Endometrial stromal cell miR-29c-3p regulates uterine contraction

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Abstract

Uterine peristalsis plays a vital role in fertility and female reproductive health. Although uterine peristalsis is thought to be correlated with some hormones and uterine pathologies, the physiological mechanisms underlying uterine peristalsis remain not quite clear. This study aimed to identify changes in miRNA in the endometrium of patients with abnormally high-frequency (hyper-) and low-frequency (hypo-) peristalsis to clarify whether miRNAs regulate uterine peristalsis. We used a miRNA microarray and RT-qPCR to identify changes in miRNA in endometrial tissue, a collagen gel contraction assay on co-cultured human endometrial stromal cells (ESCs) to analyze how the altered regulation of miRNAs influences uterine smooth muscle (USM) contraction, Western blots and other assays to elucidate the potential mechanisms involved. We found that among several differentially regulated miRNAs, miR-29c-3p was overexpressed in endometrial samples from patients with hypoperistalsis; oxytocin receptor (OXTR) expression was low in endometrial samples from patients with hyperperistalsis. Bioinformatic analysis and luciferase assays indicated that OXTR is a target of miR-29c-3p, which attenuates its expression. Additionally, downregulation of miR-29c-3p in ESC cultures increased the expression of aldo-keto reductase family 1, member C3 (AKR1C3) and increased the release of prostaglandin F2 alpha (PGF2α). Co-cultured ESCs overexpressing miR-29c-3p reduced USM cell contractions; the opposite tendency was found when ESCs were transfected with a miR-29c-3p inhibitor. To conclude, miR-29c-3p in endometrial cells regulates uterine contractility by attenuating the expression of OXTR and reducing PGF2α release.


Introduction

Uterine peristalsis is endometrial wave-like contractile activity that plays an important role in embryo implantation (Fanchin et al. 1998, Yoshino et al. 2010, Zhu et al. 2014b); it has potential as a non-invasive criterion to predict endometrial receptivity. Our previous study also indicated that uterine peristalsis of >3 waves/min before embryo transfer can influence the implantation rate in assisted reproductive technology (ART) treatment (Zhu et al. 2014a). It is also thought that dysregulated peristalsis is critical in the development of endometriosis (Leyendecker et al. 2004, Ibrahim et al. 2015, Shaked et al. 2015), dysmenorrhea, ectopic gestation, and infertility (Kunz and Leyendecker 2002). Uterine peristalsis is believed to originate from the contraction of the sub-endometrium to be influenced by factors related to uterine contractility such as estradiol and progesterin concentrations (Mueller et al. 2006b), oxytocinergic signaling (Kunz et al. 1998, Huang et al. 2017), prostaglandins, and mechanical stimuli (e.g., intruterine devices). Some uterine pathologies also influence uterine contractility, including adenomyosis, endometriosis (He et al. 2016), and submucosal leiomyoma (Yoshino et al. 2010, Kido et al. 2011). Due to the roles of uterine peristalsis in infertility and ART, proper intervention measures to avoid its negative effects become significant. Even though the luteal support may attenuate partial hyperperistalsis in ART, the hyperperistalsis still exist especially in the condition of high estradiol after controlled ovarian stimulation (Zhu et al. 2012). So the researches of the other factors influencing uterine peristalsis such as OT-OXTR signal as well as prostaglandins become significant.

The myometrial layer of the uterus has three tissue layers: the stratum supravasculare (the external-most layer); the stratum vasculare; and the stratum subvasculare (the sub-endo-myometrium). The different layers of uterine wall originate from two embryological sources, such that the endometrium and sub-endomyometrium originate from Müllerian, whereas the strata vasculare and supravasculare originate from non-Müllerian sources (Noe et al. 1999). Based on these anatomical data, it is assumed that the endo-myometrial unit acts as a single functional entity that regulates uterine peristalsis.

miRNA is a short (~22 nucleotide) non-coding RNA that regulates various physiological activities, including...
endometrial physiological functions and embryo implantation. For example, miR-222 influences the decidualization of endometrial stromal cells (ESCs) by targeting regions of CDKN1C/p57kip2 mRNAs and increasing CDKN1C/p57kip2 protein expression (Qian et al. 2009), while miR-451 takes part in regulating embryo adhesion by targeting Ankrd46 (Li et al. 2015). In goats, miR-26a regulates the expression of prostaglandin-endoperoxide synthase 2 (PTGS2) in endometrial epithelial cells (Zhang et al. 2018). Some microRNAs have also been found to take part in controlling uterine contractility in pregnant women, including those in the miR-200 family (Renthal et al. 2010); human miR-199a-3p and miR-214 both target PTGS2 and can influence the transition from a refractory to contractile state (Williams et al. 2012). However, to our best knowledge, no published information on regulation of uterine peristalsis by miRNAs was found to date. To better understand this regulation, we analyzed endometrial cell expression of miRNAs, seeking to identify differentially expressed miRNAs depending on the high or low-frequency of uterine peristalsis, and then analyzing the regulatory effects of any such miRNAs in endometrial and USM cells.

Materials and methods

Participants

Forty-one (47) women of age between 25 and 38 years who were being examined for pinopodes for individualized transplantation were recruited. All the patients had regular ovulation. The Xiangya Hospital Ethics Committee authorized the study, and all patients signed the informed consent prior to participation in the study. After gathering information on the participant's uterine peristaltic activity by performing transvaginal ultrasonography for 5 min as we did previously (Zhu et al. 2014a,b), we collected the endometrial tissue samples in the fifth day after ovulation. Within 2 min after collection, an approximately 0.5×0.5×0.3 cm³ piece of the tissue sample was placed in RNA Stabilization Reagent, and a second sample (if enough) was immersed in 4% paraformaldehyde for fixation. Each pair of samples was classified into the high-frequency group (HF) or low-frequency group (LF) according to a threshold of two contractions/min such that n = 25 LF samples and n = 22 HF samples.

MicroRNA microarray analysis

Three each of HF and LF endometrial samples (chose the samples with the three lowest and the three highest uterine peristalsis frequency from the previous 18 samples we had got) in RNA Stabilization Reagent were sent to CapitalBio (Peking, China) for the miRNA microarray assay, which used an Affymetrix miRNA 4.0 array. Sequences for the probes in the array were derived from those in SangermiRBase Release 20.0. Raw data processing and statistical analyses were conducted in the Affymetrix GeneChip Command Console program. Those miRNAs of significant change between the LF and HF groups were considered candidate miRNAs. To determine the potential relationships between miRNAs and uterine peristalsis, we used mirtargetsan.org and microrna.org to identify potential targets for candidate miRNAs.

Real-time PCR

Total RNA was extracted by a standard TRIzol-based protocol (Invitrogen) from each of the rest of 41 endometrial samples (the six for the microarray had been used up) or ESCs. Total RNA (2 µg) of each sample was reverse transcribed with the miRNA Reverse cDNA kit (GeneCopoeia, Guangzhou, China). Real-time PCR was then performed with the miScript SYBER Green PCR kit (GeneCopoeia), according to the manufacturer’s protocols. A specific forward primer was used for miR-29c-3p and U6 by GeneCopoeia, together with a universal reverse primer. All PCR reactions were performed on the ViiA™ 7 Real-Time PCR System. MicroRNAs in each sample were normalized to U6. The method of 2(−ΔΔCt) was used to analyze relative RNA expression levels.

Fluorescence assays

For immunohistochemistry (IHC), slide-mounted paraffin sections were deparaffinized in xylene (three 5-min washes) and rehydrated in descending alcohols (5 min per wash). A circle was drawn on the slide around the tissue with a hydrophobic barrier pen, and then the slide was boiled in 10 mM sodium citrate buffer (pH 6.0) for 10 min for antigen retrieval. Endogenous peroxidase activity was blocked with 3.0% hydrogen peroxide for 15 min, and non-specific binding with 5% bovine serum albumin (BSA) in PBS for 30 min at room temperature. Slides were incubated with anti-oxytocin receptor (1:167, #NBP1-28487) overnight at 4°C and then washed with 1% serum in PBS and incubated with biotinylated secondary antibody for 30 min at room temperature and washed again. We prepared the DAB solution fresh by adding the concentrated DAB to DAB diluent in the ratio of 1:50. Labeling was visualized by immersion in dianaminobenzidine solution for 3 min. The slides were counterstained with hematoxylin, dried and cover slipped. Negative control sections were processed using PBS in place of the primary antibody. The mean optical density of immunoreactivity was calculated using Image Pro-Plus 6.0 (Nikon). Four microscopy photos were taken in the same condition for each sample. The mean optical density of the brown stains covering the cells in each photo was calculated by Image Pro-Plus 6.0. Lastly, the values from the four photos are averaged to be the mean optical density of this sample.

Three each of the fixed HF and LF endometrial samples (chose randomly from each group) were sent to Servicebio (Wuhan, China) for fluorescence in situ hybridization (ISH). The procedure for ISH is as follows. Slide-mounted paraffin sections were firstly deparaffinized, then boiled with Proteinase K for 15 min at 37°C and blocked with prehybridization solution for 1 h at 37°C, mixed with miR-29c-3p probe or negative control probe (Servicebio) overnight at 4°C, washed with SSC buffer, dyed with DAPI, and lastly microscopy photos were taken of the tissue sample was placed in RNA Stabilization Reagent, and a second sample (if enough) was immersed in 4% paraformaldehyde for fixation. Each pair of samples was classified into the high-frequency group (HF) or low-frequency group (LF) according to a threshold of two contractions/min such that n = 25 LF samples and n = 22 HF samples.
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Cell culture and transfection

A human endometrial stromal cell line (ATCC® CRL-4003™) was bought from the American Type Culture Collection and cultured in a 1:1 mixture of Dulbecco’s modified Eagle’s medium and Ham’s F-12 medium with 3.1 g/L glucose and 1 mM sodium pyruvate but without phenol red (Sigma). Culture medium was supplemented with 1.5g/L sodium bicarbonate, 1% ITS-G (Gibco), 0.25g/L BSA, 1.07 mg/L linoleic acid (Sigma), and 10% fetal bovine serum (FBS) (Gibco). Oxytocin was added into culture medium in the ratio of 1 IU: 50 ml when ESCs were for experiments of prostaglandin signal analysis. Cultures were used prior to their fifteenth passage.

Primary uterine smooth muscle cells (USMCs) were cultured via directly adherent culture methods. We collected USM tissue (~1 cm) from beneath the endometrium from women of reproductive age who were being treated for uterine fibroids or CIN III by hysterectomy. Each tissue sample was put into ice cold D-hanks solution for transportation as soon as the uterus was isolated. The sample was cut into ~1 mm pieces and placed in 35 mm culture dishes that were washed clean with D-hanks solution. The pieces were washed three times with D-hanks solution and 20% FBS and then were centrifuged at 400 g for 3 min to pull down the wash solution. Isolated tissues were placed in T25 culture flasks with about 5 mm space remaining.

Figure 1 (A) The unsupervised hierarchical cluster analysis of miRNA expression in high-frequency (three on the left) and low-frequency (three on the right) peristalsis patients. The expression level of each miRNA in each sample varies from low (green) to high (red). (B) The relative expression miR-29c-3p was higher in LF ($n=22$) than HF ($n=19$). Mann–Whiney U tests were performed to show differences between groups. (C) Scatter plot of the RNA levels and frequency of uterine peristalsis shows a negative relationship between them. (D, E, F, G, H, I and J) ISH was conducted to locate the miR-29c-3p. The green light point (D) indicates the staining of miR-29c-3p, the blue light DAPI (E) shows the staining of cell nucleus. (F) is the merge of D and E. (G, H and I) are the negative controls of ISH. Those pictures indicate that miR-29c-3p was mainly expressed in the cytoplasm of stromal cell instead of glandular epithelium. (J) shows that OXTR labeling was significantly higher in the HF ($n=14$) compared with LF ($n=16$). (K) was one of the photomicrographs in LF. (L) was one of the photomicrographs in HF. (M) was the negative control of IHC. K, L show the OXTR expressed in both stromal and glandular epithelial cells both cytoplasmically and nucleary.

**$P<0.01$. $n$ indicates the sample size.

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We cultured the tissues in 5% CO_{2} at 37°C in an incubator without culture medium for 4 h. DMEM/F12 containing 20% FBS was added for continued culturing. After 7–10 days the smooth muscle cells could be identified surrounding the tissue pieces. USMs were used prior to their fifth passage.

For miRNA transfection, ESCs were seeded into six-well plates to 70–80% confluence and transfected with 50 nmol artificial miR-29c-3p mimic which was synthesized by Ribobio (Guangzhou, China) based on a human mature miR-29c-3p sequence. 100 nmol miR-29c-3p inhibitor which was anti-sense single-stranded molecular complementary to mature miR-29c-3p, 100 nmol siRNA targeting OXTR, or relative negative control RNA that they were scrambled sequences (Ribobio), using Lipofectamine® 3000 (Thermo Fisher Scientific) according to the manufacturer’s instructions. Cells were analyzed 48 h after transfection.

**Western blotting**

Total protein was extracted from cell samples using RIPA buffer (Servicebio, Wuhan, China) with protease inhibitor cocktail and the concentrations being measured by BCA assay kit (Servicebio) according to the manufacturer’s instructions. The protein was boiled at 100°C for 10 min with 5x SDS loading buffer (Servicebio). Electrophoresis was performed in a 10% SDS–polyacrylamide gel with MPOS (genscript) and the protein was transferred to a 0.4 µm membrane (Millipore). Then the membrane was blocked in 5% defatted milk in TBS buffer with 1/1000 Triton X-100 for 1 h at room temperature, followed by incubation in anti-OXTR antibody (3 µg/mL, NOVUS, #NBP1-28487), anti-AKR1C3 antibody (1:1000, Abcam, #ab209899) and anti-β-actin antibody (1:1000, Servicebio, #GB11001) overnight at 4°C. After multiple washes, the membrane was correspondingly incubated in respective horseradish peroxidase-conjugated secondary antibodies (1:5000, AbClonal, #AS029; 1:5000, AbClonal, #AS014) for 1 h at room temperature. After multiple washes, the membrane was then analyzed using hypersensitive enhanced chemiluminescence. The integrated light density and gray values were normalized to the values obtained for β-Actin in NIH ImageJ.

**Dual luciferase reporting system**

293T cells were cultured in 24-well culture plates and transfected with the miR-29c-3p mimic (see cell culture and transfection) or a negative control (NC) combined with a plasmid containing the OXTR wild-type (WT) or mutated (MUT) sequence using Lipofectamine® 3000. The OXTR WT plasmid containing 375 to 1141 of the 3’ UTR sequence, while the OXTR MUT changed OXTR WT’s sequence of TGGTGCTA (426–433) to ACCACGAT. Both kinds of plasmid contained hrRluc gene as reporter fluorescence and hLuc gene as internal reference fluorescence. After 48 h, the dual-luciferase reporter assay system (Promega) was used according to the manufacturer’s instructions. Luciferase reporter activity was collected by machine GLOMAX-MULTI (American, Promega). The ratio of Renilla luciferase values to firefly luciferase levels was averaged across three replicate wells.

**Cell (collagen) contraction analysis**

Cells were seeded in 3D gel medium in 24-well culture plates derived from rat tail collagen I (Corning). Either ESCs and USMs were seeded together at a 1:1 ratio or ESCs alone were seeded. For each well, collagen 1 (345 µL) with 1 mM NaOH (3.6 µL) was mixed on ice, and then mixed with 156 µL culture medium and 1 x 10^6 cells. 0.5 IU oxytocin was added to the culture medium. Plates were incubated at 37°C in a humidified 5% CO_{2} incubator for 1 h. When the gel had solidified it was freed from the culture plate wall by a syringe needle. Another 500 µL culture medium was added to each well. After 48 h, the area of the crystalized gel in each well was measured in ImageJ. And the ratio of the gel area to the well area was calculated to assess the contraction strength. Each group was assessed in triplicate; seeded USMs were from different tissue samples.

Laser scanning confocal microscopy was used to analyze calcium ion flux in USMs following OXTR knockdown. Three siRNA-knockdown sequences were made by Ribobio as follows: si-OXTR-1: 5’-GGAGCCGCTTCTTCTTCGT-3’; si-OXTR-2: 5’-CGGTCAAGATTTCA-3’; si-OXTR-3: 5’-GCTACGGCCTTATCAGCTT-3’. After determining knockdown efficiencies by performing RT-qPCR, si-OXTR-1 was chosen for further use. USMs were seeded onto 35 mm glass-bottomed dishes and were transfected with si-OXTR-1 or a control siRNA (si-CTRL) (scrambled sequences) after 24 h for 48 h. Cells were labeled with calcium 5 µg/mL fluo-4-AM dye (Invitrogen) diluted by DMEM/F12 for 30 min at 37 °C in a humidified 5% CO_{2} incubator. Then cells were washed twice by D-hanks solution and culture medium was added to the dishes for continuous culturing for 30 min at 37°C in a humidified 5% CO_{2} incubator to form fluo-4. The confocal microscope was then used to analyze cellular calcium ion flux by detection of fluo-4 fluorescence after 0.5 IU oxytocin was added into culture medium. The laser was fired every 15 s.

**ESC/USM co-culturing**

ESCs were seeded in six-well plates and grown to 50% confluence. A lentiviral vector with a miR-29c-3p overexpression sequence or a NC vector which was in random sequence (GeneCopoeia) was transduced into the ESCs by adding 1 x 10^6 vector and polybrene (the last concentration 5 µg/mL) for 12 h, and then we changed the culture medium for continuous culturing. Ninety-six hours later, green fluorescent protein expression was observed to assess the transfection efficiency. These well transfected ESCs were then seeded into the upper chamber of a six-well (0.4 µm) Millipore transwell (Corning) and USM was seeded into the lower chamber at 30% confluence. USMs were collected for Western blotting after 7-day co-culture.

**Enzyme-linked immunosorbent assay**

Levels of prostaglandin E2 (PGE2) and PGE2α in the culture medium of ESCs were measured by ELISA. The ESCs were transfection to overexpress or underexpress miR-29c-3p or a NC. Each sample was collected when the cells were grown to

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90% confluence. ELISA plates were provided by Elabscience (Wuhan, China). ELISAs were conducted according to the manufacturer’s instructions.

Statistical analysis

Data were reported as mean ± S.E.M. for normally distributed variables, median (interquartile range) for non-normally distributed variables. When appropriate, independent sample t-tests or Mann-Whitney U tests were performed to compare between-group difference. Rank correlation analysis (Spearman correlation) was used to test the correlation. GraphPad prism 5 (GraphPad Software) was used for data analysis. P < 0.05 was the threshold for statistical significance.

Results

Expression of endometrial miRNAs in relation to frequency of peristalsis and OXTR protein expression in endometrial samples by group

miRNA microarray analysis of three LF and three HF endometrial tissue samples demonstrated that three miRNAs were lower and 14 miRNAs were higher in LF than HF (Fig. 1A). qPCR in another 41 samples showed that miR-29c-3p were upregulated in LF (P = 0.0021) (Fig. 1B), as was found in the original microarray. Spearman correlation showed a negative relationship between the expression of miR-29c-3p and the frequency of uterine peristalsis (r = −0.52, P = 0.0004) (Fig. 1C). ISH analysis demonstrated that miR-29c-3p was primarily expressed in the cytoplasm of ESCs (Fig. 1D, E and F). Figure 1G, H and I are the NCs of ISH. Predictive target analysis indicated that OXTR contains a binding site of seed sequences for miR-29 miRNAs (Fig. 2D). Immunoreactivity for OXTR was found in both stromal and glandular epithelial cells; it appeared to be expressed mainly in the cytoplasm, although some possible nuclear labeling was identified. The optical density of OXTR labeling was significantly higher in the HF (Fig. 1L) group than in the LF (Fig. 1K) group (P = 0.007) (Fig. 1J). Figure 1M is the NC of IHC.

miR-29c-3p and ESC expression of OXTR

In cultured ESCs, combined qPCR and Western blotting for miR-29c-3p and OXTR, respectively, demonstrated the miRNA reduced the expression of receptor protein (Fig. 2A, B and C). To verify this relationship, we used a dual luciferase report system. We found that miR-29c-3p decreased luciferase activity when co-transfected with the WT-OXTR plasmid. Meanwhile, in the MUT-OXTR plasmid groups, there was no decrease suggesting that miR-29c-3p attenuated the expression of OXTR (Fig. 2E); the seeding sequence was TGGTGCT (Fig. 2D).

Smooth muscle contractility, OXTR and PGF2α expression in co-cultured ESCs/USM cells

Using the assumption that the endometrium and sub-endomyometrium functioned as a unit, we co-cultured ESCs overexpressing or downregulating miR-29c-3p or expressing a NC with USMs in 3D collagen gels.
The area ratio of the collagen gel in the miRNA-overexpressing ESCs (mean ± s.d. = 0.37 ± 0.036; n = 3) was larger (P = 0.016) than that of the NC ESCs (mean ± s.d. = 0.21 ± 0.016; n = 3) (Fig. 3A, B1 and B2). The collagen gel area ratio of the ESCs transfected with the miR-29c-3p inhibitor (mean ± s.d. = 0.11 ± 0.011; n = 3) was smaller (P = 0.0088) than the negative control-transfected ESCs (mean ± s.d. = 0.20 ± 0.016; n = 3) (Fig. 3A, B3 and B4). No difference was found between the areas of the collagen gels when USM cells were not co-cultured with the ESCs, regardless of transfection condition (Fig. 3A, B5, B6, B7 and B8). ELISAs of samples of ESC culture medium demonstrated significantly less PGF2α in ESC cultures overexpressing miR-29c-3p and increased PGF2α in inhibitor-transfected cultures (Fig. 4A). PGE2 concentrations in all cases were below detectable limits. Meanwhile, the expression of AKR1C3 was greater in ESCs overexpressing miR-29c-3p or lower in inhibitor-transfected ESCs than negative control-transfected ESCs when the oxytocin was added in the culture medium (Fig. 4B and C).

Western blots of OXTR in USM cells showed that the expression of OXTR when USM cells were co-cultured with ESCs overexpressing miR-29c-3p was lower than expression of OXTR when ESCs were transfected with the NC (Fig. 4E and F). These ESCs were transfected with lentiviral vectors for participating in a 7-day co-culturing and the efficiency of transfection was more than 70% under a under a fluorescence microscope (Fig. 4D). When si-OXTR or NC siRNAs were transfected into USM cells to analyze calcium flux, the fluorescence intensity increase rate after adding oxytocin was smaller in the si-OXTR group than it was in the control group (Supplementary Fig. 1, see section on supplementary data given at the end of this article).

Discussion

This study analyzed the miRNA-mediated molecular regulation of uterine peristalsis. This study was, to the best of our knowledge, the first to describe changes in miRNA expression related to frequency of uterine peristalsis. We demonstrated that miR-29c-3p expression in the endometrial tissue obtained from HF samples was lower than miR-29c-3p expression in the endometrial tissue from LF samples. miR-29c expression had been found lower in human late proliferative endometrium than the mid-secretory endometrium by Kuokkanen et al. (Kuokkanen et al. 2010). And the uterine peristalsis was stronger in human late proliferative endometrium than the mid-secretory endometrium. Logically, those results had consistency with our results. MiR-29c-3p played important roles in fundamental physiological activity of the cells such as proliferation, differentiation and migration. However, the factors implicated in the regulation of miR-29c-3p were still unclear. TGF-β1 may inhibit the expression of the miR-29 family (Wang et al. 2012). Because macrophage can release the TGF-β1, the inflammation may influence the expression of miR-29c-3p in vivo. Oxidative stress may increase expression of miR-29 family (Heid et al. 2017). So some life style and environment of human being may affect the expression of miR-29c-3p in vivo. miRNA often reduces or silences expression of its target genes; in this case, we found that miR-29c-3p can target OXTR and reduce its expression. Actually, Xu et al.’s study have also shown that miR-29b can target OXTR with the same the seedling sequence as we have shown and regulate OXTR in bovine corpus luteum cells (Xu et al. 2018). Whether other miRNAs such as the other miR-29 family members work in the regulation of uterine peristalsis requires further research in the future.

OXTR is a G-protein-coupled receptor in the endometrium and myometrium of the human uterus; the receptor density affects oxytocin sensitivity. It uses phospholipase C and increased IP3 and diacylglycerol as second messengers, and ultimately increases intracellular calcium ion flux. This activates myometrial contractions. The oxytocinergic system is known to function in non-pregnancy-related uterine contractions, especially those in the sub-endo-myometrium (Kunz et al. 1998, Richter et al. 2003, Huang et al. 2017). OXTR dysfunction can cause uterine contractility disorders and dysperistalsis (Mechsner et al. 2010, Huang et al. 2017). Furthermore, some clinical studies have suggested that atosiban, an oxytocin receptor antagonist, suppressed uterine peristalsic activity and improved implantation and pregnancy rates in ART treatment (Pierzynski et al. 2007, Moraloglu et al. 2010, He et al. 2016). Although OXTR is a cell membrane receptor, our data showed OXTR located cytoplasm and nucleus in endometrium, just as other’s data that the OXTRs have been found in both the endometrium and myometrium both cytoplasmically and nuclearily before (Mechsner et al. 2010). Actually, the subcellular location was provided by genecards. org including not only the plasma membrane but also nucleus, cytosol, cytoskeleton and extracellular. Maybe OXTR had a precursor in the intracellular region before becoming mature, and this antibody can label the precursor. This study only used luteal phase endometrial samples and found relatively a higher expression of OXTR in HF peristalsis relative to LF peristalsis. OXTR expressed in the endometrium may be involved in some physiological function, such as facilitating the release of PGF2α (Wilson et al. 1988, Penrod et al. 2013), participating in the coordination of the uterine peristalsis (Kunz et al. 1998) and the process of luteolysis (Mann and Lamming 2006, Oponowicz et al. 2006).

PGF2α is one of the vital factors implicated in the regulation of uterine contraction. Both PGF2α and PGE2 regulate uterine cell contraction partly by activation of G-protein-coupled receptors. Such receptors have been described previously in different regions of the pregnant.
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Although comparable data for the non-pregnant uterus are still lacking, it has been demonstrated that PGF2α took part in the regulation of uterine peristalsis (Mueller et al. 2006a, Sharif 2008). In an extracorporeal perfusion model with non-pregnant swine uteri, administering PGF2α or PGE2 or OT to the perfusion medium could cause uterine contractions and higher intrauterine pressure (Mueller et al. 2006a). AKR1C3 is an enzyme related to the PGF2α metabolism (Dozier et al. 2008). It can convert the PGH2 into PGF2α. Our data also showed that miR-29c-3p-overexpressing ESCs decreased the expression of AKR1C3 and then decreased the production and release of PGF2α when oxytocin was added to the culture medium.

**Figure 3** ESCs with altered miR29c-3p was co-cultured with USMs in 3D collagen gels. The area ratio of collagen gel was larger in the co-culturing of overexpressing miR-29c-3p ESCs (ESCs-OmiR-29c) with USMs than NC. The collagen gel area ratio of the ESCs transfected with the miR-29c-3p inhibitor (ESCs-ImiR-29c) when co-cultured with USMs was smaller than NC. P < 0.05. However, without USMs in the collagen gels, the area ratio did not change regardless of the transfection condition.

**Figure 4** (A) PGF2α concentration was lower in culture medium of ESCs-overexpressing miR-29c-3p than NC and higher in culture medium of ESCs than NC (n = 3). P < 0.05. (B) The AKR1C3 protein level was analyzed by Western blotting. (C) The variation of AKR1C3 level in ESCs transfected with miR-29c-3p mimic or inhibitor was assessed by the ratio to the relative negative control. (D) Transfection efficiency of lentiviral vectors was more than 70% under a fluorescence microscope. (E) and (F) show that the OXTR protein in USM cells co-cultured with ESCs overexpressing miR-29c-3p is lower than co-cultured with ESCs transfected with the NC.
Oxytocinergic activity can facilitate the endometrial cells to release PGF2α had been demonstrated before (Wilson et al. 1988; Burns et al. 2001), and in Burns’ study, oxytocin induces PGF2α synthesis through which oxytocin induced the phosphorylation of ERK1/2 and activated MAPK pathway, and the G(i) proteins made no difference on oxytocin-induced PGF2alpha synthesis in ovine endometrium (Burns et al. 2001). In Sun et al.’s study, AKR1C3 could also activate the MAPK pathway (Sun et al. 2016). Therefore, we think that the changes in AKR1C3 and PGF2α of miR-29c-3p-overexpressing ESCs may be caused by the following pathway: miR-29c-3p–OXTR-AKR1C3-PGF2α.

We also found that OXTRs in USM cells changed after an appropriate period when they were co-cultured with ESCs with altered miR-29c-3p levels. The molecular mechanism underlying this change requires further study, although changes in PGF2α release by the ESCs may alter OXTR expression. Previous studies have determined that PGF2α can stimulate the expression of OXTR through calcineurin/NFAT signaling in USM cells (Xu et al. 2015).

When analyzing cellular contraction in the collagen gel contraction assay, we determined that changing miRNA expression in ESCs did not alter contraction of the gel, while changing miRNA expression in ESCs co-seeding and culturing with USM cells altered the contraction of the gel, suggesting that uterine peristalsis is primarily altered by changes in contractility of USM cells in the sub-endometrial layer and ESCs may release some factors influencing the contractility of USM cells in the sub-endometrial layer. Considering that the change of the contraction of the gel can be caused in 48 h, we thought that the release of PGF2α by ESCs leading USM cells to contract may be the main reason for the change. However, in vivo, the changes of OXTR in sub-endometrial layer caused by the altered miR-29c-3p of endometrium may also be involved in the regulation of uterine peristalsis.

Some limitations also existed in this study. We only conducted cell experience in vitro to support the hypothesis of the function of endo-myometrial layers being as units in uterine peristalsis. And the co-culture model was too simple for imitating the physiological activity in vivo. In vivo experiments can be envisaged to clearly demonstrate the role of miR-29c in uterine peristalsis in the future. So the hypothesis may still need further studies to clarify the presented hypothesis. Another limitation of this study was that the USMcs were from women with either fibroids or CIN which may not represent normal myometrium.

To conclude, this study showed that miR-29c-3p can attenuate the expression of OXTR and reduce the production and release of PGF2α in ESCs, which can subsequently affect the contractility of sub-endometrial smooth muscle cells. These data also support the hypothesis of the function of endo-myometrial layers being as units in uterine peristalsis.

### Supplementary data

This is linked to the online version of the paper at https://doi.org/10.1530/REP-19-0196.

### Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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### Author contribution statement

Y L was involved in the project designing, project development and manuscript writing. L X contributed to the project designing, project development, data collection, data analysis and manuscript writing. The other authors contributed to sample collection and data analysis.

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