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# Expression and functional maintenance of volume-regulated anion channels in myometrial smooth muscles of pregnant mice

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**Abstract:** Pregnancy causes changes in the uterus, such as increased cell volume and altered water content. However, the mechanisms that protect the structure and maintain the function of uterine smooth muscle cells against these changes during pregnancy have not been clarified. This study focused on the volume-regulated anion channel (VRAC), which opens with cell swelling under low osmotic pressure and releases $Cl^-$ ions and various organic osmolytes to resist cell swelling and regulates a wide range of biological processes such as cell death. In this study, myometrial smooth muscle (MSM) tissues and cells (MSMCs) were collected from non-pregnant and pregnant mice. Using western blotting and immunocytochemistry, leucine-rich repeat containing protein 8A (LRRC8A), an essential membrane protein that constitutes part of the VRAC, was determined to be diffused throughout MSMCs including in the cell membrane. Patch-clamp experiments were performed to investigate the electrophysiology of swelling-induced $Cl^-$ currents ($I_{Cl,\ swell}$) mediated by the VRAC. No significant changes between non-pregnancy and pregnancy groups were observed in either the expression density of LRRC8A or the current density of $I_{Cl,\ swell}$, however the presence of LRRC8A on the cell membrane was significantly increased in the third trimester of pregnancy compared to the non-pregnancy. This study suggests that the VRAC may play a role, such as maintaining cellular homeostasis in the pregnant MSM.
**Key words:** leucine-rich repeat containing 8A, myometrium, pregnancy, swelling-induced $Cl^-$ current, volume-regulated anion channel

## Introduction

Pregnancy induces various changes in the uterus, including an increase in myometrial smooth muscle cell (MSMC) volume and water content, alterations in the ion concentration and electrical activity [1, 2], and changes in hormone sensitivity [3–6]. With the progression of pregnancy, bioactive molecules such as interleukins [7] are produced, and these changes become more acute. Furthermore, altered gene expression during pregnancy has been reported [8, 9]. Despite these changes, the uterus can maintain pregnancy until delivery without significantly disrupting its structure or function. However, the mechanisms involved in protecting these cells and tissues remain elusive.

The volume-regulated anion channel (VRAC), also known as the volume-sensitive outwardly rectifying (VSOR) anion channel, is ubiquitously expressed in almost all vertebrate cells [10, 11]. It opens when cells swell in response to hypoosmotic pressure, releasing

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$Cl^-$ ions and various organic osmolytes, resulting in a swelling-induced $Cl^-$ current ($I_{Cl, swell}$) and reduction in the volume of swollen cells [10, 11]. Leucine-rich repeat containing protein 8A (LRRC8A) is an essential membrane protein that constitutes the VRAC and is responsible for regulating cell volume [12, 13]. Most *in vitro* studies on LRRC8A-dependent VRAC activity have demonstrated its function in volume regulation and numerous biological processes, including cell death, proliferation, and migration, and insulin and glucose homeostasis [14–19]. Furthermore, selective VRAC blockers such as glibenclamide and 4-(2-butyl-6,7-dichloro-2-cyclopentyl-indan-1-on-5-yl) oxobutyric acid (DCPIB) have been reported to potently suppress $I_{Cl, swell}$ [20–23]. Though the function and structure of VRAC have been investigated, research on the VRAC in MSMCs is limited, and changes in the VRAC, especially during pregnancy, remain unknown.

The aim of this study was to examine whether VRAC is functionally present in mouse myometrial smooth muscle (MSM) and whether pregnancy alters its activity or expression. The functional maintenance of VRAC might be an indicator of a role of VRAC with respect to maintaining MSM structure and function in response to the considerable changes during pregnancy.

## Materials and Methods

### Animals

Wild-type female C57BL/6J mice (*Mus musculus*, 10–16 weeks old) were purchased from CLEA Japan Inc. (Tokyo, Japan). Myometrial tissues were collected from 80 non-pregnant and pregnant mice (10–22 weeks old), as previously reported [24, 25]. Among the 84 mice, 24 were used for western blotting, 20 for immunocytochemistry, and 40 for electrophysiology experiments using the whole-cell patch-clamp technique. Before tissue collection, 100 mg/kg pentobarbital sodium (Kyoritsu Seiyaku, Tokyo, Japan) was intraperitoneally administered to euthanize the mice. Fetuses removed from the pregnant mice were euthanized using the same procedure. Myometrial tissues were collected from non-parous mice in the non-pregnant group, and those from the pregnant group were collected on day 0 of gestation (when the vaginal plug was detected) as well as the first (day 7), second (day 13), and third trimesters (day 17 or 18). This study was performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All experimental protocols were approved by the institutional animal care and use committee of the Shiga University of Medical Science (2017-12-6, 2017-12-6 (H1)).

### Cell isolation

Single MSMCs were enzymatically isolated from non-pregnant (nP) and pregnant mice as previously reported [24, 25]. Briefly, the myometrium was dissected and freed from other tissues in $Ca^{2+}$-free physiological salt solution for dissociation (dPSS). This solution was then replaced with $Ca^{2+}$-free dPSS solution containing 0.1% papain (Sigma-Aldrich, St. Louis, MO, USA) and 0.1% dithiothreitol (Sigma-Aldrich) at 4°C. After 30 min of treatment, the solution was replaced with 100 $\mu$M $Ca^{2+}$ dPSS solution containing 0.1% collagenase (Wako Pure Chemical Industries, Osaka, Japan) and incubated at 37°C for 25 min. The solution was then replaced with enzyme- and $Ca^{2+}$-free dPSS solution. Subsequently, the tissue was gently triturated using a glass pipette to isolate cells. At the beginning of each experiment, a few drops of the cell suspension were placed in the recording chamber.

### Reagents and chemicals

As previously reported [23], for the patch-clamp experiment, we employed an isosmotic solution containing 100 mM NaCl, 40 mM sodium aspartate, 2.0 mM $MgCl_2 \cdot 6H_2O$, 2.0 mM $BaCl_2 \cdot 2H_2O$, 5.5 mM glucose, 10 mM HEPES, and 0.001 mM nicardipine (pH adjusted to 7.4 with 1 N NaOH, osmolality ~315 mosmol/kg $H_2O$). Cell swelling was induced using an hypoosmotic solution containing 100 mM NaCl, 2.0 mM $MgCl_2 \cdot 6H_2O$, 2.0 mM $BaCl_2 \cdot 2H_2O$, 5.5 mM glucose, 10 mM HEPES, and 0.001 mM nicardipine (pH adjusted to 7.4 with 1 N NaOH, osmolality ~230 mosmol/kg $H_2O$). Stock solutions of DCPIB (20 $\mu$M; Funakoshi, Tokyo, Japan)—prepared in ethanol—and glibenclamide (200 $\mu$M; Sigma-Aldrich)—prepared in DMSO—were diluted to the desired final concentrations just before use [20–23].

### Western blotting

Western blotting was performed as described previously [26]. Proteins were quantified using the Bio-Rad protein dye assay reagent (500-0113; Bio-Rad Laboratories Inc., Hercules, CA, USA), per the manufacturer's instructions. Equal amounts of proteins (20 $\mu$g/lane) were denatured for 60 min at 37°C in Laemmli buffer and resolved on 5–20% SDS-polyacrylamide gels (FUJIFILM, Tokyo, Japan) and transferred onto polyvinylidene difluoride membranes (Immobilon-P, IPVH00010). After blocking with 5% nonfat milk prepared in Tris-buffered saline (TBS) containing 0.1% Tween 20 (TBST; Takara Bio Inc., Shiga, Japan) for 60 min, the blots were probed overnight with rabbit polyclonal antibodies against LRRC8A (1:5,000, 56262; Abgent, San Diego, CA, USA) or glyceraldehyde 3-phosphate dehydroge-

nase (GAPDH, 1:10,000; 0312010; Cell Signaling Technology, Danvers, MA, USA) at 4°C. After washing with TBST, the blots were incubated for 2 h with horseradish peroxidase-conjugated anti-rabbit IgG (1:10,000, NA934VS; Amersham, Buckinghamshire, UK). The intensity of the protein bands was quantified using Fuji software (Fuji, Japan) and normalized to the GADPH band.

### Immunocytochemistry

Immunocytochemistry was performed as previously reported [26]. After fixing, MSMCs were permeabilized as previously reported [27] and blocked with 10% fetal bovine serum (FBS). The permeabilized cells were first probed with rabbit LRRC8A (1:1,000, 56262; Abgent) and mouse smooth muscle actin (SMA, 1:200 or 1,000, 20035047, Dako owned by Agilent Technologies, Santa Clara, CA, USA) antibodies, and then incubated with Alexa Fluor 488- or 568-conjugated secondary antibodies (1:400; Molecular Probes, Eugene, OR, USA). The nuclei were stained with 2 $\mu$g/ml 4',6'-diamino-2-phenylindole. Fluorescent images were obtained on a TSC SP8 confocal laser scanning system (Leica Microsystems, Tokyo, Japan).

### Electrophysiology and data analysis

The whole-cell patch-clamp technique [22, 23, 26] was employed to isolate MSMCs, and an EPC-8 patch-clamp amplifier (HEKA Elektronik, Lambrecht, Germany) and microscope (ECLIPSE TE-2000U; Nikon, Tokyo, Japan) were used. The isolated cells were transferred to a recording chamber and superfused continuously at 37°C with normal Tyrode solution [23]. The resistance of the patch electrode was 2.5–4.5 MΩ when filled with pipette solution [23], containing 90 mM aspartate (Sigma-Aldrich), 90 mM CsOH, 30 mM CsCl, 2 mM $MgCl_2$, 20 mM tetraethylammonium chloride (Sigma-Aldrich), 5 mM adenosine 5'-triphosphate (disodium salt; Sigma-Aldrich), 0.1 mM guanosine 5'-triphosphate (lithium salt; Sigma-Aldrich), 5 mM EGTA, and 5 mM HEPES (pH adjusted to 7.2 with 1 N CsOH, osmolality ~320 mosmol/kg $H_2O$). Using Patchmaster software (HEKA Elektronik), membrane currents were appropriately filtered and sampled at 5 kHz through an LIH-1600 analog-to-digital converter. Whole-cell membrane currents were measured using the voltage ramp protocol consisting of the following three phases: depolarization from a holding potential of –40 mV to +40 mV lasting 400 ms, hyperpolarization from +40 mV to –120 mV lasting 800 ms, and repolarization returning to –40 mV from –120 mV and lasting 400 ms (6 s interval at 2 kHz; digitized at 5 kHz). During the hyperpolarizing phase, the current-voltage (*I-V*) relationship was measured. Cell membrane capacitance ($C_m$) was calculated from the capacitive transients elicited by voltage-clamp steps (±5 mV) from a resting potential of –80 mV in 20 ms according to the following equation:

$$C_m = \tau_C I_0 / \Delta V_m (1 - I_\infty / I_0) \quad (1)$$

where, $\tau_C$ is the time constant for the decay of the capacitive current, $I_0$ is the initial peak amplitude of the current, $\Delta V_m$ is applied voltage step (5 mV), and $I_\infty$ is the steady-state current value.

### Statistical analysis

Data are presented as mean ± SEM. The number of isolated myometrial cells is expressed as "n," and the number of animals is expressed as "N." GraphPad Prism 8 (GraphPad Software Inc., San Diego, CA, USA) was used for statistical analyses. Kolmogorov-Smirnov and Shapiro-Wilk tests were used to determine whether the data were normally distributed. Significant differences in the current density between groups were examined using the one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test. Significant differences in protein content were examined using the Kruskal-Wallis test followed by Dunn's multiple comparisons test. Significant differences in the immunocytochemical signal intensity were examined using the *F* test followed by the Student's *t* test. Results with $P<0.05$ were considered significant.

## Results

### $I_{Cl,\ swell}$ during pregnancy

The whole-cell patch-clamp technique was used to measure the changes in cell membrane capacitance ($C_m$), which were used to determine changes in MSMC volume. The mean $C_m$ was 6.313 ± 0.5531 pF (n=30, N=15) in non-pregnant mice, 10.10 ± 0.9746 pF (n=17, N=4, $P$=0.4956 vs. nP) on gestation day 7, 25.84 ± 2.669 pF (n=20, N=5, $P<0.0001$ vs. nP, $P<0.0001$ vs. day 7) on gestation day 13, and 34.90 ± 1.930 pF (n=35, N=12, $P<0.0001$ vs. nP, $P<0.0001$ vs. day 7, $P$=0.0023 vs. day 13) on gestation day 18. The cell membrane volume significantly increased with pregnancy progression, especially on gestation day 18; the mean $C_m$ increased approximately 5.5-fold compared to non-pregnancy. This electrophysiologically confirmed that MSMC volume increased during pregnancy.

To confirm the presence of VRAC in MSMCs, the electrophysiological properties of $I_{Cl,\ swell}$ during pregnancy were examined using whole-cell patch-clamp. Figure 1 shows a typical patch-clamp sample obtained

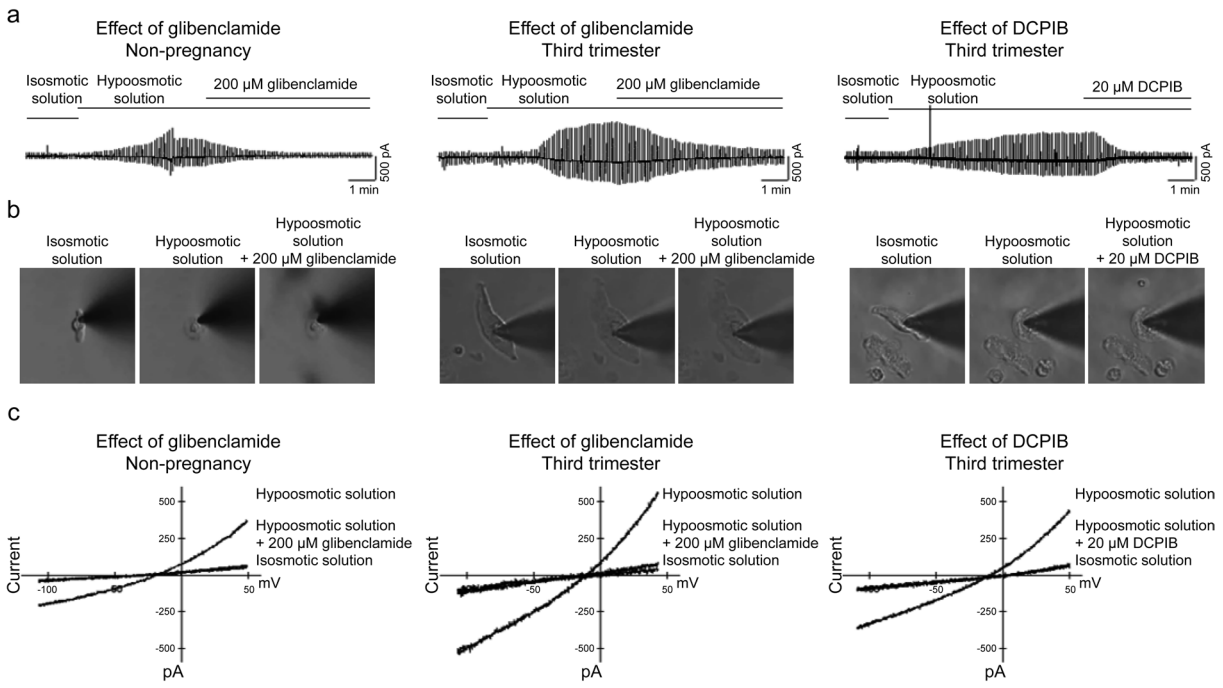


**Fig. 1.** Effects of hypoosmotic external solution and subsequent addition of volume-regulated anion channel (VRAC) blockers (glibenclamide and DCPIB) in myometrial smooth muscle cells isolated from non-pregnant and third trimester mice. a: Chart records of whole-cell current responses to voltage ramps. b: Whole-cell clamp photographs. c: Current-voltage relationships.

from MSMCs of non-pregnant and third trimester mice. The $Cl^-$ concentration in the hypoosmotic external solution was adjusted to 108 mM. Whole-cell current was monitored by applying voltage ramp testing pulse ($dV/dt$ = +0.9 V/s) between +40 and –120 mV from a holding potential (–40 mV). When the extracellular solution was changed to a hypoosmotic solution, an increase in membrane current associated with cell expansion was observed. When the increased membrane current became steady, replacement with a hypoosmotic solution including 200 $\mu$M glibenclamide or 20 $\mu$M DCPIB did not significantly alter cell swelling, but almost completely suppressed the increase in membrane current (Figs. 1a–c). These changes were similar in first and second trimester mice (not shown).

Figure 1c illustrates the *I-V* relationships of the membrane currents recorded upon the exposure of cells to isosmotic, hypoosmotic, and hypoosmotic solutions with the VRAC blocker. The increased current in the hypoosmotic solution was reversed close to the equilibrium potential of $Cl^-$ ($E_{Cl,}$) under the experimental conditions (intraelectrode fluid and cell perfusate), as calculated using the Nernst equation:

$$E_{Cl} = 61 \times \log (54 / 108) = -18.4 \text{ mV} \quad (2)$$

The difference current was designated as $I_{Cl,\ swell}$; it was calculated by digitally subtracting the current trace under isosmotic conditions, or under hypoosmotic conditions in which the VRAC blocker was administered, from the trace under hypoosmotic conditions. These results confirm that the VRAC is expressed in mouse MSMCs, and that the VRAC blockers, glibenclamide and DCPIB, inhibit VRAC function during non-pregnancy and pregnancy.

Figure 2a shows the typical current densities of $I_{Cl,\ swell}$ recorded in non-pregnant and the first to third trimester mouse MSMCs. No significant changes were observed in current density of $I_{Cl,\ swell}$ at +40 mV and –100 mV between the non-pregnancy and pregnancy groups (Fig. 2b). The current density of $I_{Cl,\ swell}$ at +40 mV was 68.15 ± 14.74 pA/pF (n=12, N=9) during non-pregnancy, 44.90 ± 12.18 pA/pF during the first trimester (n=6, N=3, *P*=0.6278 vs. nP), 44.00 ± 11.60 pA/pF during the second trimester (n=8, N=4, *P*=0.5261 vs. nP, *P*>0.9999 vs. the first trimester), and 32.74 ± 7.613 pA/pF during the third trimester (n=7, N=6, *P*=0.2373 vs. nP, *P*=0.9410 vs. the first trimester, *P*=0.9417 vs. the second trimester). The current density of $I_{Cl,\ swell}$ at –100 mV was –50.12 ± 11.07 pA/pF during non-pregnancy (n=12, N=9), –34.49 ± 8.981 pA/pF during the first trimester (n=6, N=3, *P*>0.9999 vs. nP), –30.71 ± 7.779 pA/pF during the second trimester (n=8, N=4, *P*>0.9999 vs. nP, *P*>0.9999 vs. the first trimester), and –27.48 ± 6.182 pA/pF during the third trimester (n=7, N=6, *P*>0.9999 vs. nP, *P*>0.9999 vs. the first trimester, *P*>0.9999 vs. the second trimester). These data suggest that the current density of $I_{Cl,\ swell}$

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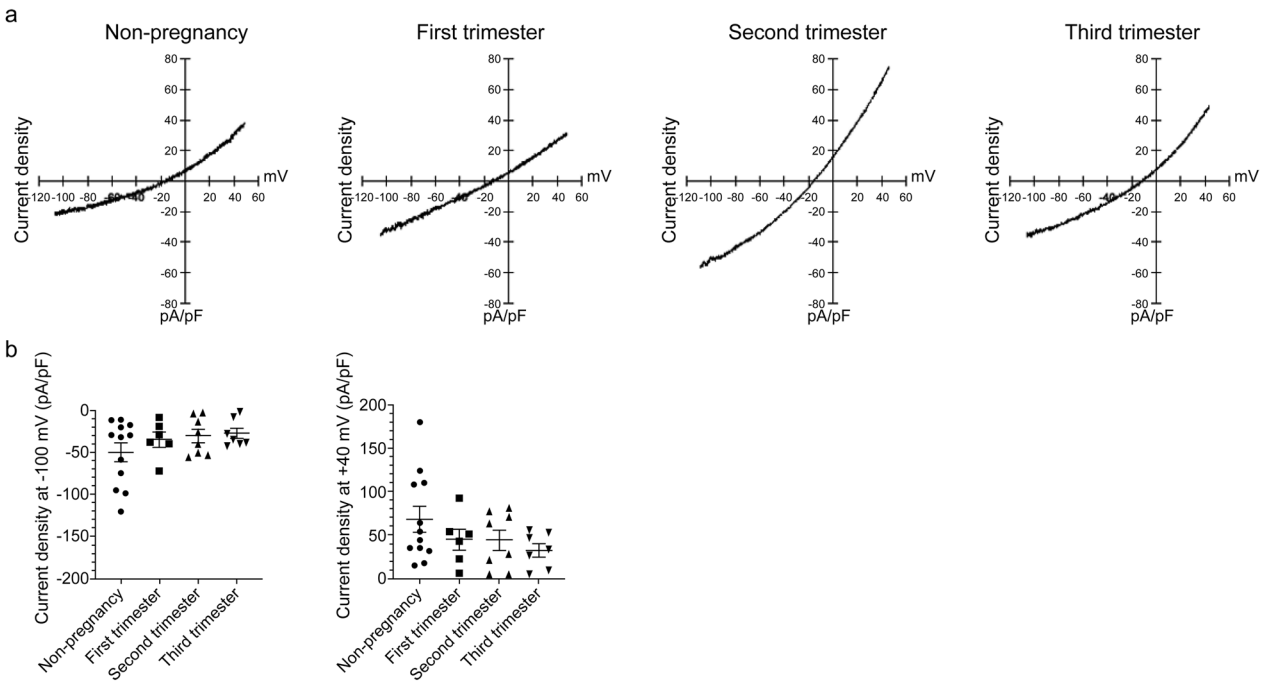


**Fig. 2.** Current density of $I_{Cl,\ swell}$ recorded in myometrial smooth muscle cells isolated from non-pregnant and first to third trimester mice. a: Current-voltage relationships. b: Summarized data of current density of $I_{Cl,\ swell}$ at test potentials of +40 mV and –100 mV.

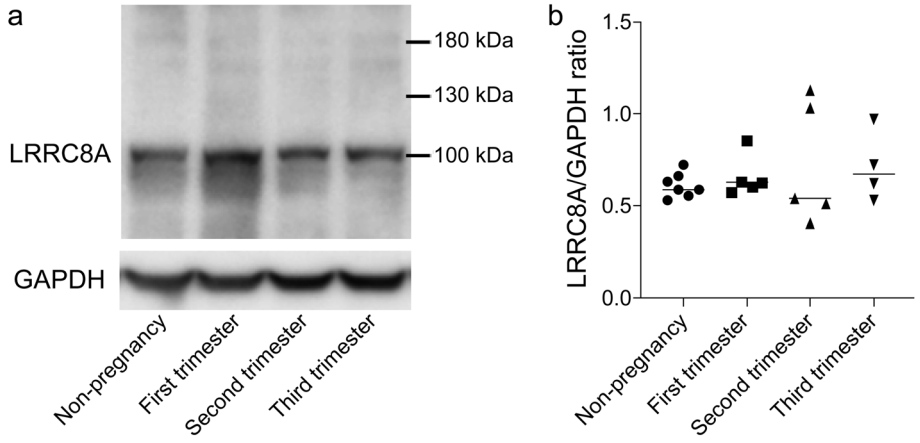


**Fig. 3.** Expression of leucine-rich repeat containing 8A (LRRC8A), a component of volume-regulated anion channel (VRAC), in the myometrium of non-pregnant and first to third trimester mice. a: Western blot analyses of LRRC8A and GAPDH in the myometrium of non-pregnant and first to third trimester mice. b: LRRC8A protein levels normalized to GAPDH.

does not change significantly during pregnancy compared to non-pregnancy.

## Intracellular localization of LRRC8A and VRAC expression during pregnancy

To histologically evaluate the pregnancy-related changes in the VRAC, the expression and intracellular localization of LRRC8A were investigated. A band was observed at approximately 100 kDa from both non-pregnant and pregnant samples, confirming the functional expression of LRRC8A (Fig. 3a). Subsequently, the ratio of LRRC8A to GAPDH was compared between non-pregnancy and each trimester of pregnancy, but no significant change was observed. The LRRC8A/GAPDH ratio was 0.6135 ± 0.02517 during non-pregnancy (N=7), 0.6588 ± 0.05019 during the first trimester (N=5, $P$>0.9999 vs. nP), 0.7257 ± 0.1489 during the second trimester (N=5, $P$>0.9999 vs. nP, $P$>0.9999 vs. the first trimester), and 0.7131 ± 0.09662 during the third trimester (N=4, $P$>0.9999 vs. nP, $P$>0.9999 vs. the first trimester, $P$>0.9999 vs. the second trimester) (Fig. 3b). This suggested that the proportion of LRRC8A to GAPDH in

mouse MSMCs does not significantly change regardless of pregnancy status.

Immunocytochemistry was used to investigate the intracellular localization of the VRAC in combination with SMA, a specific smooth muscle cell marker (Fig. 4). These results confirmed that the isolated cells were MSMCs. The fluorescent intensity of LRRC8A immunocytochemical signals was measured by the line profiles across each MSMCs in non-pregnant and third trimester mice (Figs. 4c–e). The ratio of fluorescent intensity for LRRC8A on the membrane to the whole cell area was 0.8201 ± 0.04896 during non-pregnancy (n=12, N=4) and 1.008 ± 0.02742 during third trimester (n=25, N=5, $P$<0.001 vs. nP) (Fig. 4f). This result suggests that LRRC8A was diffusely expressed in the entire cell area including the cell membrane, with increased expression on the cell membrane in third trimester compared to the non-pregnancy.

In this study, the expression of VRAC in mouse MSMCs was confirmed. Although there were no significant changes in $I_{Cl,\ swell}$ via VRAC, regardless of non-pregnancy or pregnancy and its duration, the LRRC8A expression especially on the cell membrane was significantly enhanced in third trimester compared to the non-pregnancy.

## Discussion

The VRAC is ubiquitously expressed in almost all vertebrate cells [16, 17], and LRRC8A has been identified as an essential membrane protein constituting the VRAC [18, 19]. Despite information regarding its expression in MSMCs, the function of VRAC and the localization of LRRC8A in these cells have not been elucidated, especially in the uterus during pregnancy. To the best of our knowledge, this is the first study to investigate these functions. The results revealed that LRRC8A is also expressed in MSMCs of non-pregnant mice and is expressed in the cells in a diffused manner, mainly on the membrane. VRAC-mediated $I_{Cl,\ swell}$ was also recorded in MSMCs of non-pregnant mice under low osmotic conditions. Furthermore, in MSMCs, the LRRC8A to GAPDH ratio and $I_{Cl,\ swell}$ did not significantly change regardless of pregnancy status, but the presence of LRRC8A increased on the membrane in third trimester. These results suggest that the VRAC may play a role, such as maintaining cellular homeostasis in MSM during pregnancy.

The finding that the ratio of LRRC8A to GAPDH in the whole cell, including the cell membrane, did not significantly change during pregnancy suggests that total LRRC8A expression increases with an increase in cell volume during pregnancy. Additionally, the fact that no significant change was observed in the current density of $I_{Cl,\ swell}$ during pregnancy, despite the increase in cell volume, suggests that LRRC8A expression increased on the cell membrane for maintaining the level of VRAC function per unit surface area in the membrane. This is in line with the premise that the localization of the VRAC on the membrane is a prerequisite for its function and

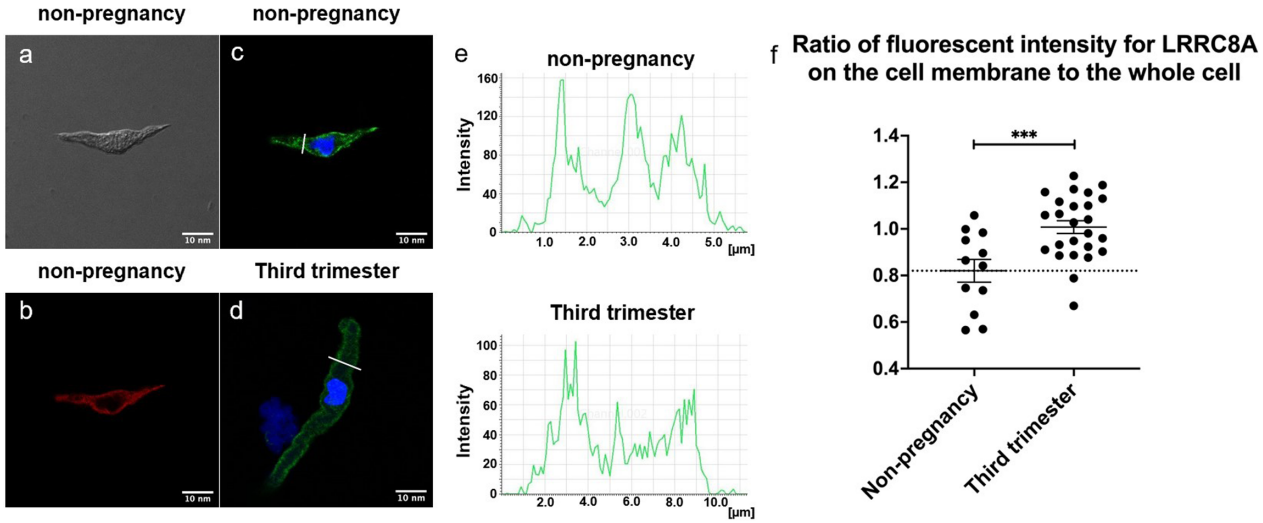


**Fig. 4.** Intracellular localization of leucine-rich repeat containing 8A (LRRC8A) in myometrial smooth muscle cells isolated from non-pregnant and pregnant mice. Immunocytochemistry revealed diffuse expression of LRRC8A in the whole cell, including the cell membrane. a: Differential interference contrast microscope images of cells during non-pregnancy. b: SMA expression (red) in cells during non-pregnancy. c, d: LRRC8A (green) and nuclei (blue) in cells during non-pregnancy and third trimester, respectively (White lines: the line profile across each cell). e: Fluorescent intensity for LRRC8A during non-pregnancy and third trimester. f: The ratio of fluorescent intensity for LRRC8A on the cell membrane to the whole cell area. ***$P$<0.001.

that the current density is the amplitude of current per capacitance ($C_m$). These results, including the intracellular localization of LRRC8A in MSMCs during pregnancy, reveal that the level of LRRC8A expressed in the cytoplasm and that transferred to the membrane increases with pregnancy. We consider that the increased expression of LRRC8A on the cell membrane might be associated to the enhanced trafficking process of LRRC8A due to various changes around MSMCs during pregnancy. This maintenance of VRAC expression on the cell membrane further supports its potential role during pregnancy.

It is known that pregnancy is accompanied by an increase in MSMC volume and uterine water content, alterations in ion concentration and electrical activity [1, 2], and changes in hormone sensitivity, which become more acute towards the end of the gestation period [3–7]. Pregnancy can be maintained until delivery without these changes significantly disrupting MSM structure or function. The mechanism protecting cells and tissues during this process has not been studied in detail. The VRAC has been reported to mediate apoptosis in cardiomyocytes and neurons exposed to ischemia-reperfusion, and the dysfunction of the mechanism of action of VRAC leads to cell death [14–16].

Based on previous reports [14–16, 28], it can be assumed that MSM is protected from pregnancy-related changes via maintenance of VRAC function in MSMCs and VRAC expression on the surface of the MSMCs. To the best of our knowledge, this is the first study concerning the maintenance of distribution, density, and function of VRAC, related to the mechanism of cell protection, in the enlarging MSMCs and MSM tissues during pregnancy. However, more detailed researches are needed to prove the association between VRAC and cell protection during pregnancy.

The VRAC opens with cell expansion under low osmotic pressure, releasing $Cl^-$ ions and various organic osmolytes, reducing the volume of expanded cells, and counteracting the increase in cell volume [16, 17]. Most previous studies have investigated the activity of VRAC in cultured cell lines [14–19]. However, in this study, the VRAC activity was examined in primary cells isolated from the mouse uterus. The VRAC activity in these cells was examined electrophysiologically by investigating changes in $I_{Cl,\ swell}$ in response to changes in osmotic pressure around the isolated MSMCs. We demonstrated that the VRAC activity did not significantly change during pregnancy, as there were no significant changes in $I_{Cl,\ swell}$, although the MSMC volume increased with the progression of pregnancy. However, experimental conditions, such as osmotic pressure and intracellular ion concentration, differed from *in vivo* conditions. The sensitivity of VRAC is reportedly not only dependent on the osmotic pressure around cells, but also on the extent of cell membrane tension, intracellular ionic strength, osmolyte concentration, and ATP concentration [27, 29–31]. For example, as MSMC volume increases during pregnancy, the degree of cell membrane tension may also change. This is a limitation of the current study as factors other than changes in pericellular osmolarity were not taken into consideration. It has been demonstrated that VRAC is formed by LRRC8 heteromers and that LRRC8A is the only essential subunit (along with at least one other LRRC8 member, LRRC8B-E) [18, 19, 32, 33]. The function and sensitivity of the VRAC changes depending on its subunit configuration [32, 33]. This study showed that LRRC8A is expressed in MSMCs and its expression on the cell membrane significantly increases during pregnancy. The composition of the VRAC subunits in MSMCs is unknown, and structural changes associated with pregnancy may alter VRAC sensitivity and function. The above descriptions might reveal that the membrane current density of VRAC does not significantly change despite the increase of LRRC8A on the cell membrane during third trimester. Another limitation of this study is its basis on a rodent model. Further studies are needed to elucidate the function of VRAC in MSMCs during pregnancy.

This study revealed that the VRAC is expressed in MSMCs and that LRRC8A, constituting the VRAC, is localized in the entire MSMC, including the cell membrane. Furthermore, the electrophysiological sensitivity of VRAC is maintained with the increasing LRRC8A expression on the membrane in third trimester. These results suggest that the VRAC in MSMCs may play a role, such as a protection for MSMCs and MSM tissues against pregnancy-related changes. Additionally, the dysfunction of VRAC in female mice is known to cause infertility and to decrease the production rate [34, 35]. By elucidating the function of VRAC in the uterus during pregnancy, this study and future studies may significantly contribute to the development of perinatal care strategies, as well as clarify the causes of mortal in uterus and of preterm birth.

## Conflict of Interest

The authors report no potential conflict of interest.

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