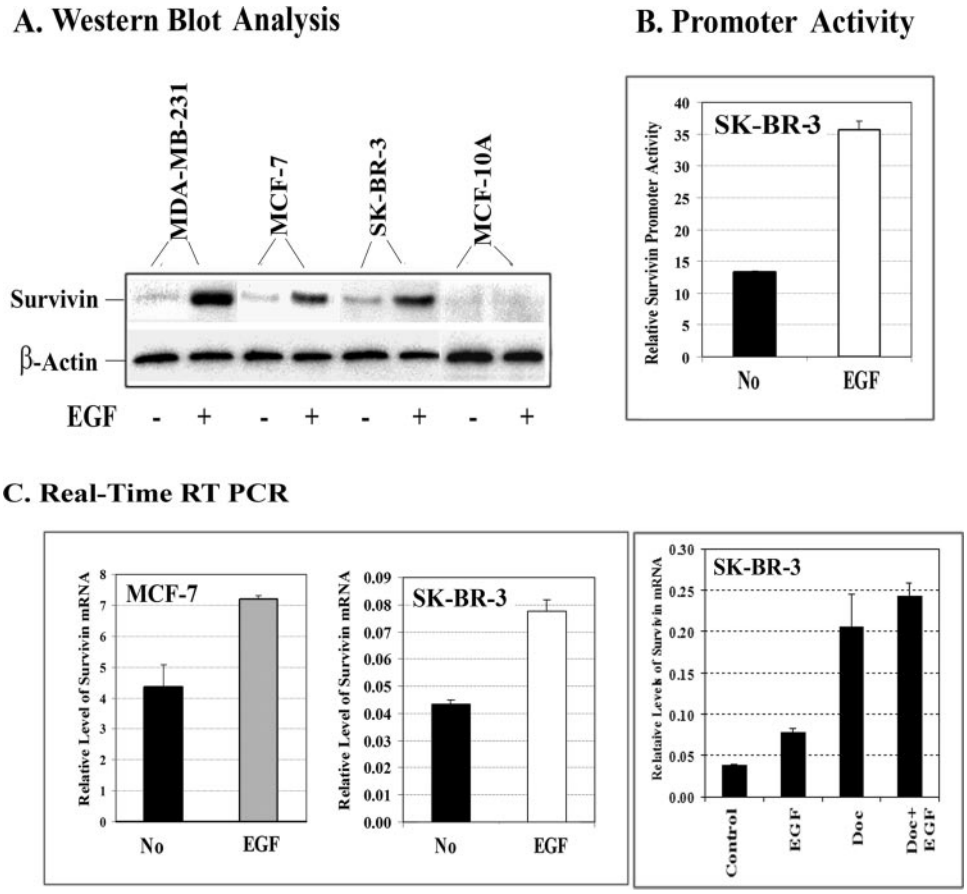


FIGURE 2. **EGF stimulation increases the levels of survivin expression in breast cancer cells.** Cells were cultured in 2% FBS medium overnight, followed by treatment with human EGF at 100 ng/ml for 45 min to 4 h. *A*, examination of the level of survivin protein (16.5 kDa) in breast cancer and normal cell lines following EGF stimulation for 45 min by Western blot analysis. *B*, EGF stimulation activates survivin promoter activity. SK-BR-3 cells were transfected with a survivin promoter luciferase reporter plasmid (pluc cyc1.2) for 24 h and then treated with EGF for 4 h. The cell lysates were collected for the luciferase assay. *C*, total RNAs were isolated and analyzed by real time reverse transcription-PCR, as described under "Experimental Procedures," to determine changes in the level of survivin mRNA after EGF treatment. EGF stimulation increases the levels of survivin mRNA in both MCF-7 and SK-BR-3 cell lines. Additionally, docetaxel treatment could also up-regulate survivin gene expression, and its expression level is further enhanced in the presence of EGF. The relative level of survivin mRNA is a ratio of the quantity of survivin to β-actin PCR products. A mean value of three repeat samples is shown.

the absence or presence of EGF for 2 days, the percentage of apoptotic cells was determined using Annexin V-PE and 7-AAD staining followed by FACScan analysis. We found that the presence of EGF markedly decreased the percentage of apoptotic cell death induced by docetaxel. EGF treatment reduced the percentage of apoptotic cells present from 16 to 9% for MCF-7 cells and from 42 to 15% for SK-BR-3 cells (Fig. 1*A*). Additionally, we found that EGF decreased the rate of spontaneous apoptosis in both cell lines (Fig. 1*A*). Consistent with the results from the apoptosis assay, we found that EGF stimulation significantly decreased sensitivity of SK-BR-3 and MCF-7 cells to docetaxel as detected by the MTT cell proliferation assay (Fig. 1*B*). Furthermore, we found that EGF-induced resistance to apoptosis was detected in breast cancer cell lines expressing both a high level of EGFR (SK-BR-3) and a low level of EGFR (MCF-7).

To elucidate the mechanism of apoptosis resistance, we examined changes in regulation of apoptotic signals in EGF-stimulated cancer cells. Since caspase activation is a critical step in induction of apoptotic cell death, we first examined caspase-3-like and caspase-9 activities in MDA-MB-231 and SK-BR-3 cell lines 2 days after docetaxel treatment. As expected, docetaxel increased these caspase activities in both tumor cell lines. However, in the presence of EGF, the docetaxel-induced caspase-3-like and caspase-9 activities were markedly inhibited (Fig. 1*C*), suggesting that the EGF-induced resistance to apoptosis by docetaxel is mediated through inhibition of caspase activity.

EGF Up-regulates Survivin Expression in Human Breast Cancer Cells—It is well known that the IAPs block apoptotic signaling through inhibition of caspase activity (27). To determine whether IAPs play a role in inhibition of caspases in tumor cells having an activated EGFR signal, we examined changes in the level of an important IAP family protein, survivin, in both breast cancer and normal cell lines following EGF treatment. Our results from Western blot analysis demonstrated that EGF stimulation did increase the level of survivin protein in three breast cancer cell lines, including MDA-MB-231, SK-BR-3, and MCF-7 (Fig. 2*A*). Interestingly, survivin was not detected in the immortalized normal human mammary epithelial cell line MCF-10A, and EGF treatment failed to induce any survivin expression (Fig. 2*A*). To

FIGURE 1. **Activation of the EGFR signaling pathway increases resistance to docetaxel-induced apoptosis in human breast cancer cells.** *A*, apoptosis assay using Annexin V staining and FACScan analysis. MCF-7 and SK-BR-3 cells were cultured for 2 days in medium containing 100 ng/ml EGF in the absence or presence of 25 nM (SK-BR-3) or 50 nM (MCF-7) docetaxel and 2% FBS. The cells were then stained with Annexin V-PE and 7-AAD and analyzed by FACScan. The percentages of apoptotic cells are shown in the *upper panel*, including both early (Annexin-V^+, 7-AAD^-) and late stage ($Annexin^+$, 7-AAD^+) apoptotic cells. *B*, EGF stimulation decreases the sensitivity of human breast cancer cells to docetaxel treatment. Cancer cells cultured in 96-well plates were treated for 3 days with the reagents as described above. The percentage of remaining viable cells was determined using a MTT cell proliferation assay. The results of this assay are expressed relative to the cell density of untreated cells. Each value in the graph represents the mean ± S.D. of five repeat samples. An *asterisk* indicates a significant difference when compared with the control value (*, $p < 0.05$; **, $p < 0.001$). *C*, activation of the EGFR signal inhibits docetaxel-induced caspase activity. The cells were treated with docetaxel in the absence or presence of EGF for 3 days. 25 μg of proteins from their total cell lysates were examined for caspase-3-like or caspase-9 activity using substrates specific for these caspases. Fluorescence intensity was measured by a fluorescence microplate reader. The *numbers* in the figure represent mean values ± S.D. from three repeat groups.

determine the mechanism of survivin up-regulation, we further examined survivin promoter activity and the level of survivin mRNA in EGF-treated cells. Using a luciferase reporter plasmid containing a 269-nt survivin core promoter fragment, we found a marked increase in survivin promoter activity in EGF-stimulated SK-BR-3 cells (Fig. 2*B*). Consistent with the level of survivin protein, survivin mRNA was elevated in the EGF-treated SK-BR-3 and MCF-7 cells (Fig. 2*C*) detected by real time reverse transcription-PCR. Additionally, we found that treatment of the cells by docetaxel increases the levels of survivin gene expression. A combined effect of docetaxel and EGF treatment further increased the level of survivin gene expression (Fig. 2*C*).

Determination of Signaling Pathways Responsible for EGF-induced Survivin Gene Up-regulation—It is well known that EGFR signaling leads to the activation of both PI3K/AKT and MAPK pathways. We examined the roles of these pathways in the EGF-induced survivin expression in breast cancer cells. As expected, we observed increases in the levels of p-AKT and p44/42 MAPK in EGF-treated cells. Although the level of survivin expression was also up-regulated in those cells, the level of another IAP protein, XIAP, remained unaffected by EGF treatment (Fig. 3).

After pretreatment of the cancer cells with either the PI3K inhibitor LY294002 or the MAPK inhibitor PD98059, we were able to achieve marked reductions in the levels of p-AKT and p44/42 MAPK present in EGF-treated SK-BR-3 and MCF-7 cells. Furthermore, we found that inhibition of p-AKT completely prevented EGF-up-regulated survivin expression in both the SK-BR-3 and MCF-7 cell lines (Fig. 3). However, we believe that activation of AKT is probably not required for maintaining a basal level of survivin expression in these cancer cells, since similar levels of survivin expression were detected in both control cells and cells treated only with PI3K inhibitor (Fig. 3).

EGF stimulation also increased the level of p44/42 MAPK in SK-BR-3 and MCF-7 cell lines, with SK-BR-3 cells showing a very high level of up-regulation (Fig. 3). Blocking the MAPK pathway with PD98059 inhibited the level of p44/42 MAPK in both cell lines. The fact that a significant reduction of survivin was seen in the MAPK-blocked, EGF-treated SK-BR-3 cells suggests that both the AKT and MAPK pathways are involved in EGF-induced survivin up-regulation (Fig. 3).

After treating the cancer cells with EGF in the absence or presence of the EGFR inhibitor AG1478, we found that AG1478 counteracted the effects of EGF on survivin expression in both SK-BR-3 and MCF-7 cells. However, AG1478 treatment alone had no significant effects on the basal levels of survivin protein in these cells (Fig. 3).

EGF-induced HIF-1α Up-regulates Survivin Expression in Breast Cancer Cells—Previous reports have shown that activation of EGFR signaling can induce HIF-1α in human tumor cells under normoxic conditions (20, 21). We previously found that survivin promoter activity is up-regulated in hypoxic tumor cells (23). To determine if the EGFR signaling-induced HIF-1α is a mediator for survivin gene expression, we first examined the changes in both HIF-1α and survivin levels in breast cancer cells after EGF treatment. Western blot analysis did show that

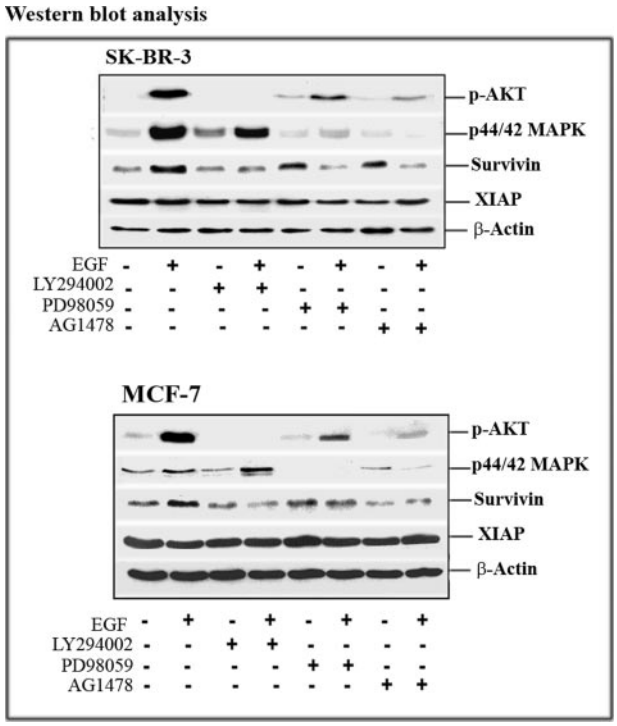


FIGURE 3. **Analysis of signal transduction pathways regulating EGF-induced survivin gene expression.** Cells were pretreated with 10 μM LY294002, PD98059, or AG1478 for 24 h in culture medium containing 2% FBS medium. Then 100 ng/ml EGF was added for 45 min. Total cell lysates (50 μg of protein) were examined by Western blot analysis to determine the levels of phosphorylated serine 473 Akt (*P-Ser473 Akt*), phosphorylated p44/p42 MAPK, survivin, and XIAP. In both breast cancer cell lines, EGF stimulation activated the AKT and MAPK signal pathways, and blocking the PI3K/AKT signal prevented EGF-induced survivin expression. On the other hand, inhibition of the EGFR signal pathway with AG1478 reduced the level of survivin protein. The level of XIAP was not affected by EGF stimulation or the PI3K/AKT, MAPK, and EGFR inhibitors.

EGF treatment induces high levels of both HIF-1α and survivin expression in breast cancer cells (Fig. 4*A*). We further found that transfection of the plasmids expressing a HIF-1α gene into cells increases the levels of HIF-1α in both breast cancer SK-BR-3 and normal breast MCF-10A cell lines. However, up-regulation of the level of survivin protein is only detected in SK-BR-3 cells. This result provides direct evidence supporting HIF-1α-mediated survivin up-regulation. Additionally, the absence of survivin expression in HIF-1α-transfected MCF-10 A cells suggests that intrinsic transcriptional inhibitory factors prevent the basal level as well as HIF-1α-induced survivin transcription in normal cells.

We then transduced the cells for 48 h with adenoviral vectors expressing either HIF-1α siRNA or control siRNA. Next, the transduced cells were treated with or without EGF for 45 min before they were collected. The resulting cell lysates were analyzed by Western blot for the levels of HIF-1α and survivin. We found that overexpression of HIF-1α siRNA markedly reduced EGF-induced HIF-1α. Meanwhile, EGF-induced up-regulation of survivin expression was completely blocked by the HIF-1α siRNA (Fig. 4*B*). To further demonstrate the critical role for HIF-1α in regulating survivin gene transcription, we co-expressed HIF-1α siRNA and HIF-1α gene plasmids in MCF-7 cells. 24 h after transfection, the cells were treated with EGF and collected for Western blot analysis. In the presence of excessive amounts of HIF-1α,

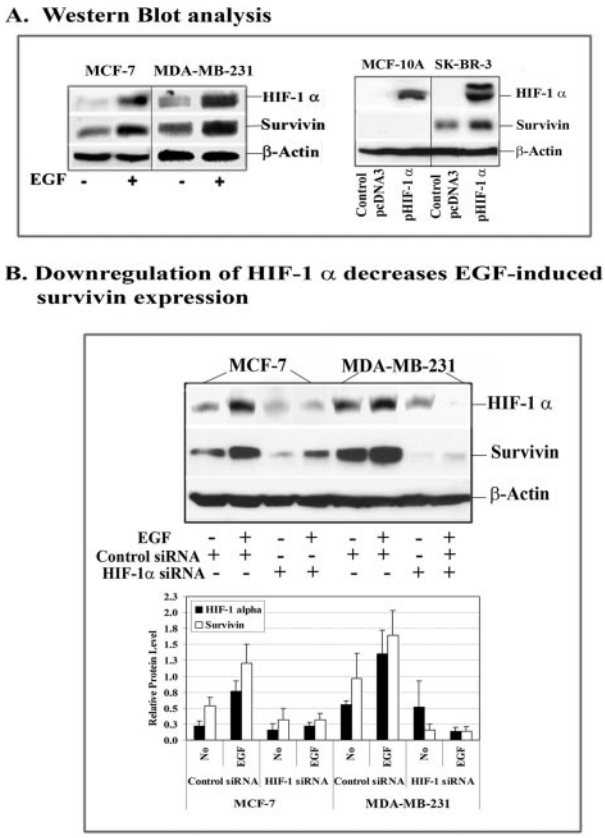


FIGURE 4. **Determination of the role of HIF-1α in survivin up-regulation in normoxic breast cancer cell lines after EGF treatment.** *A*, Western blot analysis showed that EGF stimulation induces high levels of both HIF-1α and survivin expression. Furthermore, transfection of HIF-1α gene-expressing plasmids increases the level of survivin in breast cancer SK-BR-3 cells but not in normal breast epithelial MCF-10A cells. *B*, overexpression of HIF-1α siRNA by transduction of the cells with a multiplicity of infection of 100 plaque-forming units/cell of adenoviral vectors inhibited EGF-induced as well as basal levels of HIF-1α expression in MCF-7 and MDA-MB-231 cells. The levels of survivin protein were also markedly reduced. The relative levels of HIF-1α and survivin proteins in the cells treated with control and HIF-1α siRNAs were quantified by measuring the density of protein bands using Scion Image (Scion Corp., Frederick, MD). HIF-1α or survivin protein level was calculated from a ratio of the density relative to β-actin for each sample. The *number* in the figure represents the mean value of three repeat Western blots. *C*, overexpression of the HIF-1α gene rescues HIF-1α siRNA-induced survivin down-regulation. Breast cancer MCF-7 cells were co-transfected with HIF-1α siRNA and pHIF-1α plasmids for 24 h, and the levels of HIF-1α and survivin proteins were examined by Western blot analysis. In the presence of an excessive amount of exogenous HIF-1α, expression of siRNA to HIF-1α could no longer inhibit the level of EGF-induced survivin expression. Although transfection of pSilencer HIF-1α siRNA plasmids showed less inhibitory effects on the levels of HIF-1α and survivin proteins, compared with the effect produced by using AdHIF-1α siRNA seen in this figure (*B*), it is appropriate for the design of this study, since the HIF-1α gene is expressed from a plasmid vector.

expression of HIF-1α siRNA could no longer block EGF-induced survivin down-regulation (Fig. 4*C*), supporting direct involvement of HIF-1α in EGF-activated survivin expression.

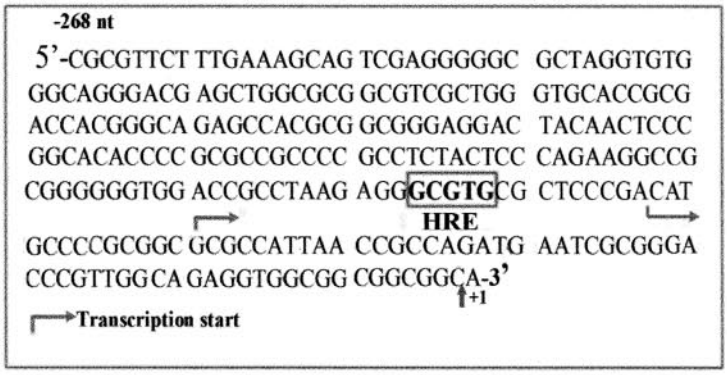
HIF-1α Activates Gene Transcription through Direct Interaction with the Survivin Promoter—At present, the mechanisms by which cellular factors may regulate survivin gene expression are not fully understood. Our previous study identifies a 269-nt DNA fragment located at the 5′-flanking region of the survivin gene that is able to activate tumor-specific gene transcription, exhibiting enhanced promoter activity under hypoxic conditions (23). To determine whether the EGF-activated survivin gene transcription is mediated by transcriptional activity of HIF-1α, we first analyzed the survivin core promoter sequences and found a putative HRE, 5′-GCGTG-3′, located at nt −81 to −85 of the 5′-flanking region of the survivin gene (Fig. 5*A*). To demonstrate the binding of HIF-1α to the survivin promoter in living cells, we performed a ChIP assay in MCF-7 cells with or without EGF treatment. In the chromatin fraction pulled down by an anti-HIF-1α antibody, we detected a higher level of the survivin promoter PCR fragments in EGF-treated cells than that in control cells (Fig. 5*B*). However, survivin promoter PCR fragments were not found in samples pulled down by a control IgG antibody.

To further confirm direct binding of HIF-1α to the survivin core promoter, we used a modified McKay assay (28) to pull down survivin promoter DNA fragments (Fig. 5*C*). Our results demonstrated that the survivin promoter fragments did bind to the HIF-1α protein and could be pulled down by HIF-1α antibody. EGF-treated cell lysates showed much higher levels of these survivin promoter fragments as compared with control groups in both breast cancer cell lines (Fig. 5*C*). Since there is a moderate basal level of HIF-1α found in those tumor cells, we also detected low to intermediate levels of survivin promoter fragments in our control groups (Fig. 5*C*).

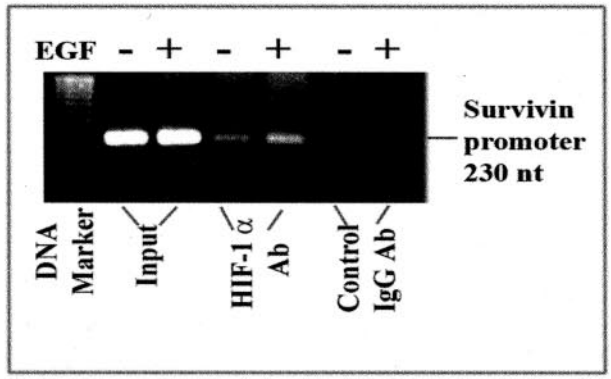
To determine whether the binding of HIF-1α to the survivin promoter actually could activate the promoter activity, we cotransfected pHIF-1α plasmid with survivin promoter reporter pluc cyc1.2 plasmid into breast normal and cancer cell lines. We found that overexpression of the HIF-1α gene markedly increased the survivin promoter activity in breast cancer MCF-7 cells but not in normal breast MCF-10A cells (Fig. 5*D*). Next, we wanted to determine if EGF-induced HIF-1α interacts with the HRE site under normoxic conditions to activate the gene transcription. We used the human breast cancer cell line MDA-MB-231, stably transfected with a luciferase reporter plasmid containing six copies of the HRE fragment of the VEGF gene. These cells were treated with EGF in normoxia, and the resultant luciferase activity was measured in cell lysates. We found that EGF-induced HIF-1α was able to activate the HRE-mediated transcription of the luciferase gene, suggesting that under normoxic conditions, HIF-1α can bind to the HRE site and activate HRE-mediated gene transcription (Fig. 5*D*).

Overexpression of the HIF-1α Gene Inhibits Docetaxel-induced Apoptosis under Normoxic Conditions—To establish a direct link between EGF-induced HIF-1α up-regulation and resistance to apoptosis, we transfected SK-BR-3 cells with pHIF-1α plasmids and then treated the cells with docetaxel in the absence or presence of EGF for 24 h. The cells were collected for Annexin V-PE staining and FACScan analysis of the

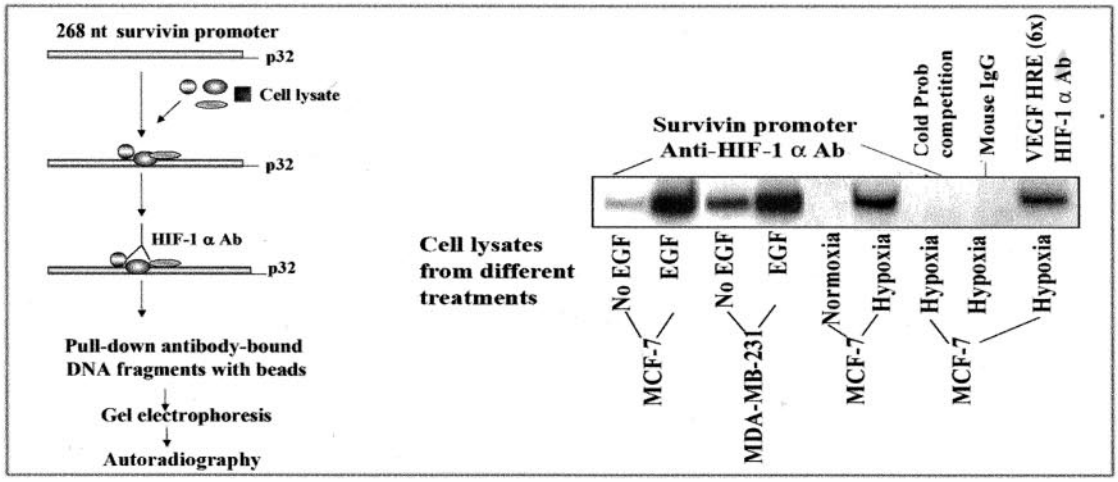
A. DNA sequence of the survivin core promoter



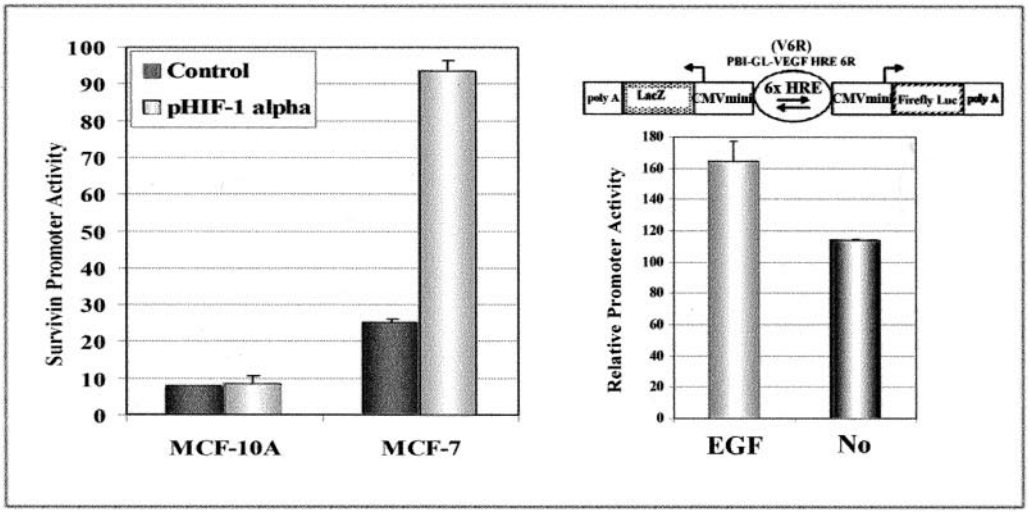
B. CHIP Assay



C. Modified McKay Assay



D. Luciferase activity assay



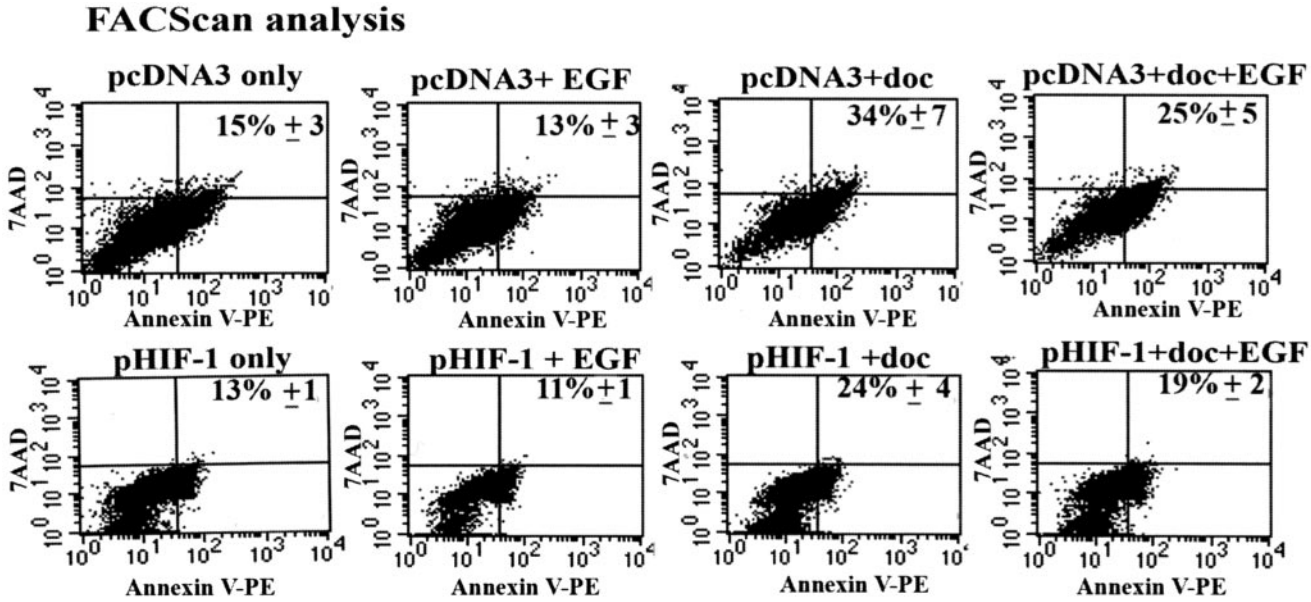


FIGURE 6. **Examination of the effect of overexpression of HIF-1α gene on docetaxel-induced apoptosis by FACScan analysis.** Breast cancer SK-BR-3 cells, transfected with pHIF-1α or control pcDNA3 plasmids for 24 h, were treated with 25 nM docetaxel in the absence or presence of 100 ng/ml of EGF for an additional 24 h. Both floating and adhesive cells were collected for Annexin V-PE and 7-AAD staining followed by FACScan analysis. Expression of the HIF-1α gene reduced the percentage of docetaxel-induced apoptosis. The combination of EGF activation with HIF-1α gene expression further increased the inhibition effects. Since the cells were cultured in the medium containing 2% of FBS due to EGF treatment, we observed a relatively high level of the basal level of apoptosis in pcDNA3 control plasmid-transfected cells. The *number* in the figure is the mean value ± S.D. from three repeat samples.

percentage of apoptotic cells. We found that overexpression of the HIF-1α gene reduced the percentage of docetaxel-induced apoptotic cells from 34 to 24%. The combination of overexpression of the HIF-1α gene with EGF treatment further enhanced the inhibitory effect on apoptosis (Fig. 6), suggesting that HIF-1α is a key mediator for EGF-induced resistance to apoptosis.

Down-regulation of the Levels of HIF-1α or Survivin Gene Expression Reverses EGF-induced Resistance to Apoptosis—To further demonstrate that EGF-induced resistance to apoptosis is indeed the result of up-regulation of HIF-1α, which consequently activates survivin gene expression, we examined the effects of HIF-1α down-regulation, using HIF-1α or survivin siRNA, on overall sensitivity to apoptosis induction. We found that expression of HIF-1α siRNA using adenoviral vectors in the no treatment control and the EGF-treated SK-BR-3 cells induced 8–10-fold increases in caspase-3-like activity as compared with control siRNA vector-containing cells (Fig. 7*A*). Furthermore, the presence of HIF-1α siRNA enhanced 2.5 times the level of active caspase-3 induced by docetaxel treatment. Although co-treatment with EGF reduced the docetaxel-induced caspase-3 activity, down-regulation of HIF-1α with siRNA completely reversed the ability of exogenous EGF to induce resistance to docetaxel (Fig. 7*A*). The changes in caspase-3 activity among the different treatment groups were also correlated with the percentage of apoptotic cell death in the cells. As shown in Fig. 7*B*, overexpression of HIF-1α siRNA significantly increased apoptotic cell death in docetaxel-treated cells by 18% ($p = 0.006$, Student's t test). EGF treatment protected the cells from docetaxel-induced apoptosis and increased the percentage of viable cells from 37% in the group without EGF to 57% in EGF-treated cells (Fig. 7*B*, $p = 0.01$). Importantly, overexpression of HIF-1α siRNA significantly reduced the EGF-induced resistance to docetaxel, since there was a 44% decrease in the percentage of viable cells in the AdHIF-1α siRNA-transduced cells when compared with control siRNA-transduced cells ($p < 0.0001$, Student's t test). We also examined the effect of direct inhibition of survivin gene expression on EGF-induced resistance to apoptosis. We found that expression of survivin siRNA significantly sensitized SK-BR-3 cells to docetaxel treatment (Fig. 7*C*, $p = 0.0004$, Student's t test). Down-regulation of survivin expression also increased docetaxel-induced cell death even in the presence of EGF stimulation (Fig. 7*C*, $p =$

FIGURE 5. **Determination of the mechanism of HIF-1α-activated survivin gene expression.** *A*, DNA sequence of the survivin core promoter. A putative HRE site, located at −81 to −85 nt, is marked in the promoter sequence. *B*, detection of the binding of HIF-1α to the survivin promoter in breast cancer cells using a ChIP assay. SK-BR-3 cells were treated with or without EGF for 4 h. A ChIP assay was then performed. 230-nt PCR products of the survivin promoter were only detectable in the samples pulled down by HIF-1α antibody and not in control IgG samples. A marked higher level of the survivin promoter PCR products was seen in the EGF-treated sample compared with the no treatment sample. *C*, modified McKay assay for detection of the binding of HIF-1α to the survivin promoter. Cancer cells were treated with or without EGF for 45 min, and nuclear extracts were incubated with 269-nt radiolabeled survivin promoter fragments, and the resulting HIF-1-DNA complexes were pulled down using anti-HIF-1α antibody-conjugated Protein A beads. As shown, a high level of HIF-1α-survivin promoter complexes was found following incubation with nuclear extracts from EGF-treated breast cancer cells. The binding specificity was further demonstrated by the absence of survivin promoter bands in samples with a 10 times excess of unlabeled (cold) survivin promoter fragments or using nonspecific mouse IgG-conjugated Protein A beads. A DNA fragment containing six repeats of the HRE fragments of the human VEGF promoter was used as a positive control. *D*, luciferase activity assay. Overexpression of the HIF-1α gene after co-transfection of pHIF-1α and pluc cyc1.2 increases survivin promoter activity in MCF-7 tumor cells but fails to induce the promoter activity in normal MCF-10A cells. EGF-induced HIF-1α was able to bind to the HRE and activate firefly luciferase gene expression under normoxic conditions, since luciferase activity was increased in the cell lysates from EGF-treated MDA-MB-231 cells stably transfected with pBI-GL-V6R plasmid, which has six repeats of VEGF HRE fragments.

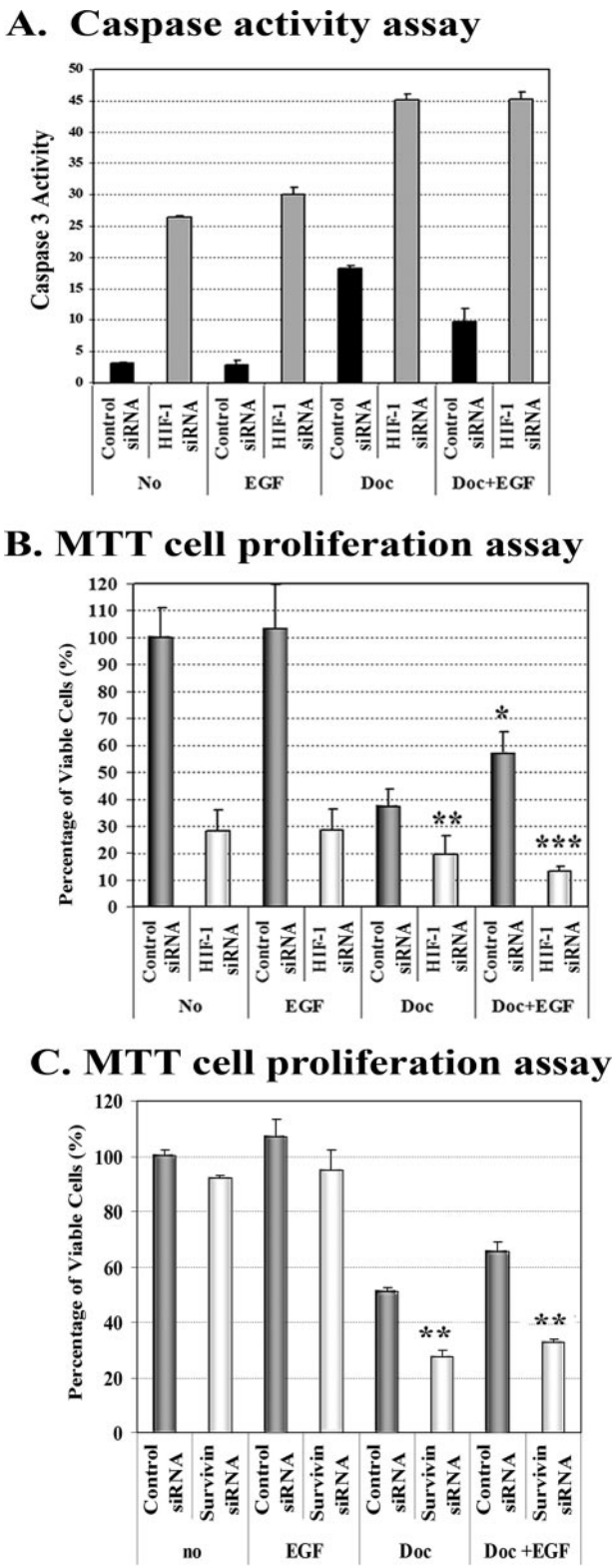


FIGURE 7. **Down-regulation of HIF-1α or survivin gene expression using siRNAs enhances docetaxel-induced apoptosis and reverses EGF signal-mediated resistance to apoptosis.** SK-BR-3 cells were transduced with AdControl siRNA or HIF-1α siRNA vectors for 24 h. The cells were then treated with 25 nM docetaxel, in the absence or presence of 100 ng/ml EGF for an additional 24 h. *A*, caspase activity was examined in the cell lysates using a caspase-3-like specific substrate. The *numbers* in the figure represent mean values of three repeat groups. Similar results were obtained from repeat experiments. As shown, inhibition of HIF-1α expression increases caspase-3 activity in tumor cells for all treatment groups. Additionally, EGF-induced inhibition of caspase-3 activity in docetaxel-treated cells was reversed by the down-regulation of HIF-1α with siRNA. *B*, results from the MTT cell proliferation assay show that down-regulation of HIF-1α significantly increased docetaxel-induced cell death and completely blocked EGF-induced resistance to docetaxel treatment in SK-BR-3 cells. On the other hand, a significant reduction of docetaxel-induced cell death is found in EGF-treated, control siRNA vector-transduced cells. *C*, transfection of plasmids expressing survivin siRNA significantly increases docetaxel-induced cell death and counteracts the protection effect of EGF stimulation. The absorbance value of cells transduced with AdControl siRNA vector or plasmids expressing control siRNA serves as a relative cell number of 100%. Each value in the *bar graph* represents a mean value ± S.D. of four repeat samples. *, $p < 0.01$; **, $p < 0.001$; ***, $p < 0.0001$.

0.0003, Student's *t* test). Therefore, our results strongly supported a role for HIF-1α-mediated survivin up-regulation in EGF-induced resistance to apoptosis in breast cancer cells.

DISCUSSION

Overexpression of EGFR and activation of the EGFR signaling pathway are found in a high percentage of human breast cancer tissues and have been associated with poor prognosis for the patients (3, 5, 7, 29). Although EGFR signaling, including activation of EGFR and Her-2/neu, is known to confer resistance to apoptosis in cancer cells (30, 31), the exact mechanism by which EGFR signaling regulates the apoptotic pathway has yet to be elucidated.

In this study, we have examined the effects of the activation of EGFR signaling on the apoptotic response in human breast cancer cell lines. We have shown that EGF stimulation reduces the sensitivity of breast cancer cells to docetaxel, a chemotherapeutic drug that is commonly used for treatment of breast cancer. We have further demonstrated that decreased drug sensitivity is the result of inhibition of the activities of caspase-3 and caspase-9, which consequently causes the observed resistance to apoptosis in EGF-treated breast cancer cells.

IAP proteins, including survivin and XIAP, are known to act as caspase inhibitors, blocking caspase activation and further inhibiting activities of the activated caspases (27, 32, 33). Recent studies, including ours, have shown that activation of EGFR leads to up-regulation of survivin expression (11–14). Additionally, it has been demonstrated that overexpression of Her-2/neu and EGFR genes increases survivin expression and apoptosis resistance in breast cancer cell lines (11). Results from examination of 195 cases of human invasive breast cancer tissues indicated that up-regulation of survivin by EGFR signaling is not just a phenomenon seen in cancer cell lines *in vitro*. In fact, about 80% of the tumors were also found to be positive for survivin, and its expression level was correlated with co-expression of Her-2/neu and EGFR (11).

Our current study has further demonstrated that EGF stimulation induces survivin expression at both the mRNA and protein levels in breast cancer cells but not in normal mammary epithelial cells. It is possible that the much higher levels of EGFR seen in many breast cancer cell lines as compared with normal cells contribute in part to the difference in the response to EGF treatment. However, lower levels of EGFR may not be the only reason for these differences, since the MCF-7 cell line expresses a low level of EGFR but exhibits a similar effect as two other tumor cell lines expressing high levels of EGFR and/or Her-2/neu (34). The intrinsic properties of breast cancer cells may determine the responsiveness to activation of EGFR and up-regulation of survivin gene

expression, since survivin is not detected in normal breast tissues, and cultured normal mammary epithelial cells either express a very low level or lack survivin gene expression (23).

It has been shown that the XIAP, a strong caspase inhibitor, prevents activation of caspase-3 by blocking caspase-9 activity as well as binding to activated caspase-3 (35). Interestingly, we found that EGFR activation did not affect the level of XIAP. Thus, up-regulated survivin expression in EGF-treated cells may play a key role in inhibiting caspase activity and creating resistance to apoptosis. Although the role of survivin in blocking caspase-9 activity has been elucidated, there is no structural evidence showing a direct interaction between survivin and caspase-3 (33). It is possible that survivin may interact with XIAP or other proteins, such as p21, to enhance the inhibitory effect on caspase-3 (36, 37).

A recent study also showed that treatment of human ovarian cells with paclitaxel transiently induced EGFR phosphorylation and PI3K activation, resulting in an increase in the level of survivin expression. Inhibition of either the EGFR or PI3K pathway was found to enhance the apoptotic cell death induced by paclitaxel (13). Up-regulation of survivin gene expression in human cancer cells after paclitaxel or docetaxel treatment has been reported by several laboratories, including ours (13, 14, 38). It was also demonstrated that treatment of human cancer cells by way of survivin siRNA down-regulated survivin expression and was able to sensitize cells to paclitaxel-induced cell death (38). Establishment of a clear link between EGFR signaling and survivin up-regulation in the apoptotic response to chemotherapy drugs will provide us with new information and a justifiable rationale for targeting this signaling pathway in the development of novel therapeutic approaches.

At present, how EGFR signaling leads to the expression of the survivin gene is still unclear. It has been shown that activation of the PI3K pathway by EGFR signaling leads to survivin up-regulation (12, 13). Results of our study also showed that inhibition of the PI3K pathway blocked EGF-induced survivin up-regulation in human breast cancer cell lines. However, the basal level of survivin expression was not affected even when the level of p-AKT was completely inhibited by a PI3K inhibitor, suggesting that the mechanism for constitutive expression of survivin may be different from EGF-induced survivin gene transcription. Although the role of PI3K/AKT in up-regulation of survivin expression has been established, we found that the effect of MAPK on survivin expression differs among breast cancer cell lines. Inhibition of the MAPK pathway blocked survivin gene up-regulation in SK-BR-3 but not in MCF-7 cells.

The PI3K/AKT pathway has been associated with important cellular pathways controlling cell proliferation and survival (39). To develop therapeutic approaches targeting EGFR signaling-induced apoptosis-resistant cancer cells, it is crucial to determine how activation of PI3K/AKT activity leads to survivin gene transcription. Previous studies have revealed another link between EGFR signaling and up-regulation of HIF-1α protein synthesis mediated by activation of PI3K/AKT (19, 40). Our laboratory recently demonstrated that survivin promoter activity was up-regulated in hypoxic tumor cells (23). By further analysis of the survivin core promoter sequences, we have now identified a putative HRE consensus 5′-GCGTG-3′ region located in −81 to −85 nt of the 5′-flanking region of the

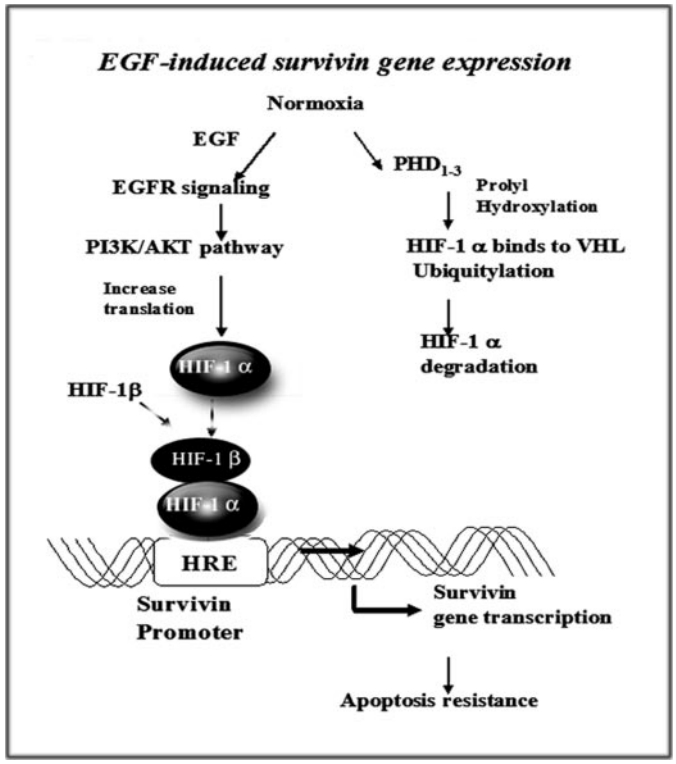


FIGURE 8. **Schematic illustration of a cross-talk between the EGFR and HIF-1α signal pathways showing up-regulation of survivin gene expression and induction of resistance to apoptosis.** Activation of EGFR signaling increases HIF-1α protein synthesis under normoxic conditions through the PI3K/AKT signal pathway. HIF-1α then directly interacts with the survivin promoter, up-regulating the level of survivin gene expression, which results in resistance to apoptosis in tumor cells.

survivin gene. Therefore, we believed it possible that transcriptional activation of survivin gene expression by EGF is mediated by HIF-1α. Our present study results demonstrated that the levels of both HIF-1α and survivin are significantly increased in EGF-treated, normoxic tumor cells. We further determined that HIF-1α is indeed a key transcription factor for EGFR signaling-activated survivin gene expression, since down-regulation of HIF-1α using HIF-1α siRNA significantly reduced the level of survivin expression in human cancer cells. Maintaining the level of HIF-1α-activated survivin seems to be very important for survival of normoxic tumor cells, since overexpression of HIF-1α siRNA alone resulted in activation of caspase activity and apoptosis in about 70% of the breast cancer cells. In addition, HIF-1α siRNA also markedly enhanced apoptotic cell death in docetaxel-treated tumor cells, even after EGF stimulation.

The results of our study have demonstrated, for the first time, that there is cross-talk between EGFR and HIF-1α signaling pathways that can up-regulate survivin gene expression and increase resistance to apoptosis. First, we showed that overexpression of the HIF-1α gene activates survivin promoter activity and the level of the protein. Using ChIP and modified McKay assays, we determined that there is a direct interaction between HIF-1α and the survivin promoter, thus finding that the transcriptional activity of HIF-1α is highly likely to activate survivin gene transcription. HIF-1α has been previously defined as a hypoxia-inducible transcriptional factor, and its role in normoxic tumor cells remained largely unclear. Our results provide

direct evidence that HIF-1α is able to bind to HRE sites and activate HRE-mediated gene transcription under normoxic conditions in human tumor cells. In another study, we found that hypoxia-induced HIF-1α up-regulation could also activate survivin gene expression using a similar mechanism.[3] Currently, the precise mechanism for HIF-1α-mediated transcriptional activation of the survivin gene is under investigation in our laboratory.

In this study, we found that EGF stimulation does not induce survivin expression in normal mammary epithelial cells. Moreover, overexpression of the HIF-1α gene activated survivin promoter activity and increased the level of survivin protein in breast cancer cell lines but not in normal cells, suggesting the presence of transcriptional inhibitory factor(s) in normal cells, preventing activation of the survivin promoter by HIF-1α. At present, we are conducting studies to identify the transcriptional factors involved in the tumor cell-specific activation of survivin gene expression.

In conclusion, we have identified a novel pathway that mediates resistance to apoptosis in EGFR signal-activated human tumor cells. Our results have demonstrated that activation of the EGFR signaling pathway leads to the up-regulation of HIF-1α through the PI3K/AKT pathway. We revealed that HIF-1α directly binds to the survivin promoter to activate gene transcription, resulting in resistance to apoptosis in normoxic, EGFR signal-activated tumor cells (Fig. 8). Since activation of the EGFR signaling pathway and the presence of hypoxic tumor regions are commonly found in solid tumors, it is possible that coexistence of activation of those two conditions makes tumor cells highly resistant to apoptosis through HIF-1α-mediated up-regulation of survivin as well as other factors. Results from our study further highlight the importance of HIF-1α-mediated expression of survivin in tumor progression and resistance to therapy. Additionally, our results provide a strong rationale for the development of novel approaches targeting HIF-1α for prevention of tumor progression as well as for cancer therapy, alone or in combination with other chemotherapeutic agents. Thus, the benefits from elucidation of this most likely common pathway that can function in both normoxia and hypoxia should eventually lead to much improved control and treatment of breast and other cancers.

Acknowledgments—We thank Dr. Hyunsuk Shim for kindly providing the pBI-V6R stable MDA-MB-231 cell line, Dr. Fengzhi Li for the pluc cyc1.2 plasmid and survivin siRNA sequence, Dr. Bert Vogelstein for the AdEasy system, Dr. Erwin Van Meir for the pBI-V6R plasmid, and Drs. Hua Zhong and Ruoxiang Wang for the pHIF-1α plasmid. We thank Nicholyn Hutchinson and Kathleen Kite-Powell for manuscript editing.

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EMORY UNIVERSITY | Institutional Review Board

Lily Yang, MD/PhD
SOM: Surgery
Room B3106
Clinic B

RE: **NOTIFICATION OF RENEWAL APPROVAL**
PI: Lily Yang, MD/PhD
IRB ID: **1039-2002**

TITLE: Early Detection of Breast Cancer Using Molecular Beacons

DATE: January 03, 2008

Renewal Review Type: Expedited

The continuing approval request referenced above was reviewed and APPROVED by the IRB. This approval is valid from **12/19/2007 until 12/18/2008**. Thereafter, continued approval is contingent upon the submission of a renewal form that must be reviewed and approved by the IRB prior to the expiration date of this study.

Any serious adverse events or issues resulting from this study should be reported immediately to the IRB and to any sponsoring agency (if any). Amendments to protocols and/or revisions to informed consent forms/process must have approval of the IRB before being implemented.

All inquiries and correspondence concerning this protocol must include the IRB number and the name of the Principal Investigator. If you have any questions or concerns, please contact the IRB office at 404-712-0720 or at email address irb@emory.edu. Our web address is http://www.emory.edu/IRB. Thank you.

Sincerely,

Colleen DiIorio PhD/RN/FAAN
Chair
Institutional Review Board

Emory University
1599 Clifton Road, 5th Floor, Room 213
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Tel 404.712.0720
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This approval is valid from 12/19/2007 until 12/18/2008.

PAGE 2 of RENEWAL APPROVAL

IRB ID: **1039-2002** DATE: January 03, 2008
TITLE: Early Detection of Breast Cancer Using Molecular Beacons

The above referenced protocol renewal was approved including the information below. Please review this information for accuracy. If there are any discrepancies, please notify your IRB coordinator immediately.

Personnel		**Human Subjects Education Certification Information**
Carlson, Grant	Co-Investigator	CITI - MED Refresher (24-Apr2006)
Yang, Lily	Main Investigator	CITI - MED Refresher (08-Dec-2006)
Lewis, Melinda	Co-Investigator	CITI - MED 1, 2, 3, 7, 12, 14, 17 (23-Jan-2007)
Smith, Mark Q.	Co-Investigator	CITI - MED 1, 2, 3, 7, 12, 14, 17 (27-Oct-2006)

Number of Approved Emory Subjects 175 **(This number indicates the number of subjects you can consent.)**

Sites
Emory University Hospital
Emory Clinic

Funding Agencies
DoD - US Dept of Defense

Protocol title:
Early Detection of Breast Cancer Using Molecular Beacons

Subtitle:
Examination of the Sensitivity of Using Molecular Beacons Targeting Survivin, Cyclin D1 and Her-2/neu mRNAs to Detect Breast Cancer Cells in Ductal Lavage and Aspirates of Fine Needle Biopsy in Breast Cancer Patients

Principal Investigator:
Lily Yang

Co-investigators:
Grant W. Carlson
Toncred Styblo
Melinda M. Lewis
Mark Q. Smith

Sponsor's Name: Department of Defense

Phase of the Study: Feasibility

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Introduction

Breast cancer is the most common type of cancer and one of the leading causes of death among women. A crucial factor to increase survival is early diagnosis. Although early screening with mammography decreases the mortality of the disease, nearly 20% of breast cancer patients are still missed by mammography. Furthermore, of all patients with abnormal mammograms, only 10 to 20% are confirmed to be breast cancer by biopsy (1). At present, there is no reliable serum tumor marker for the diagnosis of breast cancer. Therefore, development of novel approaches for the early diagnosis of breast cancer is of critical importance for successful treatment and for increasing survival. It is well known that over 90% of breast cancers arise in the epithelial cells lining the ductal system. Most breast cancers develop over a period of 8 to 10 years before being detected by standard methods. In order to detect early stage breast cancer or ductal carcinoma *in situ* (DCIS), it is important to develop novel and non-invasive approaches with high sensitivity and specificity.

It is well established that cancer cells develop due to genetic alterations in oncogenes and tumor suppressor genes. These abnormalities in gene expression provide growth advantage and metastatic potential to the cells. A novel way of achieving early detection of cancer is to identify the cancer cells through detection of mRNA transcripts that are expressed in the cancer cells but at low levels or not expressed in normal cells. We have developed a molecular beacon (MB)-based approach for direct examination of gene expression in viable and fixed cells(2, 3). The proposed study focuses on the evaluation of the feasibility of detection of breast cancer cells in ductal lavage and/or fine needle aspiration using MBs targeting survivin, cyclin D 1 and Her-2/neu mRNAs, which are tumor markers found in over 70 to 80% of DCIS tissue samples.

MBs are oligonucleotides with a stem-loop hairpin structure, dual-labeled with a fluorophore at one end and a quencher at the other. Delivering MBs into cells produces a fluorescent signal if the MBs hybridize to target mRNAs. Thus, when target mRNAs correspond to the molecular markers of a cancer, cancer cells (bright) can be distinguished from normal cells (dark)(2). We have established the structure-function relationships of MBs and experimental conditions that enable us to identify breast cancer cells using MBs.

Recently, a class of new fluorescent emitting particles, semiconductor quantum dots, has been developed for using as sensitive probes for biomolecular and cellular imaging (4,5). These quantum dots (QDs) are tiny, nanometer-scale light-emitting particles that have unique optical and electronic properties such as size-tunable light emission, improved signal brightness, resistance against photobleaching, and the ability to simultaneous excite multiple fluorescence colors (4,5). These properties are most promising for improving the sensitivity of molecular imaging and quantitative cellular analysis by 1-2 orders of magnitude. Another advantage is that multicolor QD probes can be used to image and track multiple tumor markers simultaneously in single cells. It has been shown that antibodies conjugated to QDs are able to detect several protein markers in cancer cells. Therefore, we plan to detect the levels of mRNA with MBs and protein levels using QD-labeled antibodies simultaneously in clinical samples.

Working hypothesis: Simultaneous detection of mRNA and protein levels of tumor markers, such as survivin, Her-2/Neu and cyclin D1, using MBs and QD-labeled antibodies, has a high specificity and sensitivity in identifying cancer cells. The ultimate goal is to develop a simple, sensitive and efficient clinical procedure for early detection of breast cancers.

Significance

The proposed study will be the first to apply state-of–the art nanotechnology, including both MB-based and QD methodologies for the detection of breast cancer. Survivin is a newly discovered protein in the inhibitor of apoptosis protein family (IAP). This investigation will also be the first such study to evaluate the feasibility of using survivin as an early breast cancer marker. Since MBs are highly specific in detecting target mRNAs, and MBs targeting various genes can be labeled with different fluorescent-dye molecules and delivered into single cells, expression of several tumor marker genes in a single cell can be analyzed at the same time. QD-labeled antibodies are able to detect the protein levels of several tumor markers in cancer cells. Human breast cancers contain heterogeneous cell populations with various genetic changes. Simultaneous detection of the overexpression of several tumor marker genes and proteins, especially when a single cell expresses more than one marker gene, may have a high predictive value for identifying cancer cells, and therefore increase the sensitivity and specificity of cancer detection. For example, although survivin is detected in over 70% of breast cancer tissues, about 30% of patients do not express survivin and will be missed if only survivin will be used. About 20 to 30% of DCIS tissues are also negative for cyclin D1 or Her-2/neu. We plan to use MBs targeting survivin, cyclin D1 and Her-2/neu mRNAs that are labeled with different fluorescence dye molecules to determine the relationship between the detection of the cells expressing none, one, two or three markers and the feasibility of early detection of breast cancers in ductal lavage or fine needle aspirates. Additionally, we will use the sample to detect cells expressing high levels of survivin, cyclin D1 and Her-2/neu proteins using fluorescent QD-dot labeled antibodies. Since fluorescent emission wavelength of three QDs are different from each other and are also distinguishable from fluorescent dyes in MBs, we should be to determine the mRNA and protein levels of each gene.

The results of our *in vitro* study using normal human breast and cancer cell lines has shown that delivering a mixture of survivin and cyclin D1 MBs into fixed cells produces fluorescent signals in breast cancer cells but not in normal breast cells. Interestingly, the fluorescence intensity within the cells correlated well with the level of gene expression in different tumor cell lines. Previous methods for detecting gene expression *in situ* were not quantitative since the signals were amplified by either the presence of multiple fluorescent-dye labeled nucleotides in an oligonucleotide probe or amplification of the signals with secondary antibodies to labeled nucleotides. Since each MB has only one fluorophore and unbound MBs do not fluoresce, the fluorescence intensity generated by hybridization of the MB with a specific mRNA should reflect more accurately the level of the mRNA expression. Additionally, since only a small amount of abnormal cells are present in a large amount of normal cell background in clinical samples, there is a clear advantage of direct fluorescence imaging of individual cells expressing tumor marker genes for early detection of cancer cells compared to conventional RT-PCR to amplify the expression of tumor marker genes from isolated total RNA, which may be difficult to detect the differences in the level of gene expression in a few cancer cells over the normal background.

Therefore, MB–based cancer cell detection has the potential to become a simple clinical procedure for early detection of breast cancer with a high sensitivity and specificity. Additionally, determination of protein levels of those tumor markers in the cells should further enhance the specificity and sensitivity of the detection.

Experimental procedures:

Examination of the sensitivity of using molecular beacons targeting survivin, cyclin D1 and Her-2/neu mRNAs and QD-labeled with antibodies against the above three markers to detect breast cancer cells in ductal lavage and aspirates of fine needle biopsy in breast cancer patients.

First, we will examine the specificity and sensitivity of MB- and QD- imaging cancer cells in ductal lavage or fine needle aspiration from breast cancer patients. This study will allow us to determine whether the MB and QD detections are more specific and sensitive than cytological method in detecting breast cancer cells. The proposed study will also provide us with new information regarding using survivin, cyclin D1 and Her-2/neu as tumor markers for identifying the breast cancer cells at the DCIS stage.

Study subject selection:

Eligibility Criteria:

Patients of all ages and races diagnosed with breast cancers or with the possibility of breast cancer, which come to Emory Clinic or Emory Hospital and are able to read and speak English.

Ineligibility Criteria:

Breast cancer patients who have received chemotherapy or radiation therapy for breast cancer or other cancers within the last three months, or are currently pregnant or nursing.

Recruitment methods and the informed consent process:

Breast cancer patients, who come to the Emory Clinic or Emory Hospital and are cared for by Co-PIs Drs. Carlson and Styblo in the Departments of Surgery and the Winship Cancer Institute, will be informed by their physicians about the clinical trial. If the patients are interested in participating in the study, the informed consent forms will be given to the patients. The patients and their family members will be given time to read and ask questions about the study. If the patients are unable to provide their own consent to participate the study, their legally authorized representative will be contacted. The informed consent form will be given to the representative and allowed sufficient time to read and ask questions. The patients will only be enrolled in the study after obtaining a signed informed consent form from their legally authorized representatives.

If subjects are willing to participate in the study and the consent form is signed, extra samples of a fine needle aspiration from a scheduled diagnostic procedure will be collected for the MB study. Additionally, ductal lavage will be performed right before the scheduled operation for removing the breast tumor under general anesthesia according to the procedures described in Experimental Design and Methods.

All study procedures will be conducted in compliance with the protocol, GCP and the applicable regulatory requirements.

Study protocol:

The purpose of this study is to determine whether detection of high levels of survivin, cyclin D1 and Her-2/neu mRNAs and proteins are able to identify breast cancer cells in fine needle aspirate (FNA) and ductal lavage samples which have a high probability of the presence of breast cancer cells. We will also determine the percentage of the cells that express one, two or three tumor marker genes at different stages of the disease, especially in the samples from patients diagnosed with DCIS.

Fine needle aspiration:

Fine needle aspiration is the easiest and fastest method of obtaining a breast biopsy. It is an FDA approved biopsy procedure and has been used in outpatient clinics routinely as a diagnostic procedure for breast cancer (4). It uses a thin needle on a syringe to draw fluid and/or cellular material from breast

tissues.

FNA samples will be collected during a scheduled diagnostic procedure by Dr. Lewis in the outpatient exam rooms in the Winship Cancer Institute at Emory University, Surgical Oncology Suite, Clinic C, 1365 C Clifton Road, NE, Atlanta, GA. FNA is performed under local anesthesia. Under sterile conditions, a fine hollow needle that is attached to a syringe to extract fluid from a solid lesion is inserted into the breast mass of breast cancer patients. The needle used in this procedure is very small (smaller than those used to draw blood). The procedure takes a few minutes. The FNA samples will be sent to Dr. Yang's research laboratory immediately to determine whether cells expressing tumor marker-genes are present in the FNA samples.

Recovery after the FNA procedure is generally quick and uncomplicated. Most patients are able to resume normal activity almost immediately afterwards. Pain is minimal and can usually be managed with an over-the-counter pain reliever. Complications for procedures are rare, but excessive swelling, redness, and bleeding or other drainage can indicate an infection or abnormal bleeding. The PI and CO-PIs, who usually are the physicians for these patients, should be notified immediately.

Ductal lavage:

Ductal lavage is an FDA-approved and minimally invasive procedure to collect breast ductal epithelial cells for cytopathological analysis(5, 6). This procedure is typically performed in an outpatient exam room in a clinic and takes about 30 minutes. However, for patients undergoing lumpectomy or more extensive surgery, ductal lavage will be performed by Drs. Carlson or Styblo in the operating room of the Emory University Hospital after administration of general anesthesia and before surgery.

In the operating room and under sterile conditions and general anesthesia, a microcatheter (Firstcyte ultraslim dilator, an FDA-approved Class II device from Cytyc Health Corp., MA), will be inserted 0.5 to 1.0 cm into a nipple orifice and 10 ml of sterile saline will be slowly infused. For each patient, two ducts will be lavaged. The effluent fluid from each duct will be collected and placed in separate vials that are labeled with a specific study ID number for each patient. The samples will be placed on ice and sent to Dr. Yang's research laboratory immediately to determine mRNA and protein levels of three tumor marker genes in the cellular fraction of the ductal lavage using a mixture of survivin, cyclin D1 and Her-2/neu MBs, and QD-labeled antibodies. About 13,500 cells per duct can be collected for analysis of the presence of normal, atypical, or malignant breast ductal cells. The procedure for ductal lavage under general anesthesia can be finished within 10 minutes, which will prolong the general anesthesia time for 10 minutes and may slightly increase the risk for the patients.

For each breast cancer patient, we plan to collect three samples for this study: 1) FNA samples from a scheduled diagnostic procedure (if available), 2) ductal lavage before surgery, and 3) breast cancer tissues after surgery. After examination of the FNA and ductal lavage samples from breast cancer patients, we should be able to determine which method is more sensitive for detection of breast cancer cells.

Cellular analysis of tumor marker gene expression using molecular beacons and QD-labeled antibody.

The ductal lavage and FNA samples will be processed immediately after collection to ensure the quality of RNA in the cells. After a brief centrifugation, the cell pellets will be placed on pretreated glass slides using a cytospin. About 10 to 15 cytospin slides will be obtained from one ductal lavage. After fixing the cells in ice-cold acetone, the slides will be incubated with a mixture of survivin, cyclin D1 and Her-2/neu MBs at optimized incubation conditions for 1 hour. After a brief washing, a mixture

of QDs labeled with antibodies against survivin, cyclin D1 and Her-2/Neu will be added to the slides and incubated for 1 hr. The slides will then be counterstained with Hoechst 33342 and then examined under a confocal microscope.

Breast cancer cell lines with known levels of survivin, cyclin D1 and Her-2/neu genes will be used as positive controls. Normal human mammary epithelial cell lines and human primary fibroblasts will also be used as negative controls. Images of cells from patient samples, positive and negative controls will be taken using identical instrumental sittings for confocal microscopy. The levels of fluorescent intensity for each fluorescent dye will be analyzed on all of the cells observed on the slides. The cells displaying a fluorescent intensity that is two fold-higher than negative control cells will be labeled as positive cells. In comparison with the fluorescence intensity in breast cancer cell lines, we should be able to score the fluorescence intensity in each patient sample from -, +, ++, +++ to ++++. The number of cells showing positive labeling for each MB and QD-antibody, and the number of cells having one, two or three gene expression will also be recorded. We will further compare results obtained from FNA and ductal lavage from the same patients to determine the specificity and sensitivity of detecting cancer cells.

Since the levels of mRNA and protein for the three tumor markers are labeled with different fluorescent dyes, the number of the cells overexpressing survivin, cyclin D1, Her-2/neu, two of the genes or all three genes in ductal lavage and FNA samples will be determined. We will also compare results obtained from FNA and ductal lavage to determine the specificity and sensitivity of detecting cancer cells. Since FNA samples may contain more cancer cells, this will also provide with us an additional source to evaluate the specificity of the MB and QD detection. Upon finishing the examination, the same slides will be stained with H&E and analyzed by a cytopathologist (Dr. Lewis) for the presence of benign, atypical or malignant cells. We will then compare the results of the MB and QD detection with cytological findings and pathological diagnosis after surgery to determine whether the MB and QD method is more sensitive and specific for the detection of breast cancer cells than current cytology methods. We will also determine if the MB and QD detection of the cells expressing survivin, cyclin D1 and Her-2/neu genes is able to identify cancer cells at DCIS stage. For each patient, we will obtain breast cancer and surrounding normal tissues after surgery and examine them for the expression of survivin, cyclin D 1 and Her-2/neu on frozen tissue sections by immuostaining with specific antibodies.

Current methods for identification of different cell types in ductal lavage and FNA samples is by morphological classification. Development of molecular approaches for the detection of cells at different stages of tumorigenesis will enhance the specificity and sensitivity for the early detection of breast cancer cells. Since each MB targeting a specific mRNA or QD-labeled antibody detecting a specific protein is labeled with a specific emission wavelength, a major advantage of the our approach as compared with RT-PCR and immunohistochemistry for early cancer detection is that a mixture of MBs targeting multiple tumor specific mRNAs and QD-antibodies can be delivered to a single cell at the same time, and the expression of all these markers can be observed in a single assay using a fluorescence microscope.

Adverse effect /IND safety report:

Fine needle biopsy is a minimally invasive procedure that has been used in clinic routinely for the diagnosis of breast cancer. Ductal lavage is also a minimally invasive procedure and has been used on over 500 high risk women and no serious adverse effects were found (6).

For breast cancer patients, ductal lavage will be performed during the surgery and fine needle aspirates will be obtained from scheduled biopsy. Participation in the study will not add significant risks or discomfort to the patients. It is possible that ductal lavage will prolong anesthesia time up to 10 minutes, which may potentially increase risk to the patients. Close monitoring of the patients during general anesthesia, as it done routinely, should reduce the risk of any adverse effects related to ductal lavage.

Any adverse effects related to participation in the study will be documented, whether or not the event is considered to be related to the study. This definition includes inter-current illnesses and injuries and exacerbation of pre-existing conditions. The following information will be included in the IND safety reports: subject identification number and initials; investigator's name and name of the medical treatment facility/hospital or research facility; subject's date of birth, gender, and ethnicity; date of procedure performed; signs/symptoms and severity; date of onset; date of resolution; relationship to the study; action taken; concomitant medications including dose, route and duration, and date of the last dose.

Unanticipated problems involving risk to volunteers or others, serious adverse events related to participation in the study and all volunteer deaths should be promptly reported by phone (301-619-2165), by e-mail (hsrrb@det.amedd.army.mil), or by facsimile (301-619-7803) to the Army Surgeon General's Human Subjects Research Review Board. A complete written report should follow the initial telephone call. In addition to the methods above, the complete report can be sent to the U.S. Army Medical Research and Material Command, ATTN: MCMR-RCQ, 504 Scott Street, Fort Detrick, Maryland 21702-5012.

In case of adverse effects related to the study, the PI and/or CO-PI will be notified immediately. The PI will refer the patients to Army Hospitals in Georgia, to receive treatment free of charge for injuries directly caused by the study. The primary physician and the PI of the study will follow up the patients until they are fully recovered. The Amy will not pay for transportation to and from the hospital or clinic. If the patients pay out-of pocket expenses for medical care elsewhere for injuries caused by this study, they will need to contact the PI. All questions concerning the medical care will be addressed by the PI and CO-PIs. If there are issues that cannot be resolved, the U. S. Army Medical Research and Material Command (USAMRMC) Office of the Staff Judge Advocate (legal office) at 301-619-7663/2221 will be contacted.

Proposed patient sample size:

The purpose of this protocol is to carry out a pilot study for the determination of sensitivity and specificity for the detection of breast cancer cells using the molecular beacon and quantum dot approach. We plan to use a minimum sample size that will give us information on the feasibility of detection of breast cancer cells in clinical samples. We plan to analyze samples from 50 breast cancer patients, which include at least 25 patients with DCIS. However, we will use statistical analysis such as power calculations based on results from our pilot study, which will be obtained after we start the trial, to determine the final sample size. The cancer patients will sign the informed consent forms before any procedures are performed. A total of 50 breast cancer patients will be enrolled in this study.

Statistical methods for data analysis

Statistical Power Assessment:

The statistical power analysis will be conducted using the Cox regression analysis to determine whether one or the combination of three tumor markers is better for detecting breast cancer cells.

Sensitivity and specificity for the detection of cancer cells using MBs and QD-labeled antibodies in the patient's sample will be analyzed using standard statistical analyses such as student's t-test or ANOVA analysis. Differences in the prevalence of detecting cancer cells in ductal lavage or FNA from breast cancer patients by the MB detection, QD-detection or the combination of MB and QD, and by cytology will be calculated by multi-variance ANOVA test. The prevalence of identifying tumor cells by MB- and QD-labeled antibody detection of the cells expressing each marker alone, or more than one marker will be calculated by Chi-square test or Kruskal-Wallis one-way analysis of variance on ranks. A P-value of < 0.05 will be considered statistically significant.

Data Handling:

The PI will assign all patients enrolled in the study an ID number. Drs. Carlson, Styblo and Lewis, who are co-investigators, will collect the clinical samples. All samples will be placed in sterile tubes pre-labeled with the patient's ID number and sent to Dr. Yang's research laboratory immediately. Only the PI and CO-PIs will have access to the names and hospital record numbers of the participating breast cancer patients. The research staff in the lab will only be given the ID number of each sample. All research results will be recorded using the ID numbers. The original experimental records and CDs for storing the image files will be kept in Dr. Yang's laboratory and office in locked cabinets located in the Clinic C Building, Room C-4088 and C-4038, 1365 C Clifton Road NE, Atlanta Georgia. Information on study ID numbers, patient's names and hospital numbers will only be kept in Dr. Yang's computer in her office and with a copy on a CD locked in the cabinet. All research results will be stored and kept in Dr. Yang's office for three years or until the study and all follow-up studies are finished. The paper records concerning the patient's information, hospital number and study ID number will be shredded using a paper shredder. The files on the computer hard drive and on CDs will be deleted.

The PI will inform the CO-PIs about the experimental results from all participants. Since the results are non-CLIA approved research results, the participants will not be informed about the test results.

People other than those doing the study may look at both medical charts and study records. Agencies that make rules and policy about how research is done have the right to review these records. These include the Department of Defense, the National Cancer Institute, the Emory Clinic, Emory University Hospital and the Emory University Institutional Review Board. Records can also be opened by court order. We will use a study ID number rather than patient's name on study records where we can. Patients' name and other facts that might point to the patient or normal subjects will not appear when the results of this study are presented or published.

Modification of the study protocol:

Any change in the protocol, personal, and numbers of enrollment will be submitted to Emory IRB and the HSRRB of the DOD for an approval.

Requests for termination or extension of the protocol will also be submitted to Emory IRB and

the HSRRB of the DOD for an approval.

In the event of a departure from the protocol, a subject withdraws from the protocol, or termination of a participant by the PI and/or CO-PIs, the PI will be notified and reasons for subject withdraw or termination will be recorded. The PI will report all information on the continuation application form to Emory IRB each year and in the annual report to DOD Breast Cancer Research Program.

The subject's participation may be terminated by the PI and Co-PIs if she becomes pregnant before the procedure, has received chemotherapy or radiotherapy, or has developed other illnesses that are inappropriate for conducting ductal lavage or FNA procedure.

Timeline for the study

We plan to start this sub-project in October 2006 and finish targeted enrollment by July 31 2007.

Appendix A

Qualification of the PI and Co-PIs for the study

Principal investigator: **Lily Yang**, MD. PhD is an Assistant Professor in the Department of Surgery and the Winship Cancer Institute. Dr. Yang has been working in the field of cancer research since 1986 and is trained in both cellular and molecular biology. Her PhD study involved the cellular origin of liver cancer and identification of liver stem cells. She worked on projects for the development of new approaches for cancer gene therapy including preclinical studies on the delivery of angiogenic inhibitors by adenoviral vectors for the treatment of primary and metastatic breast cancers. Dr Yang has been working on targeting survivin as a therapeutic approach for breast cancer since 1999. In May of 2001, she started working on molecular imaging of breast and pancreatic cancer cells using MBs detecting mutant K-ras, survivin, cyclin D1 and Her-2/neu. Many assays and methods for evaluation of the specificity of MBs have been established in her laboratory. She will direct all aspects of the project and is responsible for experimental design, protocol, data analysis and manuscript preparation.

Grant W. Carlson, MD is a Professor of Surgical Oncology in the Department of Surgery at Emory University. Dr. Carlson's has expertise in both breast cancer surgery and plastic surgery for breast remolding. Dr. Carlson participated in a multi-center research project on examination of cell types in ductal lavage of high risk women as an indication for tamoxifen risk-reduction therapy and has performed ductal lavage procedure. He will be responsible for performing ductal lavage in breast cancer patients, normal volunteers and women with high risk of developing breast cancer.

Toncred Styblo, MD is a Professor in the Department of Surgery**.** Dr Styblo is a leading expert in treating breast cancer. She has many years of experience operating on breast cancer patients and performed ductal lavage. Dr. Styblo will collect ductal lavage samples from the breast cancer patients.

Melinda M. Lewis, MD, FCAP is an Associate Professor, Director of Cytopathology in the Department of Anatomic Pathology and Director of Fellowship for Cytopathology. She is an expert on breast cytology and is a board-certified cytopathologist for performing FNA and characterization of cell types in FNA and ductal lavage samples. She has been responsible for cytopathology in Breast Cancer Clinic in the Winship Cancer Institute at Emory University and performed FNA daily on the breast cancer patients since 1988. Her research interests focus on the application of immunohistochemical and molecular techniques to cytologic specimens to enhance diagnostic and prognostic information. One of her research projects is on the comparison of immunohistochemistry and fluorescence *in situ* hybridization in the evaluation of Her-2/neu in image-guided breast fine needle aspiration. Dr. Lewis is also involved in a research project on the examination of cell types in ductal lavage of women with high risk of breast cancer for preventive tamoxifen treatment. In the proposed study, Dr. Lewis will be responsible for performing FNA and cytological analysis of cell types in ductal lavage and FNA samples.

Mark Q. Smith, MD is a Third year General Surgery Resident at Emory University Hospital. He has completed two years of clinical training as a general surgeon and plans on pursuing a fellowship in surgical oncology after finishing his general surgery training. He has elected to take two years off from clinical training to become involved in this research focusing on developing nanotechnology for the detection and treatment of cancer. Mark with be responsible for the characterization of MBs, QDs, cellular assays, and analysis of the ductal lavage and FNA samples. In addition, he will be involved in the molecular imaging of cancer cells.

Dr. Yang (PI) has filled a patent application, U.S. patent, 60/439,771, on January 13, 2004, entitled "Methods of detecting gene expression in normal and cancerous cells". The application is pending and under reviewing by the patent office.
Co-PIs do not have a conflict of interest for this study.

Appendix B

Medical Mediator

Ruth O'Regan, MB, MRCPI, MB FRK

Dr. Ruth O'Regan will be the medical monitor for this study. Dr. O'Regan is an Assistant Professor of Hematology and Oncology; Director, Translational Breast Cancer Research Program. Dr. O'Regan joins the WCI from Northwestern University in Chicago where she was an Assistant Professor of medicine at Northwestern Hospital specializing in breast cancer. Her areas of research include the evolution of tamoxifen therapy in breast cancer. Dr. O'Regan is studying novel selective estrogen receptor modulators-SERM, an area in which she has studied under one of the world's leading authorities, Dr. V. Craig Jordan at Northwestern. She has received numerous awards including the compassionate care award from the Women's Board of Northwestern Hospital and has several seminal publications on mechanisms of resistance of SERM's among her more than 30 peer-reviewed publications. Dr O'Regan is an Assistant Professor in the Department of Hematology and Oncology in the Winship Cancer Institute at Emory University. She is not under the supervision of the PI and Co-PIs. She doesn't have a conflict of interest for this study. Dr. Ruth O'Regan's biosketch is enclosed.

Appendix C

Case Report Form

Study Title: Early Detection of Breast Cancer Using Molecular Beacon

PI: Dr. Lily Yang,

Co-PI: Grant W. Carlson, Melinda M. Lewis, Toncred Styblo, and Mark Q. Smith

Study ID number						
Enrollment groups	**Breast cancer patients**					
Age:	**Race:**					
Physician's name						
Consent date	**Obtained by:**					
FNA date	**Performed by:**					
Ductal lavage date	**Performed by**					
Adverse effects during the procedure						
Post procedure care						
Reported adverse effects after the procedure						
Number of slides obtained						
Results of MB-detection	**Survivin**		**Cyclin D1**		**Her-2/neu**	
Total number of positive cells						
Results of QD-detection	**Survivin**		**Cyclin D1**		**Her-2/neu**	
Total number of positive cells						
Semi-quantitative analysis of levels of mRNA and protein	**MB**	**QD**	**MB**	**QD**	**MB**	**QD**
Number of cells in each level of fluorescence intensity Total number of cells presented	++++( ) +++( ) ++ ( ) + ( ) ( )	++++( ) +++( ) ++ ( ) + ( ) ( )	++++( ) +++( ) ++ ( ) + ( ) ( )	++++( ) +++( ) ++ ( ) + ( ) ( )	++++( ) +++( ) ++ ( ) + ( ) ( )	++++( ) +++( ) ++ ( ) + ( ) ( )
Number of cells expressing more than one marker genes Survivin + Cyclin D1 Survivin + Her-2/neu Cyclin D1 + Her-2/neu Survivin + Her-2+ cyclin D1						
Frozen breast cancer and normal tissues collected: yes ( ), No ( )	Immunostaining results for survivin, cyclin D1 and Her-2/neu expression in frozen tissue sections.					

	Survivin ( ), cyclin D1 ( ), Her-2/neu ( )
Pathological diagnosis • Cytopathology from ductal lavage • Cytopathology from FNA • Pathology evaluation of breast cancer tissues	
Treatments received after surgery	
Follow up information ***Breast cancer patients*** Recurrence Prognosis Contact by PI or Co-PIs by telephone Yes ( ), No ( ) If yes, any information.	

BIOGRAPHICAL SKETCH

Give the following information for all ***new*** key personnel.
Copy this page for each person.

NAME	POSITION TITLE
Ruth M. O'Regan	Assistant Professor in Hematology/Oncology

EDUCATION/TRAINING *(Beginning with baccalaureate or other initial professional education, such as nursing, and*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
University College, Dublin, Ireland	MB, BcH, BAO (MD)	1982-1988	Medicine
Royal College Physicians, Ireland	MRCPI	1992	Medicine
University College, Dublin	Medical Doctorate	2000	Oncology

A. Professional Experience

7/03-present	**Assistant Professor**	**Emory University, Atlanta, GA**
9/02-6/03	**Assistant Professor**	**Northwestern University, Chicago, IL**
7/99-8/02	**Clinical Instructor**	**Northwestern University, Chicago, IL**
7/98-6/99	**Resident (Internal Medicine PGY3)**	**Northwestern University, Chicago, IL**
7/95-6/98	**Fellow (Hematology/Oncology)**	**Northwestern University, Chicago, IL**
7/94-6/95	**Resident (Internal Medicine PGY3)**	**Medical College Wisconsin, Milwaukee, WI**
7/92-6/94	**Fellow (Oncology)**	**Mater Hospital, Dublin, Ireland**
7/91-6/92	**Resident (Internal Medicine)**	**Meath Hospital, Dublin, Ireland**
7/88-6/91	**Resident (Internal Medicine)**	**Mater Hospital, Dublin, Ireland**

Honors, Awards, and Membership

Compassionate Care Award , Womens' Board of Northwestern Hospital 1997
Coakley Medal from UCD North American Alumni 1999
NSABP Young Clinical Investigator Award 2001
American Society of Clinical Oncology
American Society of Cancer Research
University College Dublin Medical Graduates Association of North America

B. Selected Peer Reviewed Publications

1. **Shah AP, Parmar S,** O'Regan R. **Right atrial and ventricular thrombus infiltrated with osteoblastic osteosarcoma. J Cardiovasc Pharmacol Ther 8;307-311, 2003**
2. **Dardes RC**, O'Regan RM, **Gajdos C, Robinson SP, Bentrem D De Los Reyes A, Jordan VC. Effects of a new clinically relevant antiestrogen (GW5638) related to tamoxifen on breast and endometrial cancer growth in vivo. Clin Cancer Res 6;1995-2001, 2002**
3. O'Regan RM, **Gajdos C, Dardes RC, De los Reyes A, Park WC, Jordan VC. Effects of raloxifene after tamoxifen on breast and endometrial cancer growth.** J Natl Cancer Inst **20;274-83, 2002**

4. **Dardes RC, Bentrem D,** O'Regan RM**, MacGregor-Schafer J, Jordan VC. Effects of the new selective estrogen receptor modulator LY353381.HCL (Arzoxifene) on human endometrial cancer growth in athymic mice**. Clin Cancer Res **7;4149-4155, 2001**
5. MacGregor-Schafer J, Lee ES, Dardes R, **O'Regan RM**, Jordan VC. Analysis of cross-resistance of the selective estrogen receptor modulators arzoxifene (LY353381) and LY 117018 in tamoxifen-stimulated breast cancer xenografts. **Clin Cancer Res** 7;2505-12, 2001
6. **Lee, ES, MacGregor-Schafer, J, Yao, K, England G,** O'Regan RM, **De Los Reyes, A, Jordan VC**. **Cross-resistance of triphenylethylene-type antiestrogens but not ICI 182,780 in tamoxifen-stimulated breast tumors grown in athymic mice.** Clin Cancer Res **6:4893-4899, 2000**
7. **MacGregor-Schafer JI, Lee ES,** O'Regan RM**, Jordan VC. Rapid development of tamoxifen-stimulated mutant p53 breast tumors (T47D) in athymic mice.** Clin Cancer Res **6:4373-4380, 2000**
8. **Yao K, Lee ES, Bentrem D, England G, MacGregor-Schafer JI,** O'Regan RM**, Jordan VC. Antitumor action of physiologic estradiol on tamoxifen-stimulated tumors in athymic mice.** Clin Cancer **Res 6:2028-2036, 2000**
9. O'Regan RM**, Cisneros A, England GM, MacGregor JI, Muenzner HD, Assikis V, Bilimoria MM, Piette M, Dragan Y, Pitot HC, Chatterton R, Jordan VC. Growth characteristics of human endometrial cancer transplanted in athymic mice and treated with new antiestrogens, toremifene and ICI 182,780.** J Natl Cancer Inst 90:1552-1558, 1998.
10. Tonetti, DA, **O'Regan RM**, Tanjore S., England G., and Jordan, VC. Antiestrogen-stimulated human endometrial cancer growth; Laboratory and clinical considerations**. J. Ster. Biochem. Mol. Biol. 65:181-189, 1998**.
11. Eustace S, **O'Regan R,** Graham D, Carney D. Primary multifocal Hodgkin's disease confined to the bone. **Skeletal Radiology 24:61-63, 1995**
12. Kaklamani V, **O'Regan RM.** New targeted therapies in breast cancer. Sems in Onc (in press)
13. Gradishar WJ, **O'Regan RM**. Progress in systemic adjuvant therapy of early stage breast cancer. Int J Clin Oncol 8;239-247, 2003
14. Kaklamani VG**, O'Regan RM.** Breast Cancer Prevention: the risks and benefits of drug therapy. Am J Cancer 1:173-178, 2002
15. **O'Regan RM**, Khuri FR. Farnesyl Transferase Inhibitors: The Next Targeted Therapies for Breast Cancer? Endocrine Related Cancer (accepted)
16. **O'Regan RM,** Jordan VC. The evolution of tamoxifen therapy in breast cancer: selective estrogen receptor modulators and down-regulators. **Lancet Oncology** 3;207-14, 2002
17. O'Regan RM, **Jordan VC. Tamoxifen to raloxifene and beyond** Sems in Oncol **28;260-273, 2001**
18. O'Regan RM, **Jordan VC, Gradishar WJ. Tamoxifen and contralateral breast cancer**. J Amer College Surg 188:678-683, 1999
19. **Bentrem DJ,** O'Regan RM, **Jordan VC. New strategies for the treatment of breast cancer.** Breast Cancer **8;265-74, 2001**
20. O'Regan RM, **Gradishar WJ. Selective Estrogen Receptor Modulators in the year 2000.** Oncology **15;1177-85, 2001**
21. O'Regan RM, **Jordan VC. Tamoxifen, a selective estrogen receptor modulator: A prelude to breast cancer prevention**. Int Med **1999**
22. **O'Regan RM**, England GM, MacGregor JI, Yao KA, Muenzner HD, Takei H, Jordan VC. Laboratory models of breast and endometrial cancer to develop strategies for antiestrogen therapy. **Breast cancer 5:211-217, 1998.**
23. Steroid Hormone Receptors. **O'Regan RM**, Badve S, Gradishar WJ. The Breast: Comprehensive Management of Benign and Malignant Diseases (3rd Edition), Bland KI, Copeland EM III (eds.). W.B. Saunders, Philadelphia, PA, in press, 2003.
24. SERMs Other Than Tamoxifen for Chemoprevention. **O'Regan RM,** Gradishar WJ. In Managing Breast Cancer Risk, (eds.). BC Decker, Inc., Ontario, Canada, in press, 2003.
25. **O'Regan, RM,** Jordan VC. Antiestrogens and related drugs; Introduction. In Ratain, Tempero, Skosey. **Outline of Oncology Therapeutics**, 2001
26. **O'Regan RM,** Jordan VC. Chemoprevention of breast cancer. In Bergan R (ed.) **Cancer Chemoprevention** pp137-154, 2001
27. **O'Regan RM.** Chemoprevention of breast cancer. In Gradishar and Wood (eds.): **Advances in Breast Cancer Management** pp 183-207, 2000
28. **O'Regan RM**, Jordan VC. The advantages and disadvantages of tamoxifen. In Khayat and Hortobagyi (eds.): **Progress in Cancer Therapy, Vol II , 1988.**

29. Gajdos C, **O'Regan RM,** Bentrem DJ, Pappas S, Badve S, MacGregor Schafer J, De Los Reyes A, Jordan VC. Paradoxical effect of ICI 182,780 after long-term tamoxifen treatment on breast cancer growth. Proc AACR 43;950:Abs 4705, 2002
30. **O'Regan RM**, Lee E, Dardes RC, Gajdos C, De Los Reyes A, Badve S, Jordan VC. Characteristics of raloxifene-stimulated breast tumors in vivo. Breast Cancer Res Treat 69;287: Abs 443, 2001
31. Dardes RC, **O'Regan RM** and Jordan VC Low dose tamoxifen effectively controls endometrial cancer growth not previously exposed to the drug. Proc AACR 42;240:Abs:1291, 2001
32. Dardes Rc, Bentrem DJ, **O'Regan RM**, MacGregor-Schafer J, Jordan VC. Effects of the antiestrogens Tamoxifen and LY 353,381.HCL (Arzoxifene) on Endometrial Cancer Growth**. Breast Cancer Res Treat** 64;73 (Abs 269), 2000
33. O'Regan RM**, Gajdos C, Dardes R, Bentrem DJ, De los Reyes A, Jordan VC. Effects of raloxifene after tamoxifen on breast and endometrial cancer growth. Proc ASCO 20;25a:Abs 95, 2001 (oral presentation)**
34. MacGregor-Schafer JI, Lee ES, Yao K, **O'Regan RM**, Jordan VC. Cross resistance to idoxifene and the raloxifene analoque LY 117,018 in the novel tamoxifen stimulated T47D xenograft model. **AACR**, San Francisco 2000
35. **O'Regan RM,** Tonetti DA, Jordan VC (1998) Comparison of tamoxifen and raloxifene on the growth of endometrial cancer cell lines. **Breast Cancer Res Treat Abs 441:306, 1998**
36. **O'Regan RM,** Cisneros A, Jordan VC. Is there life after tamoxifen? New Anti-oestrogens. **Eur J Cancer** 34:S57, 1998
37. **O'Regan RM**, England GM, Cisneros A, Jordan VC. Relationship of tamoxifen dose and the growth of tamoxifen-stimulated tumors. **Proc ASCO Abs 419, 1998.**
38. **O'Regan RM**, England GM, Cisneros A, Muenzner HD, Jordan VC. The effects of novel antiestrogens, toremifene amd faslodex (ICI 182,780) on human endometrial cancer growth in athymic mice. **Breast Cancer Res Treat Symposium 46:215, 1997.**

C. Research Support

Ongoing Research Support

None

Completed Research Support

Avon Cosmetics Foundation 2000-2001
Avon Cosmetics Foundation 2001-2002

EMORY HEALTHCARE
EMORY HOSPITALS

Consent to Surgical or Medical Treatment

Date: ____/____/____ Time: _____ Room Number _____

Diagnosis: _____ Procedure: _____

1. I understand the following about the procedure described above:
   a. **Nature and purpose of procedure** (*Describe in laymen's terms*): _____
   b. **Material risks of procedure: DEATH, RESPIRATORY ARREST, CARDIAC ARREST, BRAIN DAMAGE, DISFIGURING SCAR, PARAPLEGIA OR QUADRIPLEGIA, PARALYSIS OR PARTIAL PARALYSIS, LOSS OR FUNCTION OF ANY LIMB OR ORGAN, SEVERE LOSS OF BLOOD, ALLERGIC REACTION AND INFECTION.** These are material risks of any surgical procedure.
      Other risks of this procedure are: _____
   c. **Likelihood of success:** ❑ Good ❑ Fair ❑ Poor
      ❑ Unknown because: _____
   d. **Practical alternatives to procedure:** ❑ None
      ❑ Other: _____
   e. **Prognosis if procedure rejected:** ❑ Good ❑ Fair ❑ Poor
      ❑ Unknown because: _____
   f. ❑ **If applicable, DNR Order or DNI/Special Code status suspended unless indicated otherwise:**

2. **Consent:** The procedure identified above has been explained to me and all of my questions have been answered. I acknowledge that no guarantees have been made concerning the outcome of the surgical or medical treatment, and I realize that the practice of medicine and surgery are not an exact science. I hereby consent to the procedure by Dr. _____ and/or any assistants who may be present. I also consent to the administration of anesthesia to be applied by or under the direction and supervision of the Section of Anesthesiology of The Emory Clinic.

3. I realize that, during the procedure, the physician/surgeon may become aware of conditions which were not apparent before the start of the procedure, or may determine that additional or different operations or procedures are necessary or appropriate. I therefore authorize and request that the above named physician/surgeon and/or any assistants who may be present to perform additional or different operations or procedures the physician/surgeon deems necessary or advisable; so long as these additional procedures do not conflict with my stated DNR or DNI/Special Code preferences as indicated above.

4. Any tissue, organ, specimen, member or implant, removed or severed in any operation or procedure, may be retained, preserved, used for scientific or teaching purposes, or disposed of by the Hospital or by the Section of Pathology of The Emory Clinic at the discretion of the Hospital or Section, except for the following:

5. If acceptable to the physician/surgeon, I authorize observers to be present during the surgery or procedure. (❑ Yes ❑ No). I further authorize the physician/surgeon, or his designee, to photograph/videotape me before, during and/or after my surgery or procedure, for the purpose related to my care and treatment and/or for the purpose of medical education (❑ Yes ❑ No).

Witness _____ Patient Signature _____

Signature of person obtaining consent _____ Signature of person authorized to consent for patient _____

❑ Check if telephone consent given Relationship to patient

10699 **Over for Blood Transfusion Consent** 34 Consent 009 10/01

For Medical Device Recipients Only

Under Federal law, a hospital must report patient information to a company which manufactures medical devices, under some circumstances, including product recalls. However, the hospital can report your social security number to a manufacturer only with your permission. Your social security number may help the manufacturer identify you in the rare case of a product recall. I ❑ do ❑ do not authorize the hospital to report my social security number to the manufacturer of the medical device I receive.

Additional Authorizations for Surgical or Medical Treatment

The purpose of this section is to authorize repeat identical operations/procedures which have already been explained on the reverse side. If there is any change in the operation or other procedure to be done or in the associated risks, another "Consent to Surgical or Medical Treatment" form is to be completed.

Date	Surgical or Medical Treatment	Patient/Person authorized to consent for patient

Consent for Administration of Blood or Blood Products

Date: ____/____/____ Time: _____

The use of blood and blood products to treat my condition has been explained to me and I have been given an opportunity to ask questions.

I understand those precautions and procedures in selecting donors and in collecting, processing, preserving and administering blood and blood products developed by the American Red Cross, American Association of Blood Banks or the Food and Drug Administration will be followed. This will include testing for hepatitis B surface antigen, hepatitis C, HTLV I and II, and antibodies to HIV (AIDS virus). I further understand that in an emergency situation it may be necessary to administer blood or blood products before all tests have been completed.

I realize that despite all precautions and procedures referred to above, adverse reactions may occur. These reactions include, but are not limited to: fever, chills, allergic reactions, shock and transmission of infection (including, among others, hepatitis infection and AIDS virus infection).

I understand there are no artificial or natural substances which can perform all functions of blood. Failure to transfuse blood or blood products when needed could cause additional medical problems or complicate existing ones. These medical problems or complications could cause serious illness or death.

Most of the time, use of blood from random donors is necessary, but in some cases it may be possible to use one of the following: directed donation (blood from donors I select); autologous donation (blood collected from me before or during surgery); fluid replacement (administration of non-blood volume maintenance fluids). These alternatives have been discussed with me and I hereby consent to receive transfusions of blood or blood products from random donors as deemed advisable by any physician involved in the management of my condition or any complications that may occur.

_____ _____

Witness Patient Signature

_____ _____

Signature of person obtaining consent Signature of person authorized to consent for patient

_____

Relationship to patient

❑ Check if telephone consent given

References:

1. Harris JR, MM, Norton L Malignant tumors of breast. Philadelphia: Lippincott-Raven; 1997. p.1557-1616
2. Tyagi, S and Kramer, FR Molecular beacons: probes that fluoresce upon hybridization. Nat Biotechnol, 1996; *14*(3)*:* 303-308.
3. Peng XH, CZ, Xia JT, Carlson W. G, Lewis MM, Wood, WC and Yang L Real-Time Detection of Gene Expression in Cancer Cells using Molecular Beacon Imaging: New Strategies for Cancer Research. Cancer Research, 2005; *65*(5).
4. Sauer, T, Myrvold, K, Lomo, J, Anderssen, KY, and Skaane, P Fine-needle aspiration cytology in nonpalpable mammographic abnormalities in breast cancer screening: results from the breast cancer screening programme in Oslo 1996-2001. Breast, 2003; *12*(5)*:* 314-319.
5. Dooley, WC, Ljung, BM, Veronesi, U*, et al.* Ductal lavage for detection of cellular atypia in women at high risk for breast cancer. J Natl Cancer Inst, 2001; *93*(21)*:* 1624-1632.
6. Khan, SA, Wiley, EL, Rodriguez, N, *et al.* Ductal lavage findings in women with known breast cancer undergoing mastectomy. J Natl Cancer Inst, 2004; *96*(20)*:* 1510-1517.

EMORY UNIVERSITY AFFILIATED HOSPITALS

ONCOLOGY RESEARCH COOPERATIVE
INFORMED CONSENT FOR CLINICAL RESEARCH

STUDY TITLE:

Early Detection of Breast Cancer Using Molecular Beacons

Subtitle: Examination of the Sensitivity of Using Molecular Beacons Targeting Survivin, Cyclin D1 and Her-2/neu mRNAs to Detect Breast Cancer Cells in Ductal Lavage and Aspirates of Fine Needle Biopsy in Breast Cancer Patients

PRINCIPAL INVESTIGATOR: LILY YANG, MD/PHD

CO-INVESTIGATORS: GRANT W. CARLSON, MD; MELINDA M. LEWIS, MD; TONCRED M. STYBLO, MD; MARK Q. SMITH, MD.

You are invited to participate in a research study conducted at Emory University by the above investigators. Your participation in this study is voluntary. You should read the information below, and ask questions about anything you do not understand, before deciding whether or not to participate.

PURPOSE OF THE STUDY

Breast cancer is the most common type of cancer and is one of the leading causes of death among women. However, if breast cancer is detected early, the chances of survival are much better. Mammograms (a special kind of x-ray of the breast) can detect breast cancer early. However, about 20% of women who have mammograms and will not have their early breast cancer detected. Of all patients who have abnormal mammograms, only 10 to 20% are confirmed to be breast cancer by biopsy. Therefore, it is important to develop new ways to diagnose breast cancer at its early stage.

Recently, a new technique has been developed to detect breast cancer cells. This technique uses very small, circular "probes" (called molecular beacons) that specifically look for tumor cells. When these probes are in the cells, they cause a fluorescent signal in breast cancer cells. Thus, cancer cells become "labeled" with bright fluorescent colors that can be distinguished from normal cells. We can identify these labeled cells by looking with a special microscope. Since above probes are used for the determination of the levels of message RNA for tumor marker genes, we

will also use fluorescent semi-conductor nanoparticle (quantum dot)-labeled antibodies to detect the levels of corresponding proteins in the same cellular fraction.

WHAT IS INVOLVED IN THE STUDY?

We are examining the possibility of detecting breast cancer cells using special techniques called ductal lavage or fine needle aspiration.

Ductal lavage is a method to collect cells of breast milk ducts. This procedure involves three steps. First, an anesthetic numbing cream is applied to the nipple area and gentle suction is used to locate the openings of the milk duct on the nipple. Then, a hair-thin catheter is inserted into a milk duct opening and two teaspoons of saline are injected slowly into the duct. Next, the ductal cell fluid is withdrawn through the catheter and collected for cellular analysis with the molecular beacon probes.

Fine needle aspiration is a routine biopsy procedure performed in the outpatient clinic that uses a thin needle on a syringe to draw fluid and/or cellular material from breast tissues. A long, thin needle is inserted through the breast into the area of breast abnormality. The cells collected from this procedure will be sent to research lab.

About Fifty (50) of women with breast cancers will be enrolled in this study to determine the feasibility of detecting breast cancer cells in ductal lavage and fine needle aspiration samples using the molecular beacon technology.

You are invited to take part in this study. Please take your time to make your decision.

During a scheduled fine needle aspiration diagnostic procedure, excess aspirates that are not needed for diagnosis, and otherwise would be discarded, will be saved and used for research. No extra tissue will be removed for this research.

Ductal lavage will be performed during scheduled surgery for removing the breast cancer. In the operating room and after administration of general anesthesia, ductal lavage is performed as described above. This procedure will extend your anesthesia time for about 10 minutes.

After removing the breast cancer, if there is excess breast cancer tissue that is not needed for pathological diagnosis, it will be collected and frozen in liquid nitrogen for the examination of the expression of tumor marker genes.

Yes, I am willing to have above procedures performed and specimen donated.
Please initial _____.

HOW LONG WILL I BE IN THE STUDY?

For you, the study will last as long as your scheduled examination, diagnostic and surgical procedures for the treatment of your diseases. Your medical records may be reviewed during the study to determine the sensitivity and specificity of the detection method. Additionally, your prognosis will be followed for three years by examining your medical records and phone calls from the PI or CO-PIs.

WHAT ARE THE RISKS OF THE STUDY?

A recent study on over 500 high risk women conducted at 19 breast cancer centers showed that ductal lavage is a well-tolerated procedure. The majority patients said that the procedure was no more uncomfortable than a mammogram and some felt the sensation as breast fullness. There was no serious side effect found in this study.

Ductal lavage will be collected during the surgery and fine needle aspirates will be obtained from scheduled biopsy. Participation in the study will not add to any discomforts or significant risk to the patients. It is possible that prolongation of general anesthesia for 10 minutes during ductal lavage may have a minimal healthy risk. However, you will be carefully monitored by surgical and anesthetic staff during the ductal lavage procedure to reduce any healthy risk.

WHAT THE PRECAUTIONS TO BE OBERVED BY THE PARTICIPATE BEFORE AND AFTER STUDY PROCEDURES

Please inform your physician if you have fever or feel pain in your breast before or after the fine needle biopsy. Please also contact your physician if you feel swelling in you breast or notice any discharges from your breast nipple after the procedure.
Your ductal lavage procedure will be performed before your surgery to remove the tumor. Your physician will inform you about all precautions for preparation of the surgery. All routine post operation care procedures will be followed and your physician will follow-up on your recovery after the surgery.

ARE THERE BENEFITS TO TAKING PART IN THE STUDY?

You should not expect your condition to improve as a result of participating in this research. However, your participation in this study may help us learn more about the diagnosis of breast cancers and help doctors better identify and care for breast cancer in the future.

WHAT OTHER OPTIONS ARE THERE?

You have the right to refuse to participate in this study. Choosing not to take part will not result in any penalty or loss of benefits to which you are entitled.

Your participation may be terminated by your physician or principal investigator if you are no longer meet the inclusive criteria for the study, such as becoming pregnant or starting chemotherapy before the procedures are performed.

WHAT ABOUT CONFIDENTIALITY?

All participants of this study will be assigned a Study ID number. We will use the study number rather than your name on study records where we can. Your name and other facts that might point to you will not appear when we present this study or publish its results.
People other than those doing the study may look at both medical charts and study records. Agencies that make rules and policy about how research is done have the right to review these records. The Department of Defense has the Congressionally Directed Medical Research Programs (CDMRP) funding research on the diagnosis and treatment of breast cancer. This study is

sponsored by this program. Those with the right to look at your study records are, the Department of Defense, the National Cancer Institute, the Emory Clinic, Emory University Hospital and the Emory University Institutional Review Board. Records can also be opened by court order. We will keep your records private to the extent allowed by law. We will do this even if outside review occurs.

WHAT ARE THE COSTS?

There is no added cost to you for taking part in this study.

Other than medical care that may be provided specially stated in the consent form, there is no other compensation available for your participation in this research.

If you are hurt or get sick because of this research study, you need to notify your physician immediately. The PI and CO-PIs will make appropriate arrangement for you to receive medical care at an Army hospital or clinic free of charge. You will only be treated for injuries that are directly caused by the research study. The Army will not pay for your transportation to and from the hospital or clinic.

If you have questions about this medical care, talk to the principal investigator for this study, (Lily Yang, 404-778-4269). If you pay out-of-pocket for medical care elsewhere for injuries caused by this research study, contact the principal investigator. If the issue cannot be resolved, contact the U. S. Army Medical Research and Materiel Command (USAMRMC) Office of the Staff Judge Advocate (legal office) at (301) 619-7663/2221.

Emory University has not set aside funds to pay for your care or to compensate you if you are injured.

WHAT ARE MY RIGHTS AS A PARTICIPANT?

Taking part in this study is voluntary. You may choose not to take part or may leave the study at any time. Leaving the study will not result in any penalty or loss of benefits to which you are entitled.

This is a feasibility study for the detection of breast cancer cells using molecular beacon. Since these results have not been validated and accepted as reportable results, the test results will not be provided to you.

During this study you will be asked to provide fine needle biopsy, ductal lavage and breast cancer tissue samples. There samples will be used for detection of breast cancer cells using molecular beacon and examination of tumor marker gene expression in breast cancer cells. They may be used for purpose that currently unknown. There is a chance that the samples that you are donating under this study may be used in other research studies and may have some commercial value. Should your donated samples lead to the development of a commercial product, Emory University will own it and may take action to patent and license the product. Emory University does not intend to provide you with any compensation for your participation in this study nor for any future value that the sample you have given may be found to have. You will not receive any notice of future uses of your samples.

WHAT IF NEW INFORMATION ABOUT THIS TREATMENT IS LEARNED?

We may learn new things during the study that you may need to know. If so, you will be notified about any new information.

WHOM DO I CALL IF I HAVE QUESTIONS OR PROBLEMS?

For questions about the study or a research-related injury, contact your physician or Dr. Lily Yang, Principal Investigator, at 404-778-4269.

For questions about your rights as a research participant, contact Colleen Dilorio, PhD, Chairman of the Emory University Institutional Review Board (IRB) at (404) 712-0720. The IRB is a group of people who review the research to protect your rights.

WHO CAN BE INCLUDED IN THIS STUDY?

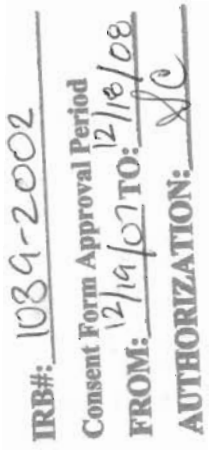
Patients of all ages and races diagnosed with breast cancers or with possibility of breast cancer, who come to Emory Clinic or Emory Hospital.

WHO SHOULD BE EXCLUSED FROM THIS STUDY?

Breast cancer patients who have received chemotherapy or radiation therapy within last three months.

Breast cancer patients who are currently pregnant or nursing.

Brest cancer patients who can not speak and read English.



CONSENT

If you agree to participate in this study, please sign below.
You are entitled to have a copy of the consent, regardless if you sign the document.

Please type your name_____

Signature _____ _____ _____
Participant Date Time

Or

Please type your name_____

Signature _____ _____ _____
Legally authorized representative Date Time

Permanent Address of the Participate

Street_____, Apt_____

City_____, State_____, Zip_____

Please type your name_____

_____ _____ _____
Person obtaining consent Date Time

_____ _____ _____
Principal Investigator (if different from above) Date Time

SIGNATURE OF WITNESS:

My signature as witness certifies that the subject signed this consent from in my presence as her voluntary act and deed.

_____
Name of Witness

_____ _____
Signature of Witness Date (Same as subject's)

Date of Approval:

Expiration Date:

IRB#: 1039-2002
Consent Form Approval Period
FROM: 12/19/07 TO: 12/18/08
AUTHORIZATION: AC

WINSHIP CANCER INSTITUTE

INFORMED CONSENT ADDENDUM

FOR

TISSUE STORAGE/FUTURE RESEARCH

As a participant in the study on "Early Detection of Breast Cancer Using Molecular Beacons", I voluntarily donate ductal lavage, fine needle aspirates and breast cancer tissue samples to Emory University. These samples will be used for examination of the presence of tumor markers using molecular beacons and quantum dot-labeled antibodies, types of tumor marker expression in breast cancer cells and studies on early detection of breast cancer. There is a possibility that these samples that I am donating under this study may be used in other research studies and may have some commercial value. Should my donated sample(s) lead to the development of a commercial product, Emory University will own it and it is possible that it will be patented and licensed by Emory University. Emory University does not intend to provide me with any compensation for my participation in this study and will not give me any notice of future uses of my sample(s).

I am informed that my identity will be confidential and a specific ID number will be used to identify my tissue. The link between that number and my name will be carefully guarded. My tissue samples will be used only for research in Dr. Lily Yang's lab, and will not be sold. If I decide now that my tissue can be kept for research, I can change your mind at any time. Just contact Dr. Lily Yang at 404-778-4269 or Lyang02@emory.edu, and let her know that I do not want her to use my tissue. She can link my name to my specimen. My donated samples can be destroyed at anytime. I may agree to participate in the research protocol, but refuse to provide the additional samples discussed above.

Am I willing to donate my left over tissue for possible future research of early detection of breast cancer?

Yes_____ No_____

After making your choice, please sign below.

Participant Date Time

Person obtaining consent Date Time

Principal Investigator (if different from above) Date

IRB#: 1039-2002
Consent Form Approval Period
FROM: 12/19/07 TO: 12/18/08
AUTHORIZATION:

EMORY UNIVERSITY AFFILIATED HOSPITALS

AUTHORIZATION TO USE AND DISCLOSE PERSONAL HEALTH INFORMATION

TITLE:

- **Early Detection of Breast Cancer Using Molecular Beacons**

PRINCIPAL INVESTIGATOR:

- Lilly Yang, MD/PhD

COINVESTIGATORS:

- Grant Carlson, MD
- Toncred Styblo, MD
- Melinda M. Lewis, MD
- Mark Q. Smith, MD

SPONSOR:

- Department of Defense

INTRODUCTION/PURPOSE:

The United States government has issued a new privacy rule to protect the privacy rights of patients. This rule was issued under a law called the Health Insurance Portability and Accountability Act of 1996 (HIPAA). The Privacy Rule is designed to protect the confidentiality of your health information. This document, called an "Authorization," describes your rights and explains how your health information will be used and disclosed for this study, named above.

This study is being conducted by the Emory University Winship Cancer Institute. The purpose of this research study is to learn if molecular beacons can help with the early detection of breast cancer.

ABOUT CONFIDENTIALITY AND PROTECTED HEALTH INFORMATION (PHI):

Protected health information (PHI) is any health information about you that identifies you or that can reasonably be used to identify you by the person to whom it is provided. The people

who are conducting the study (the "Researchers") may need to look at your medical and study records that contain PHI. In addition, government agencies that make rules and policies about how research is done, including the Office for Human Research Protections (OHRP) [and the Food and Drug Administration (FDA)] and, have the right to review these records. Sponsors who pay for the study also have the right to review records, as does the Emory University Institutional Review Board (IRB) and IRBs at other sites if the study is being conducted at more than one location. In addition, records may be disclosed pursuant to court order.

We will not use or disclose your records in any ways other than the ways we describe in this form, and we will keep your records private to the extent allowed by law. We will do this even if outside review of your records occurs. We will use a study number or other code rather than your name on study records where we can. Your name and other facts that might point to you will not appear when we present this study or publish its results.

Under the Health Insurance Portability and Accountability Act (HIPAA), a federal law enacted to protect the privacy of your PHI, before we can use or disclose your PHI, we must provide you with information about what PHI will be used and how it will be used and disclosed. This section of this form provides you with this information regarding your PHI. Specifically, it will tell you what PHI the Researchers will look at; who will collect the PHI; who will use the PHI, with whom it will be shared and the purpose of each use or disclosure; the expiration date or event, if any, after which we won't use or disclose your PHI any more; and your rights under HIPAA to ask us not to use your PHI any more. If you decide to participate in this research, then you will be agreeing to let the Researchers and any other persons, companies or agencies described below to use and share your PHI for the study in the ways that are set forth in this section, so please review this section very carefully.

WHAT PHI WILL THE RESEARCH TEAM USE?

The Researchers will look at your entire medical file, which contains all of your personal identifying information and health insurance information; health care providers notes; results of laboratory tests, x-rays and other medical tests; results of physical examinations, and any other information that your health care provider may have recorded about your health or health care. The researchers at the Winship Cancer Institute will also add your PHI to a database that they are compiling for research purposes. You will be followed-up through your doctor's office visits and telephone calls by the Researchers listed above for three years. The results of your laboratory tests, mammograms, response to treatments and prognosis will be added to your data files.

WHO WILL COLLECT THE PHI?

The Researchers will collect and copy the PHI described above during your doctor's office visits and after your operation or treatment from your records or by calling you directly. If any of the PHI is to be shared with other persons, as described later on in this section, then the Researchers also will be responsible for making these disclosures.

WHO WILL USE THE PHI; WITH WHOM WILL IT BE SHARED; AND FOR WHAT PURPOSE(S) WILL IT BE USED OR SHARED?

In order to conduct the study, the PHI that is collected regarding you will be used by or shared with the following persons, agencies or companies for the purposes listed in the chart below.

Person/Entity	***Purpose***
Researchers	*To conduct the study entitled, "Early Detection of Breast Cancer Using Molecular Beacons", the purpose of which is to learn if molecular beacons can help with the early detection of breast cancer.*
Governmental Agencies with oversight over the research being conducted, including the FDA and OHRP	*To monitor safety, efficacy and compliance with applicable laws and regulations.*
University personnel, committees and departments charged with oversight of research, including the IRB.	*To monitor safety and compliance with applicable laws, regulations and University policies and procedures.*
The US Department of Defense, the study sponsor.	*To provide oversight for the study and to perform data analysis.*
Study monitors hired by the Department of Defense	*To verify that data has been properly collected for reporting to the FDA.*

EXPIRATION DATE OR EVENT:

The Researchers will add your PHI to a database that they are compiling for research purposes. There is no date or event after which your Authorization will expire and your PHI will no longer be used for this purpose. After the study is finished and the results are published, any records connecting your personal information to results of laboratory tests, x-rays, other medical tests and physical examinations will be erased from the our database or hard copies will be destroyed.

YOUR RIGHT UNDER HIPAA TO REVOKE YOUR AUTHORIZATION AND ASK US NOT TO USE YOUR PHI ANY MORE:

Giving the Researchers your authorization to use and share your PHI is voluntary. At any time, you may choose to revoke your authorization for the Researchers to use and share your PHI. If you revoke your authorization, the Researchers may no longer be able to provide you with any research-related treatment, but your revocation will not otherwise affect your current or future health care. Further, if you revoke your authorization, there will be no penalty or loss of any benefits to which you are otherwise entitled.

If you decide that you want to revoke your authorization for us to use your PHI, you may do so by providing it to the researcher a written and signed request to do so. Once we receive your written revocation of your authorization to use your PHI, we will not make any other use of your PHI or share it with anyone else, except as follows: (a) we will let the study sponsor (if any) know that you have revoked your authorization; (b) we will not ask the study sponsor (if any) or any other parties to whom we said we would disclose data to return any data that we provided to it/them before you revoked your authorization; (c) and, even after we receive your revocation, we will still provide the study sponsor (if any) and any other parties to whom we stated that we would disclose data with any data that is necessary to preserve the integrity of the research study, and we will provide any governmental or University personnel, departments

or committees with any data that they may need in order to comply with/or investigate adverse events or non-compliance with any applicable laws, regulations or University policies.

PHI MAY BE RE-DISCLOSED:

If we disclose your PHI to one of the other parties described above, that party might further disclose your PHI to another party. If your PHI is further disclosed, then the information is no longer covered by HIPAA.

SIGNATURE AND DATE:

The researchers will ask you to sign and date this form.
You will be provided with a copy of this form after you have signed and dated it.

AUTHORIZATION

I have read this authorization form and have been given the chance to ask questions about it. I am signing this form voluntarily and I understand that by signing I will be authorizing the Researchers to use and disclose my PHI as described in this form.

_____ _____
Signature of: **Date**

____ Participant or

____ Participant's Representative
[check one]

_____ _____
Witness **Date**

IF APPLICABLE

For Personal Representatives Signing for Participants who are Unable to Sign due to Incapacity:

I certify that I _____, am over 18 years of age and that I am the personal representative of _____ ("Participant"), a person over 18 years of age, who has been invited to participate in this study but who is unable to sign this form due to physical or mental incapacity. I certify that legally I have been designated as the personal representative of the Participant because [insert description of reason for authority, e.g., "I have a court order dated 0/0/0 naming me as the Participant's legal representative"; I am named as the representative by a Durable Power of Attorney for Healthcare dated 0/0/0," etc.]. I further certify that I have full legal authority to make decisions concerning the participant, including decisions regarding health care and health care information.

IRB#: 1039-2002
Consent Form Approval Period
FROM: 12/19/07 TO: 12/18/08
AUTHORIZATION: AC

Sarah Clark

From: Sarah Clark [skclar2@emory.edu]
Sent: Tuesday, January 22, 2008 3:37 PM
To: 'lyang02@emory.edu'
Subject: IRB# 1039-2002

The renewal for your protocol "Early Detection of Breast Cancer Using Molecular Beacons" has been approved by the IRB. However, while preparing the consent documents for stamping, I noticed that the consent and HIPAA Authorization submitted with the renewal do not have the correct version dates. The consents for this protocol were revised and submitted as part of a modification in March of 2007. The currently approved consent and authorization should have a version date of 3/6/2007. Please submit the most recently approved consent and authorization. Thank you for your attention to this matter, and please let me know if you have any questions.

Sarah K. Clark
Analyst Assistant
Institutional Review Board
Emory University
1599 Clifton Rd, 5th Floor
Atlanta, GA 30322
Direct Line: 404-712-0218

EMORY UNIVERSITY AFFILIATED HOSPITALS

ONCOLOGY RESEARCH COOPERATIVE
INFORMED CONSENT FOR CLINICAL RESEARCH

STUDY TITLE:

- **Early Detection of Breast Cancer Using Molecular Beacons**

PRINCIPAL INVESTIGATOR: LILY YANG, MD/PHD
COINVESTIGATORS: WILLIAM C. WOOD, MD., GRANT W.CARLSON, MD., MELINDA M. LEWIS, MD

You are asked to participate in a research study conducted at Emory University by the above investigators. Your participation in this study is voluntary. You should read the information below, and ask questions about anything you do not understand, before deciding whether or not to participate.

PURPOSE OF THE STUDY

Breast cancer is the most common type of cancer and is a leading cause of death among women. However, if breast cancer is detected early, the chances of survival are much better. Mammograms (a special kind of x-ray of the breast) can detect breast cancer early. However, 20% of breast cancer patients had early mammograms and their cancer was not detected. And, of all patients who had abnormal mammograms, only 10 to 20% were confirmed to be breast cancer by biopsy. Therefore, it is important to develop new ways to diagnose breast cancer at its early stage.

Recently, a new technique has been developed to detect breast cancer cells. This technique uses very small, circular "probes" (called molecular beacons) that specifically look for tumor cells. When these probes are in the cells, they cause a fluorescent signal in breast cancer cells. Thus, cancer cells become "labeled" with bright fluorescent colors that can be distinguished from normal cells.

WHAT IS INVOLVED IN THE STUDY?

We are examining the feasibility of detecting breast cancer cells in ductal lavage or fine needle aspiration samples from breast cancer patients and women at high risk for breast cancer.

Ductal lavage is a minimally invasive method to collect cells of breast milk ducts. This procedure involves three steps. At first, an anesthetic cream is applied to the nipple area and gentle suction is used to locate the openings of the milk duct on the nipple. Then, a hair-thin catheter is inserted into a milk duct opening and 10 ml of saline is injected slowly into the duct. Next, the ductal cell fluid is withdrawn through the catheter and collected for cellular analysis with molecular beacon probes.

Fine needle aspiration is a routine biopsy procedure in clinic that uses a thin needle on a syringe to draw fluid and/or cellular material from breast tissues. A long, thin needle is inserted through the breast into either the area of breast abnormality (breast cancer patients) or four positions around breast (high risk women and normal volunteer). The cellular fraction from needle aspiration will be sent to research lab.

About 175 women will take part in this study at Emory. Fifty (50) of those women will have breast cancer. One hundred (100) of them will be at high risk for getting breast cancer, and 25 will be normal volunteers who are also not at high risk for developing breast cancer.

We hope to enroll three different groups of women for this study. You are being asked to take part in this study because you fall under one of these categories:

- breast cancer patients
- women at a high risk for getting breast cancer,
- healthy women at normal risk for getting breast cancer

Please take your time to make your decision.

Consent form for women at high risk for breast cancer and normal volunteers

High risk women
Ductal lavage or fine needle aspiration procedure is performed during a doctor office visit. Only one of the procedures will be performed on you. Your physician will inform you about the type of the procedure and you are asked to read and sign this consent form.

Yes, I am willing to have ductal lavage performed and specimen donated.
Please initial _____.

Or
Yes, I am willing to have fine needle aspiration performed and specimen donated.
Please initial _____.

For normal donors
Normal volunteers who have a history of low risk for breast cancer and come to Emory Clinic for annual mammograms will be recruited into the trial by physicians at Emory Clinic. Only one of the procedures will be performed on you. Your physician will inform you about the procedure you are receiving.

Yes, I am willing to have ductal lavage performed and specimen donated.
Please initial _____.

Or
Yes, I am willing to have fine needle aspiration performed and specimen donated.
Please initial _____.

HOW LONG WILL I BE IN THE STUDY?

Women in the high risk group and normal volunteers will be followed for three years to determine the significance between detecting the cells expressing marker genes and developing breast cancers.

If any abnormalities are found, we will contact you for follow-up examinations.

WHAT ARE THE RISKS OF THE STUDY?

A recent study on over 500 high risk women conducted at 19 breast cancer centers showed that ductal lavage is a well-tolerated procedure. Majority patients said that the procedure was no more uncomfortable than a mammogram and some felt the sensation as breast fullness. There was no serious side effect found in this study.

Fine needle aspiration is a routine clinical procedure for breast biopsy. It is possible for you to have a small amount of bruising in the area of breast that was sampled and to have some mild soreness afterward.

Additionally, minor discomfort may be associated with ductal lavage and fine needle aspiration. It is uncommon but a possibility for the duct lavage or fine needle aspiration to cause an infection in breasts. If this happens, you will be given medicine to fight the infection.

WHAT THE PRECAUTIONS TO BE OBERVED BY THE PARTICIPATE BEFORE AND AFTER STUDY PROCEDURES

Please inform your physician if you have fever or feel pain in your breast before or after the procedure. Please also contact your physician if you feel swelling in you breast or notice any discharges from your breast nipple.

ARE THERE BENEFITS TO TAKING PART IN THE STUDY?

This study may be beneficial for women at high risk for breast cancer since identification of the cells expressing tumor markers may provide information for monitoring, and early detection of breast cancer.

MB-detection of breast cancer cells is a new method for caner detection and the specificity of this approach is currently under evaluation in this study. It is not a validated clinical test for breast cancer. We will not report the results to your physician or you if you choose not to know. However, you also have an option of electing to find out results and follow-up mammograms based on the results of this study.

____Yes, I would like to be informed about the results.

____ No, I would not like to know the results.

WHAT OTHER OPTIONS ARE THERE?

You have the right to refuse to participate in this study. Choosing not to take part will not result in any penalty or loss of benefits to which you are entitled.

Your participation may be terminated by your physician or principle investigator if you are no longer meet the inclusive criteria for the study, such as becoming pregnant or starting chemotherapy for other cancers before the procedures are performed.

During the course of the study, you will be informed of any significant new findings (either good or bad), such as changes in the risk or benefits resulting from participation in the research or new alternatives to participation, that might cause you to change your mind about continuing in the study. If new information is provided to you, your consent to continue participating in this study will be re-obtained.

WHAT ABOUT CONFIDENTIALITY?

People other than those doing the study may look at both medical charts and study records. Agencies that make rules and policy about how research is done have the right to review these records. The Department of Defense has the Congressionally Directed Medical Research Programs (CDMRP) funding research on the diagnosis and treatment of breast cancer. This study is sponsored by this program. Those with the right to look at your study records, The Department of Defense, The National Cancer Institute, The Emory Clinic, Emory University Hospital and The Emory University Institutional Review Board. Records can also be opened by court order. We will keep your records private to the extent allowed by law. We will do this even if outside review occurs. We will use a study number rather than your name on study records where we can. Your name and other facts that might point to you will not appear when we present this study or publish its results.

WHAT ARE THE COSTS?

There is no added cost to you for taking part in this study.

Other than medical care that may be provided and any other payment specially stated in the consent form, there is no other compensation available for your participation in this research.

Any tissues or materials removed shall become the property of Emory University. Any substance, which may be derived, developed or created from it, may be used in any manner by the University, including patenting and commercialization, without further permission or compensation to the participants of this study.

If you are hurt or get sick because of this research study, you can receive medical care at an Army hospital or clinic free of charge. You will only be treated for injuries that are directly caused by the research study. The Army will not pay for your transportation to and from the hospital or clinic. If you have questions about this medical care, talk to the principal

investigator for this study, (Lily Yang, 404-778-4269). If you pay out-of-pocket for medical care elsewhere for injuries caused by this research study, contact the principal investigator. If the issue cannot be resolved, contact the U. S. Army Medical Research and Materiel Command (USAMRMC) Office of the Staff Judge Advocate (legal office) at (301) 619-7663/2221.

Emory University has not set aside funds to pay for your care or to compensate you if you are injured.

WHAT ARE MY RIGHTS AS A PARTICIPANT?

Taking part in this study is voluntary. You may choose not to take part or may leave the study at any time. Leaving the study will not result in any penalty or loss of benefits to which you are entitled.

WHAT IF NEW INFORMATION ABOUT THIS TREATMENT IS LEARNED?

We may learn new things during the study that you may need to know. If so, you will be notified about any new information.

WHOM DO I CALL IF I HAVE QUESTIONS OR PROBLEMS?

For questions about the study or a research-related injury, contact your physician or Dr. Lily Yang at 404-778-4269.

For questions about your rights as a research participant, contact Dr. James W. Keller, Chairman of the Emory University Institutional Review Board (IRB) at (404) 727-5646. The IRB is a group of people who review the research to protect your rights.

CONSENT

If you agree to participate in this study, please sign below.
You are entitled to have a copy of the consent, regardless if you sign the document.

Type_____

Signature _____ _____ _____
Participant Date Time

Or

Type_____

Signature _____ _____ _____
Legally authorized representative Date Time

Permanent Address of the Participate

Street_____, Apt_____

City_____, State_____, Zip_____

Type_____

_____ _____ _____
Person obtaining consent Date Time

_____ _____

_____ _____ _____
Principal Investigator (if different from above) Date Time

SIGNATURE OF WITNESS:

My signature as witness certifies that the subject signed this consent from in my presence as her voluntary act and deed.

_____
Name of Witness

_____ _____
Signature of Witness Date (Same as subject's)

Date of Approval:

Expiration Date:

WINSHIP CANCER INSTITUTE

INFORMED CONSENT ADDENDUM

FOR

TISSUE STORAGE/FUTURE RESEARCH

For high risk women or normal volunteers:

As a participant in Early Detection of Breast Cancer Using Molecular Beacons, your are being asked to donate any and all ductal lavage or fine needle aspirates to Emory University. These samples will be used for examination of expression of tumor marker genes, further molecular beacon study and may also be used by Dr. Lily Yang at Emory University for uses not currently known. There is a possibility that the samples that you are donating for this study may be used in other research studies and may have some commercial value. Should your donated sample(s) lead to the development of a commercial product, Emory University will own it and it is possible that it will be patented and licensed by Emory University. Emory University does not intend to provide you any compensation for this and will not give you any notice of future uses of my sample(s).

To make your identity confidential, we will use specific ID number to identify your tissue. The link between that number and your name will be carefully guarded. Your tissue samples will be used only for research in Dr. Lily Yang's lab, and will not be sold. If you decide now that your tissue can be kept for research, you can change your mind at any time. Just contact Dr. Lily Yang, and let her know that you do not want her to use your tissue. She can link your name to your specimen. It can be destroyed at anytime.

You may agree to participate in the research protocol, but refuse to provide the additional samples discussed above.

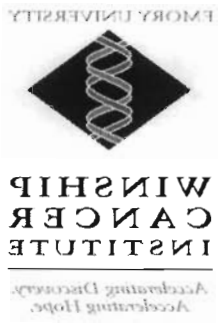
- May Dr. Lily Yang keep your left over tissue for possible future research of molecular beacons?

  Yes_____ No_____

After making your choice, please sign below.

Participant	Date	Time
Person obtaining consent	Date	Time
Principal Investigator (if different from above)	Date	

EMORY UNIVERSITY AFFILIATED HOSPITALS



AUTHORIZATION TO USE AND DISCLOSE PERSONAL HEALTH INFORMATION

TITLE:

- **Early Detection of Breast Cancer Using Molecular Beacons**

PRINCIPAL INVESTIGATOR:

- Lilly Yang, MD, PhD

COINVESTIGATORS:

- William C. Wood, MD
- Grant Carlson, MD
- Melinda M. Lewis, MD

SPONSOR:

- Department of Defense

INTRODUCTION/PURPOSE:

The United States government has issued a new privacy rule to protect the privacy rights of patients. This rule was issued under a law called the Health Insurance Portability and Accountability Act of 1996 (HIPAA). The Privacy Rule is designed to protect the confidentiality of your health information. This document, called an "Authorization," describes your rights and explains how your health information will be used and disclosed for this study, named above.

This study is being conducted by the Emory University Winship Cancer Institute. The purpose of this research study is to learn if molecular beacons can help with the early detection of breast cancer.

ABOUT CONFIDENTIALITY AND PROTECTED HEALTH INFORMATION (PHI):

Protected health information (PHI) is any health information about you that identifies you or that can reasonably be used to identify you by the person to whom it is provided. The people who are conducting the study (the "Researchers") may need to look at your medical and study records that contain PHI. In addition, government agencies that make rules and policies about how research is done, including the Office for Human Research Protections (OHRP) [and the Food and Drug Administration (FDA)] and, have the right to review these records. Sponsors who pay for the study also have the right to review records, as does the Emory University Institutional Review Board (IRB) and IRBs at other sites if the study is being conducted at more than one location. In addition, records may be disclosed pursuant to court order.

We will not use or disclose your records in any ways other than the ways we describe in this form, and we will keep your records private to the extent allowed by law. We will do this even if outside review of your records occurs. We will use a study number or other code rather than your name on study records where we can. Your name and other facts that might point to you will not appear when we present this study or publish its results.

Under the Health Insurance Portability and Accountability Act (HIPAA), a federal law enacted to protect the privacy of your PHI, before we can use or disclose your PHI, we must provide you with information about what PHI will be used and how it will be used and disclosed. This section of this form provides you with this information regarding your PHI. Specifically, it will tell you what PHI the Researchers will look at; who will collect the PHI; who will use the PHI, with whom it will be shared and the purpose of each use or disclosure; the expiration date or event, if any, after which we won't use or disclose your PHI any more; and your rights under HIPAA to ask us not to use your PHI any more. If you decide to participate in this research, then you will be agreeing to let the Researchers and any other persons, companies or agencies described below to use and share your PHI for the study in the ways that are set forth in this section, so please review this section very carefully.

WHAT PHI WILL THE RESEARCH TEAM USE?

The Researchers will look at your entire medical file, which contains all of your personal identifying information and health insurance information; health care providers notes; results of laboratory tests, x-rays and other medical tests; results of physical examinations, and any other information that your health care provider may have recorded about your health or health care. The researchers at the Winship Cancer Institute will also add your PHI to a database that they are compiling for research purposes. You will be followed-up through your doctor's office visits and telephone calls by the Researchers listed above for three years. The results of your laboratory tests, mammograms, response to treatments and prognosis will be added to your data files.

WHO WILL COLLECT THE PHI?

The Researchers will collect and copy the PHI described above during your doctor's office visits and after your operation or treatment from your records or by calling you directly. If any of the PHI is to be shared with other persons, as described later on in this section, then the Researchers also will be responsible for making these disclosures.

WHO WILL USE THE PHI; WITH WHOM WILL IT BE SHARED; AND FOR WHAT PURPOSE(S) WILL IT BE USED OR SHARED?

In order to conduct the study, the PHI that is collected regarding you will be used by or shared with the following persons, agencies or companies for the purposes listed in the chart below.

Person/Entity	***Purpose***
Researchers	*To conduct the study entitled, "Early Detection of Breast Cancer Using Molecular Beacons", the purpose of which is to learn if molecular beacons can help with the early detection of breast cancer.*
Governmental Agencies with oversight over the research being conducted, including the FDA and OHRP	*To monitor safety, efficacy and compliance with applicable laws and regulations.*
University personnel, committees and departments charged with oversight of research, including the IRB.	*To monitor safety and compliance with applicable laws, regulations and University policies and procedures.*
The US Department of Defense, the study sponsor.	*To provide oversight for the study and to perform data analysis.*
Study monitors hired by the Department of Defense	*To verify that data has been properly collected for reporting to the FDA.*

EXPIRATION DATE OR EVENT:

The Researchers will add your PHI to a database that they are compiling for research purposes. There is no date or event after which your Authorization will expire and your PHI will no longer be used for this purpose. After the study is finished and the results are published, any records connecting your personal information to results of laboratory tests, x-rays, other medical tests and physical examinations will be erased from the our database or hard copies will be destroyed.

YOUR RIGHT UNDER HIPAA TO REVOKE YOUR AUTHORIZATION AND ASK US NOT TO USE YOUR PHI ANY MORE:

Giving the Researchers your authorization to use and share your PHI is voluntary. At any time, you may choose to revoke your authorization for the Researchers to use and share your PHI. If you revoke your authorization, the Researchers may no longer be able to provide you with any research-related treatment, but your revocation will not otherwise affect your current or future health care. Further, if you revoke your authorization, there will be no penalty or loss of any benefits to which you are otherwise entitled.

If you decide that you want to revoke your authorization for us to use your PHI, you may do so by providing it to the researcher a written and signed request to do so. Once we receive your written revocation of your authorization to use your PHI, we will not make any other use of your PHI or share it with anyone else, except as follows: (a) we will let the study sponsor (if any) know that you have revoked your authorization; (b) we will not ask the study sponsor (if any) or any other parties to whom we said we would disclose data to return any data that we provided to it/them before you revoked your authorization; (c) and, even after we receive your revocation, we will still provide the study sponsor (if any) and any other parties to whom we

stated that we would disclose data with any data that is necessary to preserve the integrity of the research study, and we will provide any governmental or University personnel, departments or committees with any data that they may need in order to comply with/or investigate adverse events or non-compliance with any applicable laws, regulations or University policies.

PHI MAY BE RE-DISCLOSED:

If we disclose your PHI to one of the other parties described above, that party might further disclose your PHI to another party. If your PHI is further disclosed, then the information is no longer covered by HIPAA.

SIGNATURE AND DATE:

The researchers will ask you to sign and date this form.
You will be provided with a copy of this form after you have signed and dated it.

AUTHORIZATION

I have read this authorization form and have been given the chance to ask questions about it. I am signing this form voluntarily and I understand that by signing I will be authorizing the Researchers to use and disclose my PHI as described in this form.

_____ _____
Signature of: **Date**

____ Participant or

____ Participant's Representative
[check one]

_____ _____
Witness **Date**

IF APPLICABLE

For Personal Representatives Signing for Participants who are Unable to Sign due to Incapacity:

I certify that I _____, am over 18 years of age and that I am the personal representative of _____ ("Participant"), a person over 18 years of age, who has been invited to participate in this study but who is unable to sign this form due to physical or mental incapacity. I certify that legally I have been designated as the personal representative of the Participant because [insert description of reason for authority, e.g., "I have a court order dated 0/0/0 naming me as the Participant's legal representative"; I am named as the representative by a Durable Power of Attorney for Healthcare dated 0/0/0," etc.]. I further certify that I have full legal authority to make decisions concerning the participant, including decisions regarding health care and health care information.