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Progestin regulated miRNAs that mediate progesterone receptor action in breast cancer

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Abstract

Progesterone receptors (PR) mediate response to progestins in the normal breast and breast cancer. We identify 28 miRNAs significantly altered by 6 hrs of progestin treatment. Many progestin-responsive genes are putative targets of these miRNAs. We find that ATP1B1, a gene directly regulated by PR at the promoter, is targeted by miR-29 through a site in the ATP1B1 3'-untranslated region (UTR), thereby relieving repression of ATP1B1. Thus, PR regulates ATP1B1 through sites in both the 5' and 3'UTRs, to achieve maximal and tight hormonal regulation of ATP1B1 protein via both transcriptional and translational control. We further demonstrate that PR itself is targeted by a progestin-upregulated miRNA, miR-513a-5p, providing a novel mechanism for control of PR protein expression. These studies establish that progestin-regulated miRNAs are a functional component of PR action that can affect the degree of hormonal control of PR-regulated genes, and indeed, PR itself.

Keywords

miRNA; progestin; breast cancer; ATP1B1; progesterone receptor

1. Introduction

In the normal breast, progesterone (P4) is well known to mediate both proliferation and differentiation. The role of P4 in breast cancer is not as straightforward as that of estradiol (E2). In breast cancer cell lines in 2D culture, P4 has a biphasic effect on growth (Groshong et al., 1997); however, in normal breast cells in 3D culture and in the mouse mammary gland, P4 is clearly mitogenic (Beleut et al., 2010; Fernandez-Valdivia et al., 2008; Graham

et al., 2009; Lydon et al., 1995). In addition, women on hormone replacement therapy containing estrogen and progestin compared to estrogen alone have an increased risk of breast cancer, larger tumors of higher grade, and are more likely to succumb to their cancer (Beral, 2003; Fournier et al., 2009; Holmberg et al., 2008; Prentice et al., 2008). Recently, several groups have demonstrated in both normal and malignant breast cells, that P4 mediates the expansion of a stem cell population (Asselin-Labat et al., 2010; Graham et al., 2009; Horwitz and Sartorius, 2008; Joshi et al., 2010).

Evidence is accumulating that fluctuating hormone levels can affect miRNA expression in steroid responsive tissues. MiRNA expression profiles differ in the proliferative versus secretory stages of the human endometrium in which P4 levels vary on the background of E2 levels (Kuokkanen et al., 2010; Xia et al., 2010a; Xia et al., 2010b). Likewise, the most dramatic alterations in miRNA expression that occur during post-natal mammary gland development coincide with the most dramatic fluctuations in steroid hormone levels (Avril-Sassen et al., 2009; Sdassi et al., 2009; Silveri et al., 2006; Wang and Li, 2007). Since E2 and P4 also profoundly influence tumors arising from steroid sensitive organs, such as breast, endometrial and prostate cancer, it is logical to ask whether hormonally regulated miRNAs play a role in such cancers. There are clear differences in miRNA expression in luminal A (ER+/PR+) versus triple negative breast cancer (Blenkiron et al., 2007; Cochrane et al., 2010; Lowery et al., 2009); however, since there are many differences between these two subtypes other than hormone receptor status, it is difficult to know which alterations are directly affected by steroid hormones and their receptors. While the effect of E2 on miRNA expression in breast cancer cells has been studied (Castellano et al., 2009; Cochrane et al., 2010; Cohen et al., 2008; Klinge, 2009; Maillot et al., 2009), progestin-mediated effects have not.

We performed miRNA profiling of breast cancer cells treated with progestin for 6 and 24 hrs. We find that progestin mediates rapid effects on miRNA expression. We noted that many known progestin-responsive genes are predicted targets of the identified progestin-regulated miRNAs. As an example, we demonstrate that the Na⁺/K⁺ ATPase, ATP1B1, is regulated by progestins in two ways: 1) by liganded PR binding to the promoter to induce transcription, and 2) by progestin mediated downregulation of miR-29 to relieve repression of the *ATP1B1* transcript, since miR-29 directly targets and represses *ATP1B1* via a site in the 3'UTR. We further demonstrate in multiple cell lines that estradiol-mediated upregulation of PR protein is abrogated by miR-513a-5p, a progestin-upregulated miRNA.

2. Materials and methods

2.1 Cell culture and hormone treatments

T47D and MCF7 breast cancer cells were grown in Dulbecco's minimal essential medium (DMEM) with 10% fetal bovine serum (FBS) and penicillin/streptomycin. BT474 cells were grown in 10% FBS as above, with the addition of nonessential amino acids and insulin. ZR75-1 cells were grown in MEM containing 5% FBS, HEPES, nonessential amino acids, L-glutamine, penicillin, streptomycin, and insulin. All cells were grown in 5% CO₂ at 37°C. The identity of all cell lines was verified using Single Tandem Repeat analysis.

Hormone treatments were carried out in the base media described above; however phenol red-free media and 5% charcoal stripped serum (CSS) were used in lieu of FBS. Prior to treatment with hormones, cells were grown in CSS media for 24h. All hormones were dissolved in 100% ethanol and used at a final concentration of 10 nM unless otherwise stated in the text. The hormones used were medroxyprogesterone acetate (MPA, Sigma), progesterone (P4, Sigma), 17 β -estradiol (E2, Sigma) R5020 (Perkin-Elmer), and RU-486 (Sigma).

2.2 MiRNA profiling, data analysis

T47D cells were treated with EtOH or MPA for 6h or 24h, in duplicate for each timepoint (eight samples total). Cells were harvested and miRNA was prepared using Trizol (Invitrogen) according to the manufacturer's instructions. Labeling, hybridization to miRNA microarray slides, and feature extraction were performed by ThermoFisher using the Agilent miRNA microarray platform. Each miRNA probe is spotted in seven locations to allow relative intensity data for the multiple probes for each miRNA to be subjected to statistical filtering as follows: data analysis was performed for each timepoint to identify miRNA probes with $p < 0.05$ in at least 1 of the 4 microarrays. This resulted in 406 miRNA probes that passed the filter with the ANOVA analysis. The remaining data was inter-array scaled and transformed to log(2). This analysis found 59 differentially expressed miRNAs ($P^* 0.01$) and of these 28 had a fold change of greater than 1.5 after 6 hrs of treatment.

2.3 Real time PCR

Cells were plated as above and treated with MPA or vehicle for 6 hrs and total RNA harvested using Trizol. Sequences and probes for PCR were ATP1B1 fwd, 5'-TGCCTGGCTGGCATCTTC-3'; ATP1B1 rev, 5'-TGGGCTTAAATTCACCTGATGGTG-3'; ATP1B1 Taqman probe, 5'-6famTCGGAACCATCCAAGTGATGCTGCTamra-3'.

For the miRNA real time PCR, T47D cells were treated with 10 or 100 nM MPA for 6 hrs prior to harvesting total RNA with Trizol. Taqman miRNA Reverse Transcription kit was used to generate cDNA from total RNA using miRNA specific primers and probes (Applied Biosystems). For normalization, real time RT-PCR was performed on the cDNA using U6 small nucleolar RNA primers and probe (Applied Biosystems). The relative miRNA levels were calculated using the comparative Ct method ($-Ct$). Briefly, the Ct (cycle threshold) values for the U6 were subtracted from Ct values of the miRNA to achieve the $-Ct$ value. The 2^{-Ct} was calculated and then divided by a control sample to achieve the relative miRNA levels ($-Ct$). Reported values are the means and standard errors of four biological replicates.

2.4 DNA constructs and cloning

The *ATP1B1* promoter was obtained from genomic DNA using PCR with the following primers: ATP1B1 promoter forward, 5'-GCGAAGCTTGAATTCTCTGATATGGGGCTGGGACGTCAAGATGGG-3'; ATP1B1 promoter reverse, 5'-GCGGTCGACGGGGTAGGAGAGTCAGAGAGGG-3'. The PCR fragment was digested with HindIII and SalI and subcloned into the pSPORT vector

(Invitrogen) and sequenced. PCR was repeated with the following primers containing HindIII and SalI restriction sites respectively: pA3 Sense Primer, 5'-GATCGTCGACGGGACGTCAAGATGGGCCATGAT-3'; pA3 Antisense Primer, 5'-CTAGAAGCTTGCCCGCCCTACCTTTACTATATACC-3', the fragment was digested with HindIII and SalI and subcloned into pA3Luc. The integrity of the promoter was again confirmed by sequencing.

The 233 bp portion of the *ATP1B1* 3'UTR containing the putative miR-29a target site was amplified out of Hela genomic DNA using the following primers: forward 5'-CCACTAGTGAATGCTGTCTTGAC-3' and reverse 5'-CTCAAGCTTATTGTACAACATGCAT-3'. A 196bp fragment of the *PGR* 3'UTR containing the first miR-513a-5p site (PGR site 1) was amplified using 5'-ACGACGCGTCACAAGAAATCTATG-3' and 5'-CTCAAGCTTTCAATGCTTCTTATG-3' and a 408bp fragment of the *PGR* 3'UTR containing the second and third miR-513a-5p sites (PGR site 2) was amplified using 5'-ACGACGCGTCTGAGTTGTGCATGT-3' and 5'-CTCAAGCTTGGATGCCTCTGCTA-3'. Fragments were cloned into the 3' UTR of a firefly luciferase reporter vector (pMIR-REPORT, Ambion).

2.5 Transfections and immunoblotting

T47D cells were transfected using lipofectamine. The *ATP1B1* promoter construct was cotransfected with *Renilla* luciferase as an internal control. Cells were treated with vehicle, 10 nM R5020, or 100 nM RU486 for 24h, extracts were harvested and luciferase assays were performed as described (Ghatge et al., 2005).

Transfections of miRNA mimics were performed as described previously (Cochrane et al., 2009). Briefly, cells were plated 24 hours prior to transfection. Lipofectamine 2000 (Invitrogen) was incubated with the miRNA mimic or scrambled negative control (Ambion) at a concentration of 50 nM incubated in serum-free media for 20 min before addition to cells. Cells were incubated at 37°C for 4 h before replacement of FBS. For transfections of miR-513a-5p mimics, 24h after transfection, media was changed to CSS containing media for one day. The cells were then treated with 10 nM E2 or EtOH for 24 or 48 hrs.

Cells were harvested in RIPA lysis buffer and 50 µg of extracts were separated on SDS-PAGE and transferred to PVDF. Blots were probed with antibodies to *ATP1B1* (ab2873, Abcam), *GAPDH* (Cell Signaling), *PR* (clone 1294, DAKO), and α -tubulin (clone B-5-1-2, Sigma) followed by secondary antibodies conjugated to HRP (Sigma). Bands were detected with Western Lightning Chemiluminescence Reagent Plus (Perkin-Elmer).

2.6 Luciferase assays

T47D cells (12,500/well) were plated in a 96 well plate 24 h prior to transfection. ATPase β 1-PA3-luciferase promoter reporter vector or empty vector (196 ng/well) and pRL-SV40 (Promega; 4 ng/well) were transfected using Lipofectamine 2000. At the same time, cells were treated with ethanol vehicle (EtOH), 10 nM R5020, or R5020 plus 100 nM RU-486.

Cells were lysed 24 hours after treatment and analyzed using the Dual Luciferase Reporter assay system (Promega).

Hec50 cells were used for the *ATP1B1* 3'UTR luciferase assays. Cells (15,000/well) plated in a 96-well plate were mock transfected, transfected with negative control, 50 nM miR-29a mimic, 50 nM miR-29a antagomir (Dharmacon) alone (α 29a) or in conjunction with miR-29a (α 29a + 29a). After 24 hrs, the firefly reporter plasmid (196 ng) and a *Renilla* luciferase normalization plasmid pRL-SV40 (4 ng) were introduced using Lipofectamine 2000. Cells were harvested 48 hrs later for analysis using the Dual Luciferase Reporter assay system (Promega). For the *PGR* 3'UTR luciferase assays, 15,000 T47D cells were plated per well in a 96-well plate. The cells were transfected with 196ng of luciferase reporter constructs containing one miR-513a-5p site of the *PGR* 3'UTR (*PGR* site 1) or two sites (*PGR* site 2/3) or an empty vector (EV) and 4 ng of pRL-SV40. Twenty four hours after transfection, cells were treated with EtOH (vehicle control), 10 nM or 100 nM MPA. Twenty four hours later, cells were lysed and a Dual Luciferase Reporter assay performed.

2.7 DNA Footprinting

The PR-B isoform full length receptor was expressed in baculovirus-infected Sf9 insect cells using an expression vector encoding human PR-B fused to an N-terminal hexahistidine tag following standard protocols (Christensen et al., 1991). Solution and DNA binding properties of PR-B were previously characterized. Briefly summarized, the receptor was purified to at least 95% homogeneity and subjected to extensive sedimentation velocity and equilibrium analyses to demonstrate structural and functional homogeneity (Heneghan et al., 2005; Heneghan et al., 2006).

The *ATP1B1* promoter was excised from pSPORT to generate a 1129 bp fragment. The fragment was ³²P-end-labeled using a Klenow fill-in reaction. Footprints were carried out using DNase I as described previously (Brenowitz et al., 1986a; Brenowitz et al., 1986b; Connaghan-Jones et al., 2008), with the following modifications. All reactions were carried out in an assay buffer containing 50 mM NaCl, 20 mM Hepes, 1 mM DTT, 1 mM CaCl₂, 2.5 mM MgCl₂, 10⁻⁵ MP4, 100 µg/mL BSA, and 2 µg/mL salmon sperm DNA. Each footprinting reaction contained 20,000 cpm of freshly labeled promoter DNA. PR-B was added to each reaction mix, covering a concentration range from sub-nanomolar to micromolar, and allowed to equilibrate at 4°C for at least 45 minutes. DNase I (Invitrogen) was diluted to a concentration of 0.0058 units/µl. After the samples reached equilibrium, 5 µl of the diluted DNase I solution was added to each 200 µl reaction and digestion was allowed to proceed for exactly 2 minutes. Digestion products were electrophoresed on 6% acrylamide-urea gels and visualized using phosphorimaging.

2.8 Generation of stable cell lines

For stable expression of the miR-29a antagonist, pmiR-29a-Zip, or pGreenPuro Scramble Control (System Biosciences Inc.) lentiviral vectors were used. For generation of the stable shRNAs against *ATP1B1*, two MISSION lentiviral shRNA clones (Sigma-Aldrich, from the University of Colorado Cancer Center Functional Genomics Core) were used, clones 43333 (*ATP1B1* shRNA3) and 43335 (*ATP1B1* shRNA5) or a negative control vector. The

lentiviral plasmids were packaged in 293FT cells and virus was harvested after 48 h. Virus was added to T47D cells at a 1:1 (virus:media) ratio and selection was performed using 1 µg/ml puromycin.

2.9 Migration and invasion assays

Cells were serum starved for 12 hrs prior to performing the assay. BD Bio-Coat Control Insert Chambers 24-well plate with 8-µm pore size and BD BioCoat Matrigel Invasion Chambers were used for migration and invasion assays, respectively. After starvation, cells were trypsinized and 2.5×10^4 cells were plated in 0.5 mL MEM with 0.5% FBS in the upper chamber. In the lower chamber, 0.8 mL of 50% conditioned medium from T47D cells plus 50% DMEM with 5% FBS and L-glutamine was used as an attractant. Cells were incubated for 48 hrs at 37°C. Migrating or invading cells on the lower surface of the membranes were stained with Diff-Quik stain (Fisher) and counted.

2.10 Proliferation assays

T47D cells (1000 cells per well) were plated in a 96-well plate in phenol red-free media containing CSS. Plates were treated with 10 nM MPA or EtOH vehicle control with 6 replicate wells per treatment group. Cells were fixed with 4% paraformaldehyde at day 0 (prior to treatment as plating control) and 1, 2 and 3 days afterwards. Cells were stained in 0.1% crystal violet in 25% methanol for 1 hr and destained with water. Cells were lysed in 10% acetic acid and absorbance read at 570 nM. Absorbance readings for each of the days were normalized by dividing by the day 0 values and are reported as fold change in cell number over day 0.

3. Results

3.1 Progesterin regulation of miRNA expression

T47D (ER+/ PR+) breast cancer cells were used to examine progesterin-mediated regulation of miRNAs. Since T47D cells constitutively express robust levels of PR, they are an ideal model for PR mediated miRNA regulation in contrast to other cell lines which require estradiol treatment for the estrogen receptor to induce upregulation of PR. Cells were treated with either vehicle or MPA for 6 or 24 hrs and miRNA profiling performed using arrays interrogating 703 different miRNAs. Data were analyzed by ANOVA using a 1.5 fold cut off. Following 6 hrs of progesterin treatment, 28 miRNA were differentially regulated in T47D cells (Fig. 1A). Interestingly, the vast majority of the regulated miRNAs (20 of 28) were downregulated by progesterin treatment (Fig. 1A, right), while only 8 miRNAs were upregulated (Fig. 1A, left). While many of the miRNAs that changed at 6 hrs showed the same trend at 24 hrs (Supplemental Figure 1), none reached statistical significance and were different by more than 1.5 fold, suggesting the progesterin effect is rapid and diminishes quickly. The 6 hour progesterin regulated miRNAs and fold changes are listed in Table 1. The majority of progesterin-regulated miRNAs are located either within introns or between genes; however, miR-220b is located within an exon of Tubulin Beta 4 (TUBB4, Table 1).

We confirmed the effect of progestins on several of the progesterin-regulated miRNAs by real time PCR (Fig. 1B). We find that the three members of the miR-29 family (miR-29a,

miR-29b and miR-29c) are decreased with 6h MPA treatment in a dose dependent manner. We also confirmed that miR-141, a member of the miR-200 family, is downregulated by MPA. Members of the miR-200 family have been previously shown to be downregulated by progestins in the uterus at the onset of labor (Renthal et al., 2010). Interestingly, both miR-513a-5p and miR-513a-3p (which are derived from the same pre-miRNA) are both increased by progestin treatment by real time PCR, although only miR-513a-5p was statistically different in the array study.

3.2 Progestin regulated miRNAs are predicted to target many PR regulated genes

We previously performed gene expression profiling with 6 hrs MPA (Ghatge et al., 2005) and were therefore able to compare miRNAs regulated by progestin to mRNAs regulated by the same treatment in T47D cells. We found that approximately 50% of statistically significant mRNAs up or downregulated by at least 2 fold, are predicted to be targeted by progestin regulated miRNAs in the opposite manner. Furthermore, many progestin-regulated genes such as *ATP1B1*, *ABCA1*, *ABCG2*, *CDKN1A*, *MARCKS*, *SOX4*, *SGK* and *SEMA6A* have predicted binding sites for multiple progestin-regulated miRNA. We therefore hypothesized that some of these genes were controlled by progestins at two different levels, the first being through ligand-bound PR acting at the promoter and secondly, by a progestin-regulated miRNA exerting post-transcriptional control. Since the promoter of the ATPase, Na⁺/K⁺ transporting, beta 1 polypeptide gene (*ATP1B1*) is hormonally regulated via multiple glucocorticoid response element (GRE) sites (Derfoul et al., 1998; Pathak et al., 1990), and is P4 responsive, we chose to test our hypothesis using this particular gene.

3.3 Progestin bound PR regulates the sodium potassium ATPase ATP1B1 at both the promoter and the 3'UTR

Expression of *ATP1B1* is strongly induced at the message level (10 fold, Fig. 2A) and at the protein level (Fig. 2B) by progesterone (P4) and MPA. To test whether progestin regulation of ATP1B1 is mediated by PR via its promoter, we examined ~1kb of the *ATP1B1* promoter. The *ATP1B1* promoter is induced by progestin (~4.5 fold, Fig. 2D) and this induction is suppressed by the antiprogestin RU486, clearly showing that the effect is mediated by PR. To definitively show that PR binds to the promoter of *ATP1B1*, DNA footprinting was performed. We find three regions of protection that contain progesterone response element (PRE) half sites (Fig. 3, top). One of these sites (−1032 to −1027) contains a previously identified GRE half site (Derfoul et al, 1998). Furthermore, sequence analysis of the footprinted regions (Fig. 3, bottom) shows all three regions bound by PR contain putative PRE half sites.

One of the miRNAs with the highest fold downregulation was miR-29b (−2.5 fold, Table 1 and Fig. 1B). The *ATP1B1* gene has a well conserved putative binding site for miR-29abc located at nt 210-220 within in its 3' UTR. All three miR-29 family members share the same seed sequence (Fig. 4A) and are therefore all predicted to bind the *ATP1B1* 3'UTR equally. We find that both miR-29a and miR-29b decrease endogenous ATP1B1 levels in the absence of progestin stimulus; however, miR-29a has a slightly stronger effect compared to miR-29b (Fig 4A). To test whether miR-29a can also inhibit ATP1B1 upregulation by progesterone, we transfected a miR-29a mimic into T47D cells with and without progestin

treatment. Progesterin strongly upregulated ATP1B1 protein in the absence of the mimic and this upregulation was not affected by the negative scrambled control; however, the miR-29a mimic diminished the progesterin-mediated upregulation of ATP1B1 (Fig. 4B). Since the miR-29 family is decreased with progesterin treatment, we next sought to inhibit its function using a miR-29a antagonist (29a-ZIP) stably expressed in T47D cells. Similar to what is observed when miR-29a levels are decreased by progestins, the miR-29a antagonist increased endogenous ATP1B1 protein levels (Fig. 4C). To definitively show that miR-29a directly targets *ATP1B1*, we cloned a 233 bp portion of the *ATP1B1* 3'UTR downstream of luciferase in a reporter construct. We have previously found that Hec50 endometrial cells have high transfection efficiency, very low background luciferase and reliable luciferase expression following transfection, while still allowing for relatively quick turnover of luciferase to show regulation of the 3' UTR (Cochrane et al., 2010; Cochrane et al., 2009; Howe et al., 2011) and we used these cells for this luciferase assay. Transfection of miR-29a mimic decreased luciferase activity compared to mock transfected and scrambled negative controls. This effect is specific to miR-29a since an antagonist to miR-29a blocks the miR-29a-mediated decrease in luciferase activity (Fig. 4D). Collectively, these data clearly demonstrate that the miR-29 family plays a role in regulating ATP1B1 expression.

We present a two component model for progesterin regulation of *ATP1B1* gene expression (Fig. 5). Ligand bound PR binds to the promoter of *ATP1B1*, inducing its expression. Progestins also cause a decrease in miR-29a, which relieves repression normally exerted by this miRNA on the *ATP1B1* 3'UTR. These two components work together for maximum and tight regulation of ATP1B1 expression via hormonal control over both transcription and translation.

To determine the effect of ATP1B1 in breast cancer cells, we stably expressed shRNAs targeting *ATP1B1* to decrease its protein levels. Since miRNAs such as miR-29a can affect many genes, we used shRNAs to determine what effects are caused by specifically modulating ATP1B1 expression. Two shRNAs targeting *ATP1B1* decrease endogenous ATP1B1 protein as well as abrogate progesterin induction of ATP1B1, with shRNA 5 being the more effective (Fig. 6A). Because ATP1B1 has been implicated in polarity, we examined the effect of downregulating ATP1B1 on migration and invasion of T47D cells using Boyden chamber assays. Cells expressing shRNA 5 against *ATP1B1* have a 1.9 fold increase in migration and a 1.8 fold increase in invasion (Fig. 6B). This implies that ATP1B1 expression limits the migratory and invasive capacity of breast cancer cells. Knockdown of ATP1B1 also causes an increase in proliferation (Fig. 6C).

3.4 Progesterone receptor itself is downregulated by the progesterin upregulated miRNA miR-513a-5p

Treatment with progesterone results in a decrease in PR protein expression. Interestingly, we identified *PR* itself as a putative target of miR-513a-5p, a miRNA that is induced with progesterin treatment. The 3'UTR of the human *PR* gene contains 3 predicted miR-513a-5p binding sites (Fig. 7A). To examine whether expression of PR is regulated by miR-513a-5p, we transfected either the mimic for miR-513a-5p or a scrambled control into three luminal breast cancer cell lines (MCF7, ZR75-1, and BT474) that express PR only when it is

induced by estradiol (E2) treatment. Cells were treated with either vehicle or E2 for 24 or 48 hrs to induce PR expression (Fig. 7B). Induction of PR was weak after 24 hrs of E2; however, the strong PR induction observed after 48 hrs of E2 treatment was greatly reduced by addition of miR-513a-5p mimic in all three cell lines. MiR-513a-3p, which is derived from the same pre-miRNA as miR-513a-5p, is predicted to target 6 sites on the human *PR* 3'UTR (Fig. 7A). To determine if miR-513a-3p is also able to affect PR levels, we performed similar transfections and E2 treatments. In contrast to the strong effect of miR-513a-5p, transfection of miR-513a-3p mimic has no effect on E2 induction of PR in any of the cell lines (Fig. 7C), implying that even with 6 predicted target sites, this miRNA does not target the *PR* 3'UTR. Two regions of the *PR* 3'UTR containing one miR-513a-5p site (PGR site 1) or two sites (PGR site 2/3) were cloned into a luciferase reporter construct. Treatment with MPA causes a statistically significant decrease in luciferase activity (Fig. 7D) indicating that these regions that contain the 513a-5p sites are targeted by progestin regulated miRNAs to control PR protein expression.

We propose a model for which PR limits its estrogen induced upregulation (Fig. 8). Ligand bound ER binds the promoter of the *PR* gene, inducing its expression. Progesterone-bound PR causes an increase in miR-513a-5p which targets the *PR* 3'UTR, causing a decrease in PR protein.

4. Discussion

E2 regulates miRNAs in breast cancer cells in a modest, but repeatable manner (Castellano et al., 2009; Cochrane et al., 2010; Cohen et al., 2008; Klinge, 2009; Maillot et al., 2009). Since the effects of E2 on miRNAs peak at later time points than the effects we observe on progestin-regulated miRNAs, it is not clear if they are direct effects, or effects on the miRNA processing machinery (Yamagata et al., 2009). The exact mechanism by which progestins regulate miRNAs also remains to be determined.

Few progestin-regulated miRNAs have been identified and most studies were performed in uterine tissue. Progestin regulated miRNAs are involved in embryo implantation in the uterus (Hu et al., 2008; Xia et al., 2010a; Xia et al., 2010b), in uterine contraction at the onset of labor (Renthal et al., 2010), as well as in disease states such as leiomyoma and endometriosis (Aghajanova and Giudice, 2011; Burney et al., 2009; Ohlsson Teague et al., 2009; Pan et al., 2008; Pan et al., 2007). PR play a role in normal mammary gland development and it is interesting that the most dramatic change in global miRNA expression in the post-natal mammary gland occurs at parturition (Avril-Sassen et al., 2009; Wang and Li, 2007), when PR function decreases to allow and uterine contraction and the milk protein production in the mammary glands. We present here the first miRNA profiling study identifying progestin regulated miRNAs in breast cancer cells and demonstrate a functional role for these miRNAs in control of progestin regulated genes and indeed on PR itself. In comparison to E2 regulated miRNAs, the progestin effects on miRNA expression peak at an earlier time point (6 hrs) and are attenuated by 24 hrs, and the majority are downregulated. Many progestin-regulated genes are predicted targets of progestin-regulated miRNAs, leading us to postulate that hormonal control may be executed not only at promoters and

enhancers via PR, but also at the level of post-transcriptional control by progestin-regulated miRNA that target the 3'UTRs of progestin-regulated genes.

To test our hypothesis, we chose the *ATP1B1* gene, which is upregulated by PR-B in a manner that does not require new protein synthesis (Richer et al., 2002). The *ATP1B1* gene promoter has been extensively characterized and contains multiple hormone response elements to which members of the steroid receptor family including the glucocorticoid receptor, mineralocorticoid receptor (Derfoul et al., 1998) and PR can bind. Interestingly, there is evidence that Na^+ actually serves as a direct physiologic regulator of PR function (Connaghan et al., 2010). Consistent with this, a number of studies have demonstrated that increased intracellular Na^+ levels trigger an increase in *ATP1B1* mRNA expression and protein production (Bowen and McDonough, 1987; Pressley, 1988; Pressley, 1992; Pressley et al., 1988). We now find that the upregulation of this gene by progestin treatment may also be facilitated by progestin-mediated downregulation of miR-29, a miRNA that we demonstrate to directly target the *ATP1B1* 3'UTR. We propose that downregulation of miR-29 relieves repression of the *ATP1B1* mRNA such that it is allowed to be translated to augment progestin response. While it remains to be tested by a more global analysis, the dual regulation of genes at the 5' end by hormone receptors and the 3' end by hormone-regulated miRNAs may prove to be a common form of regulation designed to fine tune the expression of hormone-responsive genes, and indeed could also be involved in tissue- and cell type-specific responsiveness to hormones.

Na^+ , K-ATPase is a cationic pump that consists of alpha and beta subunits. The alpha subunit is responsible for catalytic function while the beta subunit performs regulatory functions. One of the isoforms of the beta subunit is the b1 (ATP1B1), which is also involved in cell-cell adhesion and in epithelial cell polarization (Bab-Dinitz et al., 2009; Rajasekaran et al., 2001; Vagin et al., 2005). Overexpression of ATP1B1 in MDCK cells decreases cell motility (Barwe et al., 2005). Here we show that in breast cancer cells, knockdown of ATP1B1 causes an increase in migration and invasion, implying that it serves to limit cell motility in these cells. We further find that knockdown of ATP1B1 causes an increase in proliferation. Since the overall effect of ATP1B1 in breast cancer cells decreases proliferation, migration and invasion, the function of ATP1B1 may be to mediate the differentiative actions of progesterone, although it may act differently if it is overexpressed and no longer under hormonal control in some breast cancers.

PR expression is regulated by liganded ER (Kastner et al., 1990) and growth factors (Cui et al., 2003). It is also regulated by ligand-dependent downregulation (Lange et al., 2000; Shen et al., 2001) and downregulates transcription of itself (Alexander et al., 1989). We present another mechanism whereby liganded PR can modulate its own levels via a progestin-regulated miRNA that targets the *PR* 3'UTR. The human *PR* transcript has a long 3'UTR (over 13kb) (Yue et al., 2010), which makes it attractive as a regulatory region for miRNAs to control PR translation. Two estrogen-regulated miRNAs have been shown to decrease PR levels, miR-181 and miR-26a (Maillot et al., 2009). Here we show that a progestin upregulated miRNA, miR-513a-5p, represses PR expression and may represent a mechanism to limit the amount of PR induced by estradiol bound ER. Furthermore, this type of hormonal control may be altered in breast cancer cells.

In conclusion, we have identified miRNAs regulated by progestins in breast cancer cells. We functionally demonstrate that progestin-regulated miRNAs can modulate PR action by controlling the expression of progestin responsive genes such as *ATP1B1*, and by controlling expression of PR itself.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ATP1B1	ATPase, Na ⁺ /K ⁺ transporting, beta 1 polypeptide
miRNA	microRNA
MPA	Medroxyprogesterone acetate
PR	progesterone receptors

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Highlights

- Progestin stimulates rapid regulation of miRNAs.
- ATP1B1 is upregulated by progesterone receptor at its promoter.
- ATP1B1 is repressed by miR-29, a progestin downregulated miRNA.
- MiR-513a-5p is increased by progestin and downregulated the progesterone receptor.

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Fig. 1.

Progestins regulate expression of miRNAs in breast cancer cells. T47D cells were treated with 10 nM MPA (dark bars) or EtOH vehicle control (open bars) for 6h. Biological duplicate samples for each treatment were hybridized to Agilent miRNA microarrays. Each miRNA probe is spotted in seven locations to allow relative intensity data for the multiple probes for each miRNA to be subjected to statistical filtering. Probes with $p < 0.05$ in at least 1 of the 4 profiles were identified. This resulted in 406 miRNA probes that passed the filter with the ANOVA analysis. The remaining data was inter-array scaled and transformed to $\log(2)$. (A) Shown are the 59 differentially expressed miRNAs ($P < 0.01$) and of these 28 had a fold change of greater than 1.5 at 6 hrs of treatment. Error bars represent the range of the duplicate samples. Left panel, upregulated miRNA, right panel, downregulated miRNA. (B) T47D cells were plated and treated with vehicle (open bars), 10 nM (grey bars) or 100nM MPA (black bars) for 6h and Taqman PCR was performed using primers and probes specific for the miR-29 family (left panel, miR-29a, miR-29b and miR-29c), miR-141 (mid panel), and miR-513a-3p and miR-513a-5p (right panel). Shown are the relative miRNA

levels, normalized to U6. Averages of quadruplicate samples are shown. Asterisk above the error bar indicates $P < 0.05$, Student's T-test.

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Fig. 2.

Progestin treatment upregulates the *ATP1B1* gene at the promoter level. (A) Taqman qRT-PCR of the endogenous *ATP1B1* gene in T47D breast cancer cells treated plus (+) or minus (–) 10nM of the progestin R5020 for 6 hrs. Error bars indicate standard deviation of the mean for three biological replicates. (B) T47D cells were treated with vehicle (EtOH), 10 nM MPA or 10 nM P4 for 12, 24 and 48 hrs prior to protein being harvested. Immunoblot for ATP1B1 and GAPDH (loading control) shows maximal increase of ATP1B1 at 24 hrs. (C) Transcriptional activity on the *ATP1B1* promoter was analyzed by transfecting T47D

cells 24 hrs after plating with the ATP1B1-PA3-luciferase promoter reporter vector or empty vector and subsequent treatment with ethanol vehicle (EtOH, open bars), 10 nM R5020 (grey bars), or R5020 plus 100 nM Ru486 (black bars). Cells were lysed 24 hrs after treatment and relative luciferase units were determined by normalizing firefly luciferase to SV40-renilla-luciferase. Error bars indicate standard deviation of the mean for five replicates.

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Fig. 3.

PR binds to the ATP1B1 promoter. Top; a representative footprint titration of the ATP1B1 promoter with concentrations of PR-B covering sub nM to sub μ M (black-filled triangle) is shown. At higher concentrations of PR-B a footprint is observed in three separate regions. Schematic to the right of the footprint image depicts the ATP1B1 promoter. Colored rectangles signify regions where PR-B:promoter binding occurs and an arrowhead shows the direction of transcription and approximate location of the start site. Numbers to the right of the schematic indicate the base pair position relative to start of transcription. Bottom; the

sequence of the three regions bound by PR-B is shown; colored nucleotides correspond to the footprinted regions. Green solid arrows denote consensus or nearly consensus PRE ½ sites (6/6 or 5/6 nt conserved). Gray broken arrows denote less conserved PRE ½ sites (4/6 nt conserved).

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Fig. 4.

The progestin downregulated miRNA miR-29a targets the *ATP1B1* 3'UTR. (A) The miR-29 family consists of three family members that share a common seed sequence (in box). T47D cells were transfected with a scrambled negative control, a miR-29a or miR-29b mimics in triplicate. Shown is an immunoblot for ATP1B1 and GAPDH as a loading control.

Quantification of the ATP1B1 protein relative to GAPDH is shown on the right. Asterisk indicates $P < 0.05$, Student's t-test. (B) T47D cells were transfected with a negative scrambled control or a mimic for miR-29a and cells were treated with vehicle (–) or MPA (+) for 48 hrs, immunoblotted and probed for ATP1B1 or GAPDH (loading control). Duplicate samples are shown. (C) Immunoblot for ATP1B1 and GAPDH of T47D cells stably expressing a miR-29a antagomir (29a-ZIP) or a scrambled negative control (scr-ZIP). (D) Region of the *ATP1B1* 3' UTR where miR-29a is predicted to bind was cloned into a luciferase reporter vector. Hec50 cells treated with transfection reagent only (mock),

scrambled negative control (neg), miR-29a mimic (29a), miR-29a in conjunction with a miR-29a antagomir (anti29a + 29a) and luciferase assay was performed. Shown are mean of five replicates and error bars indicate standard deviation of the mean. Asterisk indicates $P < 0.05$, Student's t-test.

Fig. 5.

Model for progestin regulation of ATP1B1. Ligand bound progesterone receptors (PR) bind to the promoter at the 5' end of the *ATP1B1* gene to induce transcription. Progestin treatment inhibits expression of the miR-29 family, which normally target and repress *ATP1B1* at the 3' end of the transcript.

Fig. 6.

Knockdown of ATP1B1 causes an increase in proliferation, migration and invasion. (A) T47D cells were stably infected with a negative control or shRNAs against *ATP1B1*. The stable cells were treated with EtOH vehicle (–) or 10 nM MPA (+) for 24 hrs prior to protein being harvested. Immunoblot for ATP1B1 and GAPDH are shown. (B) Boyden chamber assays to measure migration (left) and invasion (right) were performed on stable cell lines. Cells that migrated through triplicate filters were stained and counted. Asterisks indicate

P<0.05, Student's t-test. (C) Proliferation of the stable cell lines was measured using a crystal violet assay. Asterisks indicate P<0.05, Student's t-test.

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Fig. 7.

MiR-513a-5p attenuates estradiol mediated upregulation of PR. (A) Schematic of the PGR 3'UTR indicating the miR-513a-5p and miR-513a-3p sites. Constructs used for luciferase assays are shown. (B) MCF7, ZR75-1 and BT474 cells were transfected with either a negative scramble control (indicated by -) or a miR-513a-5p mimic (indicated by +). Twenty four hours after transfection, the media was changed to phenol red-free media containing CSS. The cells were treated with 10nM estradiol (E2; indicated by +) or an equal volume of ethanol as a vehicle control (indicated by -). Protein was harvested 24 hrs or 48 hrs later. Shown are immunoblots for PR and α -tubulin (as a loading control). (C) Cells were treated as described above using a miR-513a-3p mimic. (D) T47D cells were transfected with empty vector (EV) luciferase vector or a vector containing a region of the PGR 3'UTR with one putative miR-513a-5p site (PGR site 1) or two sites (PGR site 2/3). The cells were treated with MPA for 24 hours and a luciferase assay performed. * indicates $P < 0.05$, ** indicates $P < 0.01$, ANOVA.

Fig.8.

Model for attenuation of estradiol mediated upregulation of PR through miR-513a-5p.

Ligand bound ER binds to the promoter of PR causing an increase in PR protein.

Progesterone causes an increase in miR-513a-5p, which in turn targets the PR 3'UTR. The overall effect is that miR-513a-5p limits the amount of E2 mediated upregulation of PR.

Table 1

Sequence Code	Condition Factor 1 P*-value	ETOH Value	ETOH Error	MPA Value	MPA Error	Direction MPA vs. ETOH	Fold Change	Chromosome	intronic/intergenic	nearest gene
hsa-miR-921	1.35E-07	7.4107	0.0548	8.3221	0.1237	UP	1.88	1q24.1	intronic	FAM78B
hsa-miR-513b	5.25E-07	7.7079	0.0711	8.5965	0.1254	UP	1.85	Xq27.3	intergenic	miRNA cluster
hsa-miR-27b*	0.00416	6.8752	0.0876	7.7246	0.1609	UP	1.80	9q22.32	intronic	C9orf3
hsa-miR-513a-5p	0.00039	9.7766	0.0766	10.4248	0.1068	UP	1.57	Xq27.3 (-1 and -2)	intergenic	miRNA cluster
hsa-miR-634	4.04E-10	8.2757	0.0617	8.9228	0.0561	UP	1.57	17q24.2	intronic	PRKCA
hsa-miR-30c-1*	0.02356	7.2165	0.0726	7.8386	0.1391	UP	1.54	1p34.2	intronic	NFYC
hsa-miR-483-5p	3.12E-06	9.2266	0.0642	9.8467	0.0762	UP	1.54	11p15.5	intronic	IGF2
hsa-miR-412	0.00002	6.1157	0.0624	6.7085	0.0784	UP	1.51	14q32.31	intergenic	miRNA cluster
hsa-miR-487b	3.99E-13	9.5691	0.1296	7.9401	0.1335	DOWN	3.09	14q32.31	intergenic	miRNA cluster
hsa-miR-29b	9.89E-06	7.6099	0.1613	6.2743	0.1668	DOWN	2.52	1 at 7q32.3, -2 at 1q32.2	intergenic, intergenic	miR-29a, miR-29c
hsa-miR-220b	4.51E-14	9.3327	0.0993	8.0697	0.0899	DOWN	2.40	19p13.3	exonic	TUBB4
hsa-miR-141	0.00007	7.9975	0.2360	6.7506	0.1314	DOWN	2.37	12p13.31	intergenic	miRNA cluster with 200c
hsa-miR-525-5p	2.80E-08	6.2250	0.0755	5.1642	0.1271	DOWN	2.09	19q13.41	intergenic	miRNA cluster
hsa-miR-371-5p	1.34E-13	8.0910	0.0723	7.1558	0.0727	DOWN	1.91	19q13.41	intergenic	miRNA cluster
hsa-miR-301a	6.83E-06	6.7850	0.0903	5.8798	0.1449	DOWN	1.87	17q22	intronic	FAM33A
hsa-miR-212	4.51E-14	9.1641	0.0575	8.3323	0.0623	DOWN	1.78	17p13.3	intergenic	DPH1, miR-132 and HIC1
hsa-miR-32	0.00036	7.0734	0.1203	6.2740	0.0930	DOWN	1.74	9q31.3	intronic	c9orf5
hsa-miR-19b	2.27E-11	6.1929	0.0737	5.3945	0.0565	DOWN	1.74	1 at 13q31.3 and -2 at Xq26.2	intronic, intergenic	c13orf25, miRNA cluster
hsa-miR-374a	2.44E-10	6.1638	0.0451	5.3957	0.0865	DOWN	1.70	Xq13.2	intergenic	miR-545
hsa-miR-30b	4.51E-14	7.1740	0.0564	6.4596	0.0670	DOWN	1.64	8q24.22	intergenic	mir-30d
hsa-miR-1229	5.36E-07	7.8199	0.1255	7.1063	0.0504	DOWN	1.64	5q35.3	intronic	MGAT4B
hsa-miR-29c	0.00069	7.9004	0.0859	7.1924	0.1064	DOWN	1.63	1q32.2	intergenic	miR-29b-2
hsa-miR-324-5p	0.00077	7.0307	0.0892	6.3288	0.1303	DOWN	1.63	17p13.1	intronic	ACADVL
hsa-miR-370	3.25E-07	10.1624	0.0870	9.4931	0.0484	DOWN	1.59	14q32.31	intergenic	miRNA cluster

Sequence Code	Condition Factor 1 P*-value	ETOH Value	ETOH Error	MPA Value	MPA Error	Direction MPA vs. ETOH	Fold Change	Chromosome	intronic/intergenic	nearest gene
hsa-miR-130a	0.0159	9.7431	0.1001	9.1029	0.1326	DOWN	1.56	11q12.1	intergenic	SERPINC1 and YPEL1
hsa-miR-30e	0.00001	7.5535	0.0894	6.9148	0.0605	DOWN	1.56	1p34.2	intronic	NFYC
hsa-miR-101	0.02853	7.2814	0.1264	6.6782	0.1098	DOWN	1.52	1 at 1p31.1, -2 at 9p22.1	intergenic, intronic	JAK1 and AK3L1, RCL1
hsa-miR-20a	0.03163	6.3749	0.0803	5.7848	0.1290	DOWN	1.51	13q31.3	intergenic	miRNA cluster