Involvement of let-7/miR-98 microRNAs in the regulation of progesterone receptor membrane component 1 expression in ovarian cancer cells

ALEXANDRA WENDLER[1], DANIELA KELLER[1], CHRISTIAN ALBRECHT[1], JOHN J. PELUSO[2] and MARTIN WEHLING[1]

[1]University of Heidelberg, Clinical Pharmacology Mannheim, Maybachstr. 14, 68169 Mannheim, Germany; [2]The University of Connecticut Health Center, 263 Farmington Ave., Farmington, CT 06030-1941, USA

Received August 31, 2010; Accepted September 20, 2010

DOI: 10.3892/or_00001071

Abstract. PGRMC1 (progesterone receptor membrane component 1) is part of a multi-protein complex, that is highly expressed in several cancers and is involved in chemo-resistance. Although PGRMC1 plays an important role in various cancers, little is known about how PGRMC1 expression is regulated. Therefore, the present study was designed to elucidate the molecular mechanisms that influence PGRMC1 expression in ovarian cancer cells. An *in silico* approach revealed that the 3'-untranslated region of PGRMC1 contains one highly and one poorly conserved binding site for the microRNA let-7/miR-98 and one highly conserved binding site for miR-141/200a. Luciferase assays and real-time PCRs showed that the let-7 isoforms let-7i and miR-98 target PGRMC1 in SKOV-3 cells. In contrast, the conserved binding site for miR-200a/141 in the 3'-UTR of PGRMC1 is not functional. Stimulation of SKOV-3 cells with progesterone resulted in a decrease in PGRMC1 mRNA levels. Further, an analysis of endogenous let-7i levels in SKOV-3 cells revealed that let-7i expression increased after stimulation with progesterone. Therefore, progesterone may exert its effect on PGRMC1 expression in part by stimulation of let-7i. In conclusion, we propose that PGRMC1 expression is regulated by the miRNAs let-7/miR-98, which could become therapeutic targets, as PGRMC1, like many other targets of let-7, seems to be involved in cancer proliferation and chemotherapy resistance.

Introduction

Epithelial ovarian cancer is the most frequent cause of gynaecologic malignancy-related mortality of women (1). Despite advances in platinum-based chemotherapy patients typically experience disease relapse within two years of initial treatment and develop platinum resistance (2). Therefore, a better understanding of the mechanisms resulting in resistance against chemotherapy is urgently needed.

PGRMC1 expression is elevated in several cancer cells like lung, breast, and ovarian cancer (3-5). Further, in ovarian cancer PGRMC1 expression is increased in advanced cancer stages (3). The protein promotes cell survival and chemotherapy resistance (5,6) and therefore may be an interesting therapeutic target for the intervention of cancer (5-7).

Deletion of PGRMC1 in different cancer cell lines results in higher sensitivity of these cells towards the anti-cancer drugs cisplatin, doxorubicin and camptothecin, while over-expression of PGRMC1 renders cells more resistant against these drugs (3,8). *In vitro* studies revealed that PGRMC1 depleted cells show a slower rate of cell proliferation (6). These data were verified by *in vivo* studies demonstrating that PGRMC1 depleted ovarian cancer cells form tumors in fewer mice. In those mice that tumors were formed, the number of tumors was reduced by 55% compared to mice with wild-type tumors (6). Further, PGRMC1 is induced by chemotherapy (8,9) and in mouse cells with short telomeres, which suffer chromosomal damage during senescence (10).

Although PGRMC1 expression is increased in ovarian cancer and functions to promote chemotherapy resistance, little is known about the mechanisms regulating its expression. MicroRNAs (miRNAs) have been shown to regulate expression of several target genes, and therefore may influence PGRMC1 expression. MiRNAs are small RNAs 18-25 nucleotides of length, which regulate the expression of their target genes largely through translational inhibition or mRNA destabilization after binding to specific sequences in the 3'-UTR of the target mRNA. To date, more than 723 miRNAs have been identified and validated in the human genome (11). Importantly, human cancers often show a distorted expression profile of miRNAs (12). Analysis of the PGRMC1 3'-UTR revealed several binding sites for miRNAs, including two putative let-7/miR-98 binding sites and one miR-200a/141 binding site. Therefore, this study was designed to investigate the functional importance of these sites in regulating PGRMC1 expression. Additionally, the

Correspondence to: Dr Alexandra Wendler, University of Heidelberg, Clinical Pharmacology Mannheim, Maybachstr. 14, 68169 Mannheim, Germany
E-mail: alexandra.wendler@medma.uni-heidelberg.de

Key words: progesterone receptor membrane component 1, ovarian cancer, microRNA, let-7/miR-98, progesterone

Table I. Sequences of primers used for the preparation of constructs, site-directed mutagenesis, and real-time PCR.

Primer	Application	Forward	Reverse
PGRMC1 3'-UTR	Cloning of the PGRMC1 3'-UTR	gattctagaagcattcagtggaagtata	gattctagatagtgggtaaaaccattttta
let-7-1	Mutation of the first let-7 binding site	ctgcatgatttctgttttattaaagcaaatctgcagtgtt	aacactgcagatttgctttaataaaacagaaatcatgcag
let-7-2	Mutation of the second let-7 binding site	gtgaactgtgttttatgattaatcagaaaacaaaatgatgtgc	gcacatcattttgttttctgattaatcataaaacacagttcac
miR-141/200a	Mutation of the miR-141/200a binding site	tacctctaaagcaaatctgccaaagactttggtatggatta	taatccataccaaagtctttggcagatttgctttagaggta
PGRMC1 RT	Quantification of PGRMC1 mRNA	cgacggcgtccaggaccc	tcttcctcatctgagtacacag
GAPDH RT	Quantification of GAPDH mRNA	cagcctcaagatcatcagcaatgc	agaccacctggtgctcagtgtag

influence of progesterone (P4) on miRNAs regulating PGRMC1 was analyzed, since progesterone (P4) regulates PGRMC1 expression in a tissue-dependent manner (13,14), which may involve miRNAs.

Materials and methods

Cell culture. SKOV-3 cells were purchased from ATCC (Rockville, MD, USA). The cells were cultured in DMEM-F12 (Invitrogen, Karlsruhe, Germany), supplemented with 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen) at 37˚C in a humidified atmosphere of 5% CO_2.

Transfection of cells. For luciferase assays $5x10^4$ SKOV-3 cells per well were seeded in 24-well plates 24 h prior to transfection. Transfection of the various constructs was performed in quadrublicate using the transfection reagent Nanofectin (PAA, Coelbe, Germany) according to the manufacturer's instructions. Immediately prior to transfection, medium was replaced with 500 μl fresh culture medium. In each well, cells were transfected with 0.25 μg of pGL3 promoter vector, 0.25 μg pSV ß-galactosidase control vector (Promega, Mannheim, Germany) and either 5 nM of specific let-7i, miR-98, miR-200a, miR-141 mimic (Qiagen, Hilden, Germany) or control (Silencer negative control; Ambion, Darmstadt, Germany) as recommended by the supplier.

For real-time PCRs $1x10^6$ cells were seeded in 6-cm dishes and transfected with 5 nM let-7i mimic or control 24 h after seeding. In these studies, siPORT™ Neo FX™ Transfection reagent (Invitrogen) was used according to the manufacturer's recommendations.

Generation of constructs for the miRNA studies. The 3'-UTR (1212 bp) of PGRMC1 was amplified by PCR using the primers PGRMC1 3'-UTR for and PGRMC1 3'-UTR rev (for sequences see Table I) and cDNA from SKOV-3 cells as template. The PCR fragment and the pGL3 promoter vector (Promega) were both restricted with *Xba*I. The PCR fragment was ligated in the vector downstream the luciferase gene and direction of the insert was checked by restriction analysis.

The let-7/miR-98 or miR-141/200a binding sites in the 3'-UTR of PGRMC1 were deleted using the site-directed mutagenesis kit II (Stratagene, Waldbronn, Germany). The primers used for the deletions of the binding sites are shown in Table I. For the analysis of the two let-7 sites several constructs were prepared. In the construct let-7-1 the first let-7 binding site (bases: 427-448 of the PGRMC1 3'-UTR) was deleted, in the construct let-7-2 the second binding site (bases: 1149-1170) was deleted and in let-7 mut both binding sites were deleted. Accordingly, in the construct miR-141/200a the binding site for miR-141/200a was deleted. Correctness of all constructs was verified by sequencing. MiRNA mimics (chemically synthesized, double-stranded RNAs which mimic mature endogenous miRNAs after transfection into cells) were obtained from Qiagen.

Luciferase assays. Forty-eight hours after transfection of SKOV-3 cells, luciferase assays were performed with the Steady-Glo Luciferase assay system (Promega) in accordance with the manufacturer's recommendations. Luminescence was measured by the Envision 2102 multi-label-reader (Perkin Elmer, Rodgau-Jüdesheim, Germany). The ß-galactosidase assay served as normalization control. The assay was performed in phosphate buffer containing o-nitrophenyl galactopyranoside (ONPG) as substrate and was measured in the same device as the luciferase activity, but at 405 nm.

Quantitative real-time PCR. For quantitative real-time PCR, total RNA including miRNAs was isolated using the miRNeasy kit (Qiagen) 48 h after cells were transfected with 5 nM miRNA mimic or control (Silencer negative control; Ambion). RNA (1 μg) was transcribed into cDNA using the miScript reverse transcription kit (Qiagen). For the quantification of let-7i, 50 ng cDNA was used for real-time PCR analysis

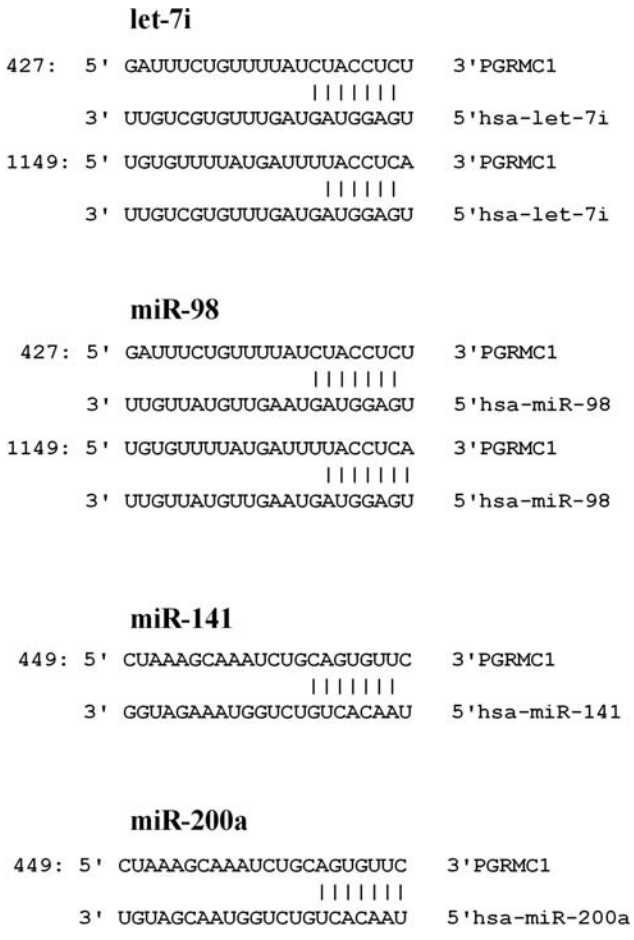

Figure 1. Alignment of the analyzed miRNA binding sites and the PGRMC1 3'-UTR.

using the miScript Sybr Green kit (Qiagen) on the LightCycler Instrument (Roche, Mannheim, Germany). Cycling conditions were: 95˚C, 15 min; 95˚C, 15 sec; 55˚C, 10 sec; 70˚C, 20 sec. Steps 2-4 were repeated 40 times. Let-7i primers (Qiagen) were used for quantification of let-7i, further primers against SNORA73A (Qiagen) were used for normalization, since SNORA73A did not show altered levels under the conditions used.

To analyze the influence of let-7i on PGRMC1 expression, real-time analysis was performed with the cDNA prepared above but with PGRMC1 primers (for sequences see Table I) and the LightCycler®FastStart DNA Master Plus SYBR Green I kit (Roche). Cycling conditions used were: 95˚C, 15 min; 95˚C, 15 sec; 58˚C, 5 sec; 72˚C 10 sec; steps 2-4 were repeated 40 times. Since no differences in GAPDH levels were detected after several treatments, GAPDH primers (Table I) were used for normalization. Serial dilutions of cDNA were used for the generation of standard curves for all primer sets. Reactions containing water instead of cDNA did not show specific amplification of the fragments with all primers and served as negative controls. The specificity of the primers was verified by analyzing the PCR products by gelelectrophoresis. For analysis of the results and relative quantification using CP values the Relquant 1.01 software was used.

Analysis of the influence of hormones on PGRMC1 and let-7i expression. SKOV-3 cells were seeded at $1x10^6$ cells per 6-cm dish in steroid-free medium. Twenty-four hours after seeding 0-1000 nM P4 (Sigma, Taufkirchen, Germany), 1000 nM estradiol (Sigma), or vehicle (0.1% ethanol) was added to the culture medium and the cells were incubated for another 24 h. Cells were harvested, RNA isolated, cDNA synthesized, and real-time PCRs quantifying the endogenous amount of let-7i and PGRMC1 mRNA were performed as mentioned above.

Statistical analysis. Data are presented as means ± SD. For experiments with only two groups, a Student's t-test was used to assess statistical signficance, while experiments with three or more groups were analyzed by an One-way ANOVA followed by a Dunnett's multiple comparison test. Data shown in Fig. 2 was analyzed using a Two-way ANOVA followed by a Bonferroni post hoc test. Regardless of the statistical analysis used, values were considered to be significantly different at $p<0.05$.

Results

The 3'-UTR of PGRMC1 contains highly conserved binding sites for let-7/miR-98, miR-141/200a and poorly conserved sites for several other miRNAs. An *in silico* analysis using TargetScan (15-17), revealed one conserved putative let-7/miR-98 binding site and a second poorly conserved let-7/miR-98 binding site in the 3'-UTR of PGRMC1 (Fig. 1). These binding sites were also predicted by miRDB (18,19), and microRNA.org (20,21). The let-7/miR-98 sites were predicted to bind the let-7 isoforms a, b, c, d, e, f, g, i and miR-98. Since let-7i has been shown to be downregulated in chemotherapy resistant ovarian cancer (22,23), this isoform was selected for further study. The other let-7 isoforms have also been reported to be downregulated in ovarian cancer (22-25), but the correlation of their expression to chemosensitivity has not been reported yet. Additionally, a conserved miR-141/miR-200a binding site (Fig. 1), was predicted by all three software programs. This binding site was also analyzed for functionality. All other predicted binding sites were poorly conserved in mammals and thus not analyzed in this study.

PGRMC1 is targeted by let-7/miR-98 but not by miR-141/200a. To determine if the let-7/miR-98 and/or miR-141/miR-200a binding sites in the PGRMC1 3'-UTR are functional, the 3'-UTR of PGRMC1 was cloned downstream of the luciferase gene into the pGL promo luciferase vector. Further, constructs were prepared in which the let-7/miR-98 or miR-141/miR-200a binding sites were deleted (Fig. 2A). Ovarian cancer (SKOV-3) cells were transfected with these constructs and luciferase assays were performed. Compared with cells transfected with the pGL promo vector (column 1, Fig. 2B), luciferase activity was reduced in SKOV-3 cells transfected with a construct additionally containing the 3'-UTR of PGRMC1 (3'-UTR, column 3) ($P<0.05$; Fig. 2B). In cells cotransfected with the 3'-UTR construct and a let-7i mimic (column 4), luciferase activity was significantly ($P<0.05$) lower than the activity observed in cells transfected with the 3'-UTR vector and control (column 3). Further, luciferase activity in cells transfected with a construct lacking both

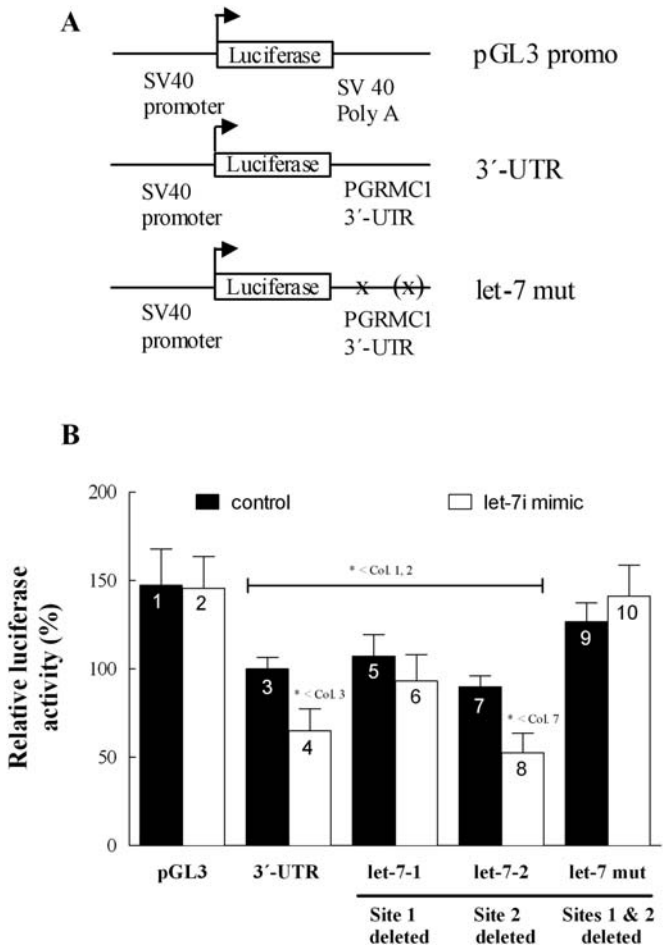

Figure 2. Validation of the let-7/miR-98 binding sites in the 3'-UTR of PGRMC1. (A) Constructs used for transfection of SKOV-3 cells to validate the functionality of miRNA binding sites. The PGRMC1 3'-UTR was cloned downstream of the luciferase gene in the pGL3 promo vector. Putative binding sites for let-7/miR-98 were deleted by site-directed mutagenesis (let-7 mut). (B) Luciferase assays were performed with crude extracts of SKOV-3 cells transfected with different luciferase constructs and let-7i mimics or negative control. Cells were transfected with: pGL3: pGL3 promoter vector; 3'-UTR: pGL3 promoter vector + 3'-UTR of PGRMC1; let-7-1: 3'-UTR, in which the first putative let-7 binding site (bases: 427-448) was deleted; let-7-2: 3'-UTR, in which the second putative let-7 binding site (bases: 1149-1170) was deleted; let-7 mut: 3'-UTR, in which both putative let-7 binding sites were deleted. All cells were cotransfected with the let-7i mimic or the negative control (Silencer negative control). Values are shown as means ± SD. *P-values that are significantly different (P≤0.05, n=4 independent experiments).

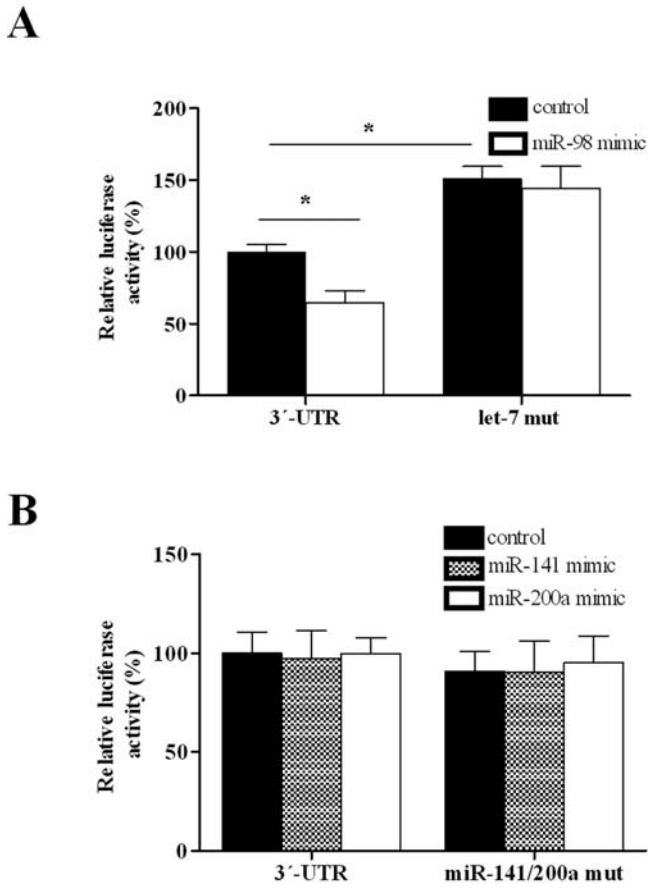

Figure 3. Analysis of the effect of miR-98 and miR-141/200a on PGRMC1 expression. Luciferase assays were performed with crude extracts of SKOV-3 cells transfected with different luciferase constructs and miRNA mimics or negative control. (A) Effect of miR-98. Cells were transfected with: 3'-UTR: pGL3 promoter vector + 3'-UTR of PGRMC1; let-7 mut: 3'-UTR, in which both putative let-7 binding sites were deleted. (B) Effect of miR-141/200a. Cells were transfected with: 3'-UTR: pGL3 promoter vector + 3'-UTR of PGRMC1; miR-141/200a mut: 3'-UTR, in which the putative miR-141/200a binding site was deleted. The cells were additionally transfected with miR-141, miR-200a mimic or negative control (Silencer negative control). Values are shown as means ± SD. *P-values that are significantly different (P≤0.05, n=4 independent experiments).

binding sites for let-7 (let-7 mut, column 9), was not significantly different from that associated with pGL3 vector (column 1). Moreover, luciferase activity in cells transfected with the let-7 mut constructs was not affected by the presence of the let-7i mimic (column 10), demonstrating that the decrease in luciferase activity in cells cotransfected with 3'-UTR and let-7i mimic is due to specific binding of let-7i to the let-7 binding sites.

In order to estimate if both predicted let-7/miR-98 binding sites are functional, constructs in which just one of the two let-7/miR-98 binding sites was deleted were used in luciferase assays (let-7-1: construct in which the binding site at bases 427-448 of the PGRMC1 3'-UTR was deleted (columns 5 and 6), let-7-2: construct in which the binding site at bases 1149-1170 was deleted (columns 7 and 8). Cotransfection of cells with the construct let-7-1 and let-7i mimic (column 6), did not significantly reduce luciferase activity compared to cells transfected with the control (column 5). If only the second binding site was deleted (let-7-2) cotransfection with the mimic (column 8) resulted in a significant downregulation of luciferase activity compared to the control treated cells (column 7) (Fig. 2B, $P<0.05$).

Since the miRNA miR-98 also belongs to the let-7 family, luciferase assays using a miR-98 mimic were performed as described for let-7. The miR-98 mimic lead to a significant downregulation of luciferase activity and luciferase activity was recovered if cells were transfected with the let-7 mut construct (Fig. 3A). In contrast, miR-200a and miR-141, which have a common, conserved, putative binding site different from the let-7 binding site, do not target PGRMC1 since the miR-200a and miR-141 mimics did not have any effect on luciferase activity (Fig. 3B).

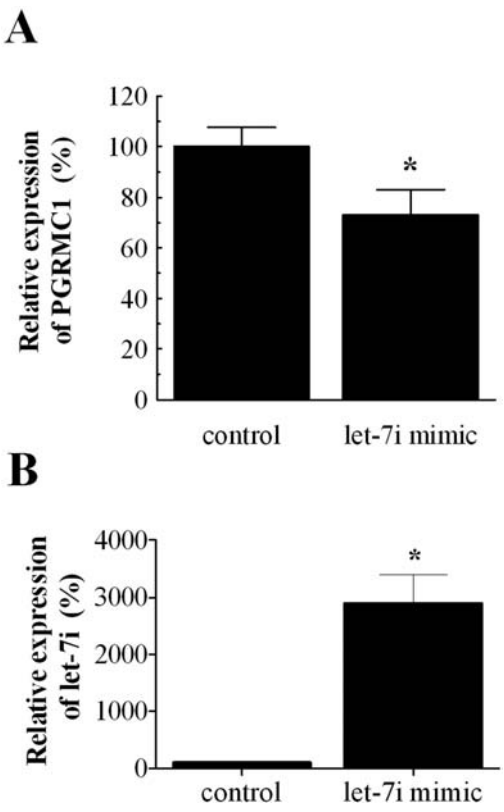

Figure 4. Analysis of PGRMC1 mRNA and let-7i levels in SKOV-3 cells transfected with the let-7i mimic or negative control. Cells were transfected with 5 nM let-7i mimic or control (Silencer negative control) 48 h prior to harvesting of cells for real-time PCR. (A) Quantification of PGRMC1 mRNA levels. GAPDH was used for normalization. (B) Quantification of let-7i levels. SNORA73A was used for normalization. Values are shown as means ± SD. *P-values that are different from controls (P≤0.05, n=3 experiments with different cDNAs, respectively).

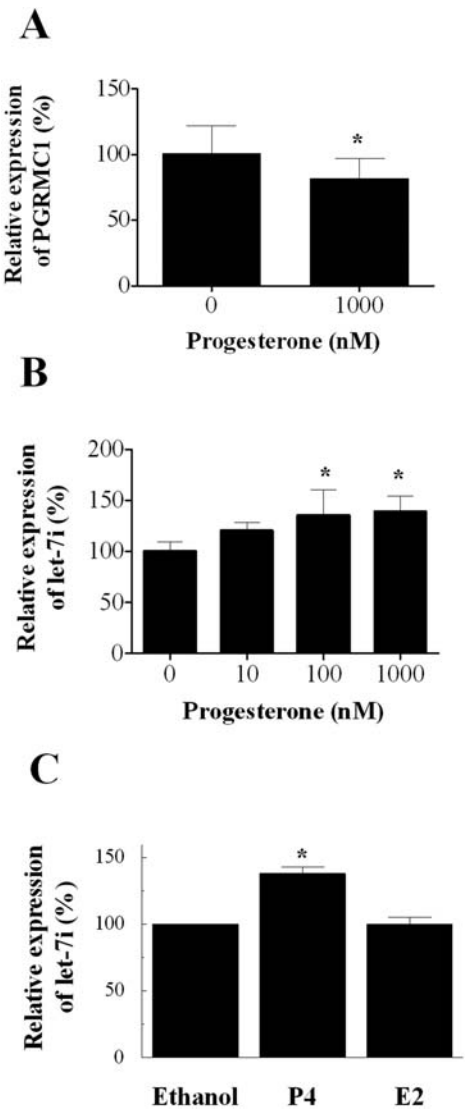

Figure 5. Effect of P4 on PGRMC1 and let-7i expression in SKOV-3 cells. (A) Quantification of endogenous PGRMC1 mRNA levels after stimulation with 0 and 1000 nM progesterone (P4). Values are shown as means ± SD. *P-values that are different from vehicle (P≤0.05, n=9, experiments were performed 3 times with the same cDNA, and three cDNAs were analyzed). (B) Concentration-dependent effect of P4 on let-7i levels. Cells were stimulated with 0, 10, 100, and 1000 nM P4, respectively and endogenous let-7i levels were determined by real-time PCR. Values are shown as means ± SD. *P-values that are different from vehicle (P≤0.05, n=3 experiments with different cDNAs, respectively). (C) Effect of 1000 nM estradiol (E2) and P4 on let-7i expression. Values are shown as means ± SD. *P-values that are different from vehicle (P≤0.05, n=3 experiments with different cDNAs, respectively).

Let-7i regulates PGRMC1 expression at the mRNA level. To confirm that let-7i regulates endogenous PGRMC1 levels in SKOV-3 cells, PGRMC1 mRNA levels were measured after transfection of cells with let-7i mimic. Here it was demonstrated that let-7i mimics suppressed endogenous PGRMC1 mRNA levels (P<0.05; Fig. 4A) compared with cells transfected with negative control. As a control of transfection, the increase in the amount of let-7i was determined, which in cells transfected with the let-7i mimic was 30-fold increased compared to control (P<0.05; Fig. 4B).

P4 suppressed PGRMC1 mRNA levels and increased let-7i levels. PGRMC1 mRNA levels are regulated by P4 in several tissues (13,14). In order to analyze the effect of P4 on PGRMC1 expression in SKOV-3 cells, cells were incubated with 0 or 1000 nM P4 for 24 h. Treatment of SKOV-3 cells with 1000 nM P4 suppressed native PGRMC1 mRNA levels by ~20% of control values (P<0.05; Fig. 5A). In the same cells P4 increased let-7i mRNA levels by 40% (P<0.05 Fig. 5B). In the case of let-7i levels, there was already a significant increase detected at 100 nM, showing that this effect is dose dependent (Fig. 5B). In order to show that the effect on let-7i is specific for progesterone, cells were also incubated with 1000 nM estradiol. These cells did not show a significant increase in let-7i levels (Fig. 5C).

Discussion

Increasing evidence is arising that PGRMC1 is involved in the promotion of tumorigenesis and chemotherapy resistance (5,6). Its expression is elevated in several cancer types such as ovarian, breast, and lung cancers (4). In this study, we showed for the first time that the regulatory mechanisms, which account for the elevated levels of PGRMC1 mRNA in ovarian tumors likely involve miRNAs such as let-7/miR-98, which are downregulated in several cancer types (22-25).

Several isoforms of let-7 have two putative binding sites in the 3'-UTR of PGRMC1. One of these sites is highly

conserved among mammals. The present study showed that the isoform let-7i represses PGRMC1 expression, mainly via the conserved let-7 binding site. This conclusion is based on four observations. First, the addition of the 3'UTR of PGRMC1 downstream of the luciferase gene in the pGL promo vector decreases luciferase activity in cells transfected with this construct compared to cells transfected with the pure pGL3 promo luciferase vector. This suggests that there are regulatory elements within this region that suppress PGRMC1 expression. Second, *in silico* analysis revealed that there are two let-7 binding sites in the 3'-UTR of PGRMC1. Third, a let-7i mimic suppresses luciferase activity from constructs containing the 3'-UTR. Finally, deleting both let-7 sites eliminates the suppressive effects of the 3'-UTR on luciferase activity.

Interestingly, the first and most highly conserved let-7 site seems to play the main role in the PGRMC1 3'-UTR, at least for let-7i, since it is sensitive to the inhibitory effects of the let-7i mimic, while the second binding site is not sensitive to this mimic. Recently, it was demonstrated that the isoforms let-7a, d, f, g, as well as i are expressed in SKOV-3 cells (23,26). These let-7 isoforms are likely able to interact with the let-7 sites in the 3'UTR region of PGRMC1 and may account for the significantly reduced luciferase activity seen in cells transfected with the vector containing the 3'-UTR of PGRMC1. Moreover, the second let-7 site might be responsive to other let-7 isoforms that are known to be expressed in SKOV-3 cells since the luciferase activity in cells transfected with the construct lacking just the first binding site is not recovered to that of cells transfected with the pGL promo vector. More studies are required in order to discern the functionality of this second let-7 site. The 30% decrease in luciferase activity seen after transfection with the let-7i mimic and 3'-UTR compared to transfection with control and 3'-UTR is similar to the effect seen for let-7 in other cell lines targeting other transcripts. For example, the transfection of a let-7a mimic in human prostate cancer cell lines transfected with a luciferase E2F2-3'-UTR construct suppressed the luciferase activity to an amount comparable to our results (27).

The let-7 family consists of 11 very closely related genes and several members of this family, i.e. let-7b (28), 7d (22,26), 7e (22), 7f (22), and 7i (23) have been shown to be down-regulated in ovarian cancer cells. Dahiya and coworkers (22) showed that 94% of the examined ovarian tumors had at least one let-7 family member downregulated by at least two-fold. Since binding sites for several other isoforms of the let-7 family are predicted in the PGRMC1 3'-UTR, the down-regulation of these miRNAs in ovarian cancer most probably will also contribute to the upregulation of PGRMC1 in ovarian cancer patients. This may result in a much more pronounced downregulation of PGRMC1 as seen in our study by let-7i alone. The downregulation of several let-7 isoforms therefore contributes significantly to the upregulation of PGRMC1 in cancer cells.

A study analyzing the NCI60 panel of human cancer cell lines showed that advanced stages of cancers have lower let-7 levels than early stages (26), which is in good correlation with the upregulation of PGRMC1 in advanced stages of ovarian cancers (3). Further, the expression of let-7i was shown to be negatively correlated with chemotherapy resistance, and a longer survival time of patients with ovarian cancer exposing a high expression of let-7i has been shown for different patient groups (23). These findings are in line with the role of PGRMC1 in chemotherapy resistance and to its induction in cancer cells (7), in which let-7 often is downregulated (22,25,26,28). Let-7 not only targets PGRMC1 but also other genes such as K-ras, HMGA2, and NF2 (23), which are involved in oncogenesis and chemotherapy resistance (6,29,30). This implies that enhanced let-7 expression could suppress tumor progression. *In vitro* studies have shown that the overexpression of let-7b results in reduced cell division of A549 lung cancer cells and HepG2 liver cancer cells (31). In addition, an *in vivo* study has demonstrated that intranasally administered let-7b reduces tumor formation *in vivo* in lungs of mice expressing a G12D activating mutation for the K-ras oncogenes (32). Therefore, it would be an interesting approach to determine whether elevating levels of let-7i and other let-7 isoforms in ovarian tumors would alter tumorigenesis using a xenograft model system. If so, this would provide another scientific basis for the therapeutic use of let-7.

In addition to the let-7 sites, *in silico* analysis predicted a highly conserved binding site for miR-141/200a. This site does not seem to be functional in the PGRMC1 3'-UTR as revealed by luciferase assays in this study. Conveniently, the context score percentile and the PCT values (probability of conserved targeting) predicted by TargetScan are much higher for the let-7/miR-98 sites (context score percentile: 88/76 for the first and the second binding site, respectively; PCT: 0.9/0.406) than for the miR-141/200a site (context score percentile: 20/15 for miR-141 and miR-200a, respectively; PCT: 0.29 for both). Additionally, these results show that the prediction of miRNA binding sites in a certain target by software programs definitely must be confirmed by experimental approaches.

Finally, our *in vitro* studies support previous studies, which show that P4 regulates PGRMC1 mRNA levels (7,13,14,33). However, the effect of P4 on PGRMC1 expression seems to be tissue specific, since a downregulation of PGRMC1 by P4 was detected in some tissues, while an upregulation was shown in others (7). For example, in secretory endometrium of rhesus monkeys P4 downregulates PGRMC1 mRNA levels (13). Further, in estradiol treated rats P4 suppressed PGRMC1 within hypothalamic nuclei (14). In contrast, in ovariectomized mice P4 induced PGRMC1 expression in the uterus (34). As miRNAs are also expressed in a tissue-dependent manner, a possible mechanism of P4-dependent PGRMC1 expression regulation is through miRNAs. In human endometrium and breast cancer cells a regulation of several miRNAs through P4 or estrogen was already shown (35,36). In the present study, a significant 20% decrease of PGRMC1 mRNA levels upon stimulation with 1000 nM P4 in SKOV-3 cells was shown. Further, we showed for the first time that miRNA let-7i is significantly upregulated through P4 in SKOV-3 cells. This may in part explain the decrease in PGRMC1 mRNA levels after stimulation of SKOV-3 cells with P4. A P4 concentration of 1000 nM is physiological in the ovary, in which even higher concentrations of P4 are present (37). The smaller effects on the PGRMC1 mRNA levels compared to effects on the let-7i levels (20% for PGRMC1 to 40% for let-7i) can be explained by the fact that

let-7i also targets many other mRNAs. Further, it should be considered that several other mechanisms may be involved in the P4 dependent and P4 independent regulation of PGRMC1. This may include other miRNAs, a regulation of PGRMC1 expression via the promoter or other regulatory mechanisms in the 3'-UTR. However, the regulatory effects of P4 on let-7i levels may be the cause for the tissue-specific effects of P4 seen on PGRMC1, since the expression level of miRNAs varies among tissues. The regulatory mechanisms by which P4 influences let-7i levels are not yet known and further studies are needed to address this issue.

In summary, PGRMC1 expression in ovarian cancer cells is regulated by a complex mechanism that involves the suppressive actions of let-7/miR-98. The high expression of PGRMC1 in ovarian and other cancers is therefore, at least in part, mediated by the downregulation of let-7. The decrease of PGRMC1 mRNA levels in SKOV-3 cells treated with P4 may in part be mediated by let-7i, which is upregulated through P4. Hormone-dependent tumors may be beneficially treated by steroid hormones including P4 (38). Therefore, P4s stimulating effect on let-7i would be in line with the earlier assumption of let-7i being an antiproliferative agent. Since let-7 plays an important role in the suppression of several genes involved in cancer progression including PGRMC1, this miRNA represents a promising target in the improvement of cancer therapies.

References

1. Jemal A, Siegel R, Ward E, Murray T, Xu J and Thun MJ: Cancer statistics, 2007. CA Cancer J Clin 57: 43-66, 2007.
2. Agarwal R and Kaye SB: Ovarian cancer: strategies for overcoming resistance to chemotherapy. Nat Rev Cancer 3: 502-516, 2003.
3. Peluso JJ, Liu X, Saunders MM, Claffey KP and Phoenix K: Regulation of ovarian cancer cell viability and sensitivity to cisplatin by progesterone receptor membrane component-1. J Clin Endocrinol Metab 93: 1592-1599, 2008.
4. Crudden G, Loesel R and Craven RJ: Overexpression of the cytochrome p450 activator hpr6 (heme-1 domain protein/human progesterone receptor) in tumors. Tumour Biol 26: 142-146, 2005.
5. Ahmed IS, Rohe HJ, Twist KE, Mattingly MN and Craven RJ: Progesterone receptor membrane component 1 (Pgrmc1): a heme-1 domain protein that promotes tumorigenesis and is inhibited by a small molecule. J Pharmacol Exp Ther 333: 564-573, 2010.
6. Peluso JJ, Gawkowska A, Liu X, Shioda T and Pru JK: Progesterone receptor membrane component-1 regulates the development and Cisplatin sensitivity of human ovarian tumors in athymic nude mice. Endocrinology 150: 4846-4854, 2009.
7. Rohe HJ, Ahmed IS, Twist KE and Craven RJ: PGRMC1 (progesterone receptor membrane component 1): a targetable protein with multiple functions in steroid signaling, P450 activation and drug binding. Pharmacol Ther 121: 14-19, 2009.
8. Crudden G, Chitti RE and Craven RJ: Hpr6 (heme-1 domain protein) regulates the susceptibility of cancer cells to chemotherapeutic drugs. J Pharmacol Exp Ther 316: 448-455, 2006.
9. Mallory JC, Crudden G, Oliva A, Saunders C, Stromberg A and Craven RJ: A novel group of genes regulates susceptibility to antineoplastic drugs in highly tumorigenic breast cancer cells. Mol Pharmacol 68: 1747-1756, 2005.
10. Franco S, Canela A, Klatt P and Blasco MA: Effectors of mammalian telomere dysfunction: a comparative transcriptome analysis using mouse models. Carcinogenesis 26: 1613-1626, 2005.
11. Griffiths-Jones S: miRBase: the microRNA sequence database. Methods Mol Biol 342: 129-138, 2006.
12. Metias SM, Lianidou E and Yousef GM: MicroRNAs in clinical oncology: at the crossroads between promises and problems. J Clin Pathol 62: 771-776, 2009.
13. Ace CI and Okulicz WC: Microarray profiling of progesterone-regulated endometrial genes during the rhesus monkey secretory phase. Reprod Biol Endocrinol 2: 54, 2004.
14. Krebs CJ, Jarvis ED, Chan J, Lydon JP, Ogawa S and Pfaff DW: A membrane-associated progesterone-binding protein, 25-Dx, is regulated by progesterone in brain regions involved in female reproductive behaviors. Proc Natl Acad Sci USA 97: 12816-12821, 2000.
15. Lewis BP, Burge CB and Bartel DP: Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell 120: 15-20, 2005.
16. Grimson A, Farh KK, Johnston WK, Garrett-Engele P, Lim LP and Bartel DP: MicroRNA targeting specificity in mammals: determinants beyond seed pairing. Mol Cell 27: 91-105, 2007.
17. Friedman RC, Farh KK, Burge CB and Bartel DP: Most mammalian mRNAs are conserved targets of microRNAs. Genome Res 19: 92-105, 2009.
18. Betel D, Wilson M, Gabow A, Marks DS and Sander C: The microRNA.org resource: targets and expression. Nucleic Acids Res 36: D149-D153, 2008.
19. John B, Enright AJ, Aravin A, Tuschl T, Sander C and Marks DS: Human MicroRNA targets. PLoS Biol 2: e363, 2004.
20. Wang X: miRDB: a microRNA target prediction and functional annotation database with a wiki interface. RNA 14: 1012-1017, 2008.
21. Wang X and El Naqa IM: Prediction of both conserved and nonconserved microRNA targets in animals. Bioinformatics 24: 325-332, 2008.
22. Dahiya N, Sherman-Baust CA, Wang TL, *et al*: MicroRNA expression and identification of putative miRNA targets in ovarian cancer. PLoS One 3: e2436, 2008.
23. Yang N, Kaur S, Volinia S, *et al*: MicroRNA microarray identifies Let-7i as a novel biomarker and therapeutic target in human epithelial ovarian cancer. Cancer Res 68: 10307-10314, 2008.
24. Iorio MV, Ferracin M, Liu CG, *et al*: MicroRNA gene expression deregulation in human breast cancer. Cancer Res 65: 7065-7070, 2005.
25. Iorio MV, Visone R, Di Leva G, *et al*: MicroRNA signatures in human ovarian cancer. Cancer Res 67: 8699-8707, 2007.
26. Shell S, Park SM, Radjabi AR, *et al*: Let-7 expression defines two differentiation stages of cancer. Proc Natl Acad Sci USA 104: 11400-11405, 2007.
27. Dong Q, Meng P, Wang T, *et al*: MicroRNA let-7a inhibits proliferation of human prostate cancer cells in vitro and in vivo by targeting E2F2 and CCND2. PLoS One 5: e10147, 2010.
28. Nam EJ, Yoon H, Kim SW, *et al*: MicroRNA expression profiles in serous ovarian carcinoma. Clin Cancer Res 14: 2690-2695, 2008.
29. Johnson SM, Grosshans H, Shingara J, *et al*: RAS is regulated by the let-7 microRNA family. Cell 120: 635-647, 2005.
30. Lee YS and Dutta A: The tumor suppressor microRNA let-7 represses the HMGA2 oncogene. Genes Dev 21: 1025-1030, 2007.
31. Johnson CD, Esquela-Kerscher A, Stefani G, *et al*: The let-7 microRNA represses cell proliferation pathways in human cells. Cancer Res 67: 7713-7722, 2007.
32. Esquela-Kerscher A, Trang P, Wiggins JF, *et al*: The let-7 microRNA reduces tumor growth in mouse models of lung cancer. Cell Cycle 7: 759-764, 2008.
33. Losel RM, Besong D, Peluso JJ and Wehling M: Progesterone receptor membrane component 1 - many tasks for a versatile protein. Steroids 73: 929-934, 2008.
34. Zhang L, Kanda Y, Roberts DJ, *et al*: Expression of progesterone receptor membrane component 1 and its partner serpine 1 mRNA binding protein in uterine and placental tissues of the mouse and human. Mol Cell Endocrinol 287: 81-89, 2008.
35. Kuokkanen S, Chen B, Ojalvo L, Benard L, Santoro N and Pollard JW: Genomic profiling of microRNAs and messenger RNAs reveals hormonal regulation in microRNA expression in human endometrium. Biol Reprod 82: 791-801, 2010.
36. Bhat-Nakshatri P, Wang G, Collins NR, *et al*: Estradiol-regulated microRNAs control estradiol response in breast cancer cells. Nucleic Acids Res 37: 4850-4861, 2009.
37. Keith Bechtel M and Bonavida B: Inhibitory effects of 17beta-estradiol and progesterone on ovarian carcinoma cell proliferation: a potential role for inducible nitric oxide synthase. Gynecol Oncol 82: 127-138, 2001.
38. Markman M: Hormonal therapy of endometrial cancer. Eur J Cancer 41: 673-675, 2005.