The nucleolar channel system reliably marks the midluteal endometrium regardless of fertility status: a fresh look at an old organelle

Eli A. Rybak, M.D., M.P.H.,[a,b] *Michael J. Szmyga, M.S.,*[a] *Gregory Zapantis, M.D.,*[b,c] *Mary Rausch, M.D.,*[d] *Victor E. Beshay, M.D.,*[e] *Alex J. Polotsky, M.S., M.D.,*[b] *Christos Coutifaris, M.D.,*[d] *Bruce R. Carr, M.D.,*[e] *Nanette Santoro, M.D.,*[b,f] *and U. Thomas Meier, Ph.D.*[a]

[a] Department of Anatomy and Structural Biology, Albert Einstein College of Medicine, Bronx, New York; [b] Department of Obstetrics & Gynecology and Women's Health, Division of Reproductive Endocrinology and Infertility, Albert Einstein College of Medicine and Montefiore Medical Center, Bronx, New York; [c] East Coast Fertility, Plainview, New York; [d] Department of Obstetrics and Gynecology, Division of Reproductive Endocrinology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania; [e] Department of Obstetrics and Gynecology, Division of Reproductive Endocrinology, University of Texas–Southwestern Medical Center, Dallas, Texas; and [f] Department of Obstetrics and Gynecology, University of Colorado, Denver, Colorado

Objective: To determine whether nucleolar channel systems (NCSs) in the midluteal endometrium are associated with overall fertility status and/or with unexplained infertility.
Design: Retrospective and prospective clinical studies.
Setting: Repository of stored specimens from prior multicenter study and private infertility center.
Patient(s): Retrospective study that included 97 women (49 fertile couples, 48 infertile couples) who had been randomized for endometrial biopsy during the midluteal or late luteal phase. The prospective study included 78 women with a variety of infertility diagnoses.
Intervention(s): Endometrial biopsies were obtained and assessed for the presence of NCSs by indirect immunofluorescence.
Main Outcome Measure(s): The presence of NCS was graded semiquantitatively and dichotomized as normal versus low or absent.
Result(s): Normal presence of NCS was significantly associated with the midluteal phase compared with the late luteal phase (80% vs. 29%). However, there was no association between presence of NCS and fertility status or between presence of NCS and unexplained infertility.
Conclusion(s): Midluteal phase endometrium consistently forms NCSs regardless of fertility status, including unexplained infertility. This indicates a possible role for the NCS in initiating the window of endometrial receptivity. However, the consistent presence of NCSs across several different types of infertility challenges the likelihood that inadequate secretory transformation is a cause of infertility. (Fertil Steril® 2011;95:1385–9. ©2011 by American Society for Reproductive Medicine.)

Key Words: Nucleolar channel system, secretory transformation, receptivity, endometrium, unexplained infertility, immunofluorescence

Fifty years ago, an enigmatic organelle associated with secretory transformation of the endometrium was discovered on the ultrastructural level, and dubbed the nucleolar channel system (NCS) (1). Precise functional and structural characterization of the NCS remains elusive. What is known a half-century later is that the NCS develops transiently in the nuclei of secretory endometrial epithelial cells (EECs) as a membranous organelle of uniform size, ~1 μm in diameter, that is associated with the nuclear envelope and often with a nucleolus. The NCS is comprised of several layers of intertwining membrane tubules embedded in an electron-dense granular matrix that, together, surround an amorphous core (2–5). In a prior work (5), we established a robust method to stain and identify NCSs at a light microscopic level through an immunofluorescence approach using an antibody directed against a subset of nuclear pore complex proteins, a major component of the NCS. Using this method, we determined that NCSs are present in roughly half of all EEC-nuclei during a period preceding and overlapping with the implantation window (i.e., cycle days 19–24 of an idealized 28-day cycle) (5). This 50% prevalence is 10-fold more abundant than previously reported from ultrastructural identification. In addition, we demonstrated that the NCS is specific to healthy, human EECs during the secretory phase. It is not present in proliferative endometrium, endometrial stromal cell nuclei, other hormonally sensitive human tissue such as breast tissue, endometrial carcinoma specimens, or in baboon endometrium (5).

Received June 18, 2010; revised September 3, 2010; accepted October 13, 2010; published online November 10, 2010.
E.A.R. has nothing to disclose. M.J.S. has nothing to disclose. G.Z. has nothing to disclose. M.R. has nothing to disclose. V.E.B. has nothing to disclose. A.J.P. has nothing to disclose. C.C. has nothing to disclose. B.R.C. has nothing to disclose. N.S. has nothing to disclose. U.T.M. has nothing to disclose.
Supported by the March of Dimes Birth Defects Foundation (1-FY09-363 to U.T.M.); CMBG Training Program (T32 GM007491 to M.J.S.); and Ferring Pharmaceuticals, Parsippany, New Jersey (to G.Z.).
Presented at the 57th annual meeting of the Society for Gynecologic Investigation, Orlando, FL, March 25, 2010.
Reprint requests: U. Thomas Meier, Ph.D., Department of Anatomy and Structural Biology, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461 (E-mail: tom.meier@einstein.yu.edu).

In addition to its temporal association with the implantation window, the NCS has received significant attention as an important part of normal uterine biology, possibly related to endometrial receptivity. Several observations, derived from multiple ultrastructural studies, support such a role. First, the NCS is induced by P in vivo, whether it is made endogenously or administered exogenously (6–9). Second, the NCS is not found in pregnancy, but remains specific to the midluteal period (2, 10). Third, oral contraceptive (OC) use and intrauterine device (IUD) insertion have been shown to interfere with NCS formation and to prematurely induce its formation during the proliferative phase (11–14). Fourth, administration of high-dose ethinyl E_2 for emergency contraception results in the specific loss of NCSs, whereas glycogen deposits and giant mitochondria, other ultrastructural hallmarks of secretory EECs, develop normally (15). Fifth, controlled ovarian hyperstimulation (COH) increased the number and size of NCSs in the endometrial epithelium of 15 women undergoing IVF compared with those of 15 control women (16). Finally, in several women with unexplained primary infertility lasting from 4.5–8 years, the absence of NCSs was the sole abnormal parameter noted in their secretory endometrium (7, 17). In other cases of unexplained infertility, the development of the NCS was delayed (18).

Endometrial receptivity during the midluteal implantation window in the human menstrual cycle requires secretory transformation of the estrogen (E)-primed proliferative endometrium (19, 20). Characteristic changes heralding secretory transformation result from progressive P exposure, and include the appearance of basal vacuolation—the first histologic evidence of ovulation (21)—and the "secretory triad" of postovulatory ultrastructural findings in the glandular epithelium, namely, glycogen accumulation, the nucleolar channel system, and giant mitochondria (22). Additional changes include pinopode expression on the luminal epithelium (23), the decline of epithelial E and P receptors, although not the stromal P receptors, which are maintained (19), and various genetic and immunohistochemical biomarkers that are specific to a secretory phase endometrium (24–26). Nevertheless, the question remains regarding the extent to which infertility can be attributed to inadequate secretory transformation hindering endometrial receptivity. The multicenter randomized controlled trial by the Reproductive Medicine Network demonstrated that women of infertile couples were no likelier to have an out-of-phase endometrial biopsy—suggestive of inadequate secretory transformation—than were women of fertile couples (27). This finding invalidated the use of classic histologic dating of timed endometrial biopsies for routine fertility investigation and the diagnosis of a luteal phase defect, but as the investigators of the study themselves noted, it did not preclude the possibility that a defect in secretory transformation might cause infertility in at least some instances. And, indeed, the use of Noyes' criteria for classic histologic dating of the secretory endometrium for diagnostic purposes has long been controversial due to the substantial intersubject, intrasubject, and interobserver variability that limit its precision, as well as concerns about the variability introduced by the endometrial sampling procedure (28–31). The availability, however, of a readily detectable, abundant marker of secretory transformation, the NCS (5), enables a fresh look at the relationship between inadequate secretory transformation and infertility.

Based on the ultrastructural data showing the NCS to be directly relevant to endometrial receptivity (6–18, 32), we hypothesized that the presence of NCS would vary by fertility status and by specific infertility diagnosis. Therefore, our objectives were as follows: first, to confirm the association of the NCS with the midluteal phase; second, to determine whether the presence of NCS is associated with overall fertility status; and third, to determine whether the presence of NCS is specifically associated with unexplained infertility.

MATERIALS AND METHODS

Participants

Endometrial biopsies were obtained from two sources. The first source is the repository of the National Institute of Child Health and Human Development–sponsored Reproductive Medicine Network (RMN) at 2 of the 12 academic centers that participated in the original study (27) and that had a research consent form allowing for future research on the specimens, site A (University of Pennsylvania) and site B (University of Texas-Southwestern Medical Center). After the study was approved by the respective institutional review boards at the Albert Einstein College of Medicine and the two RMN sites, 107 endometrial biopsies were received, 97 of which contained sufficient glands for NCS scoring. Among the site A specimens, stratification by luteal phase timing and fertility status, revealed no statistically significant differences in age, racial composition, fertility status, or biopsy timing (see Supplemental Material). Second, 78 endometrial biopsies were obtained, during a natural cycle and without hormonal medication, from patients with various infertility diagnoses from site C (East Coast Fertility, a private fertility center in Long Island, NY), with institutional review board approval. Endometrial biopsies from sites A and B were processed as previously described (27) and preserved as frozen or paraffin sections. Site C specimens were obtained using a Pipelle suction catheter, formalin fixed, and paraffin embedded, as we described previously (5). For background and cycle information see Supplemental Material.

NCS Imaging and Scoring

Immunostaining was performed essentially as described (5) (see Supplemental Material). Epifluorescent detection and scoring of NCS prevalence was performed on an Axioskop II light microscope (Zeiss, Oberkochen, Germany) using a 63×/1.4 NA planapo objective. The prevalence of NCSs was graded semiquantitatively as normal, low, and absent (Fig. 1) according to criteria established previously with a training set of biopsies, in which the absolute number of NCSs was determined (5). As observed previously, NCSs appeared and disappeared rapidly within 1 day (i.e., they were either abundant or they were low or absent) (5, 32, 33). Therefore, the data of the low and absent categories were combined for binarization. Designation as normal required the presence of NCSs in >10% of epithelial cell nuclei in at least two distinct regions of the specimen. We previously determined the 10% cutoff using absolute numbers of NCSs (5). The purpose of this study was to establish NCS presence versus absence. To quantify NCSs, stereology could be applied (34, 35), although that would be challenging for such a large sample set. Nevertheless, only a few samples approached the 10% cutoff, and most were far above or below. Specimens with fewer NCSs in an entire section with an average of 50–100 glands were graded as low. All sample preparation, immunodetection, and scoring was performed by at least two observers who were blinded to the clinical information associated with each biopsy specimen. Among the specimens (n = 175) analyzed, 10.9% received discrepant scores and were reevaluated by a third referee, also blinded, for final grading. This interobserver difference can be explained by slight variations in procedure and identification of NCSs.

Supplemental Material Online

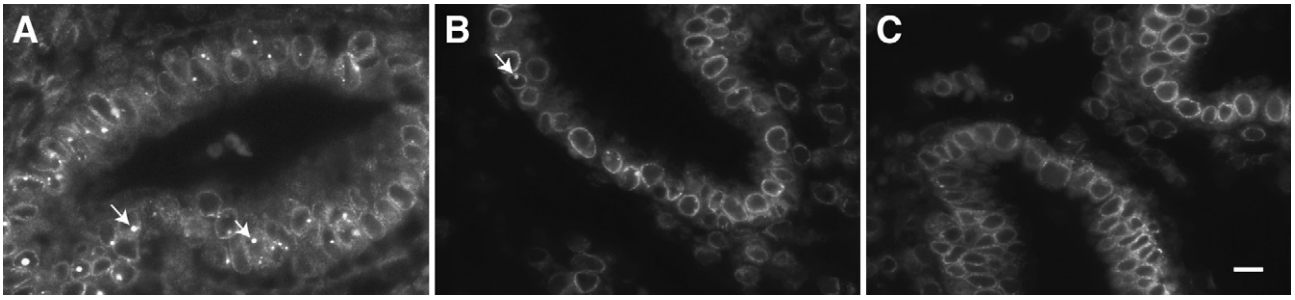
For additional Material and Methods, including outcome measures and statistical analyses, see Supplemental Material online.

RESULTS

Consistent with our prior results (5), the presence of NCS was far greater in the midluteal (80%, n = 30) compared with the late luteal phase (29%, n = 31; $P<.001$; Table 1). When these groups were stratified by fertility status, the association persisted (Table 1). Endometrial specimens from fertile compared with infertile couples demonstrated similar NCS presence (55.1% vs. 52.1%, respectively;

FIGURE 1

Representative images from site A paraffin tissue illustrating the divergent appearance of (**A**) "normal" versus (**B**) "low" versus (**C**) "absent" nucleolar channel system appearance as detected by indirect immunofluorescence with mAb414. Note the equal labeling of the nuclear pore complexes outlining the epithelial cell nuclei of all panels and the NCSs within nuclei in (**A** and **B**), some of which are indicated (*arrows*). Bar = 7 μm.



Rybak. Nucleolar channel system and infertility. Fertil Steril 2011.

P=.77; Table 2). Stratification by timing of the biopsy reinforced the lack of association between NCS presence and fertility status (Table 2). Among midluteal specimens, an identical proportion (80%) of fertile compared with infertile couples demonstrated normal NCS presence, and among late luteal specimens, the difference in normal NCS presence between fertile couples (36.8%) and infertile couples (16.7%) was not statistically significant (P=.23).

To further test these findings, we analyzed midluteal biopsies from a cohort of exclusively infertile patients with various diagnoses of infertility (n = 78, site C). Almost all of these samples (97.4%) exhibited normal NCS presence (Table 2). These 78 biopsies were then stratified by cause of infertility into two groups (Table 3): unexplained infertility (n = 21) versus infertility attributed to a known diagnosis (not unexplained, n = 57). The two groups did not differ significantly along any measured demographic or clinical parameter. Importantly, they did not differ significantly in the proportion of specimens demonstrating normal NCS presence (95.2% vs. 98.2%, respectively).

DISCUSSION

The specificity of the NCS for the midluteal phase suggests that this mysterious organelle is not merely a P-sensitive structure that appears and endures—like pinopodes (23)—once requisite levels of P are achieved but, rather, may function to promote endometrial receptivity during the implantation window. Importantly, our findings confirm the NCS as a marker of secretory transformation that reliably and ubiquitously delineates midluteal endometrium, but not as a marker for fertility status.

Counter to our hypothesis, which was based on significant ultrastructural evidence (see Introductory section; 6–13, 15–17), NCS appearance failed to discriminate by both overall fertility status and by unexplained versus not unexplained infertility. Considering this discrepancy, we note that some etiologies of infertility (e.g., tubal factor, diminished ovarian reserve) in our study are not related to NCS appearance. In addition, the grouping by the RMN study design (27) of couples with male factor infertility within the infertile group, despite having a presumably normal NCS appearance

TABLE 1

Percentage of normal presence of nucleolar channel systems among site A specimens by luteal phase timing.

	Midluteal (n = 30)	Late luteal (n = 31)	*P* value
All patients (n = 61)	80 (24/30)	29 (9/31)	<.001[a]
Fertile patients only (n = 39)	80 (16/20)	36.8 (7/19)	.006[a]
Infertile patients only (n = 22)	80 (8/10)	16.7 (2/12)	.008[b]

Note: Data are categorical and presented as percentages (proportion). Comparisons among groups with a smaller sample size were calculated by Fisher's exact test, rather than the χ^2 test.

[a] χ^2 test.

[b] Fisher's exact test.

Rybak. Nucleolar channel system and infertility. Fertil Steril 2011.

TABLE 2

Percentage of normal presence of nucleolar channel systems among all luteal phase specimens by fertility status.

	Fertile patients	Infertile patients	*P* value
Site A specimens—midluteal only (n = 30)	80 (16/20)	80 (8/10)	1[a]
Site A specimens—late luteal only (n = 31)	36.8 (7/19)	16.7 (2/12)	.23[b]
Site B specimens (approximately half midluteal and half late luteal; n = 36)	40 (4/10)	57.7 (15/26)	.46[b]
Site A and B specimens combined (n = 97)	55.1 (27/49)	52.1 (25/48)	.77[b]
Site C specimens at cycle days 19–22 (n = 78)	—	97.4 (76/78)	—

Note: Data are categorical and presented as percentages (proportion). Comparisons among groups with a smaller sample size were calculated by Fisher's exact test, rather than the χ^2 test.

[a] Fisher's exact test.

[b] χ^2 test.

Rybak. Nucleolar channel system and infertility. Fertil Steril 2011.

TABLE 3

Percentage of normal presence of nucleolar channel systems (NCSs) and clinical characteristics of site C specimens by cause of infertility.

	Specific infertility diagnosis (n = 57)	Unexplained infertility (n = 21)	*P* value
Normal NCS presence	98.2 (56/57)	95.2 (20/21)	.47[a]
Age (y)	35.9 ± 5.4	35.4 ± 3.5	.7[b]
BMI (kg/m^2)	23.5 (21.9–27.3)	25.8 (20.8–30.7)	.3[c]
E_2:P ratio on biopsy day	11.4 (7.8–15.9)	9.5 (5.7–12.9)	.35[c]
P on biopsy day (ng/mL)	10.8 ± 3.7	11.4 ± 3.2	.56[b]
E_2 on biopsy day (pg/mL)	121 (87.2–152.0)	98.8 (80.1–151.0)	.53[c]
Histologic dating, cycle day	19 (17.0–21.0)	18 (16.5–19.5)	.4[c]
LH surge dating, cycle day	20 (20.0–21.0)	20 (20.0–21.0)	.26[c]

Note: Continuous data are presented as mean ± SD (if normally distributed) or as median (interquartile range) if skewed; categorical data are presented as percentages (proportion). BMI = body mass index.

[a] Fisher's exact test.

[b] Student's *t* test.

[c] Mann-Whitney.

Rybak. Nucleolar channel system and infertility. Fertil Steril 2011.

pattern, might inaccurately inflate the rate of normal NCS appearance among infertile couples. However, these points fail to explain the similar prevalence of NCSs in the fertile versus infertile groups given that unexplained infertility is not attributable to diminished NCS appearance and, importantly, there is no evidence of a lack of NCS appearance associated with any of the various causes of infertility described by site C. Therefore, the previous underdetection of the actual prevalence of NCSs may result because ultrastructural analysis affords a more detailed, albeit focused approach, in contrast to our light microscopic approach (5), which enables a survey of larger sections of the endometrial biopsies.

Unsurprisingly, when we compared unexplained infertile couples with those having known infertility diagnoses, there were no differences in the midluteal (biopsy day) levels of P, E_2, or the ratio between them. Should an as-of-yet uncharacterized endometrial finding elucidate the pathophysiology of some cases of unexplained infertility (36, 37), it will likely be insensitive to, at least, moderate variations in circulating P and E_2 levels during the midluteal phase. Indeed, a recent study reports no correlation between circulating P levels and specific histologic, immunohistochemical, and quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) findings in the secretory endometrium (38). Specifically, the characteristic features of secretory transformation appear despite the experimental induction (through subphysiological P levels) of a luteal phase defect (38). Taken together, these findings imply that only very small quantities of P are necessary for the induction of secretory transformation, including the expression of NCSs.

The original RMN study invalidated the use of a timed secretory phase endometrial biopsy for the diagnosis of a luteal phase defect (27). However, due to potential inaccuracies involved in the process of obtaining and interpreting endometrial biopsies, the study does not definitively rule out the possibility that inadequate endometrial secretory transformation underlies at least some types of infertility. The reliable and ubiquitous specificity of the NCS for midluteal endometrium confirms ultrastructural evidence that NCS presence is a strong biomarker for secretory transformation. Inversely, diminished or absent NCS presence in the midluteal endometrium suggests inadequate secretory transformation. Our findings of equivalent rates of inadequate secretory transformation—as reflected by diminished or absent NCS presence—between fertile and infertile women and between unexplained and not unexplained infertile women, further suggest that inadequate secretory transformation does not contribute to infertility. This lack of association between inadequate secretory transformation and infertility poses a direct challenge to the existence of an endometrial cause of infertility due to lack of appropriate receptivity. At the very least, our data and the aforecited study demonstrating the minimal—if any—threshold of circulating P required for secretory transformation (38), highlight the need to better define what criteria constitute successful secretory transformation before assuming that insufficiency in that process might contribute to infertility.

Unexpectedly, the site A and B midluteal samples exhibited only an 80% NCS prevalence compared with the near 100% of the site C samples and to those we determined previously (5). The most likely explanation for this discrepancy is a slight degradation of the site A and B samples, which were procured in 1999–2002. Extended storage and transport may have contributed to a loss of NCSs and/or their detection. In contrast, all site C samples were analyzed for NCS presence within a few days to weeks of collection.

In summary, as a secretory phase structure first identified a half-century ago, the notion that the NCS might play a role in endometrial receptivity has been proffered for many years. The use of a highly sensitive immunofluorescence approach has concretized the significant and specific association between the NCS and the midluteal phase, thereby fortifying the NCS's credentials as a marker of secretory transformation, even as its presence does not discriminate by fertility status or unexplained infertility. In fact, the omnipresence of the NCS in the midluteal endometrium may mark it as a prerequisite for human fertility. Further structural and functional dissection of the NCS may provide a fresh approach in the ongoing quest to unravel the complexities of endometrial receptivity.

Acknowledgments: The authors thank Elisa Guffanti and Gayatri Sarkar for help with NCS grading of site C samples. We thank the Reproductive Medicine Network for access to its repository of timed luteal phase endometrial biopsies (at the University of Pennsylvania and the University of Texas - Southwestern Medical Center). All imaging was performed at the Analytical Imaging Facility and all slides were prepared by the Histotechnology and Comparative Pathology Facility of the Albert Einstein College of Medicine.

REFERENCES

1. Dubrauszky V, Pohlmann G. Strukturveränderungen am Nukleolus von Korpusendometriumzellen während der Sekretionsphase. Naturwissenschaften 1960;47:523–4.
2. Clyman MJ. A new structure observed in the nucleolus of the human endometrial epithelial cell. Am J Obstet Gynecol 1963;86:430–2.
3. Terzakis JA. The nucleolar channel system of the human endometrium. J Cell Biol 1965;27:293–304.
4. Spornitz UM. The functional morphology of the human endometrium and decidua. Adv Anat Embryol Cell Biol 1992;124:1–99.
5. Guffanti E, Kittur N, Brodt ZN, Polotsky AJ, Kuokkanen SM, Heller DS, et al. Nuclear pore complex proteins mark the implantation window in human endometrium. J Cell Sci 2008;121:2037–45.
6. Kohorn EI, Rice SI, Gordon M. In vitro production of nucleolar channel system by progesterone in human endometrium. Nature 1970;228:671–2.
7. Kohorn EI, Rice SI, Hemperly S, Gordon M. The relation of the structure of progestational steroids to nucleolar differentiation in human endometrium. J Clin Endocrinol Metab 1972;34:257–64.
8. Pryse-Davies J, Ryder TA, MacKenzie ML. In vivo production of the nucleolar channel system in post menopausal endometrium. Cell Tissue Res 1979;203:493–8.
9. Roberts DK, Horbelt DV, Powell LC Jr. The ultrastructural response of human endometrium to medroxyprogesterone acetate. Am J Obstet Gynecol 1975;123:811–8.
10. Demir R, Kayisli UA, Celik-Ozenci C, Korgun ET, Demir-Weusten AY, Arici A. Structural differentiation of human uterine luminal and glandular epithelium during early pregnancy: an ultrastructural and immunohistochemical study. Placenta 2002;23:672–84.
11. Azadian-Boulanger GJ, Secchi J, Laraque F, Raynaud JP, Sakiz E. Action of midcycle contraceptive (R 2323) on the human endometrium. Am J Obstet Gynecol 1976;125:1049–56.
12. Feria-Velasco A, Aznar-Ramos R, Gonzalez-Angulo A. Ultrastructural changes found in the endometrium of women using megestrol acetate for contraception. Contraception 1972;5:187–201.
13. Wynn RM. Intrauterine devices: effects on ultrastructure of human endometrium. Science 1967;156:1508–10.
14. Dockery P, Ismail RMJ, Li TC, Warren MA, Cooke ID. The effect of a single dose of mifepristone (RU486) on the fine structure of the human endometrium during the early luteal phase. Hum Reprod 1997;12:1778–84.
15. van Santen MR, Haspels AA, Heijnen HF, Rademakers LH. Interfering with implantation by postcoital estrogen administration. II. Endometrium epithelial cell ultrastructure. Contraception 1988;38:711–24.
16. Novotny R, Malinsky J, Oborna I, Dostal J. Nuclear channel system (NCS) in normal endometrium and after hormonal stimulation. Acta Univ Palacki Olomuc Fac Med 1999;142:41–6.
17. Gore BZ, Gordon M. Fine structure of epithelial cell of secretory endometrium in unexplained primary infertility. Fertil Steril 1974;25:103–7.
18. Dockery P, Pritchard K, Warren MA, Li TC, Cooke ID. Changes in nuclear morphology in the human endometrial glandular epithelium in women with unexplained infertility. Hum Reprod 1996;11:2251–6.
19. Lessey BA, Glasser S. Endometrial receptivity. In: Aplin JD, Fazleabas AT, Glasser SR, Giudice LC, editors. The endometrium. 2nd ed. NewYork: Informa Healthcare; 2008. p. 305–18.
20. Sharkey AM, Smith SK. The endometrium as a cause of implantation failure. Best Pract Res Clin Obstet Gynecol 2003;17:289–307.
21. Noyes RW, Hertig AT, Rock J. Dating the endometrial biopsy. Fertil Steril 1950;1:3–25.
22. Cornille FJ, Lauweryns JM, Brosens IA. Normal human endometrium: an ultrastructural survey. Gynecol Obstet Invest 1985;20:113–29.
23. Quinn C, Ryan E, Claessens EA, Greenblatt E, Hawrylyshyn P, Cruickshank B, et al. The presence of pinopodes in the human endometrium does not delineate the implantation window. Fertil Steril 2007;87:1015–21.
24. Mirkin S, Arslan M, Churikov D, Corica A, Diaz JI, Williams S, et al. In search of candidate genes critically expressed in the human endometrium during the window of implantation. Hum Reprod 2005;20:2104–17.
25. Achache H, Revel A. Endometrial receptivity markers, the journey to successful embryo implantation. Hum Reprod Update 2006;12:731–46.
26. Aghajanova L, Hamilton AE, Giudice LC. Uterine receptivity to human embryonic implantation: histology, biomarkers, and transcriptomics. Semin Cell Dev Biol 2008;19:204–11.
27. Coutifaris C, Myers ER, Guzick DS, Diamond MP, Carson SA, Legro RS, et al. Histological dating of timed endometrial biopsy tissue is not related to fertility status. Fertil Steril 2004;82:1264–72.
28. Murray MJ, Meyer WR, Zaino RJ, Lessey BA, Novotny DB, Ireland K, et al. A critical analysis of the accuracy, reproducibility, and clinical utility of histologic endometrial dating in fertile women. Fertil Steril 2004;81:1333–43.
29. Li TC, Dockery P, Rogers AW, Cooke ID. How precise is histologic dating of endometrium using the standard dating criteria? Fertil Steril 1989;51:759–63.
30. Gibson M, Badger GJ, Byrn F, Lee KR, Korson R, Trainer TD. Error in histologic dating of secretory endometrium: variance component analysis. Fertil Steril 1991;56:242–7.
31. Scott RT, Snyder RR, Bagnall JW, Reed KD, Adair CF, Hensley SD. Evaluation of the impact of interobserver variability on endometrial dating and the diagnosis of luteal phase defects. Fertil Steril 1993;60:652–7.
32. Dockery P, Pritchard K, Taylow A, Li TC, Warren MA, Cooke ID. The fine structure of the human endometrial glandular epithelium in cases of unexplained infertility: a morphometric study. Hum Reprod 1993;8:667–73.
33. Dockery P, Li TC, Rogers AW, Cooke ID, Lenton EA, Warren MA. An examination of the variation in timed endometrial biopsies. Hum Reprod 1988;3:715–20.
34. Sterio DC. The unbiased estimation of number and sizes of arbitrary particles using the disector. J Microsc 1984;134:127–36.
35. West MJ, Slomianka L, Gundersen HJ. Unbiased steriological estimation of the total number of neurons in the subdivisions of the rat hippocampus using the optical fractionator. Anat Rec 1991;231:482–97.
36. Aghajanova L, Altmäe S, Bjuresten K, Hovatta O, Landgren BM, Stavreus-Evers A. Disturbances in the LIF pathway in the endometrium among women with unexplained infertility. Fertil Steril 2009;91:2602–10.
37. Altmäe S, Martinez-Conejero JA, Salumets A, Simon C, Horcajadas JA, Stavreus-Evers A. Endometrial gene expression analysis at the time of embryo implantation in women with unexplained infertility. Mol Hum Reprod 2010;16:178–87.
38. Usadi RS, Groll JM, Lessey BA, Lininger RA, Zaino RJ, Fritz MA, et al. Endometrial development and function in experimentally induced luteal phase deficiency. J Clin Endocrinol Metab 2008;93:4058–64.

SUPPLEMENTAL MATERIAL

Background and Cycle Information

As described (27), site A and B specimens were obtained from women of fertile and infertile couples based on the urinary LH surge, which was considered as idealized cycle day (CD) 14. Subjects were randomized to undergo the endometrial biopsy either in the midluteal phase (idealized CD 21–22) or in the late luteal phase (CD 26–27).

Specimens obtained from site A (n = 61) had complete demographic and clinical information, whereas those from site B (n = 36) could only be stratified by fertility status. Accordingly, 97 biopsies with fertility status available from sites A and B were analyzed. Timing of the biopsy and demographic data were available only for site A specimens and results of the original histologic dating were available for only 59 of the 61 site A specimens. Among the site A specimens, stratification by luteal phase timing and fertility status revealed no statistically significant differences in age, racial composition, fertility status, or biopsy timing. Specifically, when comparing midluteal (n = 30) versus late luteal (n = 31) specimens, patient age (30.6 ± 3.6 years, mean ± SD, versus 32.0 ± 4.8 years; P=.2), proportion of specimens from non-white women (47% vs. 35%; P=.44), and proportion of specimens from infertile women (33% vs. 39%; P=.66) were similar. Expectedly, the median histologic date of midluteal specimens differed significantly from that of late luteal specimens (17 vs. 24; P<.001). When comparing fertile (n = 39) versus infertile (n = 22) specimens, patient age (31.7 ± 4.2 years vs. 30.5 ± 4.4 years; P=.32), proportion of specimens from non-white women (33% vs. 55%; P=.17), and proportion of specimens from the midluteal phase (51% vs. 46%; P=.66) were similar. In addition, the median histologic date of fertile specimens did not differ significantly from that of infertile specimens (20.5 vs. 22, P=.37).

The 78 subjects providing endometrial biopsies at site C had various established infertility diagnoses including tubal factor, endometriosis, diminished ovarian reserve, male factor, and unexplained. Specific requirements for inclusion were regular 24- to 35-day menstrual cycles and a normal uterine cavity by sonohysterogram. Thrice weekly blood monitoring or home urinary LH monitoring was used to identify the day of LH surge, considered as idealized CD 14. Biopsies were then performed 5–8 days later, corresponding to CD 19–22. Background information available for each biopsy include: specific infertility diagnosis, timing of biopsy, histologic dating using Noyes criteria (21; performed in each case by a blinded, experienced pathologist), age, body mass index (BMI), and serum E_2 and P levels on the biopsy day.

Immunostaining of Tissue Sections

Staining and slide preparation were done as described previously (5). Briefly, paraffin-embedded tissue sections (~7 μm thick) were deparaffinized, rehydrated, and (for antigen retrieval) treated with 10 mM sodium citrate (pH 6.0). In addition, to enhance nucleolar channel system (NCS) detection in older paraffin sections (sites A and B), the slides were immersed in methanol (−20°C) for 5 minutes and allowed to air dry. Frozen sections (~2 μm thick) merely required rinsing in phosphate-buffered saline (PBS) before immunostaining.

Antibodies

Mouse IgGs of monoclonal antibody 414 (mAb414, 1:5,000; Covance Research Products, Princeton, NJ) were used for detection of nuclear pore complexes and NCSs (5). Secondary antibodies used for immunofluorescence against IgGs were Cy3-conjugated donkey anti-mouse (1:200; Jackson ImmunoResearch, West Grove, PA) and DyLight 488 conjugated goat anti-mouse (1:500; Jackson ImmunoResearch). DNA was stained with 4′, 6-diaminidino-2-phenylindole dihydrochloride (DAPI; Sigma, St. Louis, MO) or propidium iodide (Sigma).

Outcome Measures and Statistical Analysis

Based on our previous results (5), we sought to demonstrate that there would be disparate normal NCS presence in site A biopsies based on when they were obtained in the luteal phase. Specifically, we expected normal NCS presence in 90% of midluteal phase (CD 21–22) biopsies versus normal NCS presence in only one-third as many late luteal biopsies (i.e., 30%). Sample size calculation set at $\alpha = 0.05$, with 80% power to detect this difference, required 13 specimens in both groups. We hypothesized preferential normal NCS presence in women of fertile couples versus women of infertile couples. Again assuming 90% versus 30% to be a clinically meaningful disparity, 13 specimens were required in both groups to maintain 80% power at $\alpha = 0.05$. Based on previous work suggesting delayed or absent NCS appearance in some cases of unexplained infertility (7, 17, 18), we explored whether normal NCS presence would occur more often—90% versus 45%—in the biopsies derived from couples of known infertility diagnoses compared with unexplained infertile couples. Sample size calculation set at $\alpha = 0.05$ and 80% power, required 20 specimens in both groups to demonstrate this outcome.

Our outcome of interest, presented in categorical fashion, was normal NCS prevalence. Proportions of biopsies with normal NCS prevalence were compared separately, and with stratification, by two independent variables: timing of biopsy (midluteal vs. late luteal phase; site A), and fertility status (fertile vs. infertile patients; sites A and B). The χ^2 test or the Fisher's exact test was used, as appropriate. For site C samples, Fisher's exact test was used to compare the proportion of biopsies with normal NCS presence between couples with specific infertility diagnoses versus those with unexplained infertility.

Associations between demographic or clinical characteristics and the independent variables were assessed using Student's t test (for normally distributed data) or Mann-Whitney U test (for skewed data) for continuous variables and χ^2 or Fisher's exact test for categorical data, as appropriate. All statistical tests used a two-tailed alpha of 0.05. Statistical analyses were performed using Stata 10.0 (Stata Corporation, College Station, TX).