Original article

Progesterone signaling/miR-200a/zeb2 axis regulates self-renewal of mouse embryonic stem cells

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A R T I C L E   I N F O

Keywords: miR-200a Zeb2 Medroxyprogesterone 17-acetate Progesterone Embryonic stem cells Self-renewal Differentiation

A B S T R A C T

Progesterone is a steroid hormone and plays an important role during pregnancy. But the regulation mechanisms of progesterone-progesterone receptor (P4-PR) signaling during pregnancy remain largely unknown. In this study, we used medroxyprogesterone 17-acetate (MPA) which is a synthetic variant of progesterone and has 20–30 times the activity of progesterone to find that at the same physiological concentration as progesterone during early pregnancy MPA had no significant influence on ES cells self-renewal. But with the increasing of dosage, MPA can inhibit the self-renewal capacity of mouse embryonic stem cells (ES cells) and promote differentiation untimely. We further determined that MPA can influence the miR-200a/zeb2 pathway by down regulating the level of miR-200a. miR-200a significantly higher expressed in ES cells to down-regulate the expression of zeb2 to inhibit the self-renewal and promote differentiation of ES cells. Then we found that the function of MPA can be rescued by over-expression of miR-200a or down-regulation of zeb2. Our findings revealed the progesterone signaling/miR-200a/zeb2 axis regulating the progesterone signaling to insure the balance of self-renewal and differentiation of ES cells. Our study also provided new insight into the dosage of progesterone and its derivant in the hormone replacement therapy for pregnant woman.

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1. Introduction

Progesterone also known as P4 (pregn-4-ene-3, 20-dione) is a C-21 steroid hormone. Progesterone plays an important role of regulating female menstrual cycle, supporting gestation and embryogenesis [1–3]. Progesterone binds to the progesterone receptor to deliver the signaling [4]. Human fetuses at 11–21 weeks of gestation expressed PR in all tissues, such as heart, liver, kidney, spleen, pancreas, intestine, thyroid and adrenal, and so on [5]. Progesterone and its derivant are widely used in hormone replacement therapy to supply the insufficient of progesterone for the pregnant woman who receives in vitro fertilization and embryo transfer [6] or suffer from threatened abortion, habitual abortion or recurrent miscarriage [7–9]. It has been reported that dydrogesteronein can reduce the risk of spontaneous pregnancy loss in women with recurrent abortion during early pregnancy [10]. Medroxyprogesterone acetate can also protect the pregnant woman from the abortion [11]. But some evidences showed that MPA has embryoxicity, which can lead to the deformity of fetus. Previous study showed that exposed to progesterone increased the risk of congenital malformations. A significantly elevated rate of malformations was found in babies exposed to either medroxyprogesterone or progesterone during pregnancy [12]. A study in rabbits showed that the progesterone group had a statistically significantly greater anogenital distance (both males and females) [13]. So progesterone and its signaling pathway should be maintained on the suitable level to meticulously to control the differentiation of embryo and development of fetus. But the regulation mechanism of progesterone signaling remains largely unknown.

Data from the NIH clinical center shows that the level of progesterone is gradually up regulated during the whole process of pregnancy. At the early-stage of pregnancy, there is a low level of progesterone. That means low level of progesterone should be maintained at the beginning of fetus development. Embryonic stem cells derived from the inner cell mass of the blastocyst, an early-stage embryo, have been used to research the early development [14]. One of the most important characteristics of ES cells is self-renewal. Self-renewal should be achieved by two important aspects. One is suppression of differentiation by high-level expression of stemness genes, such as Oct4, Sox2, Nanog.
other one is stimulation of proliferation [15]. If there were problems of anyone of these two aspects, ES cells could not maintain self-renewal. In mES cells, self-renewal is maintained by Lif/stat3 signaling pathway. So fetal calf serum (FCS) and cytokine leukemia inhibitory factor (LIF) should be added in mES cells culture medium to maintain the self-renewal [16,17]. Wnt/β-catenin signaling has been reported to be critically involved in the maintenance of pluripotency in mES cells [18–20]. Moreover, study showed that the combined pharmacological inhibition of FGFR-ERK and glycogen synthase kinase 3 (GSK3) signaling pathways promotes long-term self-renewal of ES cells even without LIF and BMP4 [21]. More and more signaling pathways that can maintain mES cells self-renewal have been reported. But the function of progesterone signaling in mES cells remains largely unknown. microRNAs (miRNAs) always plays an important role in regulating ES cells self-renewal [22]. miRNAs bind to the 3′ untranslated regions (3′UTRs) of target mRNA, which results in degradation of mRNAs, or translational repression of the encoded proteins [23]. More than 100 miRNAs are differentially expressed in human embryonic stem cells (hESCs) [24]. Previous report showed that, miR-302, miR-106a, miR-20 and miR-290 cluster miRNAs can regulate the mES cell cycle to maintain the rapid proliferation of ES cells [25]. MicroRNA-145 can regulate Oct4, Sox2, and Kif4 and represses pluripotency in human embryonic Stem Cells [18]. MicroRNA-200a regulates Grib2 and suppresses differentiation of mouse embryonic stem cells into endoderm and mesoderm [26]. More and more regulatory functions of miRNAs were discovered in ES cells. But it remains unclear that which miRNAs may be involved in the direct regulation of signaling pathway of ES cells self-renewal regulation.

In our study, we found that miR-200a was involved in the progesterone signaling in mES cells self-renewal regulation. We used Medroxyprogesterone 17-acetate (MPA) to simulate the progesterone, because it has more powerful activity than progesterone. We found that Low level of progesterone signaling of low dosage of MPA could not influence the self-renewal of mES cells. But with the increasing of dosage, especially the clinical dosage of MPA for curing habitual abortion, MPA inhibited the self-renewal capacity of mouse embryonic stem cells. We found that MPA influenced the miR-200a/zeb2 pathway by down regulating the level of miR-200a, which was significantly higher, expressed in ES cells and up regulating the expression of zeb2. It’s the first time that we have found the function of miR-200a/zeb2 pathway in ES self-renewal and differentiation. We also found that the function of MPA can be rescued by over-expression of miR-200a or down-regulation of zeb2. Our study showed that progesterone signaling/ miR-200a/zeb2 axis regulates the progesterone signaling to balance the self-renewal and differentiation of ES cells.

2. Method and materials

2.1. ES cell culture

Mouse embryonic stem ES cells (mES cells) were cultured on feeder-free, gelatin-coated plates with supplement of Lif (1:10,000; Invitrogen) in DMEM medium with 15% FBS (Gibco), 1% NEAA (Invitrogen), and 1% Glutamine (Invitrogen); 0.18% β-mercaptoethanol (sigma). mES cells were cultured at 37 °C in a humidified 5% CO2 atmosphere.

2.2. Zeb2 over-expression vector

The CDS sequence of zeb2 was amplified by the primer: forward: 5′-GGCACCGGTATGAAAGCAGGGCATCAG-3′; reverse: 5′-GCCAACATTATTCATCGCCATCTCATT-3′, and inserted into FUGW vector EcoR1 and Age1 restriction enzyme sites.

2.3. Construction of luciferase reporter

Figments of the 3′UTR of zeb2 were amplified from the cDNA of mouse embryonic stem cells by PCR, with the following primers:

- at the site of 195: forward: 5′-GGCAGGCTAGCTTTGATG-CAGTGCCCT-3′(SacI); reverse: 5′-GGCTCTAGAGCTAAAGTG-TGTGTTTACCTAGG-3′(XbaI);
- at the site of 764: forward: 5′-GGGACTGCAATGTAACG-GATAGTTAGT-3′(SacI); reverse: 5′-GGCTCTAGAACTGACCTGAGC- TAGCTGGCTAATAA-3′(XbaI).

The product was inserted into the pGL3-cm vector (Promega).

2.4. Construction of luciferase reporter of mutant UTR

Zeb2 3′UTR mutation was generated by deleting the miRNA binding site sequence. We deleted the miRNA seed sequences binding sites to construct the UTR mutant luciferase reporter vector.

2.5. Over-expression of miRNA

Over-expression of miRNA was performed by transfecting the pre-miR-200a (Biolend, China) by lipofectamineRNAiMax (Invitrogen). miRNA control is miRNA-67 (Biolend, China) which is the artificial polyribonucleotide which has no interaction of mRNA 3′UTR.

2.6. Luciferase reporter assays

Among the cells, 1 × 10^3 3T3 cells/well were transfected with 300 ng of the UTR or mutant UTR reporter, 2 ng of Renilla vector, and 25 nM chemically synthesized pre-miR-200a (Biolend, China) with Lipofectamine 2000 (Invitrogen). Lysates were harvested 48 h after transfection, and the reporter activity was measured with the Dual Luciferase Assay (Promega).

2.7. Western blotting analysis

Cells were lysed with 1 × loading lysis buffer that was diluted from 5 × loading lysis buffer (0.5 mol/L Tris–HCl [pH 6.8] 2.5 mL, DTT 0.39 g, SDS 0.5 g, bromophenol blue 0.025 g, glycerine 2.5 mL) [27]. Equal amount of protein was transferred onto the PVDF membrane (BioRad). The membrane was then incubated with primary antibodies against ZEB2 (Santa Cruz), GAPDH (Bioworld).

2.8. Quantitative real-time PCR (qRT-PCR)

The total RNA was isolated by using RNAiso (Takara). cDNA was subsequently reverse-transcribed from mRNA by M-MLV Reverse Transcriptase (Promega) mRNA. PCR included 40-cycles of amplification using the Stratagene Mx3000P system. Expression of target genes (2^−ΔΔCt) was normalized against GAPDH. The qRT-PCR primers:

- Oct4: forward: 5′-TGAAATGTGGTGTTTGGCGTTG-3′; reverse: 5′-GCGACTGCTTGCAGTATCG-3′;
- Sox2: forward: 5′-CGGACGTGAGTGGAGGTTG-3′; reverse: 5′-GGGAGGGGATTTTTATCTGTTAG-3′;
- Nanog: forward: 5′-TCCTTCTGAGTGGTCTG-3′; reverse: 5′-GGGAGGGGATTTTTATCTGTTAG-3′;
- GAPDH: forward: 5′-AGCTGCGTGAACGCATGG-3′; reverse: 5′-TCTGACCTAGTTGAGCAGTA-3′;
2.9. Quantitative real-time PCR of miRNAs

The total RNA was isolated by Trizol reagent (Sigma). miRNA was subsequently reverse-transcribed to cDNA using the miRNA specific stem-loop reverse-transcription primer (Ribobio). The amount of target gene expression \(2^{-\Delta\Delta C_{t}}\) was normalized via the endogenous small nuclear RNA U6 by using miRNA specific primers (Ribobio). The condition of reaction was preformed according to the instruction from Ribobio Co., Ltd.

2.10. Immunostaining

ES cells were fixed for 20 min by 4% paraformaldehyde (paraformaldehyd was diluted by PBS buffer). Then the cells were washed by PBS buffer for 3 times. After washing, cells were treated with 0.2% Triton X-100 for 8 min. After treating by Triton X-100, PBS containing 10% FBS (Gibico) was added into the plates for 1 h. Then discarded the PBS and added the antibodies with PBS buffer contained 10% FBS. Primary antibodies included anti-Sox2 (Santa Cruz), anti-Oct4 (Cell Signaling Technology), SSEA-1 (abcam).

2.11. Knockdown of zeb2

Transfection of siRNA-zeb2 (Santa cruz sc-38642) into mES cells to knockdown the expression of zeb2 by LipofectamineRNAiMax.

2.12. Statistical analyses

GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA) was used for all statistical analyses. Statistical significance was defined as a value of \(P < 0.05, **\) means \(P < 0.01, ***\)means \(P < 0.001\). Values are reported as mean \( \pm SD \).

3. Result

3.1. Medroxyprogesterone 17-acetate inhibits the self-renewal of mES cells and promotes differentiation untimely

Medroxyprogesterone 17-acetate (MPA) was added in the mES culture medium at concentration levels of 20 ng/mL, 200 ng/mL and 2000 ng/mL. The function of medroxyprogesterone 17-acetate was dosage dependent. ES cells maintained the clone and normal expression level of stemness gene at the MPA concentration of 20 ng/mL, which is the normal level of progesterone at the early pregnancy (Fig. 1A). But with the increasing of the MPA concentration, differentiation happened to mES cells. 200 ng/mL MPA led to differentiation of most of the ES clones. 2000 ng/mL MPA, which is the maximum value of steady plasma-drug concentration of anti-threatened abortion, could significantly inhibit the self-renewal of mES on clone morphology (Fig. 1A) and stemness gene expression detected by qRT-PCR (Fig. 1B) and immunostaining (Fig. 1C).

3.2. Progesterone signaling regulated the miR-200a/zeb2 pathway

Further, we used MPA to study the mechanism of self-renewal inhibition by progesterone signaling. We found that MPA can down-regulate the expression of miR-200a (Fig. 2A). Previous study also determined that mir-200a which was higher expressed in mES cells played an important role in maintaining self-renewal [27]. Further, we found that zeb2 was up regulated by MPA (Fig. 2B). Zeb2 had been reported to be the direct target gene of miR-200a in some kinds of cells. The prediction of miR-200a target site in the 3’UTRs of zeb2 was shown (Fig. 2C). Luciferase reporter assay was performed and determined the interaction between miR-200a and zeb2 3’UTR (Fig. 2D). In 3T3 cells, the luciferase reporters were cotransfected with miR-200a. A miR-control with no homology to the genome was used to control the nonspecific effects of expression. miRNA control did not affect the reporter activities. The miR-200a significantly reduced the luciferase activities of the wild-type zeb2 on both predicted target sites, compared to the negative controls. In contrast, mutant reporters were not repressed by miR-200a (Fig. 2D). Over-expression of miR-200a could down-regulate the level of endogenous zeb2 in mES cells on both mRNA (Fig. 2E) and protein level (Fig. 2F). These results indicated that MPA drove the progesterone signaling by regulating the miR-200a/zeb2 pathway.

3.3. miR-200a/zeb2 pathway regulates the mES cells self-renewal and differentiation

In order to determine whether the miR-200a/zeb2 pathway can regulate the mES cells self-renewal and differentiation, we over-expressed the zeb2 in the mES cells. We found that mES cells over-expressed zeb2 could not form clones and differentiated even if in the llf supplementary medium (Fig. 3A) and had lower expression level of stemness markers detected by qRT-PR (Fig. 3B) and immunostaining (Fig. 3C). We also over-expressed the miR-200a into mES cells to find that miR-200a had the opposite function of zeb2. miR-200a up regulated the expression level of stemness markers (Fig. 3D, E) and sustained the ES clones (Fig. 3F). There was no significant difference of the stemness markers expression level between cells in control group and miR-200a over-expressed group (Fig. 3D).

3.4. Progesterone signaling regulated the miR-200a/zeb2 pathway to influence the mES self-renewal

Further, we wanted to determine whether progesterone signaling aroused by MPA (200 ng/mL) can regulate the miR-200a/zeb2 pathway to inhibit the self-renewal of mES cells. We made the rescue experiments to find that the function of self-renewal inhibition of MPA could be rescued by over-expressing the miR-200a or down-regulating zeb2. We found that there was no significant difference of clone formation (Fig. 4A) and expression of stemness markers detected by qRT-PR and immunostaining (Fig. 4B, C) between the control group of mES and the mES cells treated by MPA plus the transfection of miR-200a or siRNA-zeb2.

4. Discussion

In this study, we demonstrated a new signaling pathway, progesterone signaling/miR200a/zeb2 axis, regulated the mES cells self-renewal and differentiation. During gestation, the level of progesterone is up regulated. But at the beginning of pregnancy, progesterone maintains at low level. Clinically, progesterone is regarded as tocolytic agent that can protect the gestation healthly [1,2,28]. While there is not enough progesterone in pregnant woman or woman who receives in vitro fertilization and embryo transfer, doctor may use hormone replacement therapy to supply the insufficient of progesterone [6]. But an increased spontaneous abortion rate (28.6%) was noted among women treated with progesterone suppositories [29]. Other reports also showed the embryo toxicity of progesterone and its derivant [12,13]. In our study, we found that at the same physiological concentration as progesterone during early pregnancy, MPA had no significant influence on ES cells self-renewal. But clinical dosage of MPA for anti-abortion had already inhibited the self-renewal capacity of
Fig. 1. Medroxyprogesterone 17-acetate (MPA) inhibits clone formation and stemness gene expression and promotes differentiation untimely. A. Morphology of clones in the culture medium with MPA added at concentration levels of 20 ng/ml, 200 ng/ml and 2000 ng/ml. Ctrl means alcohol, the solvent of MPA, without MPA. The scale bar represents 100 μm. B. Detection of expression level of stemness markers of mouse embryonic stem (mES) cells, Oct4, Lin28, Nanog. For all experiments n = 3, average ± SD; Student’s t-test. * P < 0.05, ** P < 0.01, *** P < 0.001. GAPDH was used as internal control. C. Immunostaining of stemness markers Oct4, Sox2 of every group of mES cells. The scale bar represents 100 μm.
mES cells and promotes the differentiation. We thought that embryotoxicity of progesterone may be caused by higher level of exogenous progesterone or its derivant treated for the pregnant woman to destroy the growth of early embryo, which leads to the failures of gestation.

The progesterone signaling can regulate the balance of self-renewal and differentiation. We found that MPA can down-regulate the level of miR-200a and up-regulate the expression of zeb2. We found that miR-200a was higher expressed in mES cells and down regulated during differentiation. mES cells come from

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**Fig. 2.** Medroxyprogesterone 17-acetate (MPA) down-regulates the expression of miR-200a and up-regulates the zeb2. A. Dose dependent of MPA down-regulates the expression of miR-200a detected by quantitative real-time PCR (qRT-PCR). For all experiments n = 3, average ± SD; Student’s t-test, **P < 0.01, ***P < 0.001. U6 was used as internal control. B. Dose dependent of MPA up-regulates the expression of zeb2 detected by qRT-PCR. For all experiments n = 3, average ± SD; Student’s t-test, *P < 0.05, **P < 0.01. GAPDH was used as internal control. C. Summary of miR-200a target sites in the 3’ untranslated regions (3’UTRs) of zeb2. D. Luciferase assays on 3T3 cells. Ctrl means pGL3 empty vector, WT-UTR means wild-type zeb2-3’UTRs, mut-UTR means mutant zeb2-3’UTR with deletion of the microRNAs (miRNAs) binding site sequence. Ctrl miRNA means miRNA control (miR-67), an artificial polyribonucleotide which has no interaction of mRNA 3’UTR. miR-200a means pre-miR-200a. E. Over-expression of miR-200a down-regulates the level of zeb2 detected by western blot. Protein level quantification was normalized to GAPDH.
Fig. 3. miR-200a/zeb2 pathway regulates the mouse embryonic stem (mES) cells self-renewal. A. Morphology of mES clones over-expressed the zeb2. The scale bar represents 100 μm. B. Expression of stemness markers of Oct4, Nanog, Lin28 in mES cells detected by quantitative real-time PCR (qRT-PCR). Ctrl means empty vector. Zeb2 means ectopic expression vector of zeb2. For all experiments n = 3, average ± SD; Student’s t-test, ** P < 0.01, *** P < 0.001. GAPDH was used as internal control. C. Immunostaining of stemness markers Oct4, Sox2 of every group of mES cells. The scale bar represents 100 μm. D. Morphology of mES clones over-expressed of miR-200a. The scale bar represents 100 μm. Ctrl means microRNAs (miRNAs) control. miR-200a means pre-miR-200a. E. Expression of stemness markers of Oct4, Nanog, Lin28 in mES cells detected by qRT-PCR. For all experiments n = 3, average ± SD; Student’s t-test, ** P < 0.01, *** P < 0.001. GAPDH was used as internal control. F. Immunostaining of stemness markers Oct4, Sox2 of every group of mES cells. The scale bar represents 100 μm.
Fig. 4. Medroxyprogesterone 17-acetate (MPA) regulates miR-200a/zeb2 pathway to inhibit the mouse embryonic stem (mES) self-renewal. Over-expression of miR-200a or knockdown of zeb2 inhibits the function of MPA in mES cells. The scale bar represents 100 μm. The expression of zeb2 was detected by quantitative real-time PCR (qRT-PCR) and western blot in rescue experiment. For all experiments n = 3, average ± SD; Student’s t-test, **P < 0.01. GAPDH was used as internal control. Ctrl means microRNAs (miRNAs) control and siRNA control plus alcohol without MPA. miR-ctrl means miRNA control and siRNA control means siRNA control. Expression of stemness markers of Oct4, Nanog, Lin28 in mES cells detected by qRT-PCR. For all experiments n = 3, average ± SD; Student’s t-test, **P < 0.01, ***P < 0.001. GAPDH was used as internal control. For all experiments n = 3, average ± SD; Student’s t-test, *P < 0.05, **P < 0.01. GAPDH was used as internal control. Immunostaining of stemness markers Oct4, Sox2 of every group of mES cells. The scale bar represents 100 μm.
the inner cell mass of the blastocyst, an early-stage embryo, and can differentiate to a body [30]. Latest study also showed that miR-200a is important for mES self-renewal and differentiation [26]. ES cells are pluripotent, that is, they are able to differentiate into a completely adult body. Low level of progesterone at the early gestation may be important for the high level of miR-200a. We demonstrated that miR-200a can directly target the zeb2 3’UTR to down-regulate the expression of zeb2 which inhibits the self-renewal and promote the differentiation of mES cells. miR-200a can target zinc finger E-box binding homebox 1 (ZEB1) known as Tcf8 and ZEB2 known as zinc finger homeobox1B and SMAD interacting protein 1 (SIP1) [31,32]. Both ZEB1 and ZEB2 are transcriptional repressors of E-cadherin (E-cad) [33]. Previous studies showed that E-cad is a cell adhesion molecule expressed in epithelia and is implicated in many developmental processes. In mouse, E-cad is necessary for the establishment of the blastocyst since E-cad null embryos fail to form trophectoderm and cannot generate the blastocyst cavity [34,35]. In ES cells culture, E-cad-coated plates maintain the pluripotency of ES Cells [36]. Additionally, during the form of induced pluripotent stem (ips) cells E-cad-mediated cell-cell contact is critical process [37]. Our finding suggested that progesterone-signaling influences the mES cells self-renewal by regulating the miR-200a/zeb2 pathway. Because of the high level of miR-200a in mES cells, progesterone receptor showed low expression level at the early-stage of pregnancy, which is associated with the low level of progesterone. So the progesterone signaling/miR-200a/zeb2 axis maybe not only regulate the self-renewal and differentiation during early pregnancy, but sustain in all the process of gestation to control the development of embryo.

Our study also showed that dosage of progesterone and its derivant used in hormone replacement therapy should be carefully controlled. The dosage of supplement to progesterone insufficient pregnant woman should be according to the individual difference. That may avoid the influence of unbalance of progesterone signaling/miR-200a/zeb2, which may lead to embryotoxicity of higher level of progesterone at the early development stage of embryo.

Disclosure of interest

The authors have not supplied their declaration of conflict of interest.

Acknowledgments

This work was supported by National Natural Science Foundation of China (81170628), national basic research program of china (973program2012CB966300), program of shanghai municipal health bureau (XBR2011064).

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