Regulation of miR-101/miR-199a-3p by the epithelial sodium channel during embryo implantation: involvement of CREB phosphorylation

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Abstract

In our previous study, we have demonstrated that the epithelial sodium channel (ENaC) mediates the embryo-derived signals leading to the activation of CREB and upregulation of cyclooxygenase type 2 (COX2) required for embryo implantation. This study aims to investigate whether microRNAs (miRNAs) are involved in the ENaC-induced upregulation of COX2 during embryo implantation. The results show that the levels of miR-101 and miR-199a-3p, two COX2 targeting miRNAs, are reduced by ENaC activation, and increased by ENaC inhibition or knock-down of ENaC subunit (ENaC α) in human endometrial surface epithelial (HES) cells or in mouse uteri during implantation. Phosphorylation of CREB is induced by the activation of ENaC, and blocked by ENaC inhibition or knockdown in HES cells. Knockdown of ENaC α or CREB in HES cells or in mouse uterus *in vivo* results in increases in miR-101 and miR-199a-3p, accompanied with decreases in COX2 protein levels and reduction in implantation rate. The downregulation of COX2 caused by knockdown of ENaC or CREB can be recovered by the inhibitors of miR-101 or miR-199a-3p in HES cells. These results reveal a novel molecular mechanism modulating COX2 expression during embryo implantation via ENaC-dependent CREB activation and COX2-targeting miRNAs.

Reproduction (2014) **148** 559–568

Introduction

The implantation of the blastocyst into the uterus is one of the most critical steps in human reproduction. Implantation failure is associated with ~75% pregnancy loss in humans (Wilcox *et al.* 1988, Norwitz *et al.* 2001), and aberrant embryo implantation can lead to adverse consequences during the later course of pregnancy such as placental insufficiency and preterm-labor in humans (Cha *et al.* 2012). Although a variety of signaling molecules associated with implantation have been identified (see reviews by Cha *et al.* (2012), Koot *et al.* (2012) and Ruan *et al.* (2014)), the mechanisms underlying implantation remain largely unclear.

Cyclooxygenase type 2 (COX2), a rate-limiting enzyme for prostaglandins (PGs) production in multiple organs and tissues (Ruan *et al.* 2011), is abundantly expressed in the endometrium during implantation period in mice and humans (Lim *et al.* 1997, Critchley *et al.* 1999, Marions & Danielsson 1999) and known to be a key factor for embryo implantation. COX2-knockout mice show multiple problems in female reproduction, including implantation failure (Lim *et al.*

1997, 1999, Matsumoto *et al.* 2002). In humans, exposure to non-steroidal anti-inflammatory drugs (NSAIDs), which inhibit cyclooxygenases, during pregnancy especially at the time of conception is associated with over 80% increased risk of miscarriage (Li *et al.* 2003). COX2-derived prostaglandin E₂ (PGE₂) is considered as one of the most important signaling molecules for the initiation and progression of embryo implantation (Lim *et al.* 1997). Women in IVF trials with repeated implantation failure are reported to have defective endometrial prostaglandin synthesis (Achache *et al.* 2010). The expression of COX2 can be induced by various factors, among which, CREB is known to be a key transcription factor that binds to COX2 promoter region for its upregulation (Miller *et al.* 1998, Tsatsanis *et al.* 2006, Pham *et al.* 2008). However, the role of CREB in the regulation of COX2 in the endometrium during embryo implantation is not well studied.

COX2 expression can also be regulated by microRNAs (miRNAs), a family of short (18–25nt) non-coding RNAs (Chakrabarty *et al.* 2007, Akhtar & Haqqi 2012). In general, miRNAs are spatiotemporally expressed and,

by binding to 3' UTRs of their targeting mRNAs to induce mRNA degradation or translation repression, they are able to fine tune the expression of a series of their target genes and influence multiple physiological and pathological processes (He & Hannon 2004, Du & Zamore 2005, Ouellet *et al.* 2006, Nilsen 2007). In fact, miRNAs are emerging as a group of gene-expression modulators critically involved in embryo implantation (Chakrabarty *et al.* 2007, Hu *et al.* 2008, Nagaraja *et al.* 2008, Revel *et al.* 2011). They have been shown to exhibit dynamic temporal and spatial expression in both the uterus and the blastocyst during implantation (Hossain *et al.* 2012, Su *et al.* 2014). Interestingly, two miRNAs predicted to target COX2, miR-101a and miR-199a-3p, have also been reported to be spatiotemporally expressed in the uterus which coincides with the expression of COX2 during implantation in mice, suggesting the potential role of these miRNAs in the process (Chakrabarty *et al.* 2007). However, how these two miRNAs are regulated during embryo implantation remains unknown. Interestingly, CREB has recently been reported to have the capacity of repressing miR-101 transcription in embryonic fibroblasts (Kottakis *et al.* 2011).

The amiloride-sensitive epithelial sodium channel (ENaC) is expressed in various epithelia, including endometrial epithelium (Chan *et al.* 2002, Yang *et al.* 2004, Ruan *et al.* 2014), which is essential to sodium and water homeostasis in the body (Hummler *et al.* 1996, Salker 2010). ENaC has been shown to exhibit cyclic expression pattern during the estrous cycle with a high expression level observed at diestrus and peri-implantation period (Chan *et al.* 2002, Yang *et al.* 2004). Like its well-known role in other epithelia, ENaC has been demonstrated to be involved in uterine fluid absorption, which is suggested to be responsible for the reduced uterine fluid volume during embryo implantation (Chan *et al.* 2000a,b, 2001, Tsang *et al.* 2001, Ruan *et al.* 2014). It has been recently recognized that ENaC plays an essential role in the initiation of embryo implantation in addition to regulation of uterine fluid volume (Ruan *et al.* 2012). ENaC is shown to be activated by an embryo-released protease, which results in a sequence of events in the endometrial epithelial cells, including membrane depolarization, activation of voltage-sensitive Ca^{2+} channel, phosphorylation/activation of CREB, upregulation of COX2, and PGE_2 release, leading to stromal cell decidualization (Ruan *et al.* 2012), a prerequisite for successful embryo implantation (Cha *et al.* 2012). Inhibition or knockdown of ENaC in mice leads to disrupted decidualization and implantation failure, indicating the essential role of ENaC in embryo implantation (Ruan *et al.* 2012). The importance of ENaC in human embryo implantation is also highlighted by the observation on clinical samples showing lowered endometrial ENaC expression levels in women with failed pregnancy during their IVF trials compared with women with successful pregnancy (Ruan *et al.* 2012).

The observed phosphorylation of CREB upon activation of ENaC and the reported capacity of CREB in repressing one of the COX2-targeting miRNAs prompted us to hypothesize that in addition to directly inducing COX2 transcription, the ENaC-dependent CREB activation may lead to the suppression of miR-101 and miR-199a-3p, which may in turn augment COX2 upregulation during embryo implantation. This study, using mouse and human endometrial epithelial cells and a mouse *in vivo* implantation model along with gene knockdown assays, demonstrates that ENaC is involved in the regulation of the two COX2-targeting miRNAs, miR-101 and miR-199a-3p, through CREB phosphorylation during embryo implantation.

Materials and methods

Materials

Trypsin (catalog #93611), amiloride (catalog #A7410), and nifedipine (catalog #N-7634) were purchased from Sigma-Aldrich. *siGENOME* siRNA-SMARTpool targeting mouse *Creb1* (catalog #M-040959-01) and *siGENOME* non-targeting siRNA pool (catalog #D-001206-14) were purchased from Thermo Scientific, Waltham, MA, USA. *Stealth* siRNA (AAA GCA AAC UGC CAG UAC AUC AUG C and GCA UGA UGU ACU GGC AGU UUG CUU U) targeting mouse ENaC α , *Stealth* RNAi Negative Control Lo GC Duplex, *Silencer* siRNAs-targeting human *CREB1* (catalog #109994), *Silencer* negative control siRNAs (catalog #AM4611), miR-101 inhibitor (ID-MH11414), miR-199a-3p inhibitor (ID-AM11779), mirVana miRNA Inhibitor Negative Control #1 (catalog #4464077), and Lipofectamine 2000 transfection reagent (catalog #11668) were purchased from Invitrogen Life Technologies.

Mice and intrauterine injection

Female imprinting control region (ICR) mice were obtained from the Laboratory Animal Service Centre of the Chinese University of Hong Kong. All animal experiments were carried out in accordance with guidelines on animal experimentation, and approval by the Animal Ethics Committee of the Chinese University of Hong Kong. The day a vaginal plug was found after mating was designated as day 1 after mating. Surgery under general anesthesia was performed on day 3. Dorsal midline skin incision was made and followed by two small incisions into the muscle wall near each ovary to expose the uterine-oviduct connecting region. siRNAs (20 pmol) was mixed with Lipofectamine 2000 (1 μl) in Opti-MEM (20 μl , catalog #31985062, Invitrogen Life Technologies) for 30 min to 1 h at room temperature before being injected into the lumen of each uterine horn at the uterotubal junction. siRNA-NC and siRNA-CREB were each injected into a separate uterine horn in each mouse. Afterwards, wounds were closed by suture and the mice were placed on a 37 °C warmer till wake-up from the anesthesia. Temgesic was given to the mice by s.c. injection 12, 24, and 48 h after surgery to reduce the pain. The mice were closely monitored for 3 consecutive days after the surgery. On day 7, the mice were killed by anesthetic overdose.

or CO₂ asphyxiation and the implanted embryo numbers were counted.

Cell culture

Mouse primary endometrial epithelial cells (mEECs) were isolated and cultured as previously described (Chan *et al.* 2000a,b). Briefly, the uteri were collected from six to eight immature ICR mice (24 days of age), sliced longitudinally, and incubated in PBS with 6.5 mg/ml trypsin and 25 mg/ml pancreatin on ice for 60 min and then 45 min at room temperature. Afterwards, the uteri were transferred to fresh PBS and gently shaken for 30 s to release the epithelial cells. The isolated cells were cultured in DMEM/F-12 with 10% (v/v) fetal bovine serum (FBS) and 1% penicillin–streptomycin (v/v) in 5% CO₂ incubator at 37 °C till confluence before other experiments. The human endometrial surface epithelial (HES) cell line was a gift from Douglas A Kniss from the Laboratory of Perinatal Research at the Ohio State University (Kniss *et al.* 1997). The HES cells were cultured in DMEM supplemented with 10% FBS (v/v) and 1% penicillin–streptomycin (v/v) in 5% CO₂ incubators at 37 °C.

Gene knockdown

Lenti-virus (LV3) packaged shRNAs-targeting human ENaC α (5'-GTG GCT CCT ACA TCT TCT-3') or scrambled non-coding shRNAs (5'-TTC TCC GAA CGT GTC ACG TTT-3') were purchased from GenePharma (Shanghai, China). The viruses (2 $\times$ 10⁷ TU/ml) were transduced into HES cells with Polybrene (5 μ g/ml). The cells were cultured in the presence of puromycin (5 μ g/ml) for three passages to select stable clones before other experiments. The siRNAs-targeting human CREB1, non-targeting siRNAs, miR-101 inhibitor, miR-199a-3p inhibitor, or miRNA inhibitor negative control (100 nM) was transfected with Lipofectamine 2000 transfection reagent into HES cells. Forty-eight hours after transfection, the cells were collected for further studies.

Intracellular calcium imaging

The HES cells grown on cover slips were washed and loaded with Fura-2 (3 μ M, Invitrogen Life Technologies) at 37 °C for 30 min in Margo solution (in mM: NaCl 130, KCl 5, CaCl₂ 2.5, MgCl₂ 1, HEPES 20, and glucose 10). Fluorescence excited at 340 and 380 nm was monitored by an inverted microscope (Nikon Ti-U).

PGE₂ ELISA

The HES cells were grown in 24-well plates till 80% confluence before 1% FBS DMEM was used for 8 h to synchronize the cells. FBS-free DMEM was used for all the treatments and collected for the EIA Kit (catalog #514010, Cayman Chemical, Ann Arbor, MI, USA).

Western blotting analysis

The cells or tissues were lysed in radioimmunoprecipitation assay (RIPA) lysis buffer (NaCl 150 mM, Tris–Cl (pH=8.0)

50 mM, NP-40 1% v/v, sodium deoxycholate 0.5% w/v, and SDS 0.1% w/v) with protease inhibitor cocktail (catalog #88266, Thermo Scientific, Waltham, MA, USA) and 1 mM phenylmethylsulfonyl fluoride. The lysates were analyzed by SDS–PAGE followed by probing antibodies. Antibodies against ENaC α (1:500, catalog #sc-22239, Santa Cruz Biotechnology, Dallas, TX, USA), ENaC γ (1:1000, catalog #ab3468, Abcam, Cambridge, UK), COX2 (1:500, catalog #160106, Cayman Chemical), CREB (1:500, catalog #9197, Cell Signaling Technology, Danvers, MA, USA), and P-CREB (1:500, catalog #9198, Cell Signaling Technology) were used. For loading controls, antibodies against β -tubulin (catalog #sc-9104, Santa Cruz Biotechnology) and GAPDH (catalog #sc-47724, Santa Cruz Biotechnology) were used. The signal was detected with HRP-conjugated antibodies (catalog #170-6515, 170-6516, Bio-Rad and catalog #sc-2020, Santa Cruz Biotechnology) and visualized by ECL detection reagents (catalog #RPN2106, RPN2232, GE Healthcare, Little Chalfont, Buckinghamshire, UK). Densitometry of western blottings was performed using the freely available ImageJ Software.

RNA extraction and real-time PCR

The cells were lysed in TRIzol reagent (Invitrogen Life Technologies) according to manufacturer's instructions. Real-time PCR assays were carried out in triplicate on an Applied Biosystems 7500 Fast Real-Time PCR System. Analysis of ENaC α (Hs00168906_m1) and COX2 (Hs00153133_m1) was performed with TaqMan probe (Invitrogen Life Technologies). 18S rRNA (catalog #4319413E, Invitrogen Life Technologies) was used as internal control. Real-time PCR for miR-101 (ID-002253) and miR-199a-3p (ID-002304) was carried out with the microRNA Assay Kit (Invitrogen Life Technologies). SnoRNA202 (ID-001232, Invitrogen Life Technologies) was used as a control for mouse miRNAs and RNU48 (ID-001006, Invitrogen Life Technologies) was used as a control for human miRNAs.

Statistical analysis

The results are shown as mean $\pm$ S.E.M. Student's unpaired *t*-test was used for two-group comparison. One-way ANOVA was used for comparing three or more groups. *P* < 0.05 was considered as statistically significant.

Results

miR-101 and miR-199a-3p expression in mouse endometrial epithelial cells is dependent on ENaC *in vitro* and *in vivo*

To test if the expression of miR-101 and miR-199a-3p could possibly be subject to the change in ENaC function or expression in endometrial epithelial cells, we used previously established *in vitro* and *in vivo* mouse models in conjunction with the use of ENaC activator/inhibitor or siRNAs (Ruan *et al.* 2012). As shown in Fig. 1A, activation of ENaC by trypsin (20 μ g/ml, 15 min) in primary cultures of mouse endometrial epithelial cells (mEECs) induced a

Figure 1 ENaC-dependent miR-101 and miR-199a-3p expression in mouse endometrial epithelial cell *in vitro* and *in vivo*. (A) Real-time PCR analysis of miR-101 and miR-199a in isolated mouse endometrial epithelial cells treated with ENaC activator, trypsin (T, 20 µg/ml), ENaC blocker, amiloride (Ami, 10 µM), or L-type Ca^{2+} channel blocker, nifedipine (Nif, 10 µM). Ctrl, control cells. * $P < 0.05$; ** $P < 0.01$ and *** $P < 0.001$, $n = 3$. One-way ANOVA. (B and C) Real-time PCR analysis of miR-101 and miR-199a-3p (B) and western blotting for COX2 (C) in mouse uteri 24 h after uterine injection of siRNAs-targeting ENaCα (siRNA-ENaCα) or siRNAs with a scrambled sequence as negative control (siRNA-NC). β-tubulin was used as a loading control. * $P < 0.05$ and *** $P < 0.001$, $n = 4$, t -test.

significant reduction in miR-101 and miR-199a-3p levels, which could be reversed by the pretreatment with amiloride (10 µM, 30 min), an ENaC blocker, suggesting the involvement of ENaC in the regulation of these two miRNAs. Since we had shown previously that ENaC activation in mEECs resulted in Ca^{2+} influx through voltage-dependent Ca^{2+} channels, we also tested the effect of nifedipine, an inhibitor of L-type Ca^{2+} channel, on the expression of the two miRNAs. Indeed, similar to amiloride, pretreatment with nifedipine (10 µM, 30 min) also reversed the trypsin-induced decreases in miR-101 and miR-199a-3p expression levels (Fig. 1A), suggesting that these changes in miR-101 and miR-199a are subsequent to the ENaC activation-induced Ca^{2+}

mobilization. Next, we determined whether ENaC could regulate these two miRNAs *in vivo*. We previously (Ruan *et al.* 2012) showed that intrauterine injection of siRNAs targeting ENaC α subunit (siRNA-ENaCα) in mice successfully knocked down uterine ENaCα level at the implantation window (day 4 after mating), which resulted in a reduction in implantation rate in mice (Ruan *et al.* 2012). We used the same mouse implantation model in this study and found that when ENaC was knocked down, the expression levels of miR-101 and miR-199a-3p in the uteri were significantly increased as compared with that in the uteri injected with control siRNAs (siRNA-NC; Fig. 1B). More importantly, the increase in miR-101 and miR-199a-3p caused by ENaC knockdown was accompanied with a decrease in uterine COX2 protein level in siRNA-ENaCα-treated mice compared with the control (Fig. 1C), further indicating the dependence of these two miRNAs and their target gene, *Cox2*, on ENaC during implantation.

ENaC regulates COX2 through miR-101 and miR-199a-3p in human endometrial epithelial cells

The observed role of ENaC in the regulation of the two miRNAs on mouse models prompted us to examine its role in the regulation of the miRNAs in a human endometrial surface epithelial cell line (HES). We first confirmed the expression of ENaC subunits α and γ by western blotting analysis (Fig. 2A). ENaC knockdown was performed on HES cells by transducing lenti-virus (LV3) packaged shRNAs targeting to ENaCα (shRNA-ENaCα), with significant reduction in both mRNA and protein levels of ENaCα observed in HES cells (Fig. 2B). Similar to that observed in mouse models (Fig. 1), shRNA-ENaCα-treated HES cells showed significant decreases in COX2 protein level (Fig. 2C) and PGE_2 release (Fig. 2D), accompanied with increases in miR-101 and miR-199a-3p expression levels (Fig. 2E), as compared with the control cells transduced with LV3-packaged shRNAs of scrambled sequence (shRNA-Ctrl). The reciprocal expression levels between COX2 and the two miRNAs in response to ENaC knockdown in both HES (Fig. 2C and E) and mouse models (Fig. 1B and C) suggest that the regulation of COX2 expression by ENaC may involve posttranscriptional modulation by these miRNAs. To prove this, we transfected specific inhibitors of miR-101 and miR-199a-3p individually to HES cells and the western blotting results (Fig. 2F) showed that the ENaC-knockdown-induced reduction in COX2 protein level was significantly reversed by these inhibitors, indicating the involvement of these miRNAs in the modulation of the ENaC-regulated COX2 expression in HES cells.

ENaC-dependent regulation of miRNAs involves CREB phosphorylation in HES cells

We next examined the possible mechanism underlying the ENaC-dependent changes in the two miRNAs.

Figure 2 Involvement of miR-101 and miR-199a-3p in the modulation of ENaC-regulated COX2 expression in human endometrial epithelial (HES) cells. (A) Western blottings show ENaC α and γ subunits expression in HES cells. (B) Real-time PCR (left) and western blottings (right) show reduced ENaC mRNA and protein levels, respectively, after lenti-virus transduction with shRNA-targeting ENaC α (shRNA-ENaC α) or control shRNA (shRNA-Ctrl). (C and D) Western blotting for COX2 (C), ELISA for PGE₂ (D), and real-time PCR of miR-101 and miR-199a-3p (E) in HES cells treated with shRNA-Ctrl or shRNA-ENaC α . (F) COX2 protein levels in HES cells treated with shRNA-Ctrl, shRNA-ENaC α without or with the inhibitor of miR-101 (anti-miR-101) or miR-199a-3p (anti-miR-199a-3p), or the inhibitor control (anti-miR-Ctrl). β -tubulin was used as a loading control. * $P < 0.05$; ** $P < 0.01$; and *** $P < 0.001$, $n = 3$, t -test.

Since ENaC activation could lead to Ca^{2+} influx and CREB phosphorylation (Ruan *et al.* 2012) and CREB had also been implicated in repressing COX2-targeting miRNA (Kottakis *et al.* 2011), we tested the possible involvement of CREB phosphorylation in the regulation of the two COX2-targeting miRNAs. In HES cells, addition of trypsin (20 $\mu\text{g}/\text{ml}$) triggered intracellular Ca^{2+} increase, which was prevented by pretreatment with amiloride (10 μM , 30 min), nifedipine (1 μM , 30 min), or removal of extracellular Ca^{2+} (Fig. 3A). Also, trypsin (20 $\mu\text{g}/\text{ml}$) induced amiloride (10 μM)- and nifedipine (10 μM)-sensitive CREB phosphorylation (Fig. 3B), COX2

up-regulation (Fig. 3C), and PGE₂ release (Fig. 3D). At the same time, the trypsin treatment caused significant decreases in miR-101 and miR-199a-3p levels in HES cells (Fig. 3E), suggesting that activation of ENaC in HES cells can initiate a Ca^{2+} /CREB/COX2 signaling pathway leading to the miRNAs changes. To specifically test the role of ENaC in activation of CREB, we knocked down ENaC α in HES cells, which caused significant reduction in phosphorylated CREB level (Fig. 3F), confirming that activation of ENaC promotes CREB activation in HES cells. As shown in Fig. 2E, ENaC α knockdown in HES cell also caused increases in miR-101 and miR-199a-3p. Thus, the results suggest that CREB may be involved in the transcription of these two miRNAs in response to the change in ENaC expression.

miR-101/miR-199a-3p and COX2 expression levels in HES cells are dependent on CREB

To further investigate the role of CREB in regulation of miR-101 and miR-199a-3p, we performed CREB knockdown in HES cells. siRNAs targeting to CREB (siRNA-CREB) were transfected into HES cells, which caused blockage of CREB protein expression in the cells in 48 h (Fig. 4A). The siRNA-CREB-transfected HES cells showed significant increases in miR-101 and miR-199a-3p levels compared with the ones with control siRNAs (siRNA-NC). Meanwhile, the protein level of COX2 in siRNA-CREB treated cells was found decreased (Fig. 4C), which was partially reversed by transfection with miR-101 or miR-199a-3p inhibitors into HES cells (Fig. 4D). These results suggested an indirect role of CREB in regulation of COX2 through miR-101 and miR-199a-3p in HES cells.

Knockdown of CREB results in the alteration of miR-101 and miR-199a-3p expression and implantation failure in mice

We next tested whether the expression of miR-101 and miR-199a-3p may be affected by CREB during embryo implantation *in vivo*. To knockdown uterine CREB, siRNA-CREB was injected into uterine lumen in mice on day 3, which resulted in a decrease in uterine CREB protein level on day 7 (Fig. 5A). The number of the implanted embryos in uteri injected with siRNA-CREB was found to be significantly less than the ones in the control uteri injected with siRNA-NC on day 7 (Fig. 5B). At the same time, the expression of miR-101 and miR-199a-3p was significantly increased (Fig. 5C), and the COX2 protein level (Fig. 5D) decreased in the uteri treated with siRNA-CREB as compared with the controls.

Discussion

This study has demonstrated for the first time that ENaC activation in mouse and human endometrial epithelial

Figure 3 Involvement of Ca^{2+} /CREB signaling in ENaC-dependent regulation of COX2-targeting miRNAs in HES cells. (A) Fura-2 measurements of intracellular Ca^{2+} changes in responses to trypsin (T, 20 $\mu\text{g/ml}$), in the absence or presence of amiloride (Ami, 10 μM), nifedipine (Nif, 1 μM), or after removal of Ca^{2+} from the bath solution (Ca-free). *** $P < 0.001$ compared with the trypsin-treated group, $n = 3-4$, one-way ANOVA. (B) Western blotting for phosphorylated CREB (P-CREB) in HES cells under different conditions. The antibody also recognizes phosphorylated ATF1. (C and D) COX2 expression (C) and PGE_2 release (D) in HES cells under different conditions. Ctrl, control. Trypsin (T, 20 $\mu\text{g/ml}$), amiloride (Ami, 10 μM), nifedipine (Nif, 10 μM). (E) Real-time PCR analysis of miR-101 and miR-199a-3p in HES cells treated with trypsin (T, 20 $\mu\text{g/ml}$). (F) Western blotting for P-CREB in HES cells transfected with shRNA-Ctrl and shRNA-ENaC α . β -tubulin was used as a loading control, $n = 3$, * $P < 0.05$ and ** $P < 0.01$; t -test.

cells may lead to alteration in two COX2-targeting miRNAs through a CREB-dependent mechanism. The CREB-induced miR-101 and miR-199a-3p repression may contribute to the uterine COX2 upregulation in response to ENaC activation by embryo-derived protease, which may be important for successful embryo implantation.

Embryo implantation is a complex process involving a large network of genes (Cha *et al.* 2012, Koot *et al.* 2012). With their known capacity in the modulation of

multiple genes expression, miRNAs have gained increasing attentions in the research into the molecular mechanisms underlying the process of embryo implantation. A number of miRNAs have been detected in the endometrium at the time of implantation in mice or at mid-secretory phase, the receptive window, in humans (Chakrabarty *et al.* 2007, Hu *et al.* 2008, Revel *et al.* 2011). Among these miRNAs, some are predicted or proved to target genes that are critical for embryo implantation, including COX2 (Chakrabarty *et al.* 2007), HOXA10 (Estella *et al.* 2012), LIF (Altmae *et al.* 2013), as well as WNT signaling (Revel *et al.* 2011, Altmae *et al.* 2013). However, despite their recognized roles in embryo implantation, how the dynamic expression of miRNAs is controlled during implantation remains elusive. Although it has been reported that miRNAs are sensitive to environmental/extracellular cues, such as osmolality (Huebert *et al.* 2011), pH (Lu *et al.* 2012), and mechanical forces (Mohamed & Boriek 2010, Qin *et al.* 2010), the exact mechanisms responsible for linking extracellular signals to changes in miRNAs are largely unexplored. Interestingly, as membrane proteins with signal transduction capacity, ion channels have been proposed to be candidate epigenetic regulators in linking extracellular signals to miRNA alterations in the cell (Jiang *et al.* 2012). Indeed, our recent study has demonstrated the important role of an anion channel, CFTR, in transducing the signal from extracellular bicarbonate into activation of miR-125b targeting p53 during embryo development (Lu *et al.* 2012). This study also provides strong evidence

Figure 4 CREB-dependent miR-101 and miR-199a-3p expression in HES cells. (A) Western blottings show CREB levels in HES cells transfected with siRNA targeting to CREB (siRNA-CREB) or siRNAs with scrambled sequences as the negative control (siRNA-NC). (B and D) Real-time PCR analysis of miR-101 and miR-199a-3p (B) and western blotting for COX2 (C) in HES cells transfected with siRNA-CREB or siRNA-NC. (D) COX2 levels in HES cells transfected with siRNA-NC or siRNA-CREB without or with the inhibitor of miR-101 (anti-miR-101) or miR-199a-3p (anti-miR-199a-3p), or the inhibitor control (anti-miR-Ctrl). β -tubulin was used as a loading control. $n = 4$, * $P < 0.05$ and ** $P < 0.01$; t -test.

Figure 5 Effect of CREB knockdown on miR-101 and miR-199a-3p expression and embryo implantation in mice. (A) CREB levels in mouse uteri 4 days after the injection of siRNA-NC or siRNA-CREB. (B and D) Implanted embryo numbers (B), miR-101 and miR-199a-3p expression levels (C), and COX-2 protein levels (D) in mouse uteri on day 7 after the injection of siRNA-NC or siRNA-CREB. The photograph in (B) shows implantation sites (arrows) in the uterine horn injected with siRNA-NC (left) as compared with the one injected with siRNA-CREB (right). GAPDH was used as a loading control. The number of animals (n) = 10 (B) and 5 (C), *** P < 0.001, t -test.

supporting an important role of ion channels, ENaC in this case, in the regulation of miRNAs. We have previously shown that COX2 transcription in mouse endometrial epithelial cells could be upregulated by activating ENaC and subsequently CREB in response to the embryo-derived protease, trypsin (Ruan *et al.* 2012). Whereas this is consistent with the well-documented direct role of CREB in the regulation of COX2 (Miller *et al.* 1998, Tsatsanis *et al.* 2006, Pham *et al.* 2008), a recent study has implicated CREB in the regulation of COX2-targeting miR-101a (Kottakis *et al.* 2011), suggesting possible additional mechanism involving miRNAs in mediating the effect of ENaC on COX2 upregulation during embryo implantation. Indeed, the results of the present study have clearly demonstrated the ability of ENaC to regulate the expression of COX2-targeting miR-101 and miR-199a-3p in mouse and human endometrial epithelial cells through CREB phosphorylation. Inhibition or knockdown of ENaC resulted in increased expression of the two miRNAs with decreased expression level of COX2. Conversely, activation of ENaC by trypsin, a protease known to be released by implanting embryo (Sawada *et al.* 1990), produced opposite effects on the miRNAs and COX2, providing the first evidence that COX2-targeting miRNAs can be regulated by the embryo-derived factor through ENaC. Taken together, these results suggest that miR-101 and miR-199a-3p are involved in the modulation of the expression of COX2 in response to ENaC activation during implantation. Interestingly, ENaC is also known to

be activated by mechano-stimuli (Fronius & Clauss 2008) and its expression in the endometrium is subject to alteration in ovarian hormones (Chan *et al.* 2002, Yang *et al.* 2004). Together with the demonstrated capacity of ENaC in the regulation of miRNAs, these suggest that ENaC might mediate other types of signals in regulation of miRNAs in different physiological contexts. For example, while 17β -estradiol (E_2) is known to down-regulate uterine ENaC in mice (Chan *et al.* 2002), it has also been reported to promote miR-199a expression in rat uterus (Xia *et al.* 2014). The promoting effect of E_2 on the miRNA might be mediated through its downregulating effect on ENaC, given the present results showing the upregulation of miR-199a in response to inhibition or knockdown of ENaC. As ENaC is expressed along the entire female reproductive tract, it would be interesting to explore whether ENaC may regulate other miRNAs

Figure 6 Working model for ENaC/CREB-dependent regulation of COX2 expression during embryo implantation. ENaC activation by serine proteases from the embryo causes epithelial cell membrane depolarization (ΔV_m) that in turn activates L-type Ca^{2+} channel with Ca^{2+} influx leading to phosphorylation of CREB, which can directly trigger the transcription of COX2 (Ruan *et al.* 2012). In addition, as demonstrated in this study, the ENaC-dependent CREB activation also suppresses miR-101 and miR-199a-3p, which in turn post-transcriptionally upregulates COX2 in human endometrial epithelial cells. The ENaC/CREB-regulated COX2 upregulation leads to PGE_2 production and release, which is essential for stromal decidualization and embryo implantation (Ruan *et al.* 2012).

targeting different genes that are important for different processes of reproduction.

This study has also demonstrated a critical role of CREB in mediating the effect of ENaC in the regulation of the two COX2-targeting miRNAs. This is evident by the observation that knockdown of CREB *in vitro* or *in vivo* led to upregulation of miR-101 and miR-199a-3p expression levels, indicating an important role of CREB in repressing these two miRNAs. Interestingly, in a previous study, CREB has been demonstrated to drive the transcription of NDY1, which in turn suppresses miR-101a expression in mouse embryonic fibroblasts (Kottakis *et al.* 2011). Whether such a mechanism also underlies the presently observed regulatory action of CREB on miR-101 and miR-199a-3p in endometrial epithelial cells awaits further investigation. It should be noted that CREB is well known for its ability to bind directly to the promoter region of COX2 and transcriptionally potentiate COX2 (Miller *et al.* 1998, Tsatsanis *et al.* 2006, Pham *et al.* 2008). We have also shown previously that ENaC-dependent CREB activation promotes COX2 transcription in mouse endometrial epithelial cells (Ruan *et al.* 2012). The present observation that the decreases in COX2 protein levels induced by knockdown of CREB or ENaC can be partially reversed by inhibiting miR-101 or miR-199a-3p (Figs 2 and 4) indicates the involvement of the miRNAs in the modulation of the ENaC/CREB-induced upregulation of COX2. This also suggests that the ENaC-dependent CREB activation, which is subsequent to the ENaC-induced membrane depolarization and Ca^{2+} mobilization (Ruan *et al.* 2012; Fig. 6) in endometrial epithelial cells, could possibly play a dual role, driving COX2 transcription directly on the one hand, and upregulating COX2 indirectly, on the other hand, through repressing the two miRNAs that post-transcriptionally modulate COX2 expression, as depicted in the working model (Fig. 6). Since COX2 is indispensable for normal implantation (Lim *et al.* 1997), the fine tuning of COX2 expression by ENaC/CREB-dependent miRNAs may be necessary for successful implantation. Of note, CREB has been reported to be activated in decidua cells (Kawarabayashi *et al.* 2012, Kusama *et al.* 2014) and implicated in pre-implantation embryo development (O'Neill *et al.* 2012). This study has demonstrated, for the first time, a critical role of CREB in embryo implantation *in vivo*, as evidenced by the CREB knockdown-induced implantation failure observed in this study. However, further study is needed to assess the exact contribution of the two miRNAs in the process.

The study on embryo implantation in humans is difficult, particularly due to the ethical concerns and technical limitation upon humans, which is in fact precluding the advance in assistant reproductive technology. The present data obtained from human endometrial cells are consistent with those from mouse models, suggesting the relevance of ENaC-dependent

regulation of miRNAs in human embryo implantation. The ability of ENaC to regulate COX2-targeting miRNAs, in addition to its previously demonstrated role in the regulation of PGE₂ production and release during embryo implantation (Ruan *et al.* 2012), supports a critical and versatile role of ENaC in regulating the process of embryo implantation. Whereas previous study has demonstrated abnormally downregulated ENaC expression in women with implantation failure (Ruan *et al.* 2012), the currently demonstrated involvement of CREB and the miRNAs in the modulation of ENaC-regulated COX2 expression during embryo implantation suggests potential molecular targets, in addition to ENaC, for diagnosis and treatment of implantation failure, which may also have applications for contraception.

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.

Funding

The work was supported in part by grants from the National Natural Science Foundation of China (81370709), National 973 project of China (2012CB944903), K.S. Lo Foundation, and the Focused Investment Scheme of the Chinese University of Hong Kong.

Author contribution statement

H C Chan conceived the study. H C Chan, H Chen, and Y C Ruan designed the experiments. X Sun, Y C Ruan, J Guo, H Chen, L L Tsang, and X Zhang performed the experiments and analyzed the data. X Jiang provided intellectual input. X Sun, Y C Ruan, and H C Chan wrote the paper.

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Received 31 July 2014

First decision 21 August 2014

Revised manuscript received 25 August 2014

Accepted 2 September 2014