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

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Abstract

Objective—To determine if 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, respectively.

Patients—Retrospective study: 97 women (49 fertile couples, 48 infertile couples) who had been randomized for endometrial biopsy during the midluteal or late luteal phase. Prospective study: 78 women with a variety of infertility diagnoses.

Intervention—Endometrial biopsies were obtained and assessed for the presence of NCSs by indirect immunofluorescence.

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

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Conflict of Interest (for all authors): NONE

Main Outcome Measures—NCS presence was graded semi-quantitatively and dichotomized as normal versus low or absent.

Results—Normal NCS presence was significantly associated with the midluteal phase compared to the late luteal phase (80% versus 29%). However, there was no association between NCS presence and fertility status or between NCS presence and unexplained infertility.

Conclusions—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.

Keywords

Nucleolar channel system; secretory transformation; receptivity; endometrium; unexplained infertility; immunofluorescence

INTRODUCTION

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 diameter, that is associated with the nuclear envelope and often with a nucleolus, and 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 prior work (5), we established a robust method to stain and identify NCSs on a light microscopic level via 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: Cycle days (CD) 19-24 of an idealized 28-day cycle (5). This 50% prevalence is ten-fold more abundant than previously reported from ultrastructural identification. Additionally, 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).

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 progesterone 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 use and intrauterine device 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 estradiol 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 increased the number and size of NCSs in the endometrial epithelium of 15 women undergoing IVF compared to those of 15 control women (16). Finally, in several women with unexplained primary infertility lasting from 4.5 to 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-primed proliferative endometrium. (19,20). Characteristic changes heralding secretory transformation result from progressive progesterone exposure, and include the appearance of basal vacuolation – the first histologic evidence of ovulation (21), the “secretory triad” of postovulatory ultrastructural findings in the glandular epithelium, namely, glycogen accumulation, the nucleolar channel system, and giant mitochondria (22); pinopode expression on the luminal epithelium (23), the decline of epithelial estrogen and progesterone receptors, although not the stromal progesterone 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 authors 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 NCS presence 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 if NCS presence is associated with overall fertility status; and third, to determine if NCS presence is specifically associated with unexplained infertility.

MATERIALS AND METHODS

Participants

Endometrial biopsies were obtained from two sources: First, from 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 (IRBs) 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, respectively, 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 IRB approval. Site A and B endometrial

biopsies 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 63X / 1.4 NA planapo objective. NCS prevalence was graded semi-quantitatively as “normal”, “low”, and “absent” (Figure 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 one 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. In order 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.

RESULTS

Consistent with our prior results (5), NCS presence 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% versus 52.1%, respectively; $P=.77$, 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% versus 98.2%, respectively).

DISCUSSION

The specificity of the NCS for the midluteal phase suggests that this mysterious organelle is not merely a progesterone sensitive structure that appears and endures – like pinopodes (23)

– once requisite levels of progesterone 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 Introduction; 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. Moreover, 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 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 under-detection of the actual prevalence of NCSs may result from the fact that 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 progesterone, estradiol, 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 progesterone and estradiol levels during the midluteal phase. Indeed, a recent study reports no correlation between circulating progesterone levels and specific histologic, immunohistochemical, and quantitative RT-PCR findings in the secretory endometrium (38). Specifically, the characteristic features of secretory transformation appear despite the experimental induction (through subphysiological progesterone levels) of a luteal phase defect (38). Taken together, these findings imply that only very small quantities of progesterone 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 versus infertile women and between unexplained versus not-unexplained infertile women, respectively, 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 aforementioned study demonstrating the minimal – if any – threshold of circulating progesterone required for secretory transformation (38), highlight the need to better define what criteria constitute successful secretory transformation prior to 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 to 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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CAPSULE

Nucleolar channel systems appear reliably in midluteal endometrium independent of fertility status, including unexplained infertility, thus further questioning the relevance of inadequate secretory transformation as a cause of infertility.

Figure 1.

Representative images from Site A paraffin tissue illustrating the divergent appearance of “normal” (A) versus “low” (B) versus “absent” (C) NCS 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 panel (A) and (B), some of which are indicated (arrows). Bar = 20 μ m.

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

Data is categorical and is presented as percentages (proportion). Comparisons among groups with a smaller sample size were calculated by Fisher's exact test, rather than Chi square.

^aChi square.

^bFisher's exact test.

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 Day 19-22 (n=78)	---	97.4% (76/78)	---

Data is categorical and is presented as percentages (proportion). Comparisons among groups with a smaller sample size were calculated by Fisher's exact test, rather than Chisquare.

^aFisher's exact test.

^bChi square.

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, years	35.9 ± 5.4	35.4 ± 3.5	.7 ^b
BMI, m/kg ²	23.5 (21.9-27.3)	25.8 (20.8-30.7)	.3 ^c
E2/P4 ratio on biopsy day	11.4 (7.8-15.9)	9.5 (5.7-12.9)	.35 ^c
P4 on biopsy day, ng/mL	10.8 ± 3.7	11.4 ± 3.2	.56 ^b
E2 on biopsy day, pg/mL	121(87.2-152)	98.8 (80.1-151)	.53 ^c
Histologic dating, cycle day	19 (17-21)	18 (16.5-19.5)	.4 ^c
LH surge dating, cycle day	20 (20-21)	20 (20-21)	.26 ^c

Note: Continuous data are presented as mean ± standard deviation (if normally distributed) or as median (interquartile range) if skewed; categorical data are presented as percentages (proportion).

^aFisher's exact test.

^bStudent's t-test.

^cMann-Whitney.