

# Immunofluorescent Localization of a Novel Progesterone Receptor(s) in a T47D-Y Breast Cancer Cell Line Lacking Genomic Progesterone Receptor Expression

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**OBJECTIVE:** To identify a novel nongenomic progesterone receptor (PR), PR-M, in T47D-Y breast cancer cells lacking genomic PR expression.

**METHODS:** Immunofluorescent staining of T47D and T47D-Y breast cancer cells with selective anti-PR antibodies and ligand binding. Transient transfection of breast cancer cells with a cDNA expressing PR-M with a carboxy terminal green fluorescent protein.

**RESULTS:** In the T47D-Y cell line, lacking expression of genomic PR, plasma membrane-bound and intracellular PR(s) are identified with anti-PR antibodies directed to the hormone-binding domain but not with an antibody directed to the amino terminus. A plasma membrane PR is also evident by immunofluorescent ligand binding. Expression of a novel truncated PR (PR-M) tagged with green fluorescent protein showed intracellular localization.

**CONCLUSIONS:** These studies support the expression of a novel, truncated PR (PR-M) in a breast cancer cell line known to lack expression of genomic PR. This observation raises the possibility of progesterone action in breast cancer cells classically considered nonresponsive due to lack of genomic PR expression. (*J Soc Gynecol Invest* 2005;12:610–6) Copyright © 2005 by the Society for Gynecologic Investigation.

**KEY WORDS:** Truncated progesterone receptor, immunofluorescent antibody staining, ligand binding, transient transfection.

Classically, progesterone has been considered a reproductive hormone that stimulates gene transcription via nuclear receptors (progesterone receptors [PR] PR-B and PR-A).<sup>1</sup> The structure of PRs include an amino terminus A/B domain that regulates transcription efficiency, a C domain or DNA binding region (DBD), a D domain or hinge region, and an E/F domain responsible for hormone binding (HBD). PR-B is composed of 933 amino acids encoded by a transcript containing eight exons, while PR-A is an N-terminally truncated form of the larger PR-B that lacks the first 164 amino acids.<sup>2,3</sup> Homo- and heterodimerization of these isoforms with ligand binding and phosphorylation leads to transcriptional regulation in progesterone-responsive tissues, traditionally considered the breast and endometrium.

Yet, studies with other tissues have suggested that functional nongenomic PR(s) may be present in the plasma membrane or cytosol. A plasma membrane-bound protein was identified in *Xenopus* oocytes, with significant homology to the human PR in the DBD and the HBD, that is responsible for a non-

genomic-induced resumption of meiosis I.<sup>4,5</sup> In mammalian tissues, a plasma membrane-bound PR induces the acrosome reaction in human spermatozoa<sup>6,7</sup> and inhibits meiosis in rat granulosa cells.<sup>8</sup> Progesterone appears to stimulate some of these nongenomic effects by modulating intracellular calcium concentrations.<sup>9–11</sup> Additionally, evidence exists that a polyproline motif in the genomic PR may function to activate various cytosolic signaling proteins via a SH3 domain.<sup>12</sup>

Two novel PRs have been described that lack homology with the genomic receptors. Thomas et al cloned, expressed, and characterized a G-protein-coupled membrane receptor showing a high-affinity, saturable, single binding site specific for progestins. Expression in MDA-MB-231 breast cancer cells showed plasma membrane localization and treatment with progestin resulted in decreased cyclic adenosine monophosphate (cAMP) levels and activation of mitogen-activated protein (MAP) kinase.<sup>13,14</sup> A second PR has been identified from porcine liver microsomes<sup>15</sup> and the cDNA identified in human liver and spermatozoa.<sup>16</sup> An antiserum to this protein inhibited both progesterone-induced calcium entry and acrosome reaction in human spermatozoa.<sup>17</sup>

We have previously reported the identification of a novel truncated PR, named PR-M. PR-M is a 38-kd protein originally cloned from a human adipose and aortic cDNA library.<sup>18</sup> The cDNA contains novel sequence, derived from the third

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intron, encoding a 5' untranslated region and sequence for the first 16 amino acids. The remainder of the cDNA is identical to exons 4 through 8 of the genomic PR. Thus PR-M contains the hinge and hormone binding domains identical to the genomic PR but lacks the amino terminus, nuclear localization signal,<sup>19</sup> and DNA binding domain. PR-M transcript and protein expression have been demonstrated in T47D, T47D-Y breast cancer cell lines and in human aortic endothelial cells.<sup>18</sup> This study utilizes immunofluorescent antibody and ligand binding in the genomic PR-negative T47D-Y cell line along with transient expression of PR-M to further analyze the localization of PR-M and possibly other yet identified non-genomic PRs.

## MATERIALS AND METHODS

### Cell Culture

T47D cells were obtained from ATCC (Manassus, VA), while T47D-Y cells were the kind gift of Dr K. Horwitz. Both cell types were cultured in phenol red-free RPMI media supplemented with 10% fetal bovine serum (FBS; Gibco BRL, Gaithersburg, MD) in a 37C incubator with 5% CO<sub>2</sub>.

### Immunofluorescent Antibody Staining

Antibody staining was performed both with adherent cells and trypsinized cells in solution. For adherent experiments, cells were grown until 80% confluence in two-well chamber slides and then processed. For cells in solution, cells were lifted from flasks with 1% trypsin, rinsed with serum-containing media, and then processed. Cells were then incubated for 30 minutes with buffer A (25 mM HEPES, pH 7.4, 120 mM NaCl, 4.6 mM KCl, 1.25 mM KH<sub>2</sub>PO<sub>4</sub>, 25 mM mM EDTA, 10 mM glucose), spun and blocked in 3% bovine serum albumin (BSA)/phosphate-buffered saline (PBS) containing 10% goat serum for 15 minutes at 4C. All subsequent steps were performed at 4C with nonpermeabilized cells. Following blocking, the cells were washed in 1% BSA/PBS and the primary antibody was added for 1 hour at a concentration of 1 μg/mL. Cells were washed with 1% BSA/PBS and the secondary antibody was added for 30 minutes. Following three washings, the cells were fixed with 4% methanol-free formaldehyde for 5 minutes, mounted in SlowFade Anti-Fade Light reagent (Molecular Probes, Eugene, OR), and observed with a Zeiss LSM 410 confocal microscope, 40–63X objective using the 488-nm spectral line of the He-Ne laser.

For permeable reactions, cells were first fixed for 25 minutes in 4% methanol-free formaldehyde in PBS and permeabilized with 0.2% Triton-X 100/PBS for 30 minutes on ice. The remainder of the steps were as above except performed at room temperature.

Selective primary antibodies included a rabbit polyclonal directed to the HBD (C19; Santa Cruz Biotechnology Inc, Santa Cruz, CA), a monoclonal antibody directed to the amino terminus (6A1; Cell Signaling Technology, Beverly, MA), and a monoclonal antibody directed to the HBD (MAB 462; Chemicon International, Temecula, CA). Respective second-

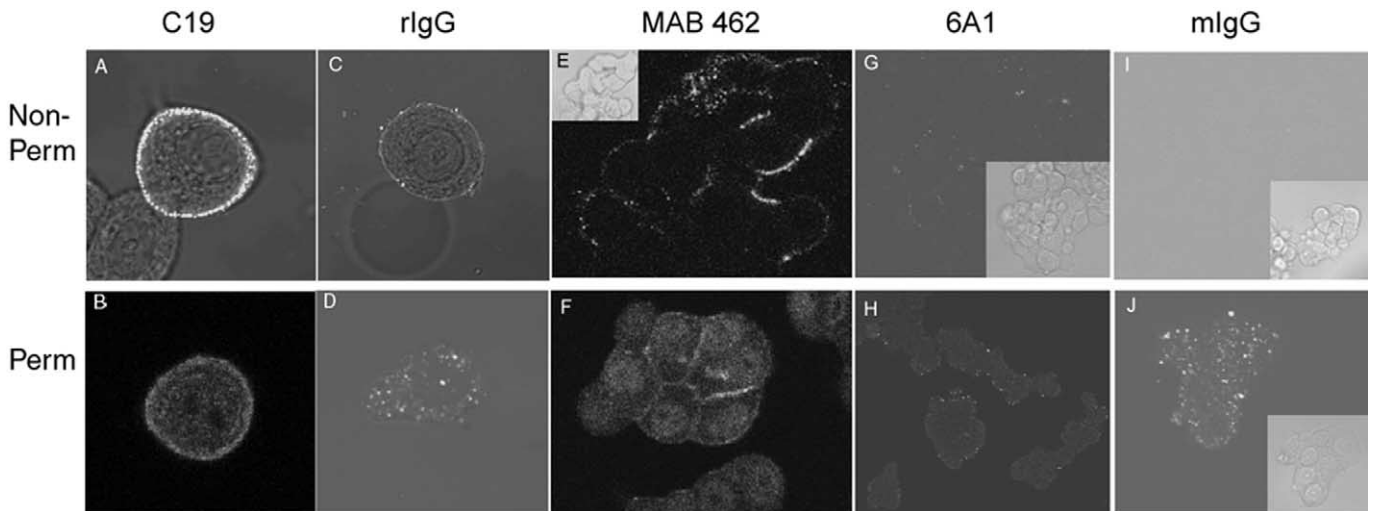
ary antibodies included goat anti-rabbit and goat anti-mouse IgG Alexa Fluor 488 antibodies (Molecular Probes). The specificity of labeling was checked by examination of samples with the primary antibody replaced with the same concentration of normal rabbit IgG or normal mouse IgG.

### Immunofluorescent Ligand Binding

Cells at 80% confluence were lifted with 1% trypsin, washed and placed in microfuge tubes at a density of 10<sup>6</sup> cells/mL. Subsequent steps were performed at 4C to decrease cell permeability. Blocking was performed for 30 minutes with 2 μM dexamethasone for all samples, and specific samples with 10<sup>-5</sup> M progesterone 3-(O-carboxymethyl)-oxime:BSA (P-CMO:BSA; Fitzgerald International Industries, Concord, MA), 10<sup>-4</sup> M medroxyprogesterone acetate (Sigma), 10<sup>-4</sup> M 17β-estradiol (Sigma), and 10<sup>-4</sup> M water-soluble progesterone (cyclo-dextrin-encapsulated, Sigma). Cells were then incubated for 30 minutes in 1% BSA/PBS with 10<sup>-6</sup> M progesterone 3-(O-carboxymethyl)-oxime:BSA-fluorescein isothiocyanate (P-CMO:BSA-FITC) or a control of 10<sup>-6</sup> M BSA-FITC (Sigma). Cells were washed with cold 1% BSA/PBS, fixed in 4% paraformaldehyde, and observed with a Zeiss LSM 510 confocal microscope, 63X oil objective using the 488-nm spectral line of the He-Ne laser.

### Transient Transfection With PR-M-GFP

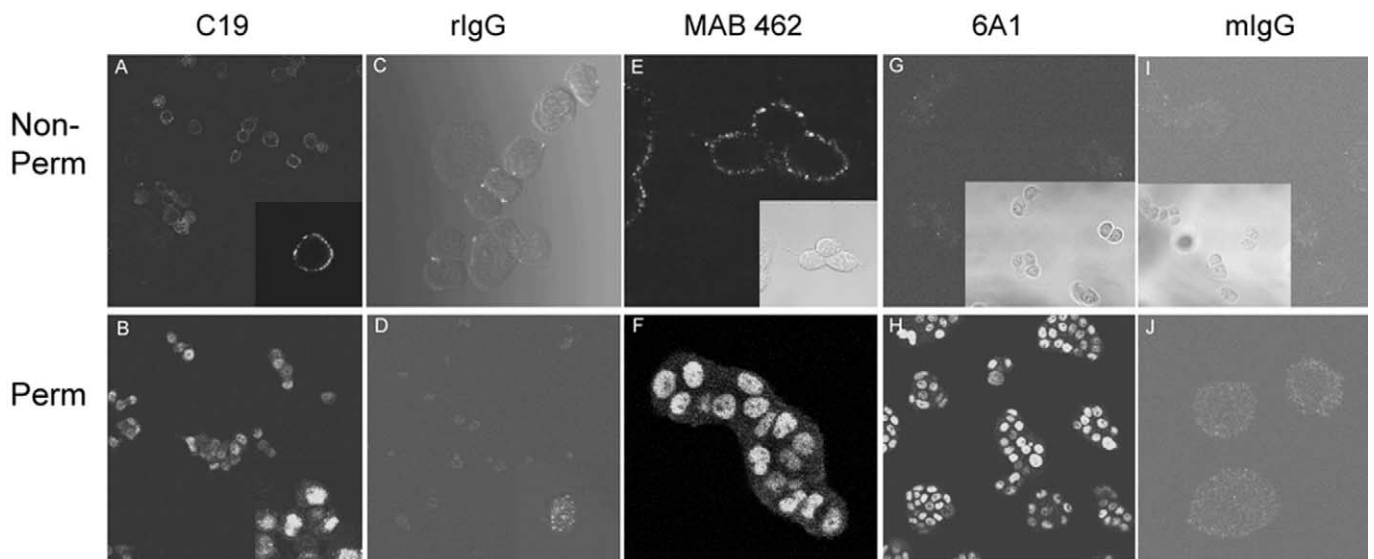
A plasmid expressing the fusion protein PR-M/green fluorescent protein (GFP) was constructed using pEGFP-N1 (Clontech, Palo Alto, CA) as the parent vector and pTarget-hPR-M as the source of the hPR-M cDNA. The pTarget-PR-M construct was made by PCR amplification of hPR-M cDNA from pIB-V5/His/TOPO-hPR-M.<sup>18</sup> For amplification, a forward primer (5'-CTATACTGGGATATGGAG-3') containing the putative translation start site was used with a reverse primer (5'-ACATGACAATAC AAATAAGATTT-3') corresponding to the OpIE2 reverse priming site of the pIB-V5/His/TOPO vector. TA cloning into the pTargetT vector (Promega, Madison, WI) was performed according to the manufacturer's instructions and standard protocols.<sup>20</sup> Restriction enzymes *NheI* and *SacI* (New England Biolabs, Beverly, MA) were used to cut pEGFP-N1 and pTarget-hPR-M at a ratio of 5 U of enzyme per μg of DNA under the manufacturer's conditions. Restriction digest products were separated by electrophoresis in 1% tris-acetate-EDTA agarose gels containing 0.5 μg/mL ethidium bromide using standard procedures.<sup>20</sup> Bands of appropriate size were gel-purified (Qiagen, Valencia, CA). Ligation of pEGFP-N1 vector and hPR-M insert DNA was performed by mixing vector and insert DNA in a 1:4 ratio with 10,000 U of ligase in 1X ligase buffer with 1 mM adenosine triphosphate (ATP) (New England Biolabs) at 4C overnight. This ligation results in insertion of hPR-M cDNA into the multiple cloning sequence of the pEGFP-N1 such that GFP is expressed on the carboxy terminal of the recombinant protein. Plasmid DNA was then transformed into XL1 Blue *Escherichia coli* (Stratagene, La Jolla, CA) according to the manufacturer's protocol. Selected colo-



**Figure 1.** Immunofluorescent staining of T47D-Y cells with selective anti-PR antibodies. Plasma membrane staining was seen in nonpermeable cell preparations with a C19 rabbit antibody directed to the HBD (A: cells in solution, 630X) and a MAB 462 mouse antibody directed to the HBD (E: attached cells, 400X with light image insert), but no staining was seen with a 6A1 mouse antibody directed to the amino terminus (G: attached cells, 400X with light image insert). Nonpermeable controls including rabbit IgG (C: cell in solution, 630X) and mouse IgG (I: attached cells, 400X with light image insert) showed negligible binding. With a permeable cell preparation, light staining was additionally seen in the cytoplasm and nucleus with the C19 (B: cell in solution, 630X) and the MAB 462 (F: attached cells, 400X) antibodies, with no specific staining seen with the 6A1 antibody (H: attached cells, 400X). Permeable controls including rabbit IgG (D: cell in solution, 630X) and mouse IgG (J: attached cells, 400X with light image insert) showed negligible binding. Images G–J have been artificially brightened to allow visualization of nonspecific staining in comparison to the other images.

nies were amplified overnight in 4 mL 37C broth culture, purified using the S.N.A.P. miniprep kit (Invitrogen, Carlsbad, CA), restriction enzyme-digested, electrophoresed, purified, and sequenced for accuracy prior to use in transfection experiments.

For transient transfection with either pEGFP-N1 or hPR-M/EGFP-N1, T47D, or T47D-Y, cells were grown to approximately 80% confluence in two-well chamber slides. Cells were treated for 40 minutes at room temperature with 50  $\mu$ L of a mixture of Fugene 6 transfection reagent (Roche, India-



**Figure 2.** Immunofluorescent staining of T47D cells with selective anti-PR antibodies. Plasma membrane staining was seen in nonpermeable cell preparations with a C19 rabbit antibody directed to the HBD (A: cells in solution, 400X with insert at 630X) and a MAB 462 mouse antibody directed to the HBD (E: cells in solution, 630X with light image insert), but no staining was seen with a 6A1 mouse antibody directed to the amino terminus (G: cells in solution, 400X with light image insert). Nonpermeable controls including rabbit IgG (C: cells in solution, 630X) and mouse IgG (I: cells in solution, 400X with light image insert) showed negligible binding. With a permeable cell preparation, intense staining was seen in the nucleus and light staining in the cytoplasm with the C19 (B: attached cells, 400X with insert at 630X), the MAB 462 (F: attached cells, 630X) antibodies, and the 6A1 antibody (H: attached cells, 400X). Permeable controls including rabbit IgG (D: cells in solution, 400X with insert at 630X) and mouse IgG (J: cells in solution, 630X) showed negligible binding. Images D, G, I, and J have been artificially brightened to allow visualization of nonspecific staining in comparison to the other images.

napolis, IN) at a concentration of 1:30 in serum-free media plus 4 ng plasmid DNA in a total of 200  $\mu$ L of media. Cells transfected with either hpPR-M/EGFP-N1 or pEGFP-N1 were observed at 48 to 72 hours with the LSM 410 confocal microscope as above.

## RESULTS

Figure 1 shows the immunofluorescent antibody staining of nonpermeabilized and permeabilized T47D-Y (genomic PR-negative) cells with selective antibodies. In nonpermeabilized cells the C19 and MAB 462 antibodies directed to the HBD showed distinct plasma membrane binding, whereas there was no binding with the 6A1 antibody directed to the amino terminus. The plasma membrane binding was heterogeneous, in that various cells showed intense staining, light staining, or no staining. Similarly, in permeabilized cells intracellular staining was noted with the HBD-directed antibodies and no staining was seen with the amino terminus-directed antibody. The intracellular staining was not abundant compared to the T47D cells and appeared to be mixed cytoplasmic and nuclear. Nonspecific staining with normal rabbit or mouse IgG was scant compared to that with the specific antibodies.

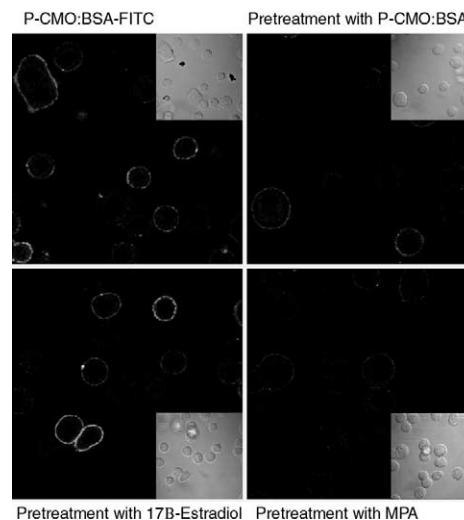
Figure 2 shows the immunofluorescent antibody staining of nonpermeabilized and permeabilized T47D (genomic PR-positive) cells with selective antibodies. As with T47D-Y cells, plasma membrane binding was seen with antibodies directed to the HBD in nonpermeabilized T47D cells. Likewise, there was no plasma membrane binding with the amino terminus-directed antibody. In contrast, staining of permeabilized cells showed intense nuclear staining with all three antibodies, consistent with nuclear genomic PR expression. Again, nonspecific staining with normal rabbit or mouse IgG was scant compared to that with the specific antibodies.

Figure 3 demonstrates immunofluorescent ligand binding in nonpermeabilized T47D-Y cells. Treatment with nonpermeable P-CMO:BSA-FITC showed heterogeneous staining with cells varying from no staining to intense staining within the same microscopic field. Specific staining was substantially decreased by pretreatment with P-CMO:BSA, medroxyprogesterone acetate and water-soluble progesterone. No decrease in specific staining was seen in cells pretreated with  $17\beta$ -estradiol. Very scant nonspecific staining was seen with BSA-FITC alone.

Figure 4 illustrates T47D cells transfected with PR-M-GFP or with GFP alone. One-micron slices of a confocal image of a transfected cell are shown. PR-M-GFP localized to the cytoplasm with no significant deposition in the nucleus or plasma membrane. PR-M-GFP appeared to aggregate in the cytoplasm suggesting microsomal localization. In contrast, the control GFP localized to the cytoplasm and nucleus in a diffuse manner. Transfected T47D-Y cells had an identical appearance (not shown).

## DISCUSSION

The predicted structure of PR-M is characterized by 16 novel amino terminus amino acids followed by sequence identical to

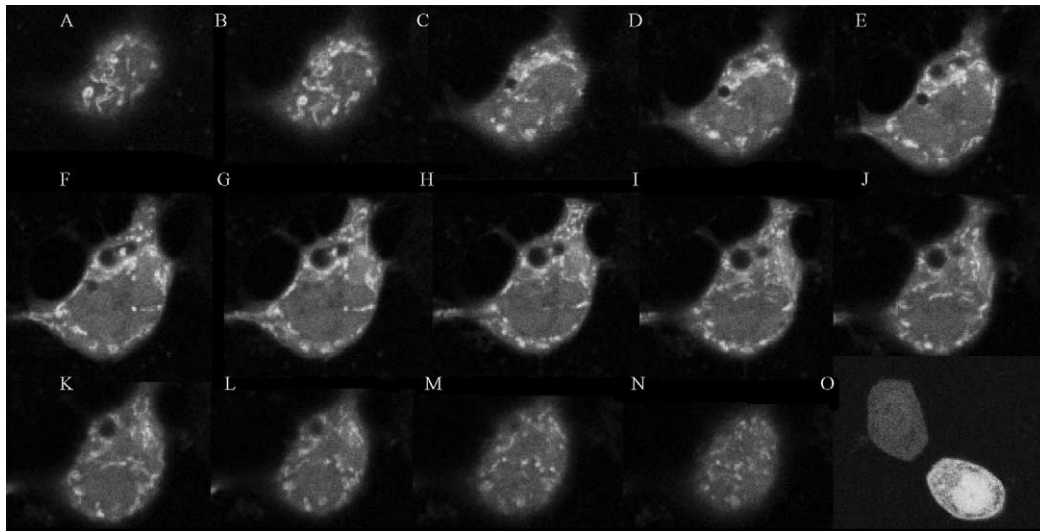


**Figure 3.** Immunofluorescent ligand binding in T47D-Y cells. Cells in solution treated with P-CMO:BSA-FITC showed varying degrees of membrane binding (top left). Pretreatment with P-CMO:BSA (top right), medroxyprogesterone acetate (bottom right) and water-soluble progesterone (not shown) followed by P-CMO:BSA-FITC showed substantial inhibition of staining. Pretreatment with  $17\beta$ -estradiol (bottom left) followed by P-CMO:BSA-FITC showed no decrease in staining. Control reactions with BSA-FITC showed very scant nonspecific staining (not shown). All cells were imaged at 630X.

the hinge and HBD of the nuclear PR.<sup>18</sup> Hydropathy analysis shows that the amino terminal amino acids are highly hydrophobic with multiple phenylalanines, suggestive but not conclusive for a signal peptide.<sup>21</sup> Signal peptides control ribosomal trafficking for secreted or membrane-bound proteins. PR-M appears to be the product of an alternative promoter for transcription in intron 3 of the genomic sequence. The 1230 bp 5' untranslated region (UTR) and the 48 bp responsible for encoding the first 16 amino acids correspond to sequence within intron 3 just prior to exon 4.<sup>22</sup> Due to the hydrophobicity and poor antigenicity of the novel amino terminus, it is not possible to make a unique antibody to recognize PR-M and not the genomic PR. Thus, in this research we have utilized a breast cancer cell line proven to lack expression of genomic PR over long-term culture,<sup>23</sup> selective anti-PR antibodies directed to different epitopes of the genomic protein, and transient expression of a recombinant protein in order to investigate the localization of PR-M and possibly other yet identified nongenomic PRs.

Use of selective antibodies shows that the PR(s) expressed in T47D-Y cells lack an amino terminus given the absence of a reaction with the monoclonal 6A1 antibody directed to the amino terminus epitope. Strong nuclear staining with this antibody in the genomic PR-positive T47D cells acts as a positive control.

The presence of a plasma membrane PR in these breast cancer cell lines is supported by both antibody and ligand binding studies. Antibody staining was performed with both adherent cells and cells in solution to ensure that brief trypsin digestion, used to lift the cells, did not eliminate the plasma membrane reaction. The heterogeneous pattern of plasma



**Figure 4.** T47D cells transfected with PR-M-GFP or GFP alone. One-micron slices of a representative cell transfected with PR-M-GFP showed intracytoplasmic aggregation (A–N). The nonfluorescent nucleus is well seen in images F–J. Control transfected cells with GFP alone showed diffuse fluorescence of cytoplasm and nucleus, often with slightly greater fluorescence in the nucleus (O). Adherent cells were visualized at 630X.

membrane staining, with some cells showing intense staining and others no reaction, suggests differential expression of this protein. This is the first immunocytochemical study demonstrating a plasma membrane PR in a genomic PR-negative breast cancer cell line. Another study demonstrated plasma membrane binding of the progesterone metabolites 5 $\alpha$ -pregnane-3,20-dione (5 $\alpha$ P) and 3 $\alpha$ -hydroxy-4-pregnen-20-one (3 $\alpha$ HP) in MCF-7 cells.<sup>24</sup> Yet, in this report radioligand binding studies after cellular fractionation showed no significant progesterone binding. The receptor responsible for this binding is yet to be identified.

It is tempting to speculate that PR-M is the plasma membrane receptor identified in these cells. This is suggested by the putative signal peptide on the amino terminus, the lack of a nuclear localization signal, and the selective antibody staining. Yet, this location is not supported by the expression studies. Expression of the PR-M-GFP construct shows definitive intracellular localization in an aggregate pattern. Possible explanations include that PR-M is an intracellular protein and that another, yet identified, plasma membrane PR is present or that the carboxy-terminus GFP interferes with proper PR-M localization. Even though the latter possibility cannot be excluded, GFP-tagged sex steroid receptors show proper localization. Genomic PR with an amino terminal GFP tag localizes to the nucleus when expressed in COS-1 cells<sup>25</sup> and genomic estrogen receptor with an amino terminal GFP tag localizes to the nucleus when expressed in multiple breast cancer cell lines.<sup>26</sup> These observations suggest that the intracellular location of PR-M represents the true location of the protein. The punctate accumulation of PR-M-GFP suggests a microsomal pattern. Other studies with cellular fractionation or costaining of intracellular organelles will be necessary to further define this point.

Nongenomic actions of progesterone in breast cancer remain to be fully elucidated. Studies have demonstrated activation of the Src/Ras/MAP kinase cytoplasmic signaling pathway by genomic PR.<sup>27,28</sup> Interaction of an amino terminal polyproline domain with the SH3 domain of c-Src may initiate the cascade.<sup>12</sup> MAP kinase activation leads to up-regulation of cyclin D and cyclin-dependent kinase 2 (cdk2) with subsequent entry into the cell cycle. Cdk2 phosphorylation may activate the genomic PR, thus providing a feed-forward regulation of genomic PR by an initial nongenomic action.<sup>27</sup> Yet, these mechanisms are only applicable to genomic PR-positive cell lines and such actions are absent in T47D-Y cells,<sup>23,27</sup> thus leaving the question as to whether PR-M and possibly other nongenomic PRs identified in these cells are functional.

Nonfunctional PRs have been proposed from the identification of variant PR cDNAs. Variant PR transcripts with deletion of exon 4 and deletion of exon 6 have been reported in several tissues, including breast cancer cells and human vascular smooth muscle.<sup>29,30</sup> Expression of the  $\Delta$ 4 protein shows no transcriptional regulation due to loss of the nuclear localization signal (NLS), hinge, and portion of the HBD, while expression of the  $\Delta$ 6 protein showed transcriptional inhibition of the genomic PR activity, but was unable to bind ligand. The actual expression of these proteins has not been demonstrated.

In contrast to these proposed aberrant proteins, lacking proper ligand binding, PR-M has a HBD identical to the genomic PR. Normal hormone binding is supported by a previous study in which a recombinant truncated PR containing only the hinge and HBD demonstrated the same ligand binding characteristics as the genomic PR.<sup>31</sup>

With the novelty of these findings, we can only speculate as to a possible function for PR-M or other nongenomic PRs in genomic PR negative breast cancer cells. As in other tissues, a

membranous PR may serve to modulate calcium mobilization. Studies of calcium regulation by progesterone in breast cancer cells have not been reported. In human spermatozoa progesterone binding to a plasma membrane receptor results in activation of phospholipase C with subsequent generation of inositol 1,4,5-trisphosphate (IP<sub>3</sub>) and diacylglycerol (DAG). IP<sub>3</sub> binding to its receptor in the endoplasmic reticulum leads to a release of intracellular calcium stores with a subsequent activation of extracellular influx via capacitative calcium entry.<sup>11,32-35</sup> In contrast, progesterone inhibits voltage-dependent calcium entry in vascular and intestinal smooth muscle cells.<sup>36,37</sup> Responses of calcium entry to progesterone may depend on tissue-specific calcium entry pathways. Given the multiple cellular processes controlled by calcium mobilization including metabolism, transcription and proliferation,<sup>38,39</sup> there remains the possibility of a nongenomic progesterone action in breast cancer cells lacking expression of genomic PR.

In summary, this research provides immunocytochemical evidence for expression of nongenomic PRs, including the recently cloned truncated receptor, PR-M, in a breast cancer cell line lacking genomic PR expression.

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