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Annexin-2, pentraxin-3, and osteopontin expressions in the endometrium of women with idiopathic recurrent pregnancy loss during the implantation window

İdiyopatik tekrarlayan gebelik kaybı olan kadınların endometriyumunda implantasyon penceresi sırasında Annexin-2, Pentraxin-3 ve Osteopontin ekspresyonları

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Abstract

Aim: Failed expression of endometrial receptivity molecules and genes during the implantation window may lead to idiopathic recurrent pregnancy loss (IRPL). The aim of this study was to investigate annexin-2 (ANXA-2), pentraxin-3 (PTX-3) and osteopontin (OPN) expressions in the endometrium of women with IRPL.

Methods: A total of 34 women with IRPL and 34 age-matched healthy women were recruited in this case control study. Serum samples were collected in the mid-luteal phase of the menstrual cycle and endometrial biopsies were harvested in the window of implantation days. The expressions of ANXA-2, PTX-3, and OPN in the endometrial biopsies according to localizations were examined by immunohistochemistry. The H-score method was used to evaluate the intensity of endometrial ANXA-2, PTX-3, and OPN immune-reactivity.

Results: The mean PTX-3 score was significantly higher in the epithelial endometrium of women with IRPL compared with control cases (2.47 (0.56) vs 1.44 (0.50), $P<0.001$). Both luminal and glandular epithelial and stromal components of the endometrium showed increased staining for PTX-3 in women with IRPL. The increase of PTX-3 expression in the epithelial endometrium correlated with the decrease of serum progesterone level ($P=0.016$). When ANXA-2 and OPN expressions in the epithelial endometrium of IRPL samples were compared with the age-matched control subjects, although there was lower expression, no statistically significant difference was observed (1.97 (0.71) vs 2.21 (0.59), $P=0.145$ and 1.97 (0.79) vs 2.12 (0.68), $P=0.418$).

Conclusion: PTX-3 expression increases in the epithelial and stromal endometrium of women with IRPL during the implantation window. As the serum progesterone level decreases, endometrial PTX-3 expression increases in glandular and luminal epithelium in women with IRPL. Endometrial PTX-3 may be a potential molecular target for IRPL.

Keywords: Annexin-2, Endometrium, Osteopontin, Pentraxin-3, Pregnancy loss

Öz

Amaç: İmplantasyon penceresi sırasında endometriyal reseptivite moleküllerinin ve genlerinin başarısız ekspresyonu, idiyopatik tekrarlayan gebelik kaybına (ITGK) yol açabilir. Bu çalışmanın amacı, ITGK'li kadınlarda endometriumda annexin-2 (ANXA-2), pentraxin-3 (PTX-3) ve osteopontin (OPN) ekspresyonlarını incelemekti.

Yöntemler: Bu vaka kontrol çalışmasına ITGK'li toplam 34 kadın ve yaşları eşleştirilmiş 34 sağlıklı kadın dahil edilmiştir. Menstruel döngüsünün orta luteal fazında serum örnekleri toplandı ve implantasyon penceresi günlerinde endometriyal biyopsiler alındı. Endometriyal biyopsilerde lokalizasyonlara göre ANXA-2, PTX-3 ve OPN ekspresyonları immunohistokimya ile incelendi. Endometriyal ANXA-2, PTX-3 ve OPN immün reaktivitesinin yoğunluğunu değerlendirmek için H-skor yöntemi kullanıldı.

Bulgular: Ortalama PTX-3 skoru, ITGK'li kadınların epitel endometriyumunda kontrol vakalarına göre anlamlı olarak daha yüksekti (2,47 (0,56)'ya karşı 1,44 (0,50), $P<0,001$). ITGK 'li kadınlarda endometriyumun hem luminal hem de glandüler epitel ve stromal bölümleri, PTX-3 için artmış boyanma gösterdi. Epitelyal endometriyumda PTX-3 ekspresyonundaki artış, serum progesteron seviyesindeki düşüş ile korelasyon gösterdi ($P=0,016$). ITGK örneklerinin epitel endometriyumundaki ANXA-2 ve OPN ekspresyonları yaşa uygun kontrol denekleriyle karşılaştırıldığında daha düşük ekspresyon görülmesine rağmen istatistiksel olarak anlamlı bir fark gözlenmedi (1,97 (0,71)'e karşı 2,21 (0,59), $P=0,145$ ve 1,97 (0,79)'a karşı 2,12 (0,68), $P=0,418$).

Sonuç: İmplantasyon penceresi sırasında ITGK'li kadınların epitel ve stromal endometriyumunda PTX-3 ekspresyonu artmaktadır. ITGK'li kadınlarda serum progesteron seviyesi düştükçe glandüler ve lüminal epitelde endometrial PTX-3 ekspresyonu artmaktadır. Endometriyal PTX-3, ITGK için potansiyel bir moleküler hedef olabilir.

Anahtar kelimeler: Annexin-2, Endometrium, Osteopontin, Pentraxin-3, Gebelik kaybı

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Introduction

Recurrent pregnancy loss (RPL) is defined as two or more consecutive failed pregnancies [1]. In obstetric practice, systematic evaluation is generally recommended after the second consecutive pregnancy loss [2]. Although anatomic, genetic, immunological, thrombophilic or endocrinological factors play a role in the development of RPL, half of cases have an unclear pathogenesis and are diagnosed with idiopathic RPL (IRPL) [3]. Couples are mostly disappointed with the process of the diagnosis of this situation, since the etiology continues to remain mostly unclear and there is a lack of evidence regarding effective treatment.

Implantation of the human embryo into the maternal endometrium is the main step in the establishment of a successful pregnancy [4]. Blastocyst adhesion in the uterine epithelium depends on endometrial receptivity, which is driven by ovarian steroids during the mid-luteal phase of the menstrual cycle [5]. The appearance of some molecules, such as integrin b3, leukemia inhibitor factor and mucin-1, in the luminal epithelium during the implantation window has been proposed as a biomarker of uterine receptivity [6]. A number of earlier studies suggested that failure of the endometrium to express a receptive phenotype is thought to be one of the causes of IRPL [7,8].

Annexins have a significant impact on the physiological and pathological processes involved in cell growth, differentiation, apoptosis, and signal transduction [9]. During the human implantation process, annexin-2 (ANXA-2) increases embryo adhesiveness, endometrial epithelial cell migration, and trophoblast overgrowth [10]. This protein has been identified as a major contributor to the human receptive endometrium [11]. ANXA-2 is strongly expressed in endometrial glands and the luminal epithelium in the mid- and late-secretory endometria for embryo adhesiveness [12].

Pentraxin-3 (PTX-3) is an acute-phase reactant and a member of the pentraxin protein family [13]. Several studies have demonstrated that PTX-3 has an important role in innate immunity, inflammation, implantation, decidualization, and placentation [14,15,16]. It has been determined that PTX-3 is expressed in the receptive endometrium, and trophoblasts have been found to affect PTX- expression in decidua [17]. PTX-3 has been proposed as a novel biomarker for the prediction of placental failure [18].

Osteopontin (OPN) is mainly involved in cell proliferation, adhesion, migration, and angiogenesis in the endometrium [19]. OPN and its receptor avB3 integrin are expressed in increasing concentrations in glandular and luminal epithelium during the secretory phase, as markers of endometrial receptivity [20]. It has been demonstrated that OPN expression is regulated by progesterone and reduces significantly in RPL patients [21].

The aim of this study was to examine the expressions of ANXA-2, PTX-3 and OPN in the endometrium of patients with IRPL and to compare these with those in a control group of subjects with a history of healthy live births.

Materials and methods

In this case-control study, 34 women were enrolled with a history of IRPL in the Infertility Clinic of Pamukkale University Hospital from June 2013 to June 2014. A control group was formed of 34 healthy and fertile, reproductive-aged women volunteers. The staining intensities of ANXA-2, PTX-3 and OPN in the endometrium during the mid-secretory phase were investigated; the serum progesterone level was measured on the same day. The study protocol was approved by the Clinical Research Ethics Committee of Medical School of Pamukkale University, with the number 11/04/2014-TPF012 and all the participants were informed about the study. The guidelines of the Declaration of Helsinki were followed.

Patients had a minimum of two consecutive pregnancy losses during the first trimester and the control subjects had at least one previous uneventful pregnancy with healthy obstetric histories. To understand the etiology of miscarriage, the patients visited the Infertility Clinic in the Medical School of Pamukkale University and underwent a full examination. They were diagnosed as IRPL with no underlying anatomic, genetic, immunological, thrombophilic, or endocrine factors. All the participants were required to meet the following inclusion criteria: Between 18–35 years of age, regular menstrual cycles (25–35 days), and no use of hormonal supplementation within the last 6 months. Cases were excluded if they had an endometrial pathology such as Asherman syndrome, endometrial polyp, and/or sub-mucous fibroids, or a diagnosis of pelvic inflammatory disease.

Each woman was followed for one menstrual cycle. Ovulation was monitored with transvaginal ultrasonography (GE Voluson® E6, IC5-9-D/GYN transducer; GE Healthcare, Zipf, Austria). The day when the luteinizing hormone (LH) surge occurred was identified by measuring LH levels in the blood samples. Endometrial biopsies were performed seven days after ovulation, during the mid-secretory phase. The endometrial samples were collected from the uterine cavity via a Karman cannula. For immunohistochemical examination, tissues were fixed in 4% formalin and finally embedded in paraffin. Histological dating was evaluated according to the criteria of Noyes [22]. All biopsies were found to be in the correct phase.

Overall, 68 biopsy samples were analyzed. Three serial sections were prepared from each biopsy sample. The 3 μm paraffin embedded sections were incubated overnight at 60°C for deparaffinization. Concentrated polyclonal antibodies against Annexin-2 (ANXA-2, Thermo Fisher, Co Ltd, US), Pentraxin-3 (PTX-3, Thermo Fisher, Co Ltd, US), and Osteopontin (OPN, Thermo Fisher, Co Ltd, US) were diluted 1:500, 1:25, and 1:50, respectively. All the immunohistochemical steps were carried out in the fully automated closed system of Ventana Benchmark XT (Roche Groups, Switzerland). Following washing with distilled water, rehydrating in xylene and a series of graded ethanol solutions at room temperature, slides were mounted with Entellan® (Merck, Darmstadt, Germany). All slides were exposed to amino ethylcarbazole chromogen, counterstained with hematoxylin, and mounted with aqueous mount. The term human placenta was used as positive control. Slides were selected at random and were examined by a single pathologist blinded to the group origin of the slide. The stained sections were

observed under a microscope (Nikon Ecclipse E200, Nikon, Japan) (10x ocular and 4x objective lenses). To evaluate the intensity of endometrial ANXA-2, PTX-3 and OPN immunoreactivity, the H-score method was used [23]. The reactivity of each antibody with the luminal epithelium, glandular epithelium, and stromal cells was assessed carefully. This is a semi quantitative method measuring the percentages of positively stained cells multiplied by a weighted intensity of staining: H-Score=Pi (i + 1), where Pi is the percentage of ANXA-2, PTX-3 and OPN stained endometrial cells in each intensity category (0–100%), and i is the intensity indicating weak (i=1), moderate (i=2), or strong staining (i=3) [24].

Serum progesterone levels were assessed using a chemiluminescent immunoassay (Liaison Assay; Diasorin, Italy). In the analysis, the day-to-day variation in progesterone level was excluded by collecting samples at a fixed time (11:00 am).

Statistical analysis

No clear data could be found in literature of ANXA-2, PTX-3 and OPN staining in the endometrial biopsy samples of patients with IRPL. Therefore, to define the sample size of a pilot study, 34 patients and 34 control subjects were selected based on the available number of cases. Data were analyzed using the Statistics Package for Social Science version 17.0 software (SPSS Inc, Chicago, IL, USA). Continuous variables were presented as mean (standard deviation (SD)) values. Differences were analyzed using the parametric Student's t-test. Correlation analysis was applied for multiple comparisons. A value of $P<0.05$ was considered statistically significant. The package used generated significant small round off errors, which had an estimated effect on the results in the order of 103.

Results

Overall, the data of 68 women (34 with IRPL and 34 without IRPL) were analyzed in this study. All menstrual cycles were ovulatory according to ultrasonographic criteria and mid-luteal serum progesterone concentration >10 ng/ml. The clinical characteristics of the women in the patient and control groups are summarized in Table 1. The mean age was 26.94 (4.57) years in the patient group and 28.82 (4.60) years in the control group ($P=0.096$). No statistically significant difference was determined between the groups with respect of body mass index (BMI), as 25.26 (2.84) kg/m^2 in the patient group and 25.74 (2.84) kg/m^2 in the control group ($P=0.497$). Women in the patient group were found to have a median of 3 previous miscarriages.

The comparisons of H-score of immunohistochemical ANXA-2, PTX-3 and OPN expression in women with IRPL and without IRPL are presented in Table 1.

In the patient group, the mean H-score of the endometrial epithelial and stromal ANXA-2 expression was similar to that of the control group (1.97 (0.71) vs 2.21 (0.59), $P=0.145$ and 1.91 (0.62) vs 2.18 (0.57), $P=0.073$, respectively). Both the glandular and luminal components of the epithelial endometrium showed decreased staining for ANXA-2 without statistical significance. The decreased ANXA-2 immunoreactivity was predominantly localized to the luminal epithelial cells (Figure 1a, 1b, 1c).

The mean H-score of the endometrial epithelial PTX-3 expression was significantly increased in the patient group compared to the control group (2.47 (0.56) vs 1.44 (0.50), $P<0.001$). Increased PTX-3 expression was detected in the cytoplasmic and membranous parts of glandular and luminal epithelial cells of the endometrium in women with IRPL. The stromal component of the endometrium showed the most increased staining for PTX-3 in women with IRPL (Figure 1d, 1e, 1f).

The mean H-scores of the endometrial epithelial and stromal OPN expressions of groups were similar (1.97 (0.79) vs 2.12 (0.68), $P=0.418$ and 1.79 (0.64) vs 2.06 (0.69), $P=0.107$). The greatest difference between the groups in OPN staining was detected in the glandular epithelium without statistical significance (Figure 1g, 1h, 1i).

The correlations between serum progesterone level and PTX-3 staining intensity scores in the patient and control groups are presented in Table 2. A negative correlation was detected between serum progesterone level and staining intensity for PTX-3 in epithelial endometrium in women with IRPL ($P=0.016$). This negative correlation was determined in both the glandular and luminal epithelium of the endometrium ($P=0.038$ and $P=0.002$). No correlation was found between serum progesterone level and stromal PTX-3 expression in women with IRPL.

Table 1: Demographic characteristics and the H-scores of the endometrial ANXA-2, PTX-3 and OPN expressions in women with IRPL and without IRPL

	Women with IRPL n=(34)	Women without IRPL n=(34)	*P*-value
Age (year)	26.94 (4.57)	28.82 (4.60)	0.096
BMI (kg/m^2)	25.26 (2.84)	25.74 (2.84)	0.497
Epithelial ANXA-2 score	1.97 (0.71)	2.21 (0.59)	0.145
Glandular	2.06 (0.64)	2.18 (0.57)	0.432
Luminal	1.79 (0.72)	2.09 (0.51)	0.060
Stromal ANXA-2 score	1.91 (0.62)	2.18 (0.57)	0.073
Epithelial PTX-3 score	2.47 (0.56)	1.44 (0.50)	<0.001*
Glandular	2.44 (0.61)	1.38 (0.49)	<0.001*
Luminal	2.50 (0.56)	1.41 (0.50)	<0.001*
Stromal PTX-3 score	2.38 (0.55)	1.38 (0.49)	<0.001*
Epithelial OPN score	1.97 (0.79)	2.12 (0.68)	0.418
Glandular	2.00 (0.77)	2.29 (0.52)	0.072
Luminal	1.74 (0.75)	1.97 (0.75)	0.203
Stromal OPN score	1.79 (0.64)	2.06 (0.69)	0.107
Progesterone (mg/dl)	11.56 (3.65)	11.97 (2.94)	0.611

ANXA-2: Annexin-2, PTX-3: Pentraxin-3, OPN: Osteopontin. Variables presented as mean (standard deviation).

Table 2: Correlations between serum progesterone level and PTX-3 scores in women with IRPL and without IRPL

	Serum progesterone level (mg/dl)	
PTX-3 scores in women with IRPL	r	*P*-value
Epithelial	-0.411	0.016*
Glandular	-0.357	0.038*
Luminal	-0.507	0.002*
Stromal	-0.200	0.258
PTX-3 scores in women without IRPL	r	*P*-value
Epithelial	0.111	0.532
Glandular	0.050	0.780
Luminal	0.111	0.531
Stromal	0.050	0.780

ANXA-2: Annexin-2, PTX-3: Pentraxin-3, OPN: Osteopontin

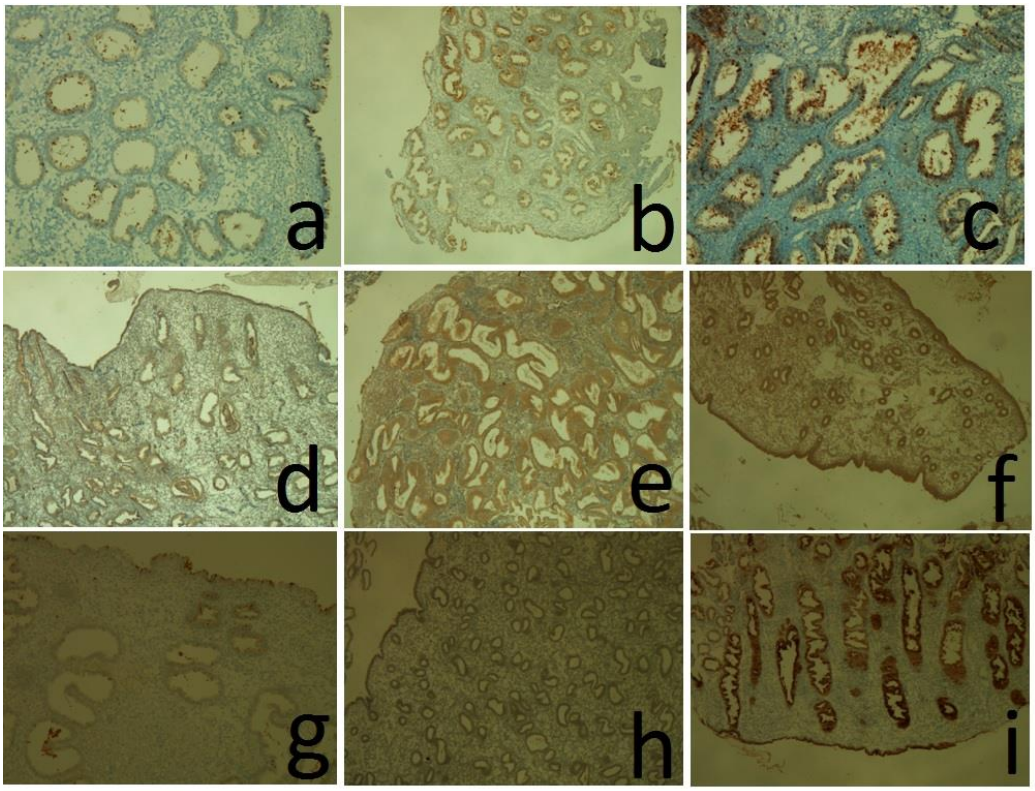

Figure 1: Antibody staining scores for endometrial localizations (ANXA-2 staining: a: score 1, luminal epithelium; b: score 2, luminal and glandular epithelium; c: score 3, glandular epithelium and stroma. ax4, bx4, cx10, PTX-3 staining: d: score 1, luminal and glandular epithelium; e: score 2, glandular epithelium; f: score 3, luminal and glandular epithelium and stroma. dx10, ex10, fx4, OPN staining: g: score 1, luminal epithelium; h: score 2, luminal and glandular epithelium; I: score 3, luminal and glandular epithelium and stroma. gx4, hx4, ix4)

Discussion

The results of the current study demonstrate that the PTX-3 expression increases in the mid-secretory endometrium of women with IRPL during the implantation window. Increased PTX-3 expression was detected in both the epithelial and stromal endometrium. However, ANXA-2 and OPN expressions in the IRPL group were similar to those of the control group. A mild negative correlation was detected between serum progesterone level and staining intensity for PTX-3 in glandular and luminal epithelial cells of mid-luteal endometrial biopsies in women with IRPL. One of the mechanisms responsible for IRPL in this study group may have been the increased PTX-3 expression in all areas of the endometrium.

Pregnancy is a well-programmed physiological process that involves a dynamic maternal and fetal crosstalk [25]. Local immune tolerance, angiogenesis, cytokine and integrin balance, cellular, and molecular trafficking are initiated with implantation and continue throughout the gestation period; deregulation of any of these processes may result in a miscarriage [26]. The proteins produced locally in the endometrial epithelium play an indisputable role in the continuity of a successful pregnancy [27]. However, the prognostic value of these proteins as biomarkers in patients with IRPL remains unclear. Therefore, with the use of immunohistochemistry, we aimed to assess the endometrium during the implantation window in the mid-secretory phase to identify a prognostic marker for IRPL diagnosis and management.

Trophoblasts and stromal cells secrete many substances into the endometrium for a successful implantation, which involves the development of placental vasculature and anchoring, while preventing rejection of semi-allograft [28,29]. The PTX-3 gene is up-regulated during the immune response in early pregnancy [30]. However, an abnormally exaggerated endometrial inflammatory response could cause RPL [31]. A recent study revealed that the level of PTX-3 in maternal serum and placenta were elevated in pre-eclampsia and intrauterine growth restriction (IUGR) [32]. In the present study, increased expression of PTX-3 in the epithelial endometrium was determined in patients with IRPL compared with the control group. Secondly, the localization of strong PTX-3 staining was detected equally in all areas of the endometrial biopsy specimens including the luminal epithelium, glandular epithelium, and stroma. In addition, all the control specimens showed weak staining with PTX-3. It reflects an exaggerated local inflammatory reaction in the whole endometrium of patients with IRPL. The third finding was that PTX-3 staining of patient samples increased with the fall in the progesterone level. Progesterone could directly affect PTX-3 expression in the implantation window or it could regulate the expression and synthesis of several integrins and cytokines indirectly. Further studies are required to clarify the exact mechanism of these interactions.

Annexins are present in the secretory luminal epithelium of endometrium and regulate the receptivity and implantation process. Fowler et al. identified annexins among the irregular proteins in women with endometriosis [33]. Genetic studies have shown that alterations of annexin haplotypes are related to RPL, IUGR, and pre-eclampsia [34,35]. It has been proven that pregnant women with anti-phospholipid syndrome often exhibit autoantibodies against ANXA-5 and the fetus is lost

spontaneously during the early stages of the pregnancy [36]. It has been postulated that ANXA-5 could be a significant auto-antigen for pregnancy loss, acting as an anti-thrombotic agent during pregnancy [37]. In the current study, the weak intensity of ANXA-2 staining in the endometrial biopsy specimens from patients with IRPL compared with the control subjects could be attributed to defective implantation, immunoregulation, or coagulation. Slightly lower ANXA-2 staining was detected in the endometrial biopsies of patients with IRPL than without IRPL, and the localization of ANXA-2 staining was the same in both groups. Despite the findings of several previous studies, these results suggest that ANXA-2 is not a distinctive protein for the pathogenesis of recurrent miscarriages.

Osteopontins are the most upregulated extracellular matrix adhesion molecules in the endometrium as it becomes receptive to implantation [38]. It was hypothesized in this study that the endometrium in the luteal phase of IRPL patients would show weak staining intensity for OPN compared with the control group because blockage of the OPN entity inhibits embryo adhesion, implantation, and the angiogenesis stage. In the immunohistochemical analysis, although the difference was not statistically significant, a slightly lower intensity of OPN staining was observed in samples from patients with IRPL compared to those of the control group. No difference was detected between the localizations of OPN staining in the specimens. In this context, the immunohistochemical examinations in a previous animal study showed that the cellular localization of OPN was mainly restricted to glandular epithelium [39]. The results obtained from the current study do not confirm that OPN deficiency in the endometrium in the luteal phase causes pregnancy loss.

Limitation

In this study, the IRPL group was considered as patients with two or more consecutive pregnancy losses. However, if patients with three and more consecutive pregnancy losses had been included, some outcomes may have been different.

Conclusion

The results of the current study demonstrated that the PTX-3 expression increases in the endometrium of women with IRPL during the implantation window. As serum progesterone level decreases, PTX-3 expression increases in the glandular and luminal epithelium and stroma. ANXA-2 and OPN expressions in the endometrium of women with IRPL are almost similar to those of women without IRPL. PTX-3 staining of endometrial biopsies can be used as a prognostic marker for IRPL and as a specific target for therapeutic implications of IRPL during the implantation window.

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