http://informahealthcare.com/gye
ISSN: 0951-3590 (print), 1473-0766 (electronic)

GYNECOLOGICAL ENDOCRINOLOGY

Gynecol Endocrinol, Early Online: 1–4
 DOI: 10.3109/09513590.2015.1127912

Taylor & Francis
Taylor & Francis Group

ORIGINAL ARTICLE

Endometrial LGR7 expression and implantation failure

Maria Rosaria Campitiello, Francesca Caprio, Daniela Mele, Diletta D'eufemia, Nicola Colacurci, and Pasquale De Franciscis

Department of Obstetrics, Gynaecology and Reproductive Sciences, Outpatient Fertility Clinic, Second University of Naples, Largo Madonna Delle Grazie 1, Naples 8, Italy

Abstract

Implantation failure is considered as a major cause of infertility in women with recurrent pregnancy loss (RPL) and in otherwise healthy women with unexplained infertility. Preliminary data in primates suggested that relaxin (RLX) is involved in endometrial preparation for implantation. In a prospective observational study, the endometrial RLX receptor (LGR7) expression was assessed in three groups of patients with regular ovulatory cycle and normal uterine cavity: 23 with RPL (Group A), 23 with unexplained infertility undergone at least three cycles of failed *in vitro* fertilization (IVF) reporting good oocyte and embryo quality (Group B), 23 with proven fertility (Group C). Assessment of LGR7 expression was performed with both polymerase chain reaction (PCR) analysis and immunohistochemistry on endometrial samples obtained with hysteroscopic biopsy performed in the secretory phase of the menstrual cycle. Endometrial LGR7 was less expressed in group A and B versus C, both by PCR analysis ($p = 0.024$) and immunohistochemistry. The decreased expression of the endometrial RLX receptor in women with implantation failures, both *in vitro* fertilization failure and recurrent pregnancy loss, suggests that RLX may play a crucial role in the structural and functional changes of the endometrium during the window of implantation.

Keywords

Endometrial biopsy, implantation failure, relaxin receptor, window of implantation

History

Received 28 November 2015
Accepted 1 December 2015
Published online 12 January 2016

Introduction

Implantation failure is considered as a major cause of infertility in women with recurrent pregnancy loss (RPL), otherwise in healthy women with unexplained infertility, and in several repeated unsuccessful IVF cycles. The human endometrium undergoes complex proliferative and secretory changes in each menstrual cycle and exhibits only a short period of receptivity known as ''window of implantation'' [1], depending on paracrine signals from stromal cells underlying the luminal epithelium [2]. The timing and duration of the ''window of implantation'' are major endometrial determinants of the likelihood of reproductive success. A large variety of molecules, including adhesion molecules, cytokines, growth factors, lipids and others, are detected at high levels during this ''window'' and it has been postulated that they may be involved in the implantation process [3]. Indeed, the clinical use of these markers of endometrial receptivity has been hypothesized to improve the management of recurrent abortion and the implantation rate in women with previous failed IVF cycles [4].

Relaxin (RLX) is a peptide hormone produced by the corpus luteum during the luteal phase and in the first trimester of pregnancy. It belongs to the family of insulin-like growth factor and is composed of two peptide chains, A and B, of 24 and 29 amino acids respectively, linked by disulfide bridges. RLX LGR-7, the classical RLX receptor, is expressed in many tissues, including human endometrium [5–7]. Preliminary data in primates suggested that RLX is involved in endometrial preparation for implantation [8]. In a previous study, we showed increased LGR7 expression in the early secretory phase confirming the involvement of RLX in the physiology of human endometrium and suggesting a role for RLX in implantation [9]. Other studies showed that RLX levels are impaired in women with early pregnancy loss [10] and that granulosa cell production of RLX is predictive of pregnancy outcome in IVF cycles [11]. In this view, the aim of our study was to assess the RLX receptor expression in the endometrium of women with implantation failure.

Address for correspondence: Maria Rosaria Campitiello, Department of Obstetrics, Gynaecology and Reproductive Sciences, Outpatient Fertility Clinic, Second University of Naples, Largo Madonna Delle Grazie 1, 80138 Naples 8, Italy. E-mail: maracampitiello@gmail.com

Materials and methods

Patients

Two groups of patients referring to the outpatient infertility Clinic of the Second University of Naples were enrolled: 23 patients with three or more consecutive pregnancy losses before 12 weeks gestation (Group A) and 23 patients with unexplained infertility after having undergone at least three cycles of failed IVF and with reported fresh transfer of good quality embryos based on a morphological analysis [3] (Group B). All the women were aged >18 years and ≤ 35 years, had regular ovulatory cycle, normal pelvic examinations; hormone determinations were normal on day 3 of the menstrual cycle at the time the study was performed (FSH ≤ 10 mIU/ml, LH ≤ 10 mIU/ml, E2 >50 pg/mL), transvaginal ultrasound showed normal ovaries, hysteroscopy excluded endometrial pathologies, karyotype was normal, and a laparoscopy excluded endometriosis and confirmed a normal

pelvis; semen analysis was normal. A homogeneous comparable group of 23 women was enrolled with proven fertility (defined as at least 2 spontaneous successfully delivered term pregnancies) and normal uterine cavity found at hysteroscopy performed because of ultrasound findings suspicious for endometrial pathology (Group C: control group). All patient presented similar demographic and baseline characteristics.

Exclusion criteria were as follows: PCOS, hyperprolactinemia, thrombophilic conditions, ovarian or adrenal disease, previous uterine surgery within 3 months, abnormal uterine bleeding, leiomyoma in or near the uterine cavity, known or suspected endometriosis, chronic diseases (e.g. systemic lupus erythematosus and diabetes mellitus), abnormal semen analysis of male partner; in addition, poor ovarian response (<3 oocytes previous pick up) in previous ART cycles for group B. All patients did not receive hormones for 6 months before sample collection. Institutional review approval was obtained for the study. Written consent was obtained from the patients for the collection and analysis of data.

Endometrium collections

An endometrial biopsy was obtained from all the patients in the secretory phases [12–14] of the menstrual cycle confirmed by serum progesterone assay (>5 mU/ml). All the patients were requested to abstain from unprotected intercourse in the cycle the hysteroscopy was planned. The endometrial biopsy was performed during the hysteroscopic procedure on both anterior and posterior aspect of the uterine cavity with a 5 Fr grasping forcep. Two endometrial specimens were obtained from all the biopsies: one was fixed in formalin and subsequently paraffin embedded for immunochistochemistry analysis and one was immediately immersed in RNA later and stored at −20° for PCR analysis.

Histological analysis

The specimens were considered adequate according to the routine criteria for adequacy of endometrial biopsies applied by the consultant pathologists in the routine Biopsy Service. Histological examination was performed on sections stained with hematoxylin and eosin [15]. Immunohistochemistry was carried out as previously described [16]. Slides were then incubated at 4°C overnight with antibodies raised against human LGR7 (sc-50328, rabbit polyclonal IgG, Santa Cruz Biotechnology) at a 1:100 dilution. Negative controls were prepared by substituting the primary antiserum with nonimmune IgG, and positive controls by using the antibody on a tissue section where the protein had already been demonstrated. Three observers evaluated the staining pattern separately, then scored each specimen for staining intensity signal: 0 (absent immunopositivity); 1 (low immunopositivity); 2 (moderate immunopositivity); 3 (intense immunopositivity). An average of 22 fields was observed for each tissue.

Detailed protocols for RNA extraction, cDNA amplification with PCR method using specific oligonucleotides raised against the human LGR7 sequence, electrophoresis of PCR products and densitometry of each sample are reported in detail in Supplemental Digital Content (S1).

Statistical analysis

All analyses were performed with R software, version 2.9.1 (Development Core Team. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing. 2009). For both PCR analysis and immunohistochemistry, the data reported represent the mean and median values of each group. A Kruskal–Wallis test for multiple comparisons was performed to compare LGR7 within each groups and between different groups.

Results

For PCR analysis, a remarkable difference between groups was found ($p = 0.02422$). Mean values and medians were 1.10 and 1.12 for group A, 0.94 and 0.95 for group B, 1.70 and 1.68 for group C (detailed data of LGR7 expression value are reported in Supplemental Digital Content S2). Multiple comparison tests show a significant difference between group B versus C and A versus C (Figure 1). Regarding immunohistochemistry, in groups A and B RLX LGR7 was expressed at a low level, whereas a significantly higher immunopositivity (moderate/intense) was present in samples of group C ($p = 0.05$) (Figures 2 and 3).

Discussion

Our study shows a decreased expression of the endometrial receptor LGR7 in women with implantation failure, suggesting that RLX plays a critical role in the complex mechanisms of endometrial preparation to embryo implantation. Such endometrial development resulting in endometrial receptivity during the window of implantation requires subtle collaboration of an extremely large number of different factors. Many of them have been described and are highly detected [3]. It has been suggested that these factors could be used as markers of endometrial receptivity: this could lead to their potential usefulness in the treatment of female infertility secondary to implantation failure [17].

Impaired decidualization of the endometrium prior to conception is a relevant mechanism underlying RPL, either by prolonging the implantation window, thereby disabling natural embryo selection or by disrupting the maternal responses to embryonic signals [18]. Endometrial failure to express a receptive phenotype is thought to be a major cause of infertility in IVF treatment failure [19]. The average implantation rate in IVF is around 25% [20] and two-thirds of implantation failures recognized as a trigger inadequate uterine receptivity, whereas the embryo itself is responsible for only one-third of these failures [12,21]. In addition, prolonged endometrial receptivity facilitates implantation of delayed or compromised embryos and has a strong association with early pregnancy loss [13,18]. To increase implantation rates, an increased knowledge of the factors involved in embryo implantation is required. A better understanding of the mechanisms regulating embryo implantation may improve the ability of clinicians to treat infertility, to prevent early pregnancy loss and improve IVF outcome. Evaluation of the implantation markers with endometrial biopsy samples may help to detect occult implantation deficiency and predict pregnancy outcome [14]. In the future, optimizing endometrial receptivity in fertility treatment and manipulating the expression of key endometrial genes with medical, surgical or future cell and gene-based therapies may improve implantation rates.

Although many molecules enhance progesterone-induced decidualization, only cAMP and RLX are known to stimulate human endometrial stromal cells (HESC) decidualization *in vitro*, independent of progesterone [22]. A large body of data that support a role for RLX in human endometrial decidualization has been generated in *in vitro* models. RLX is an extremely potent stimulator of the secretion of various hormones and growth factors, including insulin-like growth factor-binding protein and prolactin. hallmarks of decidualization. The effects of RLX on implantation and the onset of pregnancy were studied in vivo in Macaca monkeys, showing a plausible hormone's role in implantation processes [23]. To date, there are few data obtained in humans. Some studies on humans have demonstrated that RLX levels were impaired in women with early pregnancy loss [10] and that granulosa cell production of RLX was predictive of pregnancy outcome in IVF patients [11]. Our previous study [9] showed that LGR7 transcript is detectable in the human

Figure 1. A box plot of the quantified expression value for LGR7 in different groups.

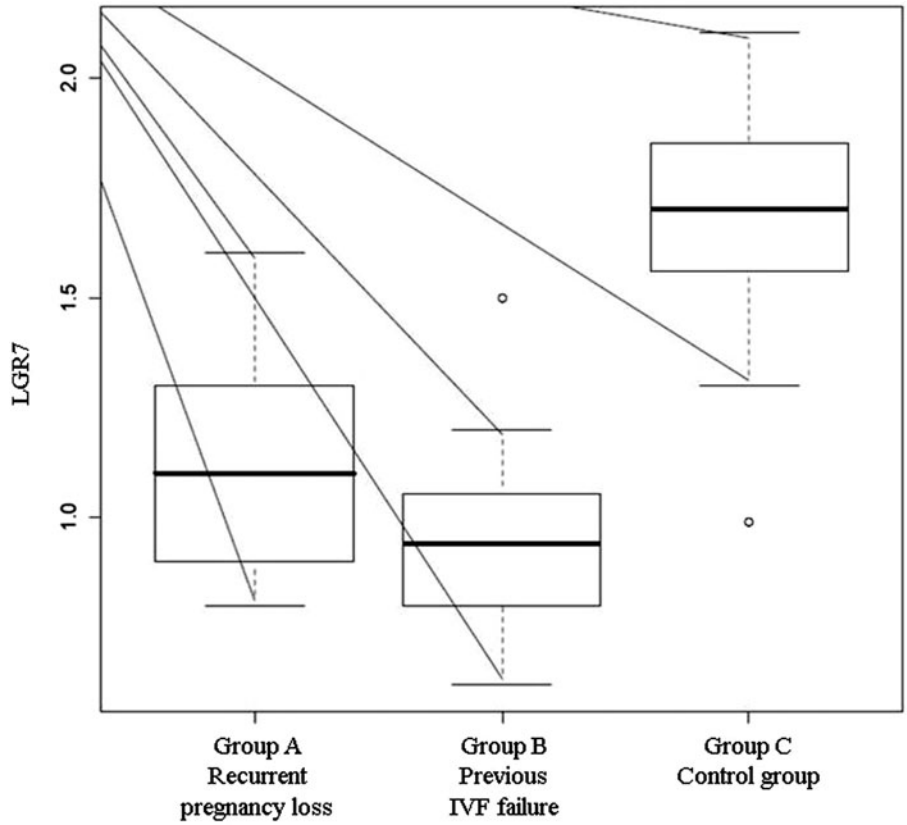
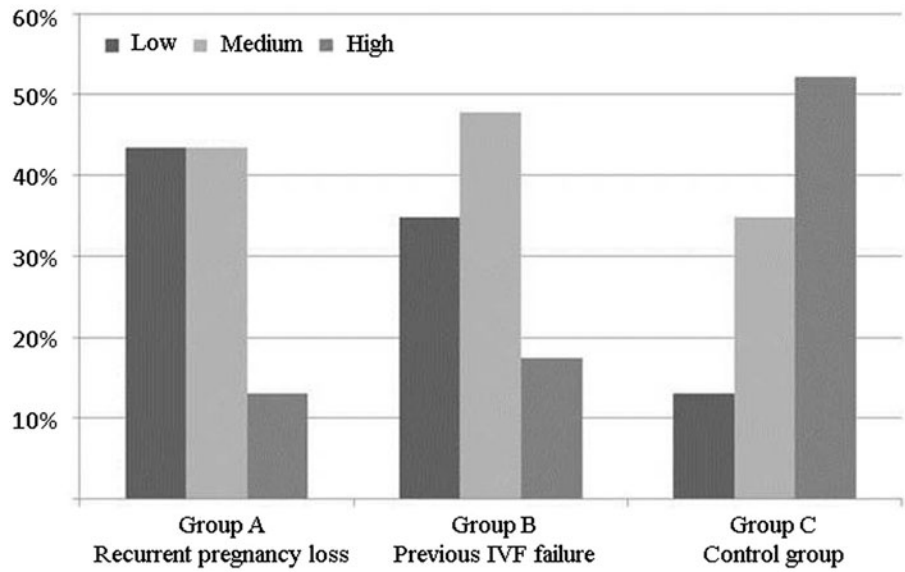


Figure 2. Expression of immunopositivity of LGR7 in endometrium samples.



endometrium in all the four phases of menstrual cycle (early and late proliferative phase, early and late secretory phase) and that LGR7 expression shows a striking significant increase in the early secretory phase, suggesting a role for RLX in implantation. To date, data on correlation between impaired function of endometrial RLX and defective implantation are lacking.

The current study describes for the first time the expression of LGR7 in the biopsies of human endometrium obtained from women with different reproductive history: women with proven fertility, with RPL and with repeated IVF failures. In the latter group, only those patients who reported a good oocyte and embryo quality in the previous failed IVF cycles were considered to avoid including failures of IVF due to gametes alterations. The decreased expression of the endometrial receptor LGR7 in women with implantation failures, both IVF failure and RPL, suggests which RLX may play a crucial role in the structural and functional changes of the endometrium during the ''window of implantation''. In addition, the reduced expression of LGR7 in women affected by RPL leads to hypothesize a role of RLX in the timing of the ''window of implantation'', assuming that a deficient action of this hormone can contribute to a desynchronization of events that lead to endometrial receptivity, with the result of an increased abortion rate [13,18]. Since an increase in mRNA expression does not mean that this is associated with the presence of a functional protein, we also performed an assessment of the tissue expression of the protein by immunohistochemistry. Results confirmed a lower presence of LGR7 in endometrial samples of patients with implantation failure. Indeed, our study has some limitations: in

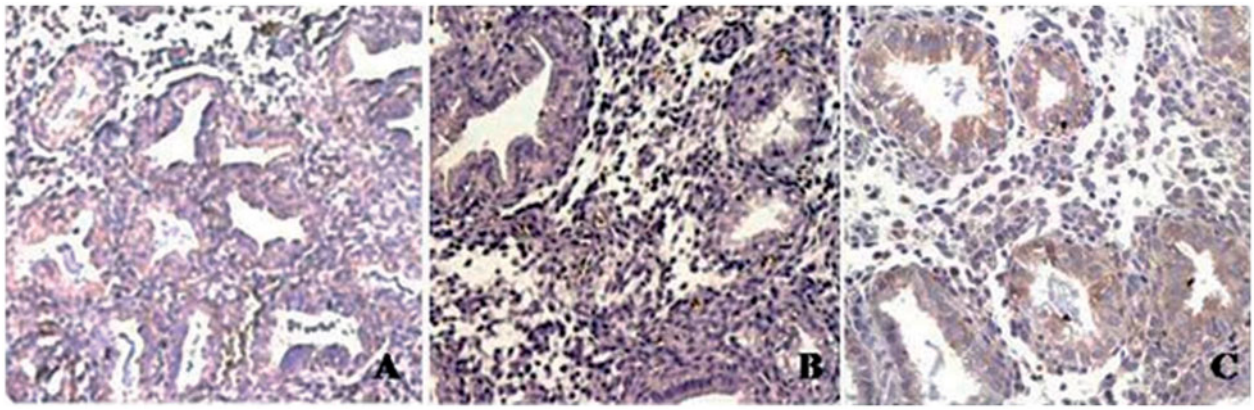


Figure 3. Immunostaining localization of LGR7 in endometrium samples.
Low immunopositivity of LGR7 in glandular and stroma of endometrium samples of group A and B, elevated immunopositivity of LGR7 in glandular compartment and moderate immunopositivity in stroma compartment of endometrium samples of group C. Magnifications: × 10 (A), × 20 (B), × 40 (C).

the control group the time elapsed between the last pregnancy and the sample collection and the possible occurrence of confounding factors were not considered. Moreover, like all *in vivo* studies, our study was performed in nonconception cycles, and cyclic expression of endometrial factors is thus unrelated to a subsequently ensuing pregnancy.

Establishing an etiology for preimplantation and preclinical losses is not easy, but the one proven explanation is morphologic abnormalities in the early embryo. It is presumed that most are due to chromosomal abnormalities, but such chromosomal abnormalities have not been evaluated in the study because not allowed by Italian law at the time of the study. In conclusion, our work opens up new possibilities to study and understand the mechanisms underlying embryo implantation, underscoring the importance that RLX through the action of its receptor LGR7 plays in endometrium in order to ensure a successful implantation.

Acknowledgements

We thank Simona Signoriello and Giuseppe Signoriello for their statistical support.

Declaration of interest

The authors report no declarations of interest

References

1. Strowitzki T, Germeyer A, Popovici R, von Wolff M. The human endometrium as a fertility-determining factor. Hum Reprod Update 2006;12:617–30.
2. Dey SK, Lim H, Das SK, et al. Molecular cues to implantation. Endocr Rev 2004;25:341–73.
3. Achache H, Revel A. Endometrial receptivity markers, the journey to successful embryo implantation. Hum Reprod Update 2006;12: 731–46.
4. Patel BG, Lessey BA. Clinical assessment and management of the endometrium in recurrent early pregnancy loss. Semin Reprod Med 2011;29:491–506.
5. Bond CP, Parry LJ, Samuel CS, et al. Increased expression of the relaxin receptor (LGR7) in human endometrium during the secretory phase of the menstrual cycle. J Clin Endocrinol Metab 2004;89: 3477–85.
6. Lowndes K, Amano A, Yamamoto SY, Bryant-Greenwood GD. The human RLX receptor (LGR7): expression in the fetal membranes and placenta. Placenta 2006;27:610–18.
7. Mazella J, Tang M, Tseng L. Disparate effects of RLX and TGFbeta1: RLX increases, but TGF beta1 inhibits, the RLX receptor and the production of IGFBP-1 in human endometrial stromal/ decidual cells. Hum Reprod 2004;19:1513–18.
8. Stewart DR, Celniker AC, Taylor Jr CA, et al. Relaxin in the periimplantation period. J Clin Endocrinol Metab 1990;70:1771–3.
9. Campitiello MR, De Franciscis P, Mele D, et al. Endometrial LGR7 expression during menstrual cycle. Fertil Steril 2011;95:2511–14.
10. Stewart DR, Overstreet JW, Celniker AC, et al. The relationship between hCG and RLX secretion in normal pregnancies vs periimplantation spontaneous abortions. Clin Endocrinol 1993;38:379–85.
11. Stewart DR, VandeVoort CA. Relaxin secretion by human granulosa cell culture is predictive of in-vitro fertilization-embryo transfer success. Hum Reprod 1999;14:338–44.
12. Simon C, Moreno C, Remohi J, Pellicer A. Cytokines and embryo implantation. J Reprod Immunol 1998;39:117–31.
13. Teklenburg G, Salker M, Heijnen C, et al. The molecular basis of recurrent pregnancy loss: impaired natural embryo selection. Mol Hum Reprod 2010;16:886–95.
14. Revel A. Multitasking human endometrium: a review of endometrial biopsy as a diagnostic tool, therapeutic applications, and a source of adult stem cells. Obstet Gynecol Surv 2009;64:249–57.
15. Noyes RW, Hertig AT, Rock J. Dating the endometrial biopsy. Am J Obstet Gynecol 1975;122:262–3.
16. Cobellis L, Caprio F, Trabucco E, et al. The pattern of expression of Notch protein members in normal and pathological endometrium. J Anat 2008;213:464–72.
17. Von Grothusen C, Lalitkumar S, Boggavarapu NR, et al. Recent advances in understanding endometrial receptivity: molecular basis and clinical application. Am J Reprod Immunol 2014;72:148–57.
18. Salker M, Teklenburg G, Molokhia M, et al. Natural selection of human embryos: impaired decidualization of endometrium disables embryo-maternal interactions and causes recurrent pregnancy loss. PLoS One 2010;5:e10287.
19. Evers JL. Female subfertility. Lancet 2002;360:151–9.
20. De los Santos MJ, Mercader A, Galan A, et al. Implantation rates after two, three, or five days of embryo culture. Placenta 2003;24: S13–19.
21. Lédée-Bataille N, Lapree-Delage G, Taupin JL, et al. Concentration of leukaemia inhibitory factor [LIF] in uterine flushing fluid is highly predictive of embryo implantation. Hum Reprod 2002;17: 213–18.
22. Fei DT, Gross MC, Lofgren JL, et al. Cyclic AMP response to recombinant human RLX by cultured human endometrial cells: a specific and high throughput in vitro bioassay. Biochem Biophys Res Commun 1990;170:214–22.
23. Hayes ES, Curnow EC, Trounson AO, et al. Implantation and pregnancy following in vitro fertilization and the effect of recombinant human RLX administration in Macaca fascicularis. Biol Reprod 2004;75:1591–7.

Supplementary materials available online