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ORIGINAL ARTICLE

Steroidal regulation of uterine miRNAs is associated with modulation of the miRNA biogenesis components Exportin-5 and Dicer1

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Abstract MicroRNAs (miRNAs) are small, non-coding RNA molecules which post-transcriptionally regulate gene expression. We have previously demonstrated that within the uterus, miRNA expression is under steroidal control and that disruption of *Dicer1*, the enzyme which generates mature miRNAs, leads to abnormalities in the development and function of the female reproductive tract. Despite the apparent importance of miRNAs and the enzymes which lead to their generation, little to no information exists on the mechanisms which regulate the expression of this system in the female reproductive tract. The objective of the current study was to examine steroidal regulation of the miRNAs biogenesis enzymes, *Drosha*, *Dgcr8*, *Exportin-5* and *Dicer1* in the mouse uterus. The results of this study indicate that estrogen and progesterone significantly increased *Exportin-5* mRNA expression while only progesterone increased *Dicer1* expression. We conclude from these studies that the miRNA biogenesis components *Drosha*, *Dgcr8*, *Exportin-5* and *Dicer1* are expressed in the mouse uterus and that *Exportin-5* and *Dicer1* appear to be the major steroid regulated components in the miRNA biogenesis pathway. These observations suggest that in addition to steroids modulating miRNA expression at the level of transcription, they may also influence miRNA expression by regulating the expression of the miRNA biogenesis components necessary for their processing to the mature cytoplasmic form.

Keywords Uterus · MicroRNA · Dicer · Exportin-5 · Estrogen · Progesterone

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Introduction

Within the uterus, the female sex steroids estrogen and progesterone play pivotal roles in the establishment of a suitable environment for embryo implantation and pregnancy. More specifically, these steroids regulate a multitude of cellular processes that include cell proliferation cell differentiation, regulation of vascular permeability, angiogenesis, and adenogenesis all of which are essential for proper development and function of the adult uterus [1–7]. In order to bring about these changes, estrogen and progesterone must appropriately modulate the expression of a variety of factors which includes growth factors, cytokines, extracellular matrix proteins, and adhesion molecules [1–7]. In recent years, microRNAs (miRNAs) have been identified as a novel class of small, non-coding RNAs which also regulate gene expression by affecting mRNA translation and/or stability [8, 9].

miRNAs are transcribed from specific genes which reside between protein-coding genes, within introns of genes, within exons or which make up the entire intron [10, 11]. miRNA biogenesis begins with the transcription of primary RNA transcript (pri-miRNA) which then is processed to a 70–100 nucleotide precursor miRNA (pre-miRNA) by a protein complex composed of Drosha (a class 2 RNAse III-type enzyme), DGCR8 (a RNA-binding protein also known as DiGeorge syndrome critical region 8), and p68/72 DEAD-box RNA helicase [12].

Pre-miRNAs are then exported from the nucleus to the cytoplasm through exportin-5 [13] where they are subsequently processed to the mature 19–23 nucleotide miRNA by Dicer [14]. Mature miRNAs form a RNA-induced silencing complex (miRISC) along with Dicer, TAR RNA-binding protein, and Argonaut proteins [15]. The miRISC then binds target mRNA and regulates their translation by either cleaving and degrading the target transcript, deadenylating and degrading the target transcript, inhibiting mRNA translation initiation, inhibiting mRNA translation elongation, or mRNA translational enhancement [8, 9].

MicroRNAs (through tissue specific knockdown experiments) have been shown to play a role in the normal development of the lung [16], limbs [17], skeletal muscle [18], female germline [19], embryo [20], oviduct [21–23], and uterus [22, 23]. Recently, post-transcriptional regulation and miRNAs have been proposed to play a role in embryo implantation [24, 25] as well as in the human endometrium [26, 27] and in the pathophysiology of the female disease endometriosis [26, 28–30]. Collectively, these studies suggest that miRNAs play a pivotal role in the molecular regulation of multiple organ systems, including reproductive function and specifically uterine implantation and uterine pathophysiological conditions. Unfortunately, beyond these limited studies there is no information on the regulation of key miRNA biogenesis components in vivo. The objectives of the current study were to examine steroidal regulation of uterine *Drosha*, *Dgcr8*, *Exportin-5* and *Dicer1* expression and to demonstrate that regulation of these miRNA biogenesis components occurs through steroid-receptor mediated pathways.

Results

Estrogen regulation of *Dicer1*, *Drosha*, *Dgcr8*, and *Exportin-5* transcript was assessed by qRT-PCR (Fig. 1). Estrogen had a minimal affect on the expression of *Dicer1* and *Drosha* as their levels of expression did not significantly change in response to estrogen treatment. In contrast, estrogen induced a modest (1.6-fold) but significant increase in *Dgcr8* expression at 8 h post steroid administration with levels decreasing to basal values at 24 h. Of the four miRNA biogenesis pathway components assessed, *Exportin-5* (*Xpo5*) was the most highly regulated by estrogen. Estrogen significantly increased *Exportin-5* expression at both 4 and 8 h post steroid administration with levels returning to 0 h values by 24 h post estrogen treatment.

Progesterone also regulated the expression of specific components of the miRNA biogenesis pathway (Fig. 2). *Dicer1* expression was significantly increased by progesterone at 8 h post steroid administration, while *Drosha* and *Dgcr8* expression was not affected by progesterone treatment. *Exportin-5* expression was also markedly regulated by progesterone. Progesterone significantly increased *Exportin-5* expression at 8 and 24 h post steroid administration.

Administration of estrogen and progesterone in combination resulted in a significant increase in all four

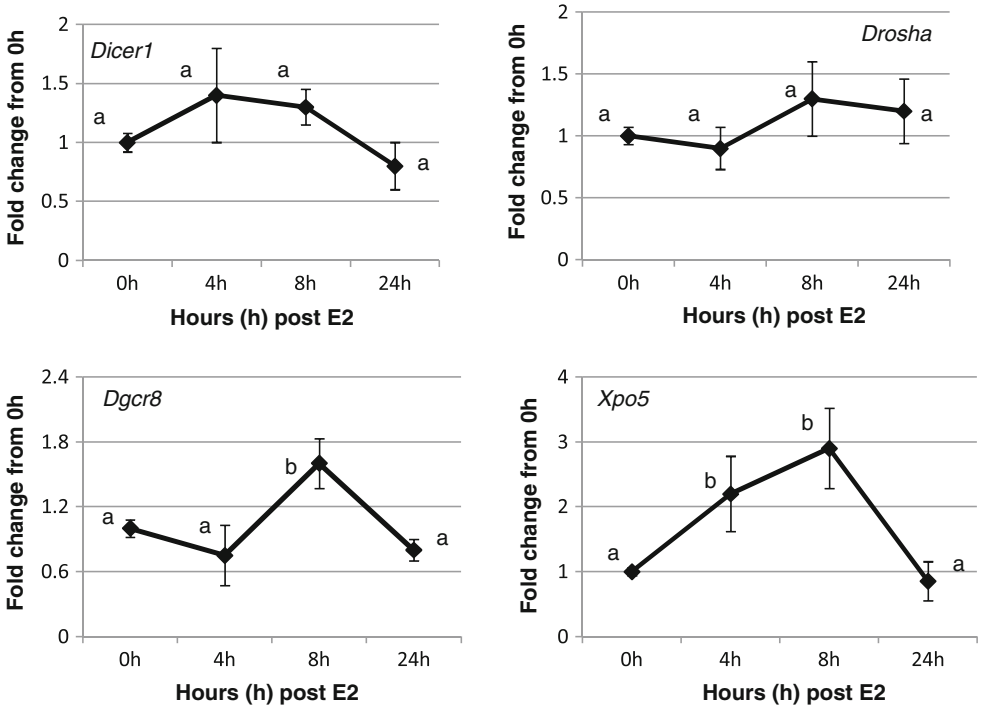

Fig. 1 Estrogen regulation of uterine miRNA biogenesis components. *Dicer1*, *Drosha*, *Dgcr8* and *Exportin-5 (Xpo5)* levels were quantitated as described in "Materials and methods" by qRT-PCR and normalized to 18S rRNA levels. Data are displayed as the mean ± SD and are representative of four independent samples per time point per treatment ($N = 4$). Data was analyzed by one-way ANOVA for comparison across time points and expressed as fold-change from 0 h values. When an F test indicated statistical significance, post hoc analysis was made using the Tukey HSD procedure. Significance was set at $P < 0.05$ for all comparisons. Different *letters* indicate statistical significance as determined by one-way ANOVA

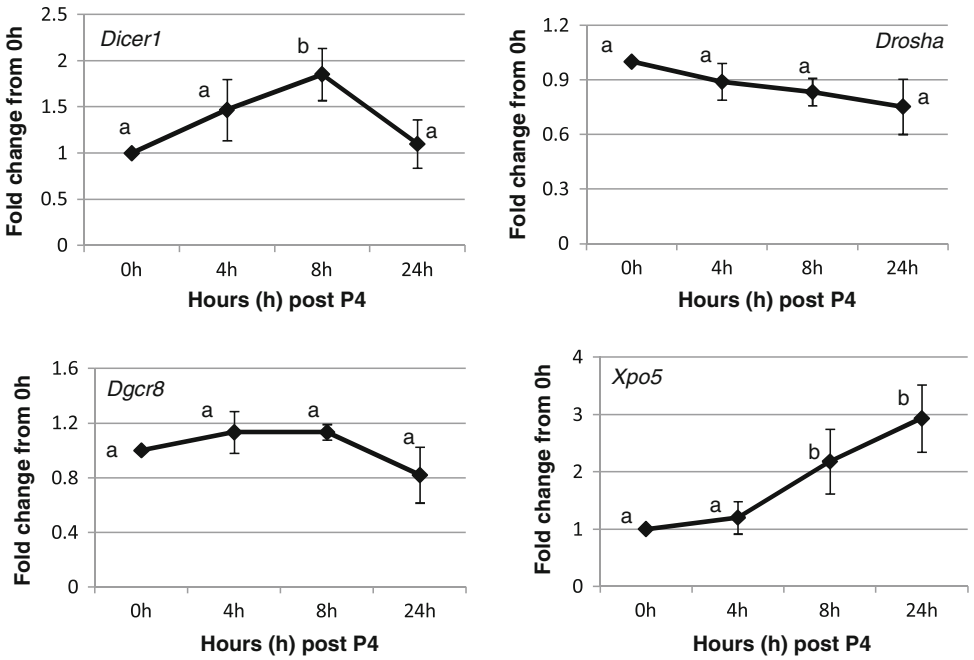

Fig. 2 Progesterone regulation of uterine miRNA biogenesis components. *Dicer1 (Dicer), Drosha, Dgcr8* and *Exportin-5* (*Xpo5*) levels were quantitated as described in "Materials and methods" by qRT–PCR and normalized to 18S rRNA levels. Data are displayed as the mean ± SD and are representative of four independent samples per time point per treatment ($N = 4$). Data was analyzed by one-way ANOVA for comparison across time points and expressed as fold-change from 0 h values. When an F test indicated statistical significance, post hoc analysis was made using the Tukey HSD procedure. Significance was set at $P < 0.05$ for all comparisons. Different *letters* indicate statistical significance as determined by one-way ANOVA

components of the miRNA biogenesis pathway (Fig. 3). *Dicer1* expression was significantly increased at both 4 and 8 h post steroid treatment while *Drosha* was increased at 8 h post treatment. *Dgcr8* expression was also significantly increased at 8 h post steroid treatment similar to estrogen alone (see Fig. 1). Similar to regulation by either estrogen or progesterone alone, concurrent administration of both steroids significantly increased the expression of *Exportin-5*. Estrogen and progesterone together induced a 2-fold increase in *Exportin-5* expression compared to either steroid alone (6-fold maximal increase versus 3-fold increase; see Figs. 1, 2) and this increase was maintained from 4 h post steroid administration through 24 h post steroid administration.

In order to determine if estrogen regulation of *Exportin-5* and *Dgcr8* was mediated through the cognate estrogen nuclear receptor pathway, ICI 182,780 antagonistic studies were employed. As displayed in Figs. 4 and 5, respectively, the estrogen-induced increase in both *Exportin-5* (*Xpo5*; Fig. 4a) and *Dgcr8* (Fig. 5) was significantly reduced by pre-treatment with ICI followed by estrogen challenge. ICI alone had no significant effect on either *Exportin-5* or *Dgcr8* transcript expression. Assessment of protein expression for Exportin-5 by Western analysis revealed that estrogen regulation of protein levels was similar to that of Exportin-5 transcript expression (Fig. 4b). Specifically, estrogen significantly ($P < 0.05$) induced Exportin-5 expression over vehicle controls approximately 2.2-fold and this induction by estrogen was blocked by pre-treatment with ICI. ICI alone had no effect on Exportin-5 protein expression.

In order to determine if the progesterone induced expression of *Dicer1* and *Exportin-5* were mediated through the cognate progesterone nuclear receptor pathway, RU-486 progesterone receptor antagonist studies were conducted (Figs. 6, 7). Pre-treatment with RU-486 blocked the progesterone induction of both *Dicer1* (Fig. 6) and *Exportin-5* (Fig. 7) while RU-486 alone had no significant affect on expression of either miRNA biogenesis component. Assessment of protein expression for Dicer1 and Exportin-5 by Western analysis revealed that progesterone regulation of their protein levels was similar to that for their transcript expression. Specifically, progesterone induced a modest but significant increase (approximately 1.5-fold; $P < 0.05$) in Dicer1 expression over vehicle controls and this progesterone induction was blocked by pre-treatment with RU-486 (RU; Fig. 6). RU-486 alone had no effect on Dicer1 protein expression (Fig. 6). Similarly, progesterone induced a significant ($P < 0.05$; 1.8-fold) increase in Exportin-5 protein expression which could be inhibited by pre-treatment with RU-486 (Fig. 7). Similar to Dicer1 protein, RU-486 alone had no significant effect on Exportin-5 protein expression.

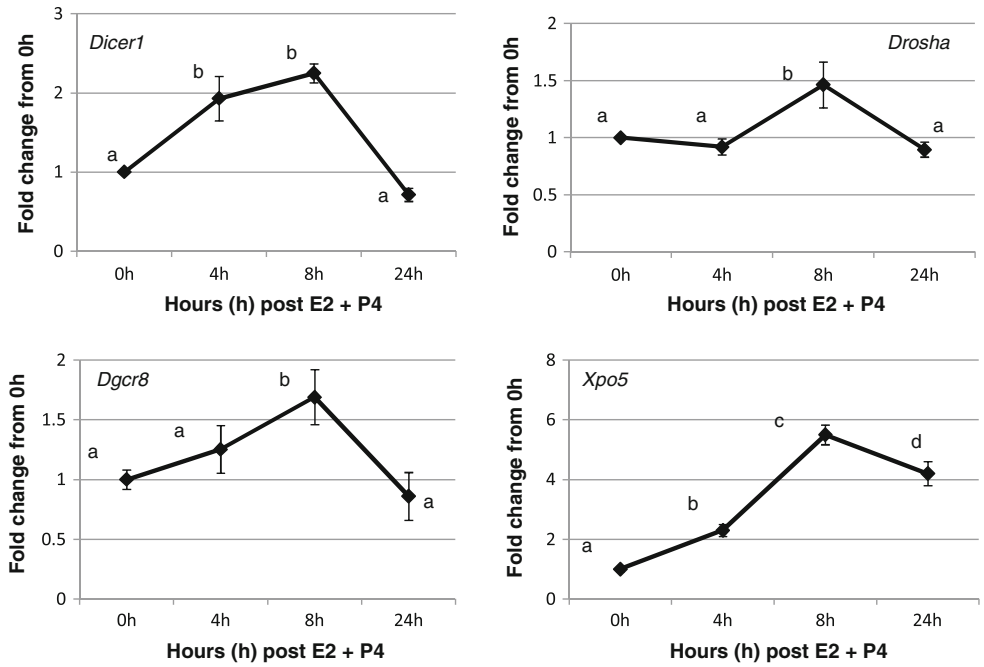

Fig. 3 Combined effect of estrogen plus progesterone on the regulation of uterine miRNA biogenesis components. *Dicer1* (*Dicer*), *Drosha*, *Dgcr8* and *Exportin-5* (*Xpo5*) levels were quantitated as described in "Materials and methods" by qRT–PCR and normalized to 18S rRNA levels. Data are displayed as the mean ± SD and are representative of four independent samples per time point per treatment ($N = 4$). Data was analyzed by one-way ANOVA for comparison across time points and expressed as fold-change from 0 h values. When an F test indicated statistical significance, post hoc analysis was made using the Tukey HSD procedure. Significance was set at $P < 0.05$ for all comparisons. Different *letters* indicate statistical significance as determined by one-way ANOVA

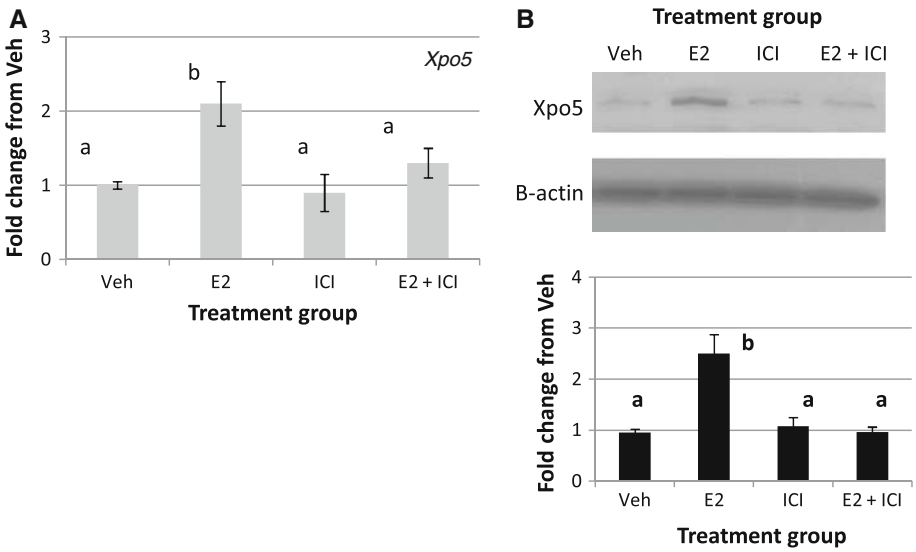

Fig. 4 Uterine *Exportin-5* expression in response to estrogen (E2) administration is regulated via cognate estrogen receptor pathways. Mice were treated with estrogen receptor antagonist ICI 182,780 (ICI) 30 min before steroidal treatments and killed at 8 h post steroidal administration. *Exportin-5* mRNA expression was analyzed as by qRT–PCR (**a**) while protein expression was analyzed by Western analysis (**b**) as described in "Materials and methods". Transcript and normalized protein data are displayed as the mean ± SD with three independent observations ($N = 3$) per treatment. Data was analyzed by one-way ANOVA for comparison across treatment groups and expressed as fold-change from vehicle (veh) values. When an F test indicated statistical significance, post hoc analysis was made using the Tukey HSD procedure. Significance was set at $P < 0.05$ for all comparisons. Different *letters* indicate statistical significance as determined by one-way ANOVA

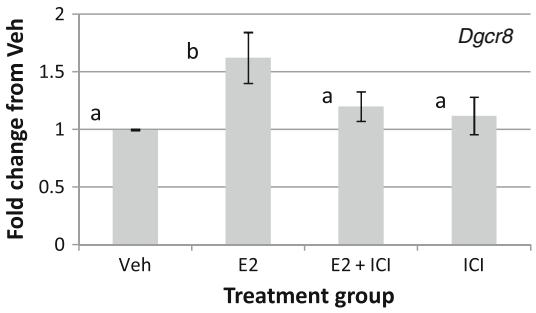

Fig. 5 Uterine *Dgcr8* expression in response to estrogen (E2) administration is regulated via cognate estrogen receptor pathways. Mice were treated with estrogen receptor antagonist ICI 182,780 (ICI) 30 min before steroidal treatments and killed at 8 h post steroidal administration. *Dgcr8* mRNA expression was analyzed as described in "Materials and methods" by qRT-PCR analysis. Data are displayed as the mean ± SD with three independent observations ($N = 3$) per treatment and expressed as fold-change from vehicle (veh) values. Data was analyzed by one-way ANOVA for comparison across treatment groups. When an F test indicated statistical significance, post hoc analysis was made using the Tukey HSD procedure. Significance was set at $P < 0.05$ for all comparisons. Different *letters* indicate statistical significance as determined by one-way ANOVA

In order to verify that these steroid-induced changes in Exportin-5 and Dicer1 were associated with changes in liberation of mature miRNAs, we assessed *miR-451* expression. We selected *miR-451* as we had previously demonstrated that *miR-451* is induced in uterine tissue in response to estrogen treatment [31]. *miR-451* expression was assessed by qRT-PCR and results are summarized in Fig. 8. *miR-451* expression was significantly increased by estrogen alone (peaking at 4 h post-estrogen administration), progesterone alone (peaking at 8 h post-progesterone administration), and by both steroids together (peaking at 8 h post steroid administration). These patterns of induction are consistent with that of the miRNA biogenesis components and their induction by these steroids (see Figs. 1, 2, 3). Steroidal modulation of *miR-451* expression through the estrogen receptor and progesterone receptor pathways was confirmed by antagonist studies. Pre-treatment with ICI blocked the estrogen induction of *miR-451* while pre-treatment with RU-486 blocked the progesterone-induced expression of *miR-451* (Fig. 9). Collectively, these studies demonstrate that the liberation of mature *miR-451* expression coincides with the induction of the miRNA biogenesis components and that this induction occurs through the cognate estrogen and progesterone receptor pathways.

Discussion

miRNAs are proposed to be involved in diverse developmental and pathological processes. It is predicted that 1–5% of genes encode for miRNAs, and they regulate the expression of as many as 30% of mRNAs [32, 33]. Not surprising, miRNAs have been proposed to regulate stability of mRNAs within the uterus that play a role in normal physiological processes which include cell

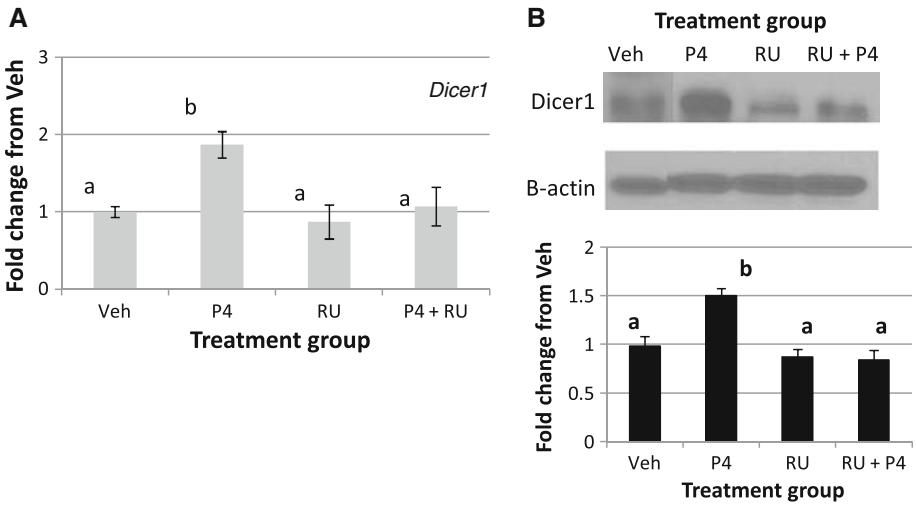

Fig. 6 Uterine *Dicer1* expression in response to progesterone (P4) administration is regulated via cognate progesterone receptor pathways. Mice were treated with progesterone receptor antagonist RU486 (RU) 30 min before steroidal treatments and killed at 8 h post steroidal administration. *Dicer1* mRNA expression was analyzed as by qRT-PCR (**a**) while protein expression was analyzed by Western analysis (**b**) as described in "Materials and methods". Data are displayed as the mean ± SD with three independent observations ($N = 3$) per treatment and expressed as fold-change from vehicle (veh) values. Data was analyzed by one-way ANOVA for comparison across treatment groups. When an F test indicated statistical significance, post hoc analysis was made using the Tukey HSD procedure. Significance was set at $P < 0.05$ for all comparisons. Different *letters* indicate statistical significance as determined by one-way ANOVA

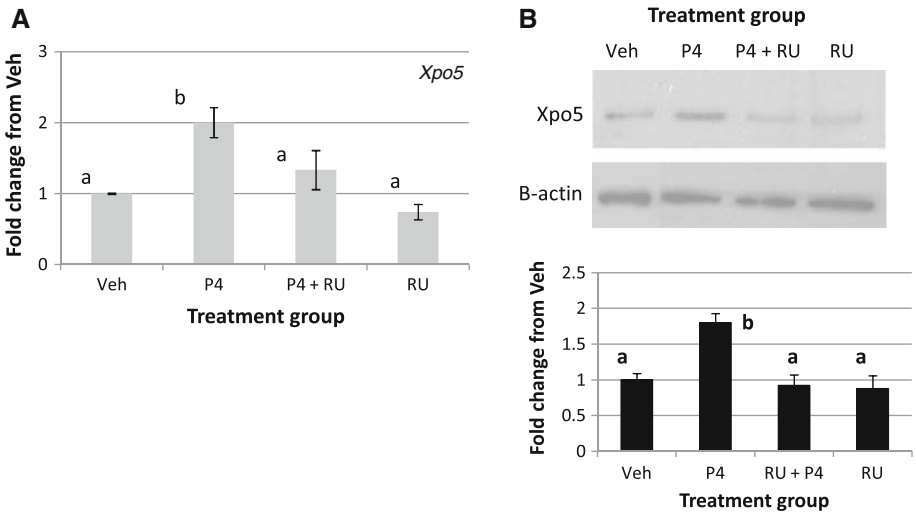

Fig. 7 Uterine *Exportin-5* expression in response to progesterone (P4) administration is regulated via cognate progesterone receptor pathways. Mice were treated with progesterone receptor antagonist RU486 (RU) 30 min before steroidal treatments and killed at 8 h post steroidal administration. *Exportin-5* mRNA expression was analyzed as by qRT–PCR (**a**) while protein expression was analyzed by Western analysis (**b**) as described in "Materials and methods". Data are displayed as the mean ± SD with three independent observations ($N = 3$) per treatment and expressed as fold-change from vehicle (veh) values. Data was analyzed by one-way ANOVA for comparison across treatment groups. When an F test indicated statistical significance, post hoc analysis was made using the Tukey HSD procedure. Significance was set at $P < 0.05$ for all comparisons. Different *letters* indicate statistical significance as determined by one-way ANOVA

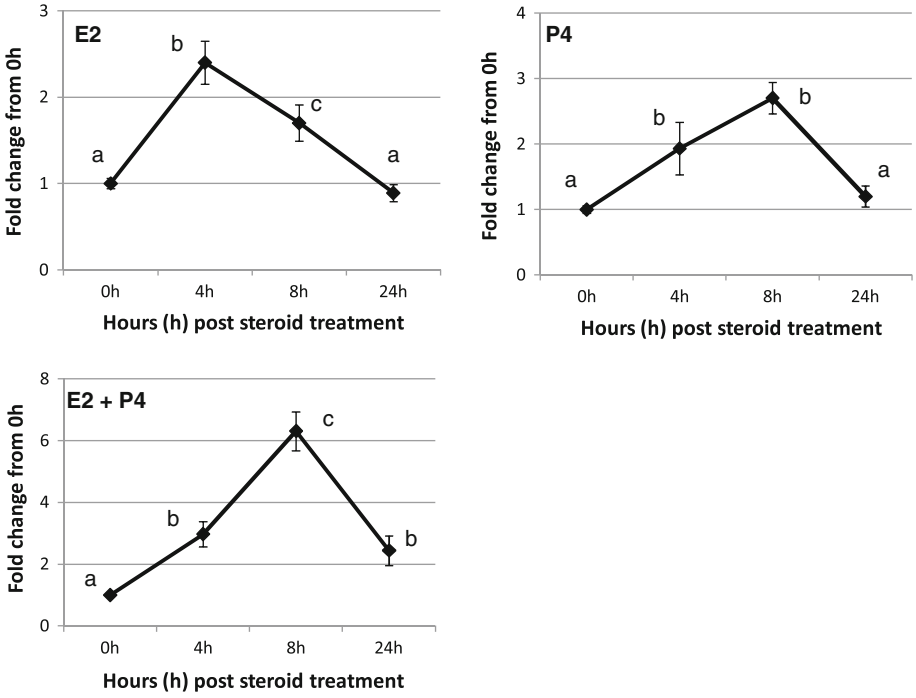

Fig. 8 Uterine *miR-451* expression in response to estrogen and progesterone administration. Mice were treated with estrogen (E2), progesterone (P4) or both steroids (E2 + P4) as described in the text. *miR-451* expression was evaluated by qRT-PCR and data are displayed as the mean ± SD with four independent observations per time point per treatment ($N = 4$) and expressed as fold-change from 0 h control values. Data was analyzed by one-way ANOVA for comparison across treatment groups. When an F test indicated statistical significance, post hoc analysis was made using the Tukey HSD procedure. Significance was set at $P < 0.05$ for all comparisons. Different *letters* indicate statistical significance as determined by one-way ANOVA

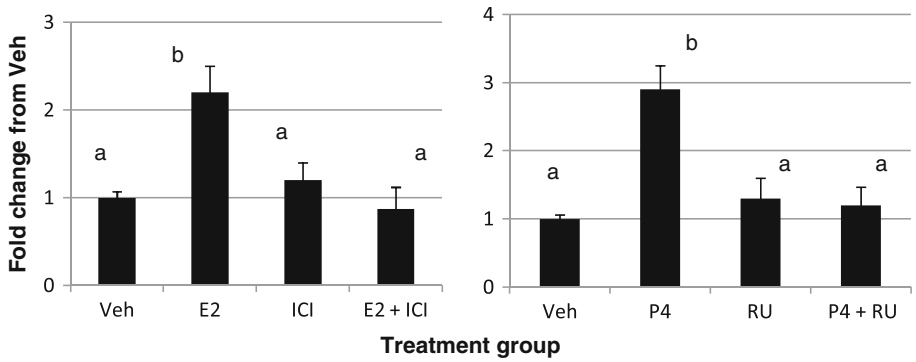

Fig. 9 Estrogen and progesterone regulation of uterine *miR-451* is mediated via their cognate receptor pathways. **a**. Mice were pre-treated with either ICI 182,780 (ICI) or vehicle and then challenged with E2 30 min later. **b**. Mice were pre-treated with either RU-486 (RU) or vehicle and then challenged with P4 30 min later. All mice were killed at 8 h after respective steroid treatment and *miR-451* expression was evaluated by qRT–PCR and normalized to U6 levels. Data are displayed as the mean ± SD with three independent observations ($N = 3$) per treatment and expressed as fold-change from vehicle (veh) values. Data was analyzed by one-way ANOVA for comparison across treatment groups. When an F test indicated statistical significance, post hoc analysis was made using the Tukey HSD procedure. Significance was set at $P < 0.05$ for all comparisons. Different *letters* indicate statistical significance as determined by one-way ANOVA

proliferation, cell differentiation, apoptosis, angiogenesis, and inflammation. In contrast, alterations in the normal pattern of expression of uterine miRNAs have been postulated to play a role in endometrial pathologies such as endometriosis [26, 28–30] as well as in human endometrial cancer [26, 34–36] and animal models of endometrial carcinoma [37].

Expression profiles for uterine miRNAs in normal and pathological conditions provide the initial basis for determining which miRNAs may actively impact the uterine genome. Within the uterus, miRNAs have been shown to be regulated by the ovarian steroids estrogen and progesterone. Previous study from our lab [31] demonstrated that estrogen both increases and decreases the expression of specific uterine miRNAs in vivo. The ability of estrogen to modulate uterine miRNAs has also been examined in vitro using isolated endometrial epithelial and stromal cell cultures. Pan and colleagues [28] have demonstrated that estrogen induces specific miRNAs as well as represses the expression of others and that this regulation is mediated through the estrogen receptor-dependent pathway. In the same study, progesterone was also shown to modulate endometrial miRNA expression through its cognate receptor pathway. While these studies demonstrate that both estrogen and progesterone modulate the expression of specific miRNAs and that this regulation occurs through their respective receptor-mediated pathways, the precise mechanism by which these steroids modulate miRNA expression remains poorly understood.

Mature miRNAs are first generated through transcription from the genome in the form of primary transcripts (pri-miRNAs). Pri-miRNAs are then processed within the nucleus by DGCR8 and Drosha, exported from the nucleus by Exportin-5 and processed in the cytoplasm by Dicer1 to liberate the mature miRNAs which are capable of regulating mRNA transcript stability. It is postulated that estrogen and progesterone induce transcription of pri-miRNAs through steroid receptor–promoter interactions but little to no data on this possibility has been presented.

Estrogen response elements have been identified in specific miRNA [38] and this group has proposed that direct binding of the estrogen receptor to the regulatory regions of the miRNAs lead to their estrogen-induced expression. Additional possibilities proposed by Bhat-Nakshatr and colleagues include estrogen-inducible expression of mRNA-encoding genes that harbor miRNA genes within their intronic regions and the possibility that estrogen regulates the expression of transcription factors which control the expression of miRNAs. Collectively, these mechanisms would all lead to an increase in miRNA expression through an increase in the levels/expression of pri-miRNAs. The findings from the current study add an additional mechanism by which steroids may increase the levels of mature miRNAs. We demonstrate that concurrent exposure to estrogen and progesterone increase expression of *Drosha*, *Dgcr8*, *Exportin-5*, and *Dicer1* with the later two being the most significantly regulated components of the miRNA biogenesis pathway. Induction of *Exportin-5* may provide a mechanism by which steroids can increase the shuffling or export of pre-miRNAs to the cytoplasm. Once in the cytoplasm, the exported pre-miRNA population can be processed to the mature miRNA through the increased levels of *Dicer1* and then in turn modulate target mRNA stability.

In summary, the current study demonstrates for the first time that the expression of the miRNA biogenesis components *Drosha*, *Dgcr8*, *Exportin-5,* and *Dicer1* is significantly up-regulated by ovarian steroids in mouse uterine tissue and that this modulation occurs through their

respective cognate receptor pathways. Steroidal modulation of these enzymes may provide an additional level of regulation of miRNA generation which in turn could impact uterine gene expression and uterine physiology.

Materials and methods

Animals and treatments

All animal studies were approved by the University of Kansas Medical Center Institutional Animal Care and Use Committee (IACUC). Mature female mice (CD1; Harlan Laboratories, Inc., Indianapolis, IN, USA), 2–4 months of age, were ovariectomized, rested 2 weeks and treated s.c. with either vehicle (0.1 ml sesame oil), estradiol-17β (E_2, 10 μg/kg BW), progesterone (P_4, 100 mg/kg BW) or E_2 + P_4 (previous doses). Mice were then killed at 0 h (no treatment), 4, 8, and 24 h after injection. Mice were killed by cervical dislocation and uteri were removed and trimmed of fat. One-half of each uteri was placed into RNalater (Ambion, Austin, TX, USA) at −20 °C until processed for RNA isolation while the remaining half was snap-frozen in liquid nitrogen until utilized for protein extraction.

A second study was conducted to examine whether steroidal regulation of uterine miRNA biogenesis components was mediated through the estrogen and progesterone nuclear receptors. To do so, mice were injected s.c. with estrogen receptor antagonist ICI-182,780 (ICI; Tocris Cookson Inc., Ellisville, MO; 20 mg/kg dissolved in 100% ethanol and resuspended in sesame oil) or the progesterone receptor antagonist RU486 (Mifepristone; Dr. A. F. Parlow, NIDDK's National Hormone and Pituitary Program, Torrance, CA; 20 mg/kg dissolved in 100% ethanol and resuspended in sesame oil) 30 min before each steroidal treatment. Additional groups consisted of mice receiving only vehicle, steroid alone (previous doses), or receptor antagonist alone. All mice were killed 8 h after steroid or vehicle administration. We selected the 8-h time point as this had been shown in the first study to be the optimal time point for both E_2 and P_4 induction of miRNA biogenesis components as well as a time point for significant increase in *miR-451* expression.

Quantitative real-time RT-PCR for miRNA processing components and miR-451

Quantitative RT-PCR (qRT-PCR) was performed as previously described [39]. Briefly, total RNA was isolated using miRNA easy kits (Qiagen, Valencia, CA, USA) according to recommendations of the manufacturer. Total RNA (250 ng in 5 μl) was reverse transcribed using reverse transcription (RT) kits (Applied Biosystems; Foster City, CA, USA) following the manufacturer's protocol with the following modifications. Briefly, mRNAs were reverse transcribed in a single reaction using 2 μl of each mRNA specific 5 × RT primers. Primers for the miRNA biogenesis components *Drosha*, *DGCR8*, *Exportin-5*, and *Dicer1* were designed using Primer Express 3.0 software and synthesized by Integrated DNA Technology (IDT, Coralville, IA, USA; see Table 1). Resulting material was then used for independent qRT-PCR for each mRNA. RT-PCR was carried out on an Applied Biosystems HT7900 Sequence Detector. In order to account for differences in starting material, human 18S primers and probe reagents were used for miRNA biogenesis components while U6 was used for *miR-451*. Primers for both U6 and miR-451 were purchased from Applied Biosystems. A standard curve was run in each assay, with an arbitrary value assigned to the highest standard and corresponding values to the subsequent dilutions. Each cDNA sample was run in triplicate and the relative abundance of each target divided by the relative abundance of 18S (or U6) to normalize for the starting quantity of cDNA. Each primer set included a minus RT control. The delta–delta CT method was used to calculate the fold-change values among samples.

Table 1 Primer sequences for reverse transcription and qRT-PCR of murine miRNA biogenesis components

Drosha (Rnasen) f: gaagtcaccgtggagctgagta
r: atcattgcatgctgacagacatc
Dgcr8 f: tcaaggtccgccctgtttat
r: gaggcaccaaaaggctcactt
Xpo5 f: gacgcagaacatggaaagaatct
r: tgtcttcatttgttggtacttgtttaca
Dicer1 f: cagctctggaccataacacaattg
r: aggtcgcccctgatctgat

f = forward primer, r = reverse primer

Western analysis

Western analysis was performed as previously described [39]. Briefly, total protein was extracted from frozen uteri using RIPA buffer (20 mM Tris [pH 7.5], 150 mM NaCl, 1% IGEPAL CA-630, 0.5% sodium deoxycholate, 1 mM EDTA, 0.1% SDS) containing a protease/phosphatase inhibitor cocktail (0.1 mg/ml PMSF, 30 μl/ml aprotinin, 5 μg/ml leupeptin, 1 mM sodium orthovanadate; Sigma, St. Louis, MO, USA). Protein concentration in each sample was determined using the *DC* Protein Assay (Bio-Rad Laboratories, Richmond, CA, USA). The same amount of protein (40 μg) was subjected to 4–12% Bis–Tris (Invitrogen, Carlsbad, CA, USA) gel electrophoresis and electroblotted onto nitrocellulose membranes (Bio-Rad

Laboratories, Richmond, CA, USA). Rabbit anti-Dicer1 (1:500; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), rabbit anti-Exportin-5 (1:500; Santa Cruz Biotechnology, Inc.), and goat anti-rabbit secondary antibody (1:5000, Jackson Immunoresearch Laboratories, Inc., Westgrove, PA, USA) were used. Stripping and reprobing for β-actin (Santa Cruz Biotechnology, Inc.) was conducted to normalize protein expression levels. Immunodetection was carried out using an enhanced chemiluminescence (ECL) kit (Amersham Biosciences, Piscataway, NJ, USA). Expression of each protein was normalized to that of β-actin and expressed as a fold-change from vehicle.

Statistical analysis

Unless otherwise indicated, all data were analyzed by one-way ANOVA for comparison across time points. When an F test indicated statistical significance, post-hoc analysis was made using the Tukey HSD procedure. Significance was set at $P < 0.05$ for all comparisons.

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