



Expression of the epithelial sodium channel (ENaC) in the endometrium – Implications for fertility in a patient with pseudohypoaldosteronism

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ABSTRACT

Pseudohypoaldosteronism type 1 (PHA) is a syndrome of unresponsiveness to aldosterone. The severe form of this disease results from mutations in the genes that encode for the epithelial sodium channel subunits, SCNN1A, SCNN1B, and SCNN1G. A PHA patient under our care failed to conceive after many years and IVF trials. Our earlier studies had shown that ENaC is expressed in the female reproductive tract. We hypothesized that a defective ENaC expression may be responsible for the infertility of the patient. To test this hypothesis we examined ENaC expression in endometrial Pipelle biopsy samples from three healthy women and the PHA patient with an Arg508X mutation in the SCNN1A gene. The formalin fixed samples were reacted with anti-ENaC (alpha subunit) antisera, followed by secondary antibodies to visualize ENaC expression by immunofluorescence. Confocal microscopy imaging of the samples showed strong ENaC immunofluorescence along the luminal border (apical membrane) of the epithelial cells in Pipelle samples from healthy women. In contrast, none of the samples from the PHA patient showed ENaC immunofluorescence. The Arg508X mutation interrupts the transport of ENaC subunits to the cell surface, yet it would not be expected to disrupt ENaC localization in the cytoplasm. In contrast to endometrium where ENaC is localized in the apical membrane of the epithelial cells, in keratinocytes ENaC is expressed in cytoplasmic pools. Therefore, we examined ENaC immunofluorescence in plucked hair follicles. As expected, ENaC immunofluorescence was detected in the cytoplasm of keratinocytes of both normal and PHA samples. Our results support the hypothesis that lack of expression of ENaC on the endometrial surface may be responsible for the infertility of the PHA patient.

1. Introduction

In mammals, the mineralocorticoid hormone aldosterone regulates body electrolyte and fluid levels by inducing a series of ion transporters and channels in the kidney tubules as well as other target organs responsive to aldosterone [1–6]. Yet, the reabsorption of sodium in the kidney tubules is the major determinant of salt homeostasis. In the extracellular fluid Na⁺ is the major electrolyte. Hence, the uptake of Na⁺ modulates osmolarity of the body fluid compartments and affects blood fluid volume and consequently blood pressure [7].

Pseudohypoaldosteronism type I (PHA) is a disease that is characterized by sodium wasting despite high levels of aldosterone. The reduction in sodium levels stimulates the renin-angiotensin-aldosterone system resulting in the classical hormonal profile [8,9]. In 1991 we reported for the first time that PHA is observed in two distinct forms; a

mild form with autosomal dominant inheritance, and severe form with autosomal recessive inheritance [9]. The severe form was later found to be a result of mutations in three genes (SCNN1A, SCNN1B, and SCNN1G) that code for the epithelial sodium channel, ENaC [10–13]. The severe form of PHA (OMIM #264350, PHA1B) has been called as the multi-system and systemic form of PHA, because in contrast to the mild form it affects several tissues including sweat and salivary glands in addition to kidney [4,9,14].

ENaC is composed of three subunits (α , β , and γ) encoded by the three genes noted above [15,16]. Studies from our lab and others have demonstrated that the expression of a functional ENaC on the cell surface is dependent on all three subunits [13,16,17]. A mutation in a single subunit may inhibit or prevent the assembly of a functional channel on the cell surface depending on the severity of the mutation [17,18].

Abbreviations: ENaC, epithelial sodium channel; PHA, pseudohypoaldosteronism

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Table 1

Diagnosis of subjects and histology of uterine Pipelle samples analyzed by immunofluorescence.

N	Age (years)	Diagnosis	Histology
1	49	Menorrhagia	Benign endometrium (glandular and stromal breakdown of endometrium)
2	43	Infertility	Proliferative endometrium
3	46	Metrorrhagia	Proliferative endometrium
4	29	PHA, infertility	Benign endometrium?

The oldest PHA patient we have had under our care carries a homozygous mutation in the SCNN1A gene leading to the substitution of a stop codon for Arg508 [11,19]. After she married, she could not conceive for many years despite many IVF treatments. An unrelated 28-year old patient (from the United Kingdom) with a homozygous mutation in the SCNN1A gene became spontaneously pregnant after one year of trying to conceive. Yet, placental insufficiency led to early delivery at 28 weeks, and the authors hypothesized that placental insufficiency was a consequence of the maternal PHAIB [20].

In an extensive review of the literature, Ruan et al. have concluded that ion channels play crucial roles in regulating endometrial receptivity and embryo implantation [21]. In pregnant women, plasma renin activity and aldosterone levels significantly increase and these changes are associated with plasma volume expansion [22]. Immunofluorescence and immunohistochemical studies have shown that ENaC is predominantly located on the apical membrane of epithelial cells in the endometrium of both women and mice [23,24]. Chronic ENaC blockade by benzamil in pregnant rats was shown to lead to a decrease in maternal blood pressure and fetal growth restriction emphasizing the

importance of ENaC activity during pregnancy [25].

In view of the background summarized above, we raised the hypothesis that the inability of our patient to conceive might be related to a dysfunctional ENaC in the reproductive tract. To check this hypothesis we examined the expression of ENaC in endometrial biopsy samples from healthy women and the PHA patient. Our results showed that the expression of ENaC on the luminal surface of endometrial epithelia could be easily detected in control Pipelle endometrial biopsy samples. Yet, in the Pipelle samples of the PHA patient, ENaC expression could hardly be detected. These results support the hypothesis that the Arg508X mutation blocks the assembly of ENaC on the endometrial luminal membrane, and the dysfunction of ENaC on the endometrial surface may be a major cause of the infertility of the PHA patient.

2. Methods

2.1. Subjects and biopsy samples

The endometrial biopsy samples were obtained from three women and a PHA patient using a Pipelle endometrial suction curette at a single time. The three healthy women (aged 40 to 49 years) were referred for the evaluation of infertility and menorrhagia / metrorrhagia (Table 1). None of them had malignancy.

The PHA patient was the index case in the study that identified the characteristic differences between systemic (multi-system) PHA and renal PHA [9]. Analysis of her genomic DNA revealed a nonsense mutation (Arg508X) in the SCNN1A gene [11]. After marriage, she failed to conceive despite nine IVF attempts over six years. In each procedure, 1–4 embryos were transferred. Her reproductive tract was intact. Except for persistently high aldosterone and renin typical of PHA, the

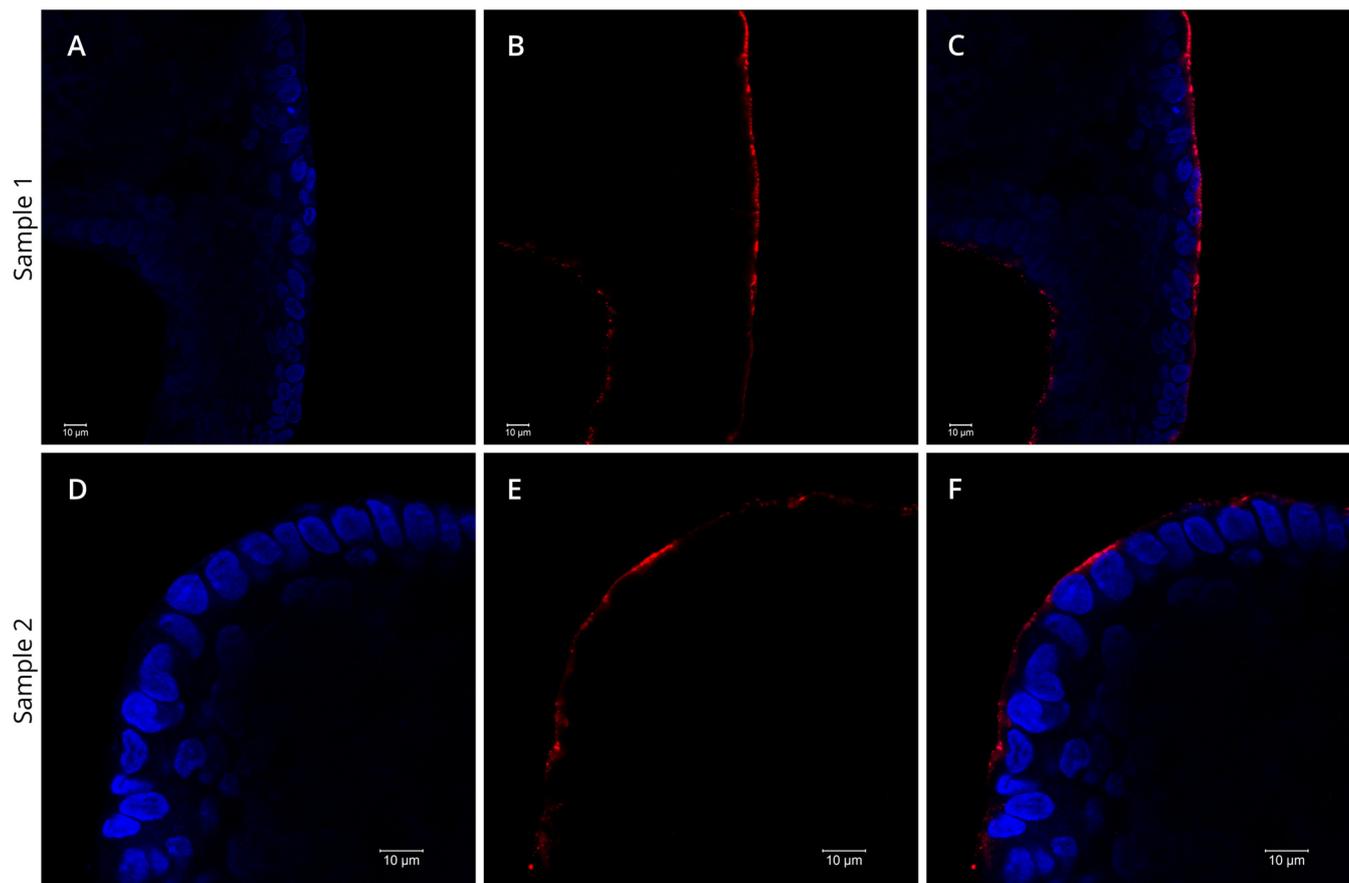


Fig. 1. Confocal microscopic imaging of immunofluorescence of endometrial Pipelle samples from subject 1 (see Table 1). The samples were reacted with anti-ENaC antiserum followed by secondary antibodies, and DAPI as described in Methods. A and D) DAPI immunofluorescence of nuclei. B and E) ENaC immunofluorescence. C and F) Merged image showing that ENaC immunofluorescence is located on the apical side of the epithelial cells.

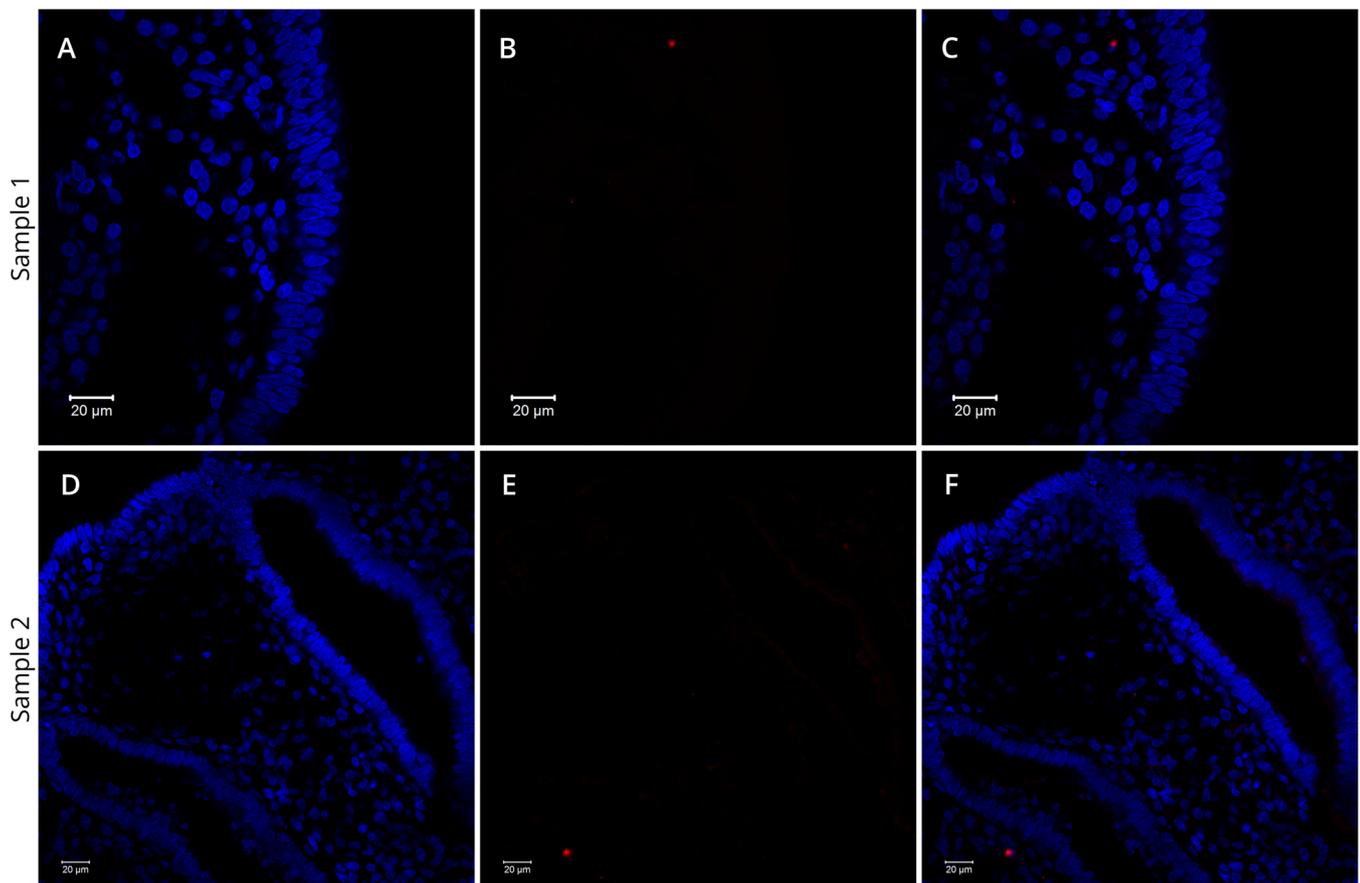


Fig. 2. Confocal microscopic imaging of immunofluorescence of endometrial Pipelle samples from the patient with pseudohypoaldosteronism (#4 in Table 1). The samples were reacted with anti-ENaC antiserum followed by secondary antibodies, and DAPI as described in Methods. A and D) DAPI immunofluorescence of nuclei. B and E) ENaC immunofluorescence. C and F) Merged image.

endocrine evaluation was normal. At 29 years of age, her plasma aldosterone at rest was 145.4 nmol/l (normal 0.138–0.42) and plasma renin activity was 11.2 ng/ml/h (normal 0.7–2.8). The parameters of husband's spermogram were within normal limits. Embryo development capacity of oocytes fertilized by husband's sperm was normal. Hence, the failure to conceive was considered to be due to a failure in implantation. The endometrial mucosal samples were obtained from this patient as part of the evaluation process during the course of IVF.

Adequacy of Pipelle samples was defined by the presence of both endometrial glands and stroma [26]. The study protocol was approved by the ethics committee at the E. Wolfson Medical Center (Date of approval: March 13, 2017. Protocol number: 0221-10-WOMC).

2.2. Immunofluorescent labeling and confocal microscopy

The Pipelle endometrial samples were fixed in 4% paraformaldehyde for 20 min and permeabilized in 0.1% Tween-20 (Sigma-Aldrich) in PBS for 10 min. The samples were then washed three times in PBS for 5 min each, incubated with 4% BSA (Sigma-Aldrich) for 20 min and washed in PBS for 5 min. The samples were then incubated overnight at 4 °C with rabbit anti- α -ENaC antiserum (1:25 dilution) that was generated against an engineered segment (residues 107–561) of the extracellular domain of human ENaCA [23]. The specificity of this antiserum has been validated in reactions with female and male reproductive tract and epidermal tissue samples, and additional analyses [14,23,27]. This antiserum has been registered in the antibody registry (antibodyregistry.org) (ID: Israel Hanukoglu; Ariel University; Israel, Cat# Hanukoglu_ENaCA, RRID:AB_2728747). The Pipelle samples were then washed three times in PBS (5 min each) and incubated with Alexa Fluor 594 goat anti-rabbit IgG (H*L) secondary antibodies in

PBS containing 2% BSA (Invitrogen, 1:100 dilution) for 1 h. After three washings in PBS (5 min each), nuclear staining was performed using DAPI (4'6-diamidino-2-phenylindole) for 1 min. Sections treated with primary and secondary antibodies separately were used as controls. After drying on pre-coated slides (Leica's adhesive slide), samples were mounted using glycerol solution containing n-propyl gallate (Sigma-Aldrich) in 0.1 M phosphate buffer (pH 7.2). All the experiments were performed in replicas in order to check staining consistency and repeated thrice.

High-resolution fluorescent images were captured using an LSM 700 confocal microscope (Carl-Zeiss, Germany) as previously described [14].

3. Results

3.1. Epithelial structure and ENaC immunofluorescence in Pipelle samples

Consistent with uterine histology, the blue colored DAPI staining of nuclei in Pipelle endometrial samples from all three healthy women showed the usual image of simple columnar epithelial cells at the edge of the endometrial stroma (Fig. 1). We had shown that the apical membrane of many cells in the endometrial glands is crowded with many copies of ENaC [23]. Similarly, the luminal border (apical membrane) of the epithelial cells in Pipelle samples from the healthy women showed strong red-colored immunofluorescence of secondary antibodies binding to anti-ENaC antibodies (Fig. 1).

DAPI staining of the Pipelle endometrial samples from the PHA patient also showed the usual simple columnar epithelial cells at the edge of the endometrial stroma (Fig. 2). However, in contrast to the samples from the healthy women, none of the samples from the PHA

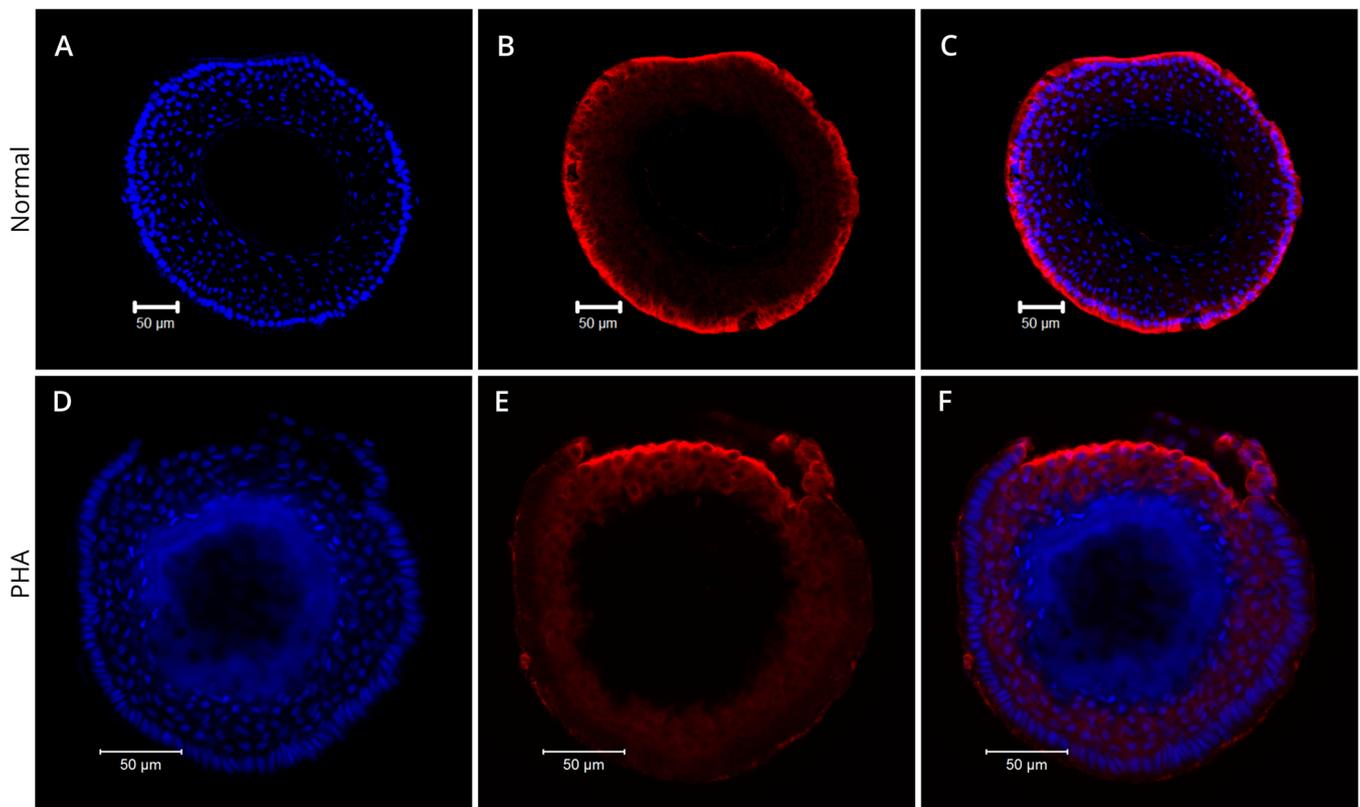


Fig. 3. Confocal microscopic imaging of ENaC immunofluorescence in a cross-section of a hair-follicle from a healthy subject and the PHA patient. A and D) DAPI immunofluorescence of nuclei. B and E) ENaC immunofluorescence. C and F) Merged image. Note that in the sample from a healthy subject (C) the outermost layer showed the strongest red ENaC immunofluorescence. In contrast, in the PHA sample (F) red ENaC immunofluorescence is weak in the outermost layer, but visible in keratinocytes especially strongly in patches where the outermost layer of cells was stripped or broken. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

patient showed strong ENaC immunofluorescence (Fig. 2). At high magnification, a very faint staining could be visualized at the epithelial border.

3.2. ENaC immunofluorescence in hair follicle samples

In an earlier study, we showed that ENaC is expressed in the cytoplasm of keratinocytes in the epidermis and hair follicles [14]. Here, we also examined ENaC localization in the hair follicles of our PHA patient versus healthy women. In a hair cross-section, the youngest cells are located at the outermost layer. As new layers are added, the inner layers of keratinocytes are flattened and keratinized [28].

The blue colored DAPI staining marks the nuclei in the hair follicle samples (Fig. 3). In the samples from both healthy women and PHA patient, ENaC immunofluorescence was detected in the cytoplasm with sharply decreasing intensity towards the central hair shaft. In the samples from healthy women, the strongest ENaC immunofluorescence was detected at the outermost layer of cells (Fig. 3, top layer). In contrast, in the PHA sample, red ENaC immunofluorescence was very low in the outermost layer, but visible strongly in patches where the outermost layer of cells was stripped or broken (Fig. 3F).

4. Discussion

Our results for the first time showed that ENaC expression in the endometrium can be detected by confocal microscopy of immunofluorescently labeled samples obtained from women using Pipelle suction (Fig. 1). In the endometrium, ENaC expression is observed specifically on the luminal side of endometrial epithelial cells [23]. ENaC immunofluorescence observed in the Pipelle samples from all three

healthy women is highly similar to the images we obtained with endometrial tissue sections (Fig. 4 and 5 in reference [23]).

In contrast to samples from healthy women, the endometrial samples obtained from the PHA patient (Table 1) showed that the expression of ENaC on the endometrial luminal surface is drastically reduced and hardly detectable (Fig. 2).

4.1. Reasons for the reduced expression of ENaC in PHA1B

Previous studies demonstrated that the transport and assembly of ENaC heterotrimer on the cell surface are dependent on three intact subunits [16,17]. We showed that mutations in any one of the ENaC subunits can most significantly reduce the cell surface expression of ENaC [18,29]. Specifically, the Arg508X nonsense mutation present in our patient reduces ENaC expression to about 5% of the level observed with wild-type subunits in the heterologous *Xenopus* oocyte expression system [30,31]. Our results on greatly reduced ENaC immunofluorescence in the Pipelle endometrial sample of the PHA patient is consistent with these earlier observations in the *Xenopus* expression system.

We examined ENaC immunofluorescence in hair follicles to serve as a type of positive control. Our previous study on ENaC expression in the epidermis showed that in keratinocytes ENaC is located mainly in cytoplasmic pools [14]. Thus, we hypothesized that the truncated alpha ENaC subunit with an Arg508X mutation (that could not be transported to the cell surface [17]) should be detectable in the cytoplasm. Indeed, in hair follicles from the PHA patient, ENaC immunofluorescence was abundant in the cytoplasm of the keratinocytes except for the outermost single layer of cells (Fig. 3). These results are consistent with our hypothesis that the Arg508X mutation interrupts the transport of the

ENaC subunit to the cell surface, but does not prevent its localization and accumulation in the cytoplasm of keratinocytes. This served as a positive control for the recognition of the mutated subunit by our anti- α -ENaC antiserum [23]. The abundance of mutant Arg508X- α -ENaC intracellularly in these cells indicates that the antisera to protein representing the WT ENaC sequence do indeed bind efficiently to the mutant form, Arg508X- α -ENaC from the PHA1B patient. Thus, the reduced immunofluorescence observed on the endometrial surface of the PHA1B patient compared to healthy controls, is due to drastically reduced abundance or absence of the mutant channel and not to reduced or inefficient recognition of the mutant Arg508X- α -ENaC by the antibodies raised against a WT ENaC sequence.

4.2. Importance of the renin-angiotensin-aldosterone system and ENaC during pregnancy

As noted in the Introduction, the renin-angiotensin-aldosterone axis plays a major role during pregnancy. PHA is characterized by major changes in the levels of these hormones throughout patient's lifetime [17]. Yet, based on the results reported here, we suggest that the major cause of the failure of embryo implantation in the PHA patient is severely reduced expression of ENaC in the reproductive tract. Apparently, ENaC has to be counted among ion channels that play an important role in endometrial receptivity [21]. The report on another systemic PHA type 1 patient who conceived but gave birth to an infant with severe intrauterine growth retardation [20] is also indicative of the importance of ENaC function throughout pregnancy including maintenance of placental function.

Finally, from a general perspective, our results show that endometrial samples obtained by Pipelle biopsy can be used for the identification of key proteins by immunofluorescence. The methods used here may be adapted for examining the expression of other proteins critical for female fertility.

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