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Activation of the epithelial Na^+ channel triggers prostaglandin E_2 release and production required for embryo implantation

Ye Chun Ruan[1,2,6], Jing Hui Guo[1,6], Xinmei Liu[3], Runju Zhang[3], Lai Ling Tsang[1], Jian Da Dong[1,2], Hui Chen[1], Mei Kuen Yu[1], Xiaohua Jiang[1,4], Xiao Hu Zhang[1], Kin Lam Fok[1], Yiu Wa Chung[1], Hefeng Huang[3], Wen Liang Zhou[2] & Hsiao Chang Chan[1,4,5]

Embryo implantation remains a poorly understood process. We demonstrate here that activation of the epithelial Na^+ channel (ENaC) in mouse endometrial epithelial cells by an embryo-released serine protease, trypsin, triggers Ca^{2+} influx that leads to prostaglandin E_2 (PGE_2) release, phosphorylation of the transcription factor CREB and upregulation of cyclooxygenase 2, the enzyme required for prostaglandin production and implantation[1–3]. We detected maximum ENaC activation, as indicated by ENaC cleavage[4], at the time of implantation in mice. Blocking or knocking down uterine ENaC in mice resulted in implantation failure. Furthermore, we found that uterine ENaC expression before *in vitro* fertilization (IVF) treatment is markedly lower in women with implantation failure as compared to those with successful pregnancy. These results indicate a previously undefined role of ENaC in regulating the PGE_2 production and release required for embryo implantation, defects that may be a cause of miscarriage and low success rates in IVF.

Embryo implantation, a rate-limiting step in human reproduction, is initiated by embryo attachment to endometrial epithelium followed by the so-called decidualization process in the stroma beneath the epithelium into large, round or polygonal decidual cells. The stromal deicidualization is considered a maternal prerequisite for embryo implantation and successful pregnancy[5]. Ultrastructural studies of early stages of implantation have demonstrated a physical interaction between the embryo and endometrial epithelium[6,7] that is facilitated by the uterine 'closure' resulting from the removal of luminal fluid by transepithelial electrolyte and fluid reabsorption during the peri-implantation period[8,9]. It has long been observed in rodents that decidualization can be induced, in the absence of the embryo, by mechanical stimuli such as intraluminal injection of oil or air[10], or even scratching on the endometrium[11], resulting in pseudopregnancy. Notably, artificially stimulated decidualization in rodents cannot occur when the endometrial epithelium is destroyed or removed[12], suggesting the involvement of endometrial epithelium in decidualization. Clinical studies have shown that local injury to the endometrium (such as endometrial biopsy) promoted the implantation rate in women who had repeated implantation failure after IVF cycles[13]. Apart from physical signals, many different molecules have also been implicated as chemical signals for embryo implantation[14,15], among which serine proteases are abundantly found in the embryo-endometrium interface, released by the invading embryo and essential or implantation[14,16]. At the epithelium-stroma interface, prostaglandins, which are synthesized from the enzymatic conversion of phospholipase A_2 (PLA_2)-derived arachidonic acid by cyclooxygenases[1], have been shown to be essential decidualizing molecules[2,3]. However, it remains unclear how signals from the embryo (either physical or chemical) at the endometrial surface are converted into chemical signals such as prostaglandins, leading to decidualization in stromal cells that do not have direct contact with the embryo.

The amiloride-sensitive ENaC, encoded by *SCNN1* genes within the degenerin/ENaC superfamily[17], is localized in the apical membrane of a wide variety of epithelia, including endometrial epithelium[18]. We have previously observed upregulation of endometrial ENaC expression during the peri-implantation period in mice, and, given the well-established role of ENaC in electrolyte and water reabsorption[17], we have proposed that this ENaC upregulation is responsible for the disappearance of uterine fluid, or the closure of the uterine lumen, normally observed during this period[18]. Notably, ENaC has also been shown to be activated by mechanical force and serine proteases in various tissues[4,19,20]. In addition, both mechanical stimulation and serine proteases have been reported to promote prostaglandin release from esophageal and airway epithelial cells[21–23]. Therefore, we asked

[1]Epithelial Cell Biology Research Centre, School of Biomedical Sciences, Faculty of Medicine, The Chinese University of Hong Kong, Hong Kong. [2]School of Life Science, Sun Yat-sen University, Guangzhou, China. [3]Department of Reproductive Endocrinology, Key Laboratory of Reproductive Genetics of Ministry of Education of the People's Republic of China, Women's Hospital, Zhejiang University School of Medicine, Hangzhou, China. [4]Key Laboratory for Regenerative Medicine (Ji Nan University–The Chinese University of Hong Kong), Ministry of Education of the People's Republic of China, China . [5]Sichuan University–The Chinese University of Hong Kong Joint Laboratory for Reproductive Medicine, West China Second University Hospital, Chengdu, China. [6]These authors contributed equally to this work. Correspondence should be addressed to H.C.C. (hsiaocchan@cuhk.edu.hk), W.L.Z. (lsszwl@yahoo.com.cn, lsszwl@mail.sysu.edu.cn) or H.H. (huanghefg@hotmail.com).

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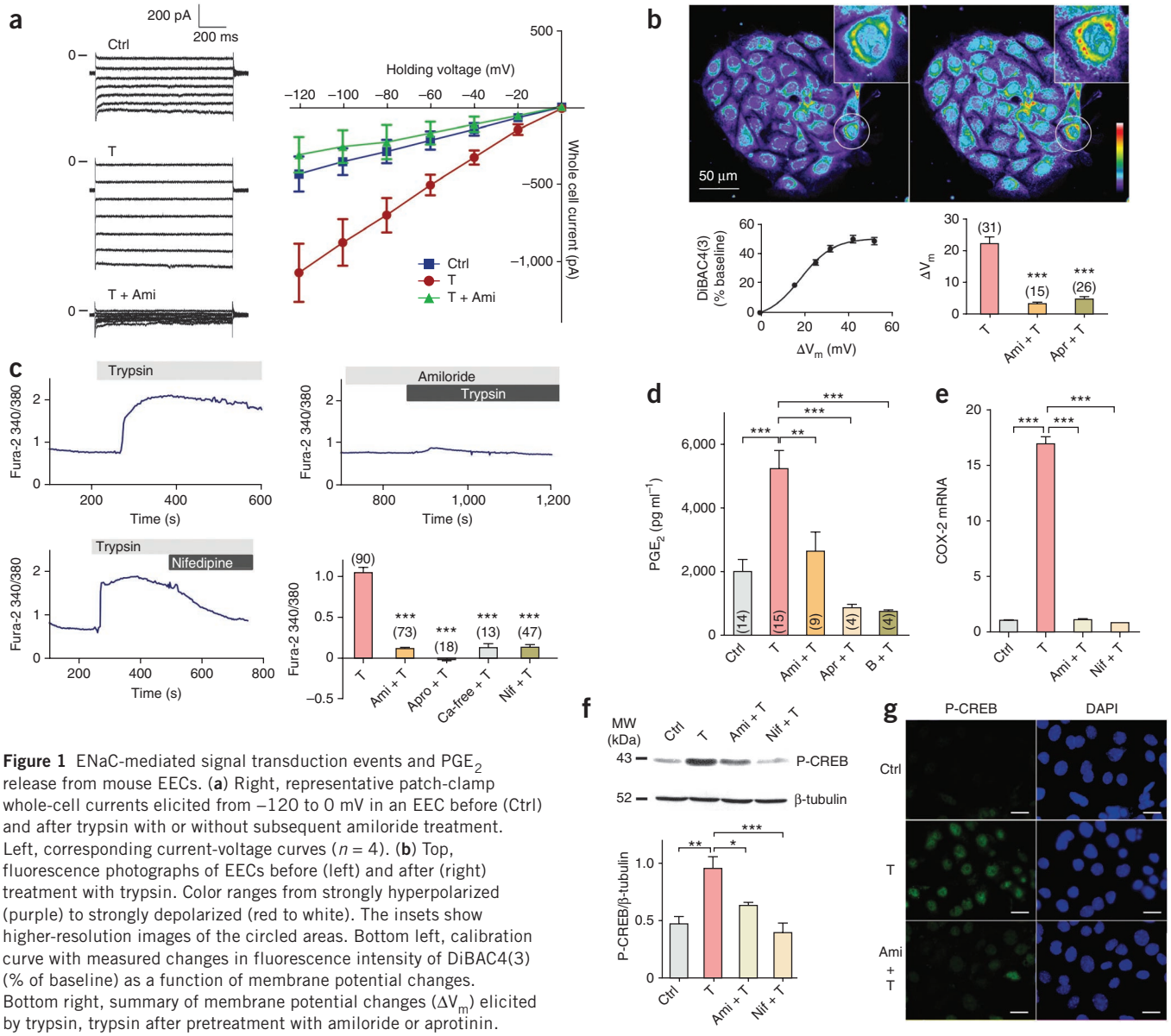

Figure 1 ENaC-mediated signal transduction events and PGE_2 release from mouse EECs. (**a**) Right, representative patch-clamp whole-cell currents elicited from −120 to 0 mV in an EEC before (Ctrl) and after trypsin with or without subsequent amiloride treatment. Left, corresponding current-voltage curves ($n = 4$). (**b**) Top, fluorescence photographs of EECs before (left) and after (right) treatment with trypsin. Color ranges from strongly hyperpolarized (purple) to strongly depolarized (red to white). The insets show higher-resolution images of the circled areas. Bottom left, calibration curve with measured changes in fluorescence intensity of DiBAC4(3) (% of baseline) as a function of membrane potential changes. Bottom right, summary of membrane potential changes (ΔV_m) elicited by trypsin, trypsin after pretreatment with amiloride or aprotinin. Number of measurements is shown in each column (***$P < 0.001$ compared to the trypsin-treated group). (**c**) Representative time-course changes in intracellular Ca^{2+} levels (indicated by 340/380 ratio of Fura-2) in EECs in response to trypsin in the presence of amiloride or nifedipine, with corresponding statistic analysis (***$P < 0.001$ compared to the trypsin-treated group). (**d**) Summary of trypsin-induced PGE_2 release from 3-d cultures of EECs (**$P < 0.01$, ***$P < 0.001$). (**e**) Quantitative PCR analysis of COX-2 mRNA levels in EECs under different conditions ($n = 4$, ***$P < 0.001$). (**f**) Western blot analysis of protein amounts of phosphorylated CREB (P-CREB). Data are presented as the ratio of P-CREB level to the level of β-tubulin ($n = 4$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). (**g**) Fluorescence labeling of P-CREB in EECs with DAPI-labeled nuclei (scale bars, 20 µm). T, trypsin (20 µg ml^{-1}); Ami, amiloride (10 µM); Apr, aprotinin (20 µg ml^{-1}); Nif, nifidipine (10 µM); B, BAPTA/AM (50 µM). Data are means ± s.e.m.

whether the activation of ENaC, which is sensitive to mechanical stimulation and proteases and whose expression is upregulated in the endometrium during peri-implantation, could trigger a sequence of events leading to the production and release of prostaglandins required for stromal decidualization and thus embryo implantation.

Using a primary culture of mouse endometrial epithelial cells (EECs) in conjunction with the patch-clamp technique, we observed that trypsin (20 µg ml^{-1}), a serine protease known to activate ENaC[24,25] and be released by invading embryos[16], induced an increase in inward whole-cell currents, which was abolished by subsequent addition of amiloride (10 µM), an ENaC inhibitor (**Fig. 1a**), indicating Na^+ influx. The immediate consequence of Na^+ influx upon ENaC activation would be membrane depolarization, and, indeed, trypsin (20 µg ml^{-1}) induced membrane depolarization (22.24 mV ± 2.2 mV) in EECs, which was abrogated by either amiloride (10 µM) or aprotinin (20 µg ml^{-1}), a protease inhibitor (**Fig. 1b**). Voltage-sensitive Ca^{2+} channel activities, which are known to be activated by membrane depolarization, have been suggested as a requirement for decidualization in mice[26]. Also, Ca^{2+} mobilization has been reported to facilitate cyclooxygenase-2 (COX-2)-dependent production of PGE_2 (ref. 27). We thus hypothesized that the serine protease–induced, ENaC-mediated membrane depolarization could result in Ca^{2+} mobilization leading to PGE_2 production or release. Addition of trypsin (20 µg ml^{-1}) induced sustained Ca^{2+} elevation in EECs,

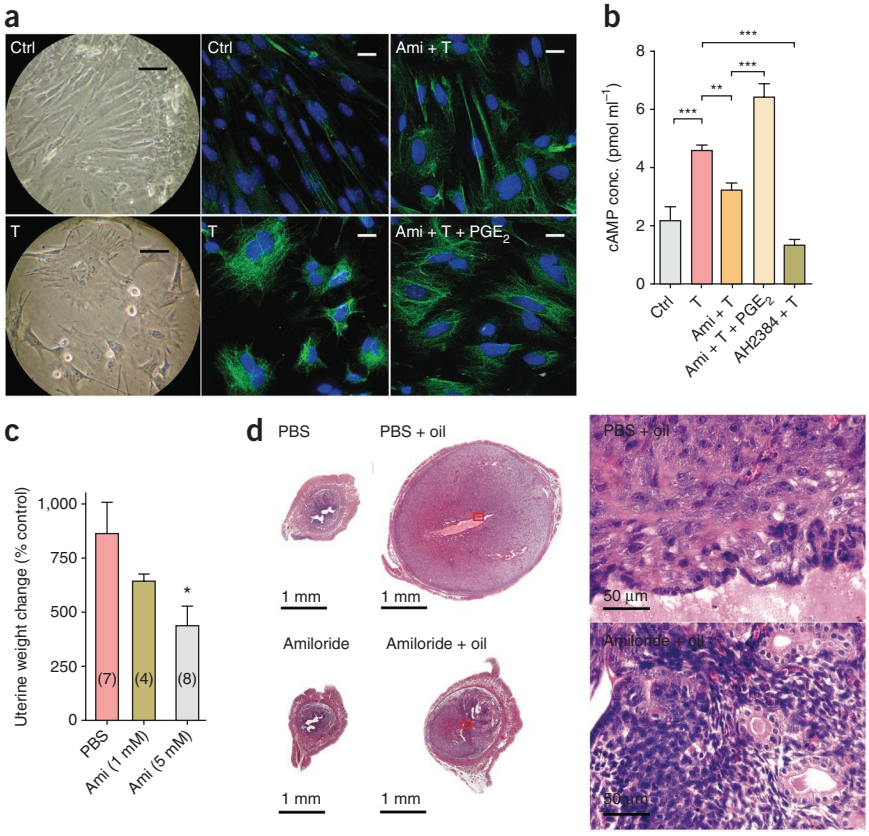

Figure 2 Involvement of ENaC in decidualization. (**a**) Bright-field photographs (scale bars, 50 μm) show the morphology of the cocultured stromal cells before (Ctrl) and after treatment with trypsin (T, 20 μg ml^{-1}). Fluorescence photographs (scale bars, 20 μm) show signal from fluorescently labeled antibody to desmin in the cocultured stromal cells before and after trypsin treatment, in the absence or presence of amiloride (Ami, 10 μM) or PGE_2 (10 μM). (**b**) Summary of intracellular cAMP concentration in cocultures in the presence or absence of trypsin, amiloride (Ami, 10 μM) or the EP4 receptor antagonist AH2384 (AH, 10 μM). $n = 6$, $^{**}P < 0.05$, $^{***}P < 0.001$. (**c**) Effect of amiloride on uterine weight change 3 d after oil-induced decidualization in pseudopregnant mice. Data are shown as percentage of controls without oil injection; the number of mice examined is shown in each column, $^{*}P < 0.05$. (**d**) H&E-stained micrographs showing uterine cross-sections from pseudopregnant mice 3 d after intrauterine injection of PBS, PBS with oil, amiloride (5 mM) or amiloride (5 mM) with oil. Higher-magnification micrographs show the framed areas. Data are means ± s.e.m.

which was inhibited by amiloride (10 μM) and aprotinin (20 μg ml^{-1}) (**Fig. 1c**). The trypsin-induced Ca^{2+} rise was largely lessened in Ca^{2+}-free solutions, suggesting Ca^{2+} influx through Ca^{2+} channels (**Fig. 1c**). Consistent with this notion, nifedipine (10 μM), a blocker of the voltage-dependent Ca^{2+} channel, abolished the trypsin-induced Ca^{2+} rise (**Fig. 1c**). We also detected L-type voltage-dependent Ca^{2+} channel mRNA expression and functional channel activities in EECs by the patch-clamp technique (**Supplementary Fig. 1**), indicating the involvement of L-type Ca^{2+} channel in the process. Using ELISA, we found that trypsin (20 μg ml^{-1}) could induce a nearly threefold increase, as compared to untreated controls, in PGE_2 release from the epithelial culture that could be attenuated by pretreatment with either amiloride (10 μM) or aprotinin (20 μg ml^{-1}) (**Fig. 1d**). Moreover, the trypsin-induced PGE_2 release from EECs was abolished by BAPTA/AM (50 μM), an intracellular calcium chelator (**Fig. 1d**), suggesting the involvement of Ca^{2+} in triggering PGE_2 release. Taken together, these results suggest that activation of ENaC by trypsin may result in membrane depolarization, which in turn leads to Ca^{2+} influx, resulting in PGE_2 release from EECs.

It has been suggested that continuous elevation of prostaglandin expression is required for decidualization[28]. Does ENaC also have a role in prostaglandin production during implantation, for example,

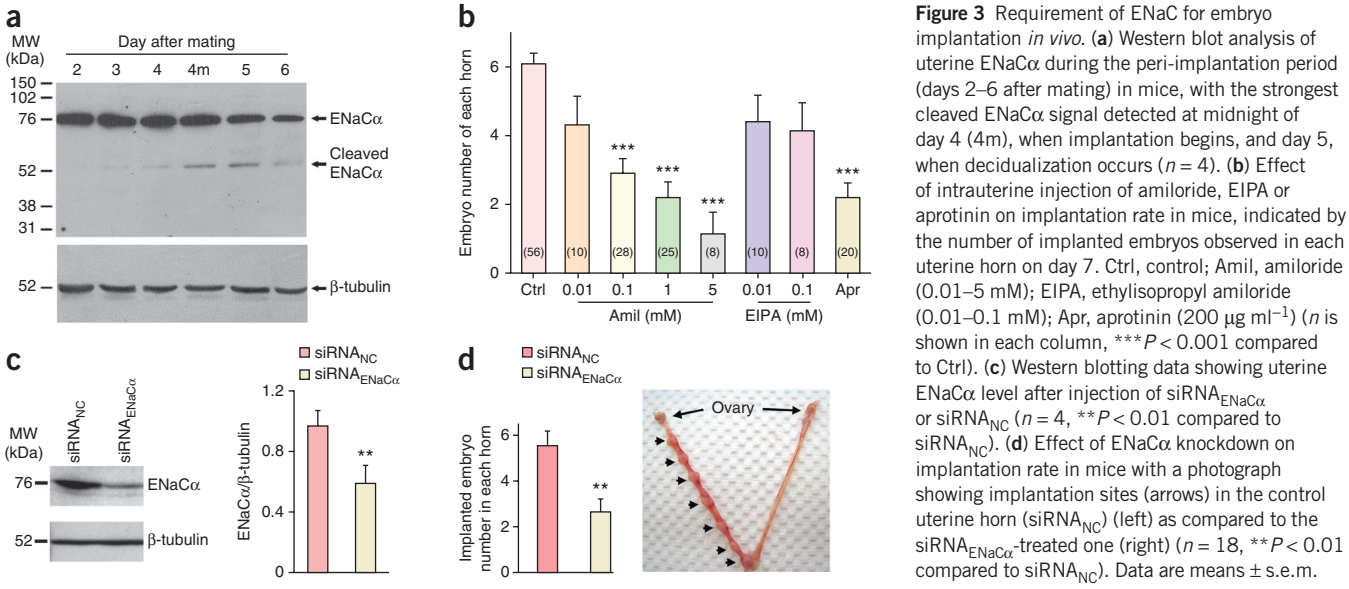

Figure 3 Requirement of ENaC for embryo implantation *in vivo*. (**a**) Western blot analysis of uterine ENaCα during the peri-implantation period (days 2–6 after mating) in mice, with the strongest cleaved ENaCα signal detected at midnight of day 4 (4m), when implantation begins, and day 5, when decidualization occurs ($n = 4$). (**b**) Effect of intrauterine injection of amiloride, EIPA or aprotinin on implantation rate in mice, indicated by the number of implanted embryos observed in each uterine horn on day 7. Ctrl, control; Amil, amiloride (0.01–5 mM); EIPA, ethylisopropyl amiloride (0.01–0.1 mM); Apr, aprotinin (200 μg ml^{-1}) (n is shown in each column, $^{***}P < 0.001$ compared to Ctrl). (**c**) Western blotting data showing uterine ENaCα level after injection of $siRNA_{ENaC\alpha}$ or $siRNA_{NC}$ ($n = 4$, $^{**}P < 0.01$ compared to $siRNA_{NC}$). (**d**) Effect of ENaCα knockdown on implantation rate in mice with a photograph showing implantation sites (arrows) in the control uterine horn ($siRNA_{NC}$) (left) as compared to the $siRNA_{ENaC\alpha}$-treated one (right) ($n = 18$, $^{**}P < 0.01$ compared to $siRNA_{NC}$). Data are means ± s.e.m.

by regulating the expression of COX-2, a key enzyme in prostaglandin synthesis? As shown by quantitative PCR (**Fig. 1e**), we found a significantly elevated mRNA level of COX-2 in EECs after a 15-min treatment with trypsin (20 μg ml^{-1}) that was abolished by pretreating the cells with either amiloride (10 μM) or nifidipine (10 μM). These results suggest that upregulation of COX-2 in EECs may be induced by ENaC activation–dependent Ca^{2+} influx. In this regard, transcription of COX-2 has been reported to be activated by the phosphorylation of cAMP/Ca^{2+} response element–binding protein (CREB), a transcription factor known to regulate prostaglandin production[29]. Indeed, western blot analysis showed that amounts of the phosphorylated form of CREB (P-CREB) were significantly increased in EECs 15 min after treatment with trypsin (20 μg ml^{-1}), which was attenuated by the pretreatment with amiloride (10 μM) or nifidipine (10 μM) (**Fig. 1f**). Immunofluorescence also showed an increased accumulation of P-CREB in the nuclei of cultured EECs after treatment with trypsin that was blocked by amiloride (**Fig. 1g**). These results suggest that ENaC activation may regulate transcriptional activities of COX-2.

Next, we tested whether the activation of endometrial ENaC by trypsin could indeed lead to stromal cell decidualization. We established an epithelial-stromal coculture and found that treatment of the coculture with trypsin (20 μg ml^{-1}, 4–6 h) induced substantial morphological changes of the stromal cells from elongated into polygonal and multinuclear cells (**Fig. 2a**), characteristic of decidual cells[30]. In addition, the expression of desmin, a reported marker of decidual cells[30], was upregulated in the trypsin-treated coculture, as indicated by immunostaining (**Fig. 2a**). Pretreatment with amiloride (10 μM) attenuated the effect of trypsin, whereas the inhibitory effect of amiloride could be reversed by adding PGE_2 (10 μM) to the coculture (**Fig. 2a**). As a decidualizing molecule[28], PGE_2 is believed to activate EP2 or EP4 receptors on stromal cells[31], which in turn leads to the accumulation of intracellular cAMP ($[cAMP]_i$)[32] and thus decidualization. We tested whether ENaC activation could lead to EP2 or EP4 receptor-mediated elevation of cAMP in EECs. We added trypsin (20 μg ml^{-1}, 20 min) to the EEC-stroma coculture and found elevated $[cAMP]_i$, which could be attenuated by pretreatment with amiloride (10 μM) (**Fig. 2b**). Consistent with the morphological result (**Fig. 2a**), adding PGE_2 could also reverse the inhibitory effect of amiloride on the trypsin-induced $[cAMP]_i$ in the coculture. Moreover, the EP4 receptor antagonist, AH2384 abolished the enhancing effect of trypsin on $[cAMP]_i$ in the coculture (**Fig. 2b**), confirming the involvement of the prostaglandin receptor in mediating the effect of ENaC activation on intracellular cAMP production. These results clearly demonstrated that the activation of ENaC by trypsin could lead to stromal decidualization involving PGE_2 and its receptor.

We further tested whether blocking ENaC could interfere with decidualization *in vivo* using a pseudopregnant mouse model with oil-induced decidualization[33]. Intrauterine injection of oil with PBS caused an ~8.5-fold higher uterine weight as compared to the weight in mice injected with PBS alone, suggesting successful induction of decidualization in these mice (**Fig. 2c**,**d**). Injection of amiloride (5 mM) with oil significantly ($P < 0.05$) reduced the uterine weight change, as well as the number of decidual cells (**Fig. 2c**,**d**). Both *in vitro* and *in vivo* results indicate a crucial role for ENaC in decidualization.

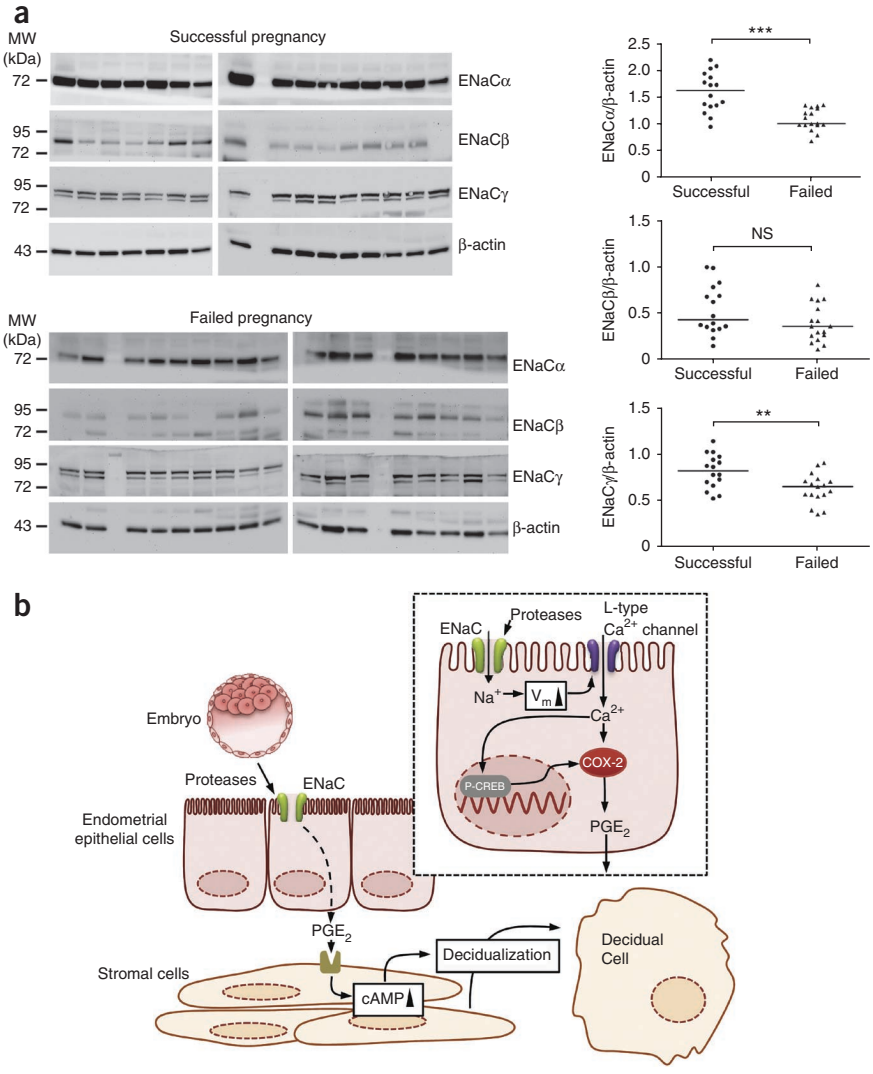

Figure 4 Abnormal ENaC expression in women with IVF failure and working model of ENaC in initiating decidualization. (**a**) Western blot analysis of protein levels of ENaC α, β and γ subunits from endometrial biopsy samples collected before the cycle of IVF and embryo transfer. Data are presented as the ratio of ENaC α, β and γ subunit levels to the level of β-actin. $n = 16$ (successful pregnancy) and 17 (failed pregnancy). $^{**}P < 0.01$; $^{***}P < 0.001$; NS, $P > 0.05$. Other parameters of the patients are not significantly different between the two groups, as shown in **Supplementary Table 2**. (**b**) Schematic drawing of ENaC involvement in decidualization. ENaC activation by serine proteases from the embryo causes epithelial cell membrane depolarization that activates L-type Ca^{2+} channel and Ca^{2+} influx leading to release of PGE_2 and phosphorylation of CREB that may upregulate the expression of COX-2. The endometrial epithelial released-PGE_2 in turn activates cAMP-related pathways in stromal cells, leading to stromal decidualization.

We next aimed to verify the involvement of ENaC in embryo implantation. We first tried to detect ENaC cleavage, an indication of ENaC activation[4], during the peri-implantation period in mouse uteri (days 2–6 after mating). By western blot analysis, we were able to clearly detect cleaved ENaCα (around 52 kDa) beginning approximately at midnight of day 4, when embryo implantation occurs in mice, and it persisted on day 5, when decidualization begins (**Fig. 3a**). We next tested whether interfering with ENaC function *in vivo* could impair implantation. Intrauterine injection with amiloride (100 μM–5 mM) induced a dose-dependent reduction in the number of implanted embryos in the amiloride-treated uterine horns compared with that in the vehicle-treated control horns (**Fig. 3b**). To exclude a possible non-specific effect of amiloride on the sodium-proton exchanger (NHE), we also applied ethyl isopropyl amiloride (EIPA), a specific blocker of NHE. Unlike amiloride injection, injection with EIPA (100 μM) showed no significant effect on implantation rate in mice (**Fig. 3b**). To confirm that serine proteases, which activate ENaC, are required for implantation, we also applied the protease inhibitor aprotinin to the pregnant mice. Similar to amiloride, aprotinin (200 μg ml^{-1}) also significantly reduced the number of implanted embryos (**Fig. 3b**). Together with the observed ENaC cleavage (or activation) at the time of implantation, the impaired implantation resulted from interfering with ENaC activation, either by protease inhibitor or ENaC blocker treatment, indicates that ENaC activation is required for embryo implantation.

The involvement of ENaC in implantation was further confirmed by knocking down ENaC *in vivo* in mice using siRNAs targeting ENaCα ($siRNA_{ENaC\alpha}$) (**Fig. 3c**), and the results showed that the implanted embryo number counted on day 7 of pregnancy in ENaC-knockdown uterine horns (**Fig. 3d**), as well as the number of decidual cells (**Supplementary Fig. 2a**), was significantly lower compared to the control treated with negative control siRNAs ($siRNA_{NC}$), demonstrating the requirement of ENaC for implantation. The expression of three implantation markers, HoxA10, IgF2 and Lif, was also drastically reduced when ENaC was inhibited or knocked down (**Supplementary Fig. 2b**), suggesting that ENaC activation is a key upstream event during implantation.

Despite major improvements in assisted reproduction techniques (ARTs), the clinical pregnancy rate per embryo transfer in fresh ART cycles, or successful rate of IVF, remains at a low rate of 31% (ref. 34). Given that ENaC is required for embryo implantation, as we observed here in mice, alteration in ENaC expression or function may be a cause of implantation failure during IVF in humans. To test this, we examined the expression levels of the rate-limiting channel subunit ENaCα in a cohort of human endometrial samples obtained before IVF treatment and compared them between women with successful ($n = 16$) and failed ($n = 16$) pregnancy. The demographic and clinical data of the subjects showed no significant differences in age, ovarian hormonal profiles, endometrial thickness or oocyte quality between the two groups (**Supplementary Table 1**). However, the endometrial samples from women with failed pregnancy after IVF and embryo transfer showed a significantly lower average level of ENaCα subunit expression as compared to those from women with successful pregnancy ($P < 0.05$, **Supplementary Fig. 3**). In another cohort of samples from successful ($n = 16$) and failed ($n = 17$) pregnancy after IVF and embryo transfer, we examined the expression of all three ENaC subunits (α, β and γ). In addition to a significant difference observed for ENaCα ($P < 0.001$), the levels of ENaCγ, the subunit that has the protease activation site[35], were also significantly lower in the failed group than in the successful group ($P < 0.01$), whereas no significant difference observed for ENaCβ (**Fig. 4a**). These results, together with the results obtained from mice, suggest that ENaC activation is required for implantation and that abnormal ENaC expression could lead to implantation failure in humans.

We have demonstrated a crucial role for ENaC in signal transduction leading to stromal decidualization and thus embryo implantation, in addition to its previously reported role in regulating uterine fluid volume during the peri-implantation period[18]. As ENaC expression is subject to regulation by ovarian hormones[36], its normal expression pattern may be altered during IVF with ovarian overstimulation, which may contribute to the low pregnancy rate achieved through IVF. Thus, defects in ENaC, either in its expression or its function, may be one of the underlying mechanisms for spontaneous miscarriage and implantation failure during IVF. Of note, a recent study has reported that deregulation of SGK1, the serum- and glucocorticoid-inducible kinase and key regulator of ENaC, in the endometrium could interfere with embryo implantation[37], supporting a key role of ENaC in the process.

Taken together, the present results have revealed a previously unsuspected role of ENaC in regulating prostaglandin production and release required for embryo implantation (**Fig. 4b**). Given that ENaC is mechanosensitive[20], the presently demonstrated involvement of ENaC in decidualization also provides an explanation for the long-observed induction of decidualization by mechanical stimuli in animal models and improved implantation rate in women undergoing IVF after endometrial scratch[13]. The ability of ENaC to regulate PGE_2 production and release, as we showed here, may have far-reaching implications beyond embryo implantation or reproduction, as ENaC is widely distributed throughout the body and PGE_2 is a versatile regulator of many physiological functions and pathological processes[38].

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

H.C.C. and Y.C.R.: conception and design; Y.C.R., J.H.G., X.L., R.Z., L.L.T., J.D.D., H.C., M.K.Y., X.J., X.H.Z., K.L.F. and Y.W.C.: experiments and/or data analysis; H.H.: clinical materials and consultancy; W.L.Z.: intellectual input and supervision; Y.C.R. and H.C.C.: article writing with contributions from other authors.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Mice and intrauterine injection. Female Imprinting Control Region (ICR) mice were purchased from the Laboratory Animal Service Centre of the Chinese University of Hong Kong. All mouse experiments were conducted in accordance with the university guidelines on animal experimentation, and approval by the Animal Ethics Committee of the Chinese University of Hong Kong was obtained for all related procedures. The day a plug was found after mating was designated as day 1 after mating. Intrauterine injection was done on day 3 around 6:00–8:00 p.m. Uteri were exposed during abdominal surgery under general anesthesia. PBS (20 μl) was injected into the lumen of each uterine horn close to the uterotubal junction. Embryo numbers were counted on day 7. The oil-induced decidualization mice model was adapted as previously reported[33]. Pseudopregnancy was induced by mating females with vasectomized males, and the day a plug was observed was designated as day 1 of pseudopregnancy. Intrauterine injection of 20 μl PBS was made 10 min before injection of 10 μl oil on day 4 of pseudopregnancy, and the uteri were weighed on day 7.

Patients and endometrial sample collection. Female patients who were diagnosed with infertility without hydrosalpinx syndrome and who sought IVF treatment at Women's Hospital of School of Medicine of Zhejiang University, were recruited and gave their written informed consent. The study was approved by the Ethics Committee of Women's Hospital of School of Medicine of Zhejiang University. The endometrial samples were collected during endometrium biopsy examination on day 21 (mid-secretory phase) of the menstrual cycle before IVF and embryo transfer. Western blotting was performed on 16 samples, each randomly selected from the sample pools of successful pregnancy and failed pregnancy, according to the pregnancy outcome after IVF and embryo transfer. A detailed statistic analysis of patient parameters is listed in **Supplementary Tables 1** and **2**.

Primary culture. EECs were isolated and cultured as previously described[39]. Briefly, uteri were obtained from immature ICR mice, sliced longitudinally and treated with 6.5 mg/ml trypsin and 25 mg/ml pancreatin in PBS at 0 °C for 60 min and at room temperature for another 45 min. The uteri were transferred to fresh PBS and gently shaken by hand for 30 s to release epithelial cells. After collection of the released epithelial cells, the remaining uterine tissue was washed with fresh PBS twice, incubated in 0.1% trypsin at 37 °C for 15 min, transferred to fresh PBS and shaken for 30 s to release stromal cells. For the cocultures, the epithelial cells were mixed with the stromal cells at ratio of 1:1 and incubated in DMEM/F-12 with 10% (v/v) FBS, 1% (v/v) nonessential amino acids, 100 IU/ml penicillin and 10 μg/ml streptomycin at 37 °C.

Patch-clamp. Whole-cell currents were obtained as previously reported[40,41]. A bath solution containing (in mM) Na-gluconate (145), KCl (2.7), $CaCl_2$ (1.8), $MgCl_2$ (2), glucose (5.5) and HEPES (10) (pH 7.4), and a pipette solution containing (in mM) K-gluconate (135), KCl (10), NaCl (6), $MgCl_2$ (2) and HEPES (10) (pH 7.2) were used for ENaC currents. To record Ca^{2+} channel currents, cells were bathed in the solution containing (in mM) NaCl (130), $BaCl_2$ (10), $CaCl_2$ (0.2), TEACl (3), $MgCl_2$ (0.6), $NaHCO_3$ (14), NaH_2PO_4 (1), HEPES (33), glucose (5.5) (pH 7.2) with pipettes filled with a solution containing (in mM) NaCl (10), TEACl (100), $MgSO_4$ (2), EGTA (5.5), $CaCl_2$ (0.5) and HEPES (10) (pH 7.2).

Membrane potential measurement. Epithelial cells on coverslips were washed with a bath solution containing (in mM) NaCl (135), KCl (5.8), $MgCl_2$ (1.2), $CaCl_2$ (2.5), HEPES (10) and glucose (5) (pH 7.4), and then loaded with the voltage-sensitive dye DiBAC4(3) (1 μM). Fluorescence (495/520 nm excitation/emission) was monitored and calculated to membrane potential change by adding K^+-gluconate (5 to 60 mM) in the presence of valinomycin (2 μM) as previously described[27,42,43].

Intracellular calcium measurement. Epithelial cells on cover slips were loaded with Fura-2 (3 μM) at 37 °C for 30 min. Fluorescence excited at 340 and 380 nm was monitored.

PGE_2 measurement. Epithelial cells were grown on Transwell-Col membranes (0.4 μm) for 3 d. The FBS concentration in the culture medium was reduced to 1% 12 h before the experiment. Immediately before starting the experiment, the cells were changed into no-FBS medium. After treatment, cell-free supernatant was collected and PGE_2 content was measured using an EIA kit (Cayman Chemical).

cAMP measurement. After treatment, cocultured cells were lysed in 0.1 mM HCl and 0.1% Triton-200 for 10 min. cAMP content was measured using an ELISA kit (Assay Design).

Immunostaining. Cells on cover slips were fixed with 4% paraformaldehyde in PBS for 15 min, permeabilized with 1% Triton-200 in PBS for 5 min and incubated in PBS containing 5% rabbit serum for 30 min for blocking. Primary antibodies against desmin (Abcam, ab8592, 1:100) and P-CREB (Cell Signaling, 9198L, 1:100) were applied on cells for overnight at 4 °C.

Antibodies for western blot. Antibodies against P-CREB (Cell Signaling, 9198L, 1:250) and ENaCα (Santa Cruz, sc-22239, 1:500) were used for mouse samples. For human samples, antibodies against ENaC subunits α (Abcam, ab65710, 1:500), β (Chemicon, AB3532P, 1:250) and γ (Abcam, ab3468, 1:500) were used.

***In vivo* RNAi.** Stealth RNA duplex oligoribonucleotides (AAA GCA AAC UGC CAG UAC AUC AUG C and GCA UGA UGU ACU GGC AGU UUG CUU U) targeting ENaCα ($siRNA_{ENaC\alpha}$), StealthTM RNAi Negative Control Lo GC Duplex ($siRNA_{NC}$) and Lipofectamine 2000 were purchased from Invitrogen. The intrauterine injection surgery was as described above, done on day 3 of pregnancy. Twenty picomol $siRNA_{ENaC\alpha}$ or $siRNA_{NC}$ combined with Lipofectamine was injected into each uterine horn.

Statistical analyses. Data are presented as mean ± s.e.m. (*n* is the number of tissue preparations, cells or experiment times). For two groups of data, two-tail Student's *t* test was used. For three or more groups, data were analyzed by one-way analysis of variance with Dunnett's *post hoc* test. A value of $P < 0.05$ was considered to be statistically significant.

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