# Timing the window of implantation by nucleolar channel system prevalence matches the accuracy of the endometrial receptivity array

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**Objective:** To test if nucleolar channel system (NCS) prevalence matches the accuracy of the endometrial receptivity array (ERA) for identification of the window of endometrial receptivity.
**Design:** Comparative retrospective study, May 2008–May 2012.
**Setting:** University-affiliated infertility clinic.
**Patient(s):** Forty-nine healthy oocyte donors, regularly cycling, aged 20–34 years with a body mass index of 19–25 kg/m$^2$.
**Intervention(s):** Endometrial biopsies were collected throughout the menstrual cycle. All samples underwent transcriptomic signature identification by ERA testing (performed in a prior study) and quantification of NCS prevalence by using indirect immunofluorescence (performed in the present study).
**Main Outcome Measure(s):** Concordance of ERA results determining the window of implantation with NCS prevalence was statistically analyzed using the kappa index. Based on dating according to the luteinizing hormone surge, specimens were dichotomized into receptive ($n = 24$) and nonreceptive ($n = 25$). The NCS prevalence was expressed as percentage of NCSs per endometrial epithelial cells in each endometrial biopsy.
**Result(s):** Concordance of ERA and NCS dating vs. luteinizing hormone yielded comparable kappa indices of 0.878 and 0.836, respectively. Direct comparison of ERA and NCS dating resulted in a kappa index of 0.796.
**Conclusion(s):** Prevalence of NCS identifies the window of endometrial receptivity previously identified by their transcriptomic signature using the ERA. (Fertil Steril® 2014;102:1477–81. ©2014 by American Society for Reproductive Medicine.)
**Key Words:** Nucleolar channel system (NCS), endometrial receptivity array (ERA), endometrium, receptivity, window of implantation (WOI)

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Human endometrium poses an efficient barrier throughout most of the menstrual cycle except during a few days when it becomes permissive to attachment of blastocysts, during the window of implantation (WOI). Accurate identification of the WOI is of utmost importance to assisted reproductive technologies (ART) for timing of embryo transfer. Nevertheless, endometrial dating for the past 60 years relied primarily on histologic evaluation (1), the accuracy of which has been questioned, leaving a void (2, 3).

More recently, the molecular signatures underlying the physiological changes of the endometrium throughout the cycle were determined by multiple groups (4). Using the transcriptomic signature of the WOI, the endometrial receptivity array (ERA) was developed (5). The ERA is a customized array composed of 238 differentially expressed genes coupled to a computational predictor that

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diagnoses whether an endometrial sample was obtained during the receptive, pre-receptive, or post-receptive stage of a given patient, independent of histologic appearance (5). When the ERA was compared to histological dating relative to the luteinizing hormone (LH) peak, ERA testing far outperformed the assessment of 2 pathologists with a concordance of 0.922 (kappa index) vs. 0.618 and 0.685, respectively (6). Additionally, the ERA was completely reproducible 29–40 months after the first test (6). Recent application of the ERA in a cycle preceding embryo transfer (ET) in patients with repeated implantation failure (RIF) demonstrated that the personalized WOI is displaced in 1 of 4 patients suffering from RIF, leading to the concept of personalized ET as a therapeutic approach (7). A recent pilot study in ovum recipients with RIF further confirms the validity and clinical application of this diagnostic test (8). In summary, the ERA is presently the most accurate method of WOI determination.

Nucleolar channel systems (NCSs) are 1-micron–sized membranous organelles that develop transiently in the nuclei of secretory-phase endometrial epithelial cells (EECs) (9–11). Discovered on the ultrastructural level over a half-century ago, NCSs have a robust midluteal presence, are sensitive to progesterone, and are absent during pregnancy. This and other evidence suggested early on that this human-specific organelle might be involved in endometrial receptivity (11–18).

More recently, we developed a light, microscopic, quantitative detection method for NCSs, taking advantage of their enrichment in certain nuclear pore complex proteins (nucleoporins) (19, 20). Applying our simplified NCS detection method in several studies, we determined that about half of the EECs harbor an NCS at their peak, midluteal presence, and that they are distributed uniformly throughout the upper uterine cavity. Although their appearance is independent of fertility status, it is dependent on a 4-ng/ml progesterone threshold (21, 22). Our recent finding that premature formation of NCSs was linked to advanced endometrial maturation after controlled ovarian hyperstimulation further supported an association of NCS presence with the WOI (23). Despite all this information, the function of these curious organelles remains as enigmatic as when they were discovered. Here, we assessed NCS prevalence in blinded fashion in the identification of receptive vs. nonreceptive endometrial specimens previously diagnosed as such by their transcriptomic signature using the ERA test.

## MATERIALS AND METHODS

### Endometrial Biopsies

Endometrial biopsies were collected throughout the menstrual cycle during a prior study (6). The Ethics Committee of the Instituto Valenciano de Infertilidad, Valencia, Spain, where the biopsies were performed after participants provided written informed consent, approved collection of the biopsies. For direct comparison with the prior study (6), all 49 biopsies (the dating set) were analyzed for NCS prevalence. Secretory biopsies were timed according to LH surge and proliferative specimens according to CD. The samples were defined as receptive (LH+7; n = 24) or nonreceptive (n = 25). The latter were further subdivided into proliferative (CD8 to CD14; n = 6); pre-receptive (LH+1 to LH+5; n = 8); and post-receptive (LH+11 to LH+13; n = 11). All biopsies were from healthy Caucasian volunteers, aged 19–40 years with a normal body mass index (BMI; 19–25 kg/m$^2$) and with regular menses. Additional details are as described (6). The ERA test was performed and evaluated as described previously (6).

### NCS Immunohistochemical Detection

Endometrial tissue was fixed in 4% formaldehyde, embedded in paraffin, and sectioned as described (6). For NCS quantification, randomly numbered tissue sections mounted on glass slides were shipped to Albert Einstein College of Medicine, Bronx, NY. Immunostaining was performed as detailed previously (20). Briefly, the sections were deparaffinized, rehydrated, and treated with 10-mM sodium citrate (pH 6.0) for antigen retrieval. For NCS detection, indirect immunofluorescence was employed with monoclonal antibody 414 (Covance, Princeton, NJ) directed against a subset of nuclear pore complex proteins followed by DyLight488-labeled secondary antibodies (Jackson ImmunoResearch, West Grove, PA). Individual EECs were identified by their nuclei through double labeling of their deoxyribonucleic acid (DNA) with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma, St. Louis, MO).

### NCS Imaging and Quantification

The NCSs were quantified by an observer-independent method described previously (20) that has since been applied repeatedly (21, 23). Briefly, an observer who was blinded regarding the CD of biopsy collection analyzed all samples. The stained sections were imaged on a DeltaVision Core system (Applied Precision, Issaquah, WA). Specifically, 10 fields with glands were randomly selected based on DAPI staining (ensuring blindness to NCS presence) and imaged across the entire thickness of the section in 0.3-$\mu$m Z-steps. All EECs, based on nuclear staining, and NCSs were counted as described (20). Overall, 1,656 NCSs in 39,067 EECs were counted in 49 endometrial biopsies assessing between 355 and 1,237 EEC nuclei in each (797 $\pm$ 187, mean $\pm$ standard deviation). For each biopsy, the output measure was NCS prevalence (percentage of NCSs per EECs). NCS prevalence of 1% was set as a cut-off to determine the status of each specimen ($\geq$1% = receptive, $<$1% = nonreceptive). This threshold allowed discounting samples with very few NCSs that were either in the process of appearing or disappearing and accounted for minor background staining that could not be assigned unambiguously.

Two independent analyses led to the 1% cut-off. First, it derived from a biphasic sample distribution, those that exhibited $\geq$1 NCS in $\geq$6 of the 10 fields imaged per sample (receptive) vs. those with 1 NCS in $\leq$3 of the 10 fields (nonreceptive). Adding the twofold standard deviation of the mean to the mean NCS prevalence of the nonreceptive samples yielded a value of 0.5%, which included all those samples, and which we doubled to safely avoid false positives. Second,

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