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14. ABSTRACT This proposal proves a new molecular mechanism whereby progesterone via a mitochondrial receptor, named PR-M, influences the growth of breast cancer cells by enhancing cellular respiration. For this purpose we developed an RNAi assay to silence expression of PR-M in T47D breast cancer cells. We have demonstrated with this assay a decrease in PR-M transcript levels by qRT-PCR and a decrease in protein levels with western blot analysis. Functionally, we then demonstrated a decrease in progesterone/progestin induced mitochondrial membrane potential with silencing of PR-M expression. We then sought to determine the influence of PR-M silencing on the metabolomic pathway of these cells. These studies are still ongoing but initially suggest an increase in lipid catabolism with increased levels of acylcarnitines. These results suggest that progesterone increases cellular energy production by influencing fatty acid oxidation via a unique mitochondrial progesterone receptor. This provides a new mechanism whereby progesterone influences the growth and survival of breast cancer cells.				
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INTRODUCTION

We have discovered a novel progesterone receptor (PR-M) that localizes to the mitochondrion. PR-M was originally cloned from human aortic and human adipose cDNA libraries obtained from Clontech using rapid amplification of cDNA ends (RACE).

The cDNA contains an approximate 1230 bp 5' untranslated region (UTR) followed by two open-frame putative ATG translation start sites separated by 18 base pairs. Translation using the first ATG start site, characterized

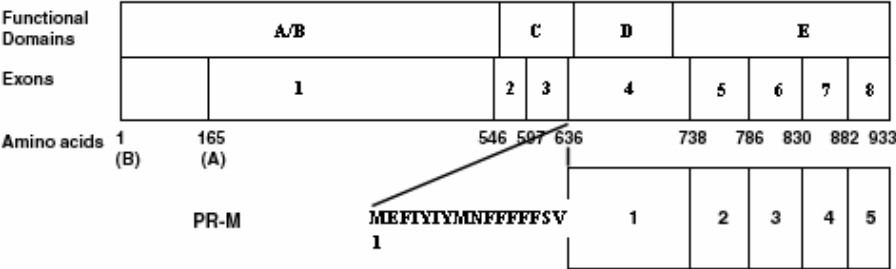


Fig 1. cDNA Structure of PR-M

by a Kozak sequence, encodes for 16 novel N-terminus amino acids compared to the nuclear PRs. Hydropathy analysis shows these 16 amino acids to be strongly hydrophobic with multiple phenylalanines consistent with a transmembrane domain (TMD). Following these 16 amino acids, PR-M contains deduced amino acid sequence identical to exons 4 through 8 of the nuclear PRs. Compared to that of the nuclear PR-B and A; PR-M lacks the amino terminal A/B regions, the DNA binding domain (DBD) and the nuclear localization signal (NLS) but contains the hinge region and hormone-binding domain (HBD) identical to that of the nuclear PRs. **Due to the strong hydrophobic and poor antigenic nature of the novel 16 a.a. sequence, it is not possible to make a specific antibody to PR-M.**

PR-M appears to be derived from an alternate promoter within the 3rd intron of the PR gene. The unique 5' sequence of PR-M is identical to intronic sequence just prior to exon 4.

The cDNA for PR-M has been expressed in both a Sf9 insect ovarian cell line with a carboxy V5 epitope tag and in *E. coli* with an amino HIS tag revealing a 38 kDa protein by western blot analysis (1).

Localization of PR-M to the mitochondrion was shown with 3 different techniques. First, lipid-mediated transient transfection of a PR-M-GFP fusion protein in Cos-1, HeLa and HepG2 cells showed solely mitochondrial localization. Another fusion protein, lacking the initial 16 a.a. (PR-M(-MLS)-GFP) showed no evidence of mitochondrial localization, suggesting that the amino-terminus sequence is a MLS (2). The amino-terminus sequence structure is similar to that of other mitochondrial proteins, such as TOM 70, which bind the outer mitochondrial membrane by an amino-terminus MLS (3). Second, western blot analysis performed with purified human heart mitochondrial protein showed an intense 38 kDa band consistent with PR-M using selective commercial antibodies. Positive reactions were seen with a rabbit polyclonal antibody directed to the HBD and a monoclonal antibody directed to the HBD, but no reaction was seen with a monoclonal antibody directed to the amino-terminus of nuclear PR, which is not present in PR-M. Third, PR-M was localized to the mitochondrial fraction after cellular fractionation with differential centrifugation of T47D-Y cells (2). T47D-Y cells are known to lack expression of nuclear PR (4).

Appropriate ligand binding to PR-M is supported by the identical hormone binding domain of the nuclear PR and previous studies. We have shown similar binding of PR-M expressed in Sf 9 insect cells to nuclear PR positive T47D cells using the progestin R5020 (unpublished). A previous study investigating functional domains of the nuclear PR showed that a construct of the hinge plus HBD, which would be equivalent to PR-M without the unique MLS, has the same ligand binding characteristics as the native nuclear PR (5).

PR-M is expressed in both benign and tumorigenic breast cell lines and primary tumors, independent of nuclear PR (nPR) expression. Progesterone/progestin treatment of MCF-10A benign breast epithelial cells, shown to express PR-M (6), but lack expression of nPR (7), reveals an increase in cellular respiration as shown by an increase in mitochondrial membrane potential (MMP) and total cellular ATP levels. This increase in mitochondrial activity occurs at physiological doses of progesterone, is inhibited by a specific PR antagonist, and occurs in the presence of the translation inhibitor cycloheximide. In addition, this progestin increase in cellular respiration decreases Fas ligand induced apoptosis (8). Other investigators have shown a synergistic effect between progestin and peptide growth factors in the growth of nPR negative breast cells (9). From these observations we believe that progesterone enhanced cellular respiration may play a role in cell growth, survival or mechanics depending upon other factors acting upon the cell. This likely represents a mechanism whereby energy production is increased during pregnancy to meet the increased metabolic demands of many tissues. In this study we sought to further elucidate the role of PR-M in the control of mitochondrial activity in cells with and without nPR expression.

BODY

Task 1: To develop siRNA knockdown protocol for PR-M in T47D and T47D-Y breast cancer cell lines.

This task took much longer than anticipated. We started with T47D-Y cells, which in hind-sight was a mistake. The T47D-Y cell line has a lower expression level of PR-M compared to T47D cells. Fairly high expression levels of a protein are key to the success of RNAi using siRNA duplex transfections. After approximately 6 months, we decided to switch to the T47D cells. Since T47D cells express both PR-M and nPR we had to develop a strategy to silence both proteins separately. This is also necessary in

TABLE 1	
SiRNA duplex sequence	Exon location in PR-M
GTAGTCAAGTGGTCTAAAT	1
AGATAACTCTCATTCAGTATT	1
GAGATGAGGTCAAGCTACATT	2
AGCGTTTCTATCAACTTA	4

that PR-M is a truncated version of nPR, with exons 4-8 of nPR being identical to exons 1-5 of PR-M. Thus, it is possible to silence nPR without affecting PR-M using siRNA duplexes to exons 1-3 of nPR, but silencing PR-M may also affect expression of nPR. Table 1 shows the sequence of 4 duplexes used to silence PR-M and the exons to which they were directed. Table 2 shows the sequence of 2 duplexes used to silence nPR and the exons to which they were directed.

TABLE 2	
SiRNA duplex sequence	Exon location in nPR
TCCTGCCTCTCAATCACGCCTTATT	1
GGTCTTCTTTAAGAGGGCAATGGAA	2

Figure 2A and 2B show the results of the RNAi assay developed for PR-M. Specific transcript levels for PR-M versus nPR were determined by realtime RT-PCR reaction after 48 hrs. Starting quantities of transcripts (SQ) were determined using a standard

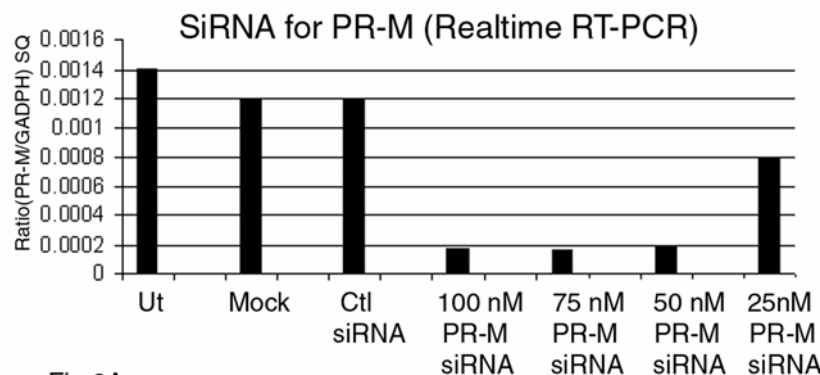


Fig 2A

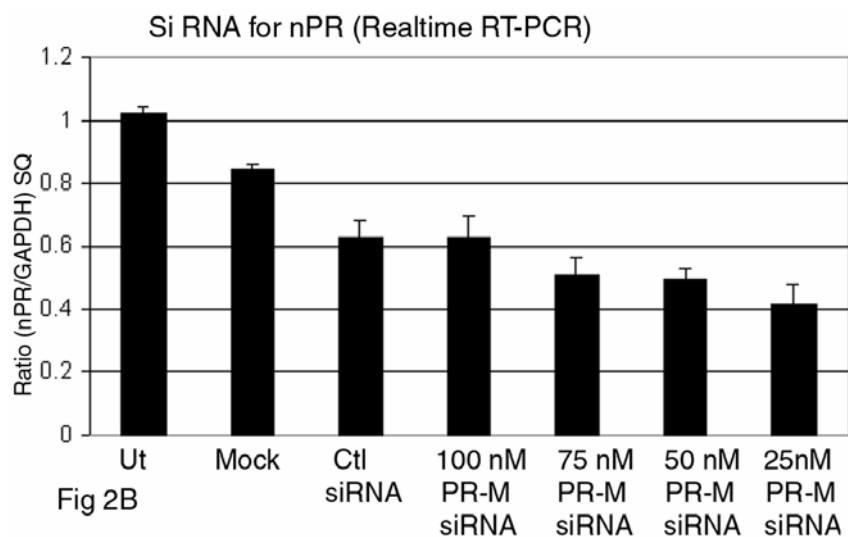


Fig 2B

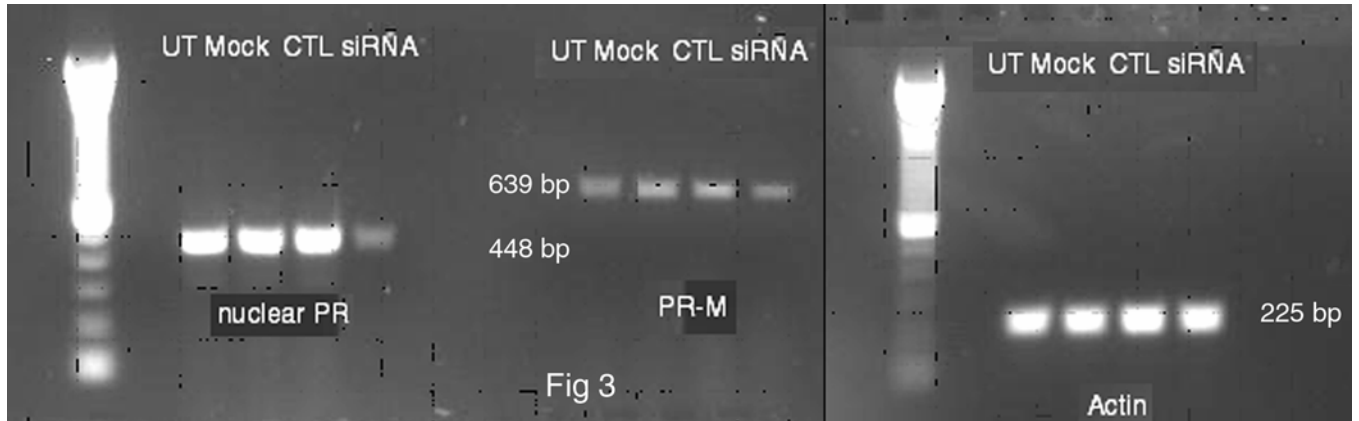
curve of known concentrations of isolated PCR product. Amplification of GAPDH transcript was used as a loading control. Figure 2A demonstrates a dose-dependent decrease in PR-M transcript with varying concentrations of siRNA duplexes.

Since the siRNA duplexes used to knockdown PR-M also recognize PR-A&B we determined transcript levels for nPR with specific primers excluding PR-M sequence. Fig 2 B shows a small decrease in nPR transcript levels compared to scrambled siRNA control, but not nearly as dramatic as that seen for PR-M.

From the above results of PR-M and nPR transcript levels we elected to use an siRNA concentration of 50 nM for subsequent experiments.

Next we designed a RNAi assay to knockdown the expression of nPR without affecting expression of PR-M. In this case 2 siRNA duplexes directed to exons 1 and 2 of nPR (which would be absent in PR-M) were used. Fig 3 shows a representative agarose

gel after semi-quantitative RT-PCR. Two separate experiments were performed with the same results. A dramatic knockdown of nPR expression is seen without effect on PR-M expression. Treatment groups included untransfected cells (Ut), transfection agent alone (Mock), scrambled control siRNA (CTL) and 40 nM nPR siRNA.



Next, we verified a decrease in protein associated with the decrease in transcript. Cells were grown for 72 hrs after transfection. Figure 4A shows a composite of 3 western blots performed showing a decrease in the 38 kDa PR-M protein after siRNA treatment for 72 hrs. The blot was probed with a monoclonal mouse antibody (MAB 462) directed to the HBD of PR along with an antibody to β -actin. A decrease in PR-M expression is seen after treatment of T47D cells with 50 nM siRNA.

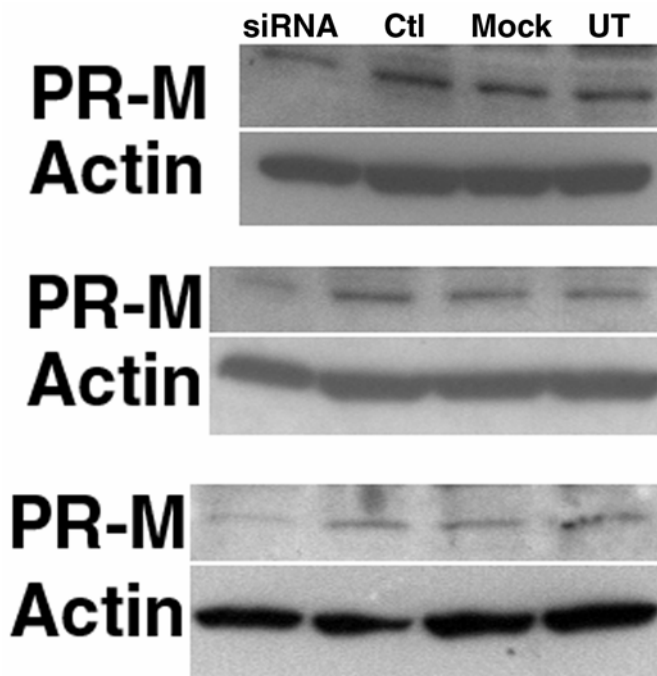
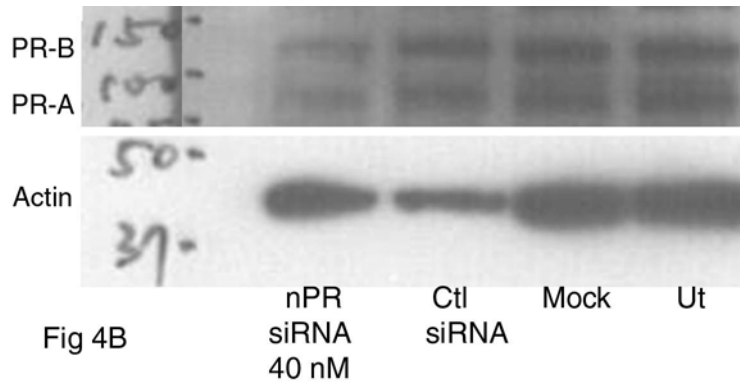
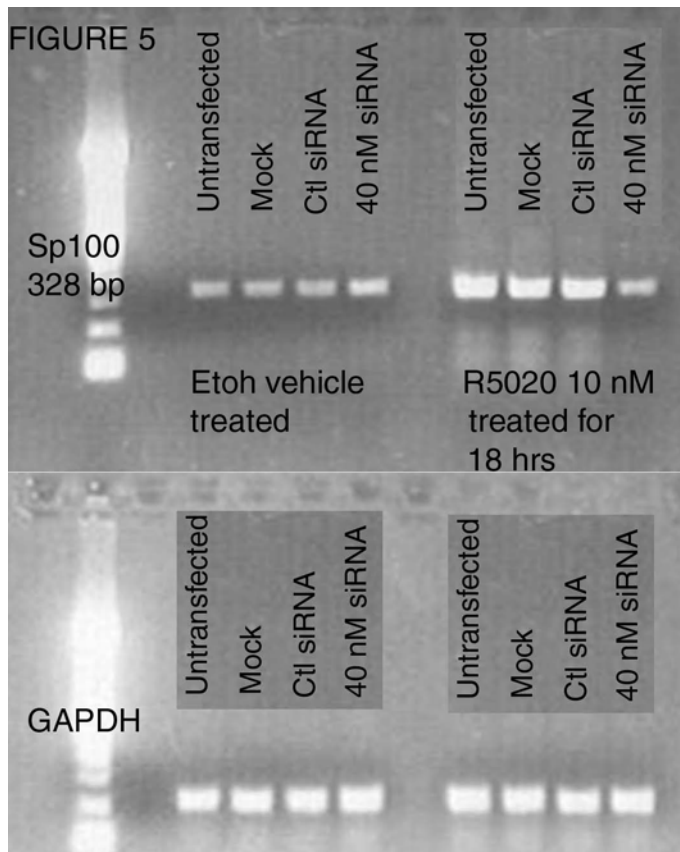


FIG 4A

Next, we determined the decrease in protein with siRNA duplexes directed to nPR. Figure 4B shows a representative western blot of 2 experiments, in which nPR expression was decreased after a 72 hr treatment with 40 nM siRNA. A significant decrease in PR-B and A was seen.

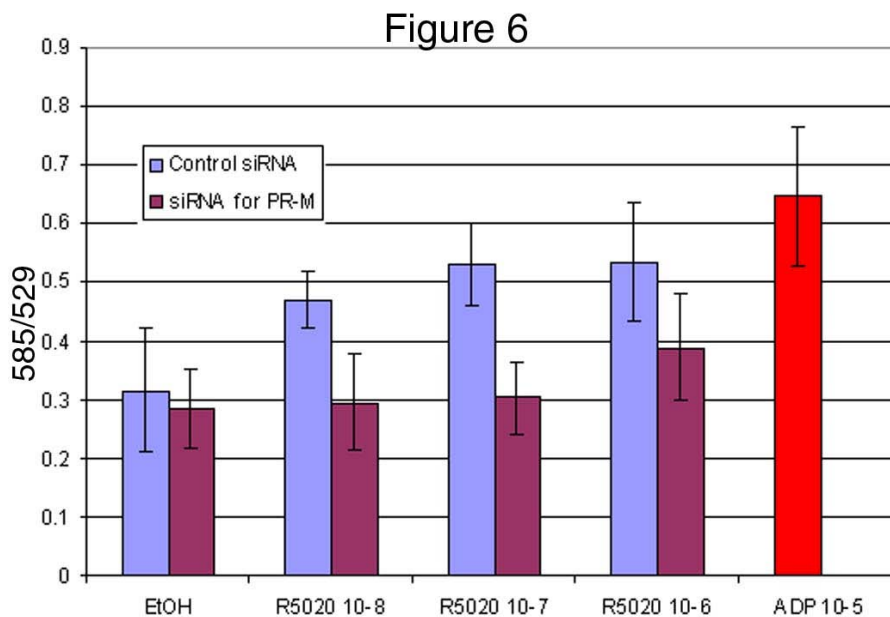


Task 2: To determine changes in cellular physiology after knockdown of PR-M in T47D and T47D-Y breast cancer cell lines

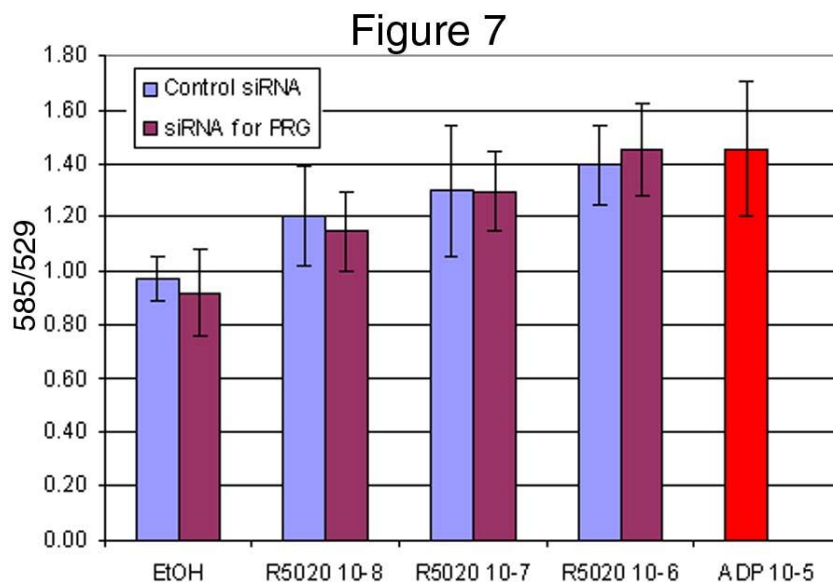


Next we demonstrated a change in function as a result of the siRNA treatment. In regards to nPR function we chose to investigate a change in the induction of the transcript for the Sp100 nuclear antigen. T47D cells were grown for 72 hrs after transfection with 40 nM siRNA directed to exons 1 and 2 of the nPR; and then SP100 transcript determined by semi-quantitative RT-PCR. To induce the SP100 transcript, cells were treated with the PR agonist, R5020, at a concentration of 10 nM for 18 hrs. The ethidium bromide stained gel in figure 5 shows a dramatic inhibition of SP100 induction with siRNA treatment.

For the function of PR-M we chose to investigate mitochondrial membrane potential (MMP). As discussed in the introduction, we have previously demonstrated a progesterone dependent increase in MMP in MCF-10A breast epithelial cells, shown to



express PR-M but lack expression of nPR (8). As described above, T47D cells were grown for 72 hrs after transfection with 50 nM siRNA directed to exons 1-5 of PR-M. Cells were then placed in a modified Krebs-Ringer-HEPES buffer (KRH) containing 25 mM Na-HEPES, 115 mM NaCl, 5 mM KCl, 1 mM KH_2PO_4 , 1.2 mM MgSO_4 , 0.5 mM CaCl_2 and 5 mM glucose at pH 7.4 for 2 hr prior to experimentation. Cells were treated for 30 mins with differing concentrations of R5020 or vehicle control. MMP was determined by the fluorescent emission ratio (585/529) of the dye, JC-1 in a fluorescent plate reader. The fluorescent emission ratio (585/529) of the dye, JC-1 indicates MMP, with an increase in the ratio reflecting increased MMP while a decrease in the ratio reflecting decreased MMP. MMP reflects the rate of oxidative phosphorylation. Figure 6 shows the results of 6 experiments (mean $\pm$ SD). A statistically significant decrease in MMP is seen with all



concentrations of R5020 treatment after siRNA knockdown. ETOH serves as a vehicle control while ADP serves as a positive control.

Next we demonstrated that siRNA knockdown of nPR did not affect MMP. Figure 7 shows the results of 3 assays in which T47D cells were treated for 30

mins with differing concentrations of R5020 after siRNA knockdown of nPR with duplexes directed to the 1st and 2nd exons of PR-A and B. No significant difference is seen with R5020 concentrations of 10⁻⁸ M to 10⁻⁶ M.

KEY RESEARCH ACCOMPLISHMENTS

- **Development of a RNAi assay for PR-M in T47D breast cancer cells.**
- **Development of a RNAi assay for nPR in T47D breast cancer cells.**
- **Demonstration that progesterone/progestin induced increase in mitochondrial membrane potential is mediated by PR-M.**

REPORTABLE OUTCOMES

- **Results from this grant have been awarded an oral presentation at the annual meeting of the Society of Gynecological Investigation to be held in March 2010.**
- **Results from this grant were used in a DOD concept application submitted in November 2009.**
- **Results from this grant are to be used as preliminary data for an R01 application to be submitted February 2010.**

PLANS DURING NO COST EXTENSION

- **During the NCE, our primary goal is to complete task 2C.**

Determine the change in cellular levels of amino acids, acylcarnitines and organic acids in untransfected, mock transfected and siRNA transfected T47D cells after progesterone treatment.

We will not have the funds to be able to complete

Task 2b: Determine the change in total cellular ATP in untransfected, mock transfected and siRNA transfected T47D cells after progesterone treatment and

Task 2d: Determine the change in proliferation rates of a combination of epidermal growth factor (EGF) and progesterone in untransfected, mock transfected and siRNA transfected T47D cells after progesterone treatment.

CONCLUSIONS

This work suggests that progesterone via a novel mitochondrial progesterone receptor (PR-M) increases cellular respiration. Other data from our lab shows this receptor to be expressed in multiple breast cancer cell lines and primary tumors independent of nuclear ER or PR status. Thus, progesterone may affect breast cancer cell survival regardless of nPR status, routinely determined by immunocytochemical staining in clinical practice. This is very important given that most breast cancers in pre-menopausal women are nPR negative and thus considered by clinicians to be unaffected by female sex hormones. It is also important as many breast cancers in pre-menopausal women over-express Her-2/neu. The combination of growth factor stimulation and progesterone enhanced cellular respiration may significantly enhance growth and

survival. Thus, we believe that combination therapy of a PR modulator (antagonist at the breast) and a peptide growth factor inhibitor such as Trastuzumab may prove superior in the treatment of Her-2/neu positive, nER/nPR negative breast cancers in pre-menopausal women.

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SUPPORTING DATA

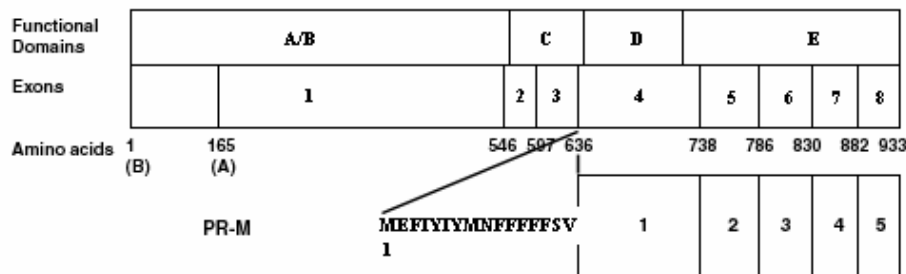


Fig 1. cDNA Structure of PR-M

Figure 1: cDNA structure of PR-M. PR-M cDNA contains a 5'UTR and initial coding sequence consistent with a mitochondrial localization signal derived from the distal 3rd exon of the PR gene. The remainder of the cDNA is identical to exons 4 through 8 of the nuclear PR-B and A cDNAs.

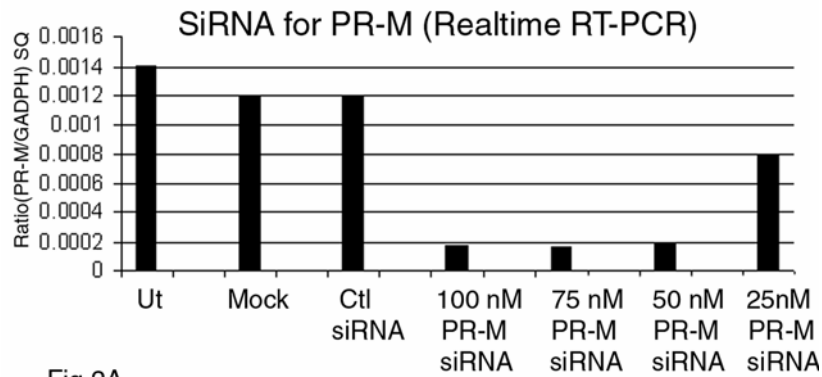


Fig 2A

Figure 2A: RNAi assay was performed with 4 siRNA duplexes targeted to exons 1-5 of PR-M. Results of a realtime RT-PCR reaction show a dramatic decrease in PR-M transcript levels with siRNA concentrations of 50, 75 and 100 nM. Amplification of GAPDH was used as a control.

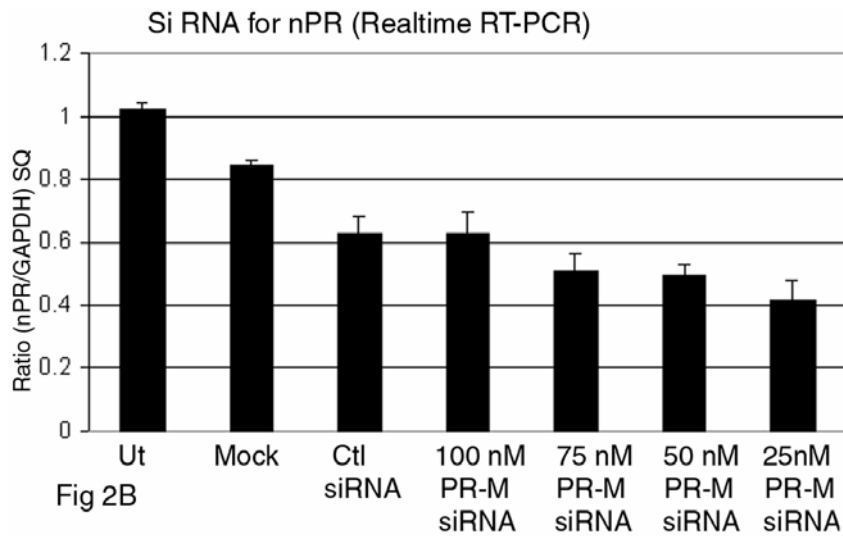


Fig 2B

Figure 2B: Figure 2b shows the results of two realtime RT-PCR experiments to determine nPR transcript levels after siRNA treatment with the same duplexes used to knockdown PR-M. A modest but significant decrease in nPR transcript was seen with the 75, 50 and 25 nM duplexes. Amplification of GAPDH was used as a control

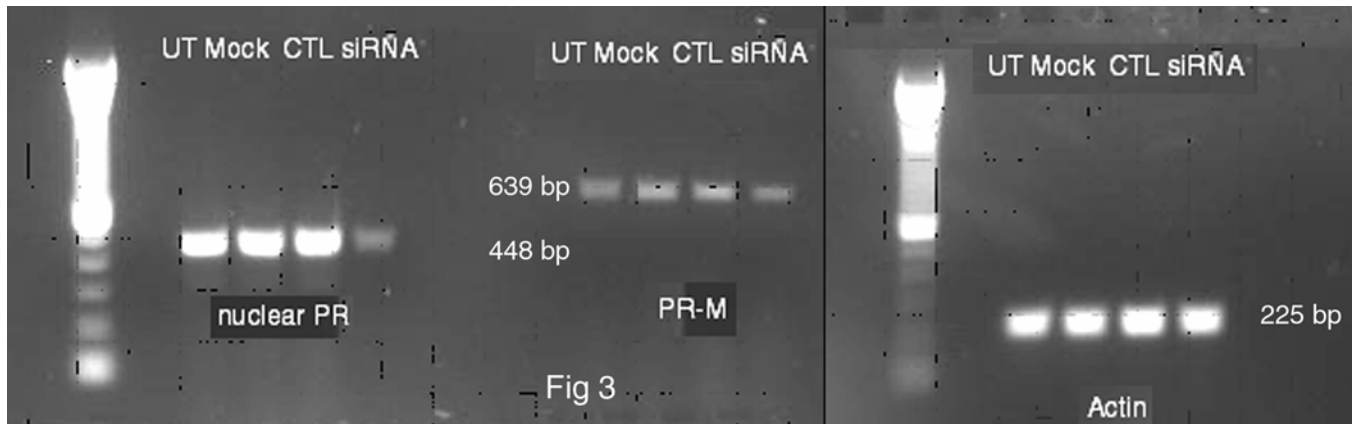


Figure 3: Ethidium bromide stained gel from qRT-PCR reaction after RNAi assay targeting nPR. A dramatic decrease in nPR transcript is seen while the transcript levels for PR-M and actin are unaffected.

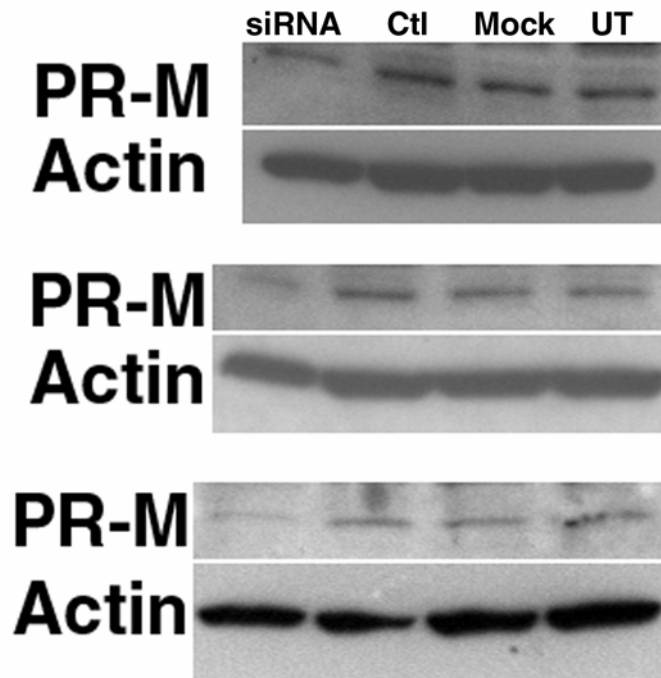


FIG 4A

Figure 4A: Three western blot analyses after RNAi assay for PR-M. The blots were hybridized with a mouse monoclonal antibody to the HBD of PR (MAB 462). In each blot a decrease in PR-M protein is seen by a band at 38 kDa.

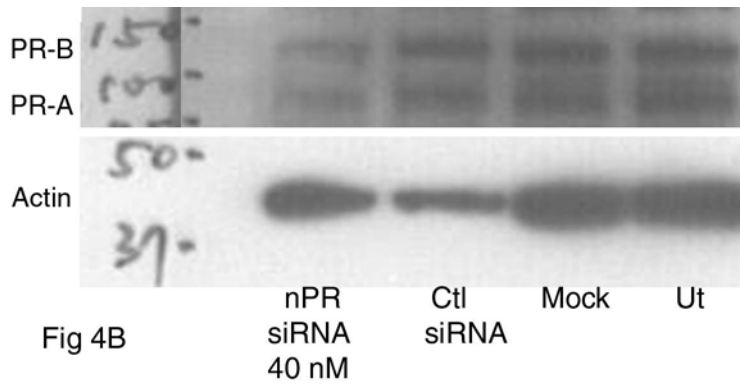


Figure 4B; Western blot analysis after RNAi assay for nPR with a mouse monoclonal antibody to the HBD of PR (MAB 462). A decrease in PR-B (~118 kDa) and PR-A (~92 kDa) are seen.

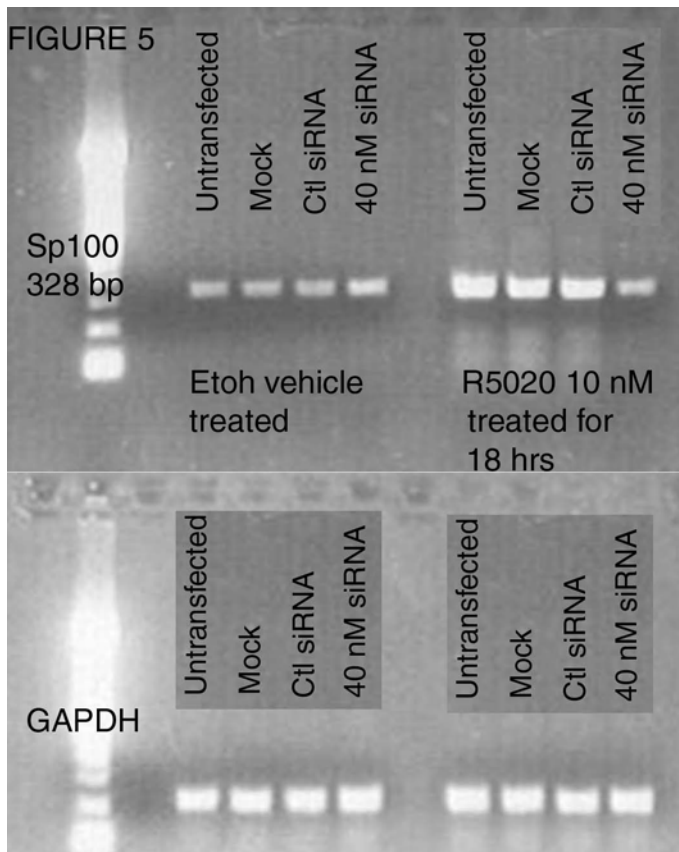


Figure 5: Ethidium bromide stained gel of qRT-PCR reaction after RNAi assay for nPR. Sp100 expression is regulated by progesterone via nPR. After siRNA transfection, cells were treated for 18 hrs with 10^{-8} M R5020. A significant decrease in Sp100 transcript is seen.

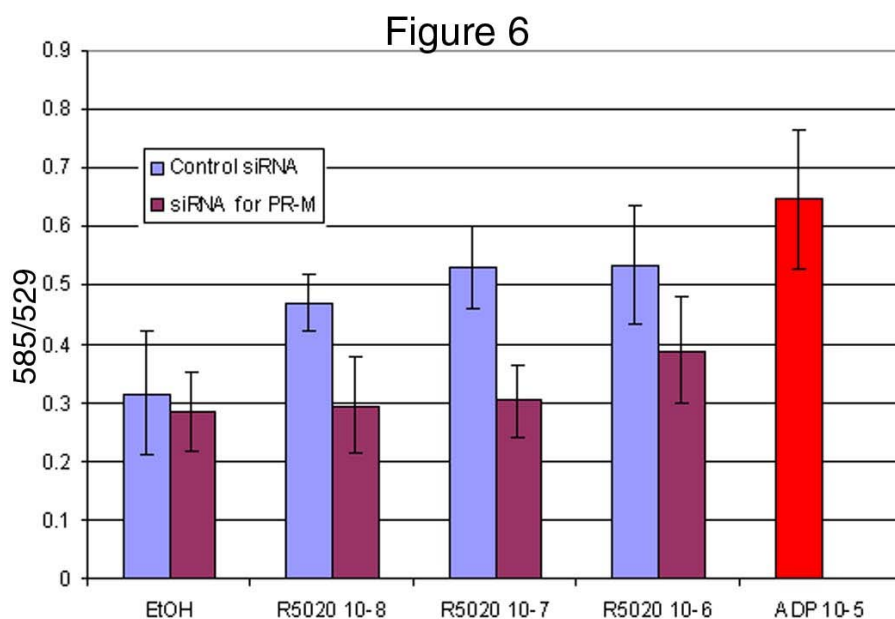


Figure 6: Determination of MMP by JC-1 emission ratio after RNAi assay with siRNA duplexes to PR-M. 72 hrs after siRNA transfection, cells were treated with R5020 for 30 mins. A progestin dose dependent increase in MMP is seen in cells transfected with control siRNA while the increase is diminished in cells transfected with siRNA to PR-M.

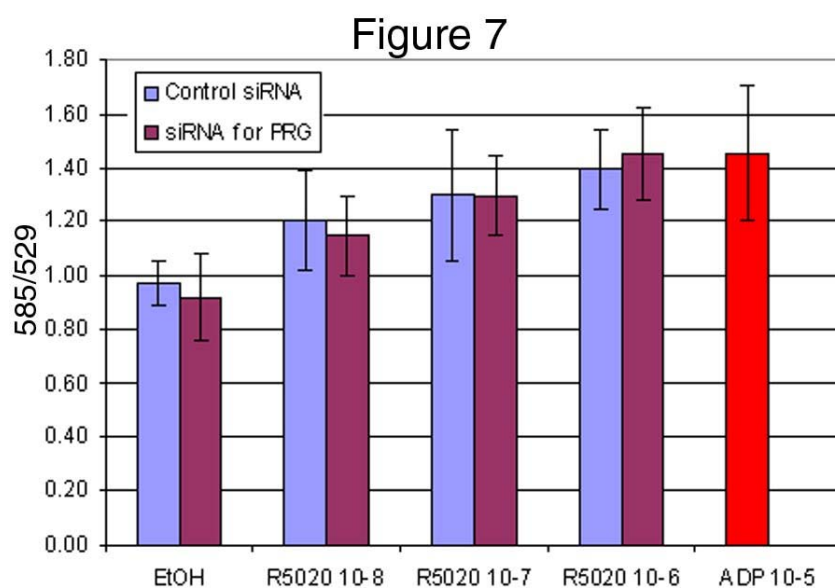


Figure 7: Determination of MMP by JC-1 emission ratio after RNAi assay with siRNA duplexes to nPR. 72 hrs after siRNA transfection, cells were treated with R5020 for 30 mins. No difference was seen in the progestin dose dependent increase in MMP in cells transfected with control siRNA or siRNA directed to nPR.