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Analysis of Epitopes on Endometrial Epithelium by Scanning Immunoelectron Microscopy[1]

Andrew W. Horne,* John O. White,* El-Nasir Lalani,† Margaret A. Mobberley,‡ Raul A. Margara,* Geoffrey H. Trew,* and Timothy A. Ryder‡,[2]

**Institute of Reproductive and Developmental Biology, Wolfson and Weston Centre for Family Health, and †Department of Histopathology, Faculty of Medicine, Imperial College, Hammersmith Hospital, Du Cane Road, London W12 0NN, United Kingdom; and ‡Electron Microscopy Unit, Department of Histopathology, Charing Cross Hospital, Fulham Palace Road, London W6 8RF, United Kingdom*

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Scanning immunoelectron microscopy was applied to human endometrial epithelium for the first time to simultaneously determine epitope localisation and cellular architecture. The method was established using HMFG1, an antibody to a glycoform of the MUC1 mucin. This was chosen because of the potential importance of MUC1 in connection with endometrial receptivity. Biopsies of mid-secretory phase endometrium were labelled using HMFG1 and silver-enhanced, gold-conjugated secondary antibody was then visualised by back-scattered electron imaging. The method provided a highly specific localisation of the HMFG1 epitope to the ciliated and 'ciliogenic' cells of the endometrial surface. In contrast, no reactivity was evident on the microvillous cells and endometrial pinopodes. The potential to integrate the study of the molecular and ultrastructural changes that occur in the endometrium by using scanning immunoelectron microscopy offers a powerful means of expanding our understanding of the adaptation of the endometrium in preparation for embryo implantation.

Key Words: **immunohistochemistry; microscopy; electron; scanning; endometrium; MUC1 mucin peptide.**

A systematic study of the cellular architecture and molecular expression pattern of the endometrial surface is crucial to the understanding of endometrium/blastocyst interaction at implantation. Scanning electron microscopy (SEM) studies in isolation have demonstrated a number of changes that occur during the period of endometrial receptivity in the mid-secretory phase (1–3). A transient decrease in luminal ciliated cells, flattening of the microvilli and generation of large apical protrusions of the epithelium called pinopodes have all been associated with successful implantation (4, 5). Uterine pinopode formation is the most dramatic morphological change occurring at this phase (6, 7).

At a biochemical level, immunological labelling and light microscope studies have indicated that changes in the expression of proteins on the cell surface may also contribute to the conversion of the endometrial surface from a non-receptive to a receptive state (6–8). One particular glycoprotein, the MUC1 mucin, has been extensively studied (9–11). Although MUC1 mucin expression has been shown to be conserved throughout the menstrual cycle, it has been suggested that localised changes in the glycoform pattern of MUC1 in the mid-secretory phase might allow the embryo to implant (12–14). One limitation of SEM is that it provides only structural information. This can be circumvented by the use of scanning immunoelectron microscopy, which combines the architectural detail of SEM with the precise antigen mapping of monoclonal antibody technology. This provides a powerful technique for investigating the co-ordinated changes of cellular anatomy and MUC1 expression (15).

Although this technique has been available for a number of years (16), its main application has been in isolated leucocytes (17, 18) and cells in culture (19, 20). However, there are scant reports of its use with whole tissue (21–23), and to our knowledge, this is the first report of its application to a complex epithelium such as the human endometrial surface.

In this study, scanning immunoelectron microscopy was performed with HMFG1, an antibody to a MUC1

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[2] To whom correspondence and reprint requests should be addressed at Electron Microscopy Unit, Department of Histopathology, 6th Floor, Laboratory Block, Charing Cross Hospital, Fulham Palace Road, London W6 8RF, UK. Fax: 00 44 208 383 0551. E-mail: t.ryder@ic.ac.uk.

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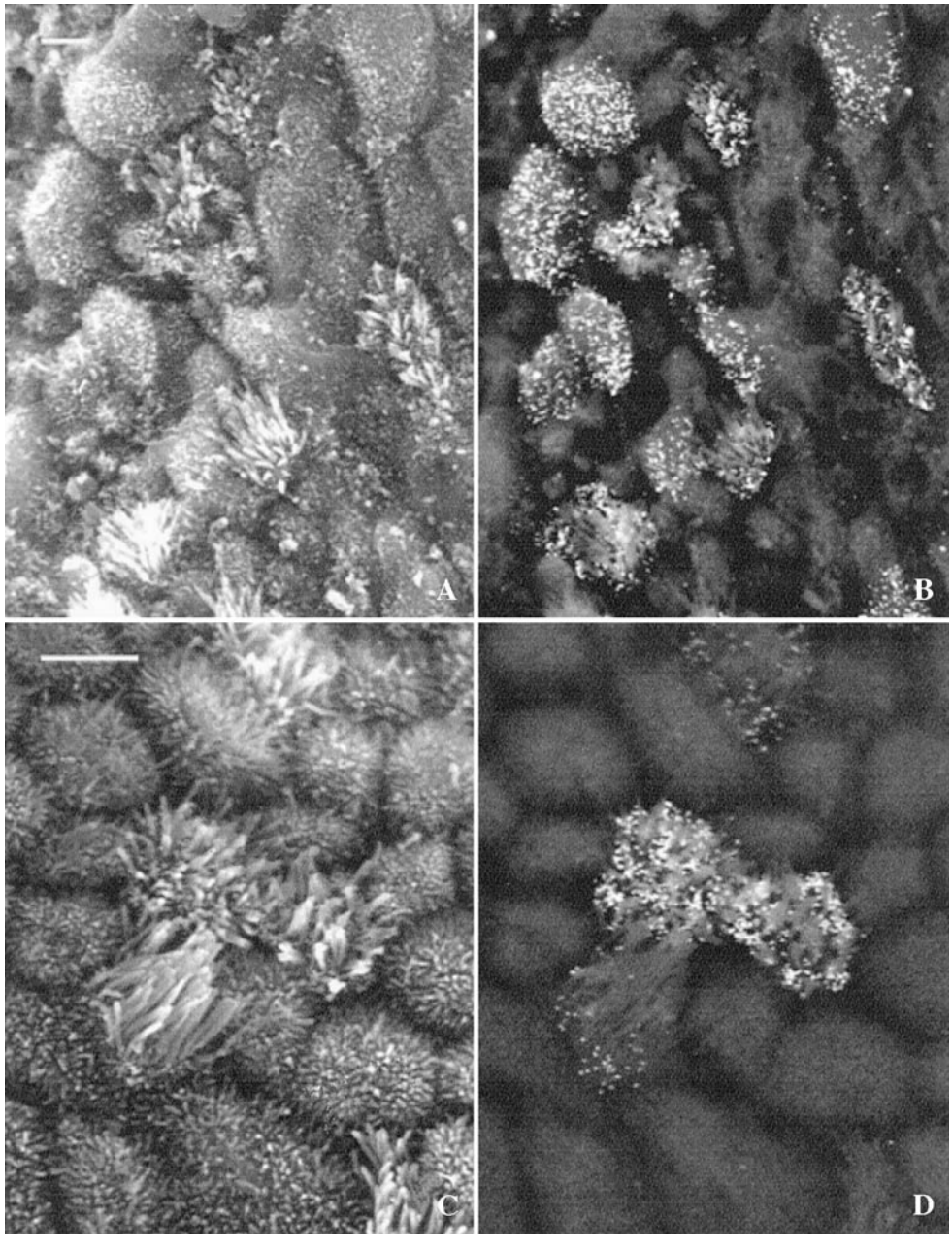

FIG. 1. Paired scanning electron microscope images of the endometrial surface of biopsies from the mid-secretory phase of the menstrual cycle. (A and C) Secondary electron images showing the morphology of the surface epithelium. (B and D) Corresponding back-scattered images where HMFG1 immunolabelling, i.e., areas of high mass, show as white against a black background. Note the positive labelling of the ciliated and 'ciliogenic' cells and absence of marker on the surrounding microvillous cells. Bars, 5 μm.

glycoform, using endometrial specimens from the mid-secretory phase.

MATERIALS AND METHODS

Tissue. Endometrial tissue was obtained by Pipelle biopsy from the uterine fundus of 10 healthy, regularly cycling women between the ages of 27 and 40 years during a routine fertility examination of women with unexplained infertility (IVF Unit, Hammersmith Hospital, London). The women underwent serial ultrasound assessments (follicular tracking) and peripheral blood sampling. Biopsies were performed 7 days after the LH surge and divided into two portions. One portion was fixed in 10% formalin and paraffin-embedded for light microscopy, and the other portion was placed in neutral buffered formalin (both BDH Laboratory Supplies, Poole, UK) for SEM. The paraffin-embedded biopsies were haematoxylin and eosin stained (24) and then dated histologically by an experi-

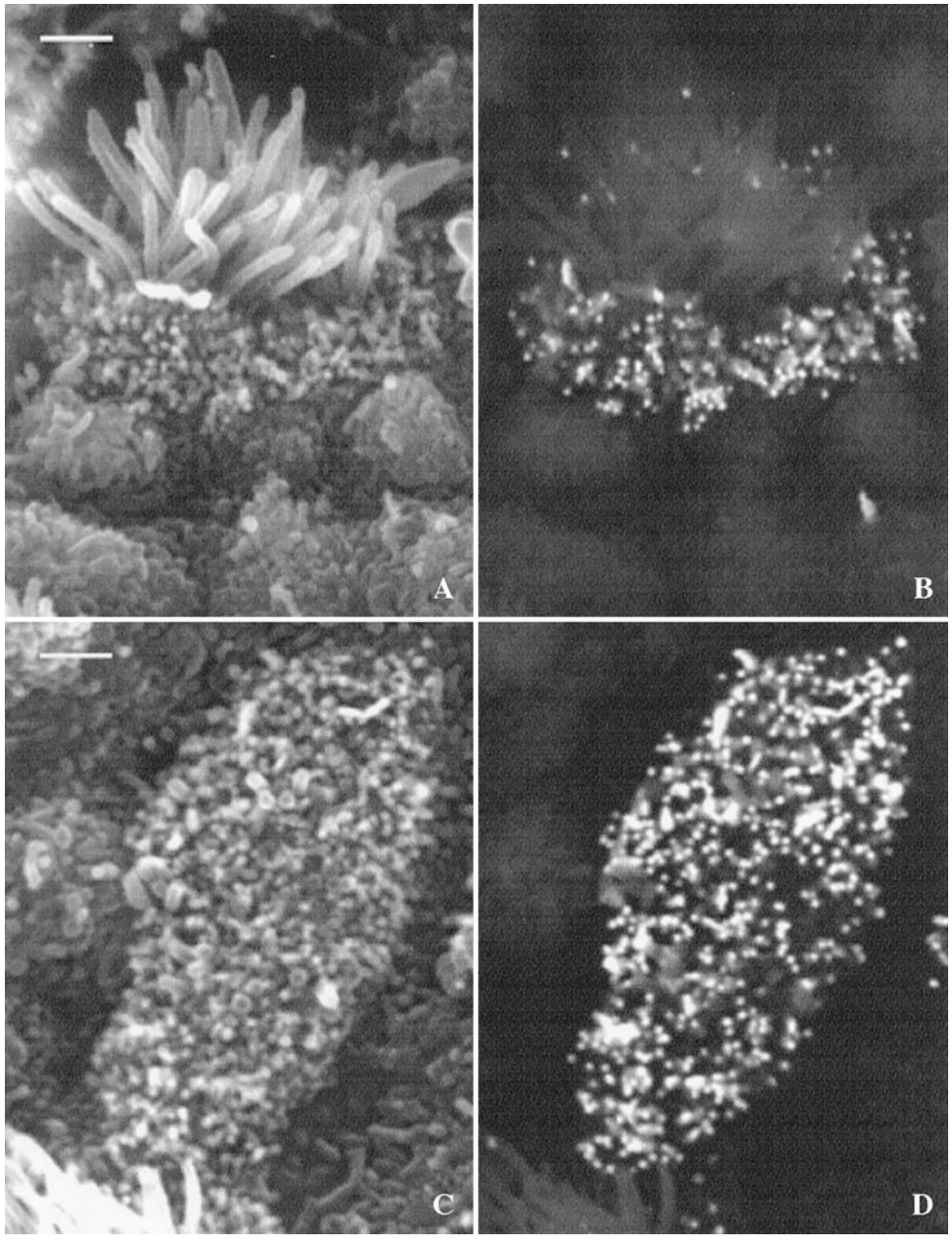

FIG. 2. Higher power paired scanning electron microscope images of the endometrial surface of biopsies from the mid-secretory phase of the menstrual cycle. Images (A) and (B) demonstrate that the HMFG1 immunolabelling is confined to the microvilli of the ciliated cell. Images (C) and (D) show a 'ciliogenic' cell that is strongly positive for HMFG1. Bars, 1 μm.

enced pathologist using Noye's criteria. The specimens were all in phase and showed no pathological features.

Antibodies. HMFG1 (Skybio Ltd., Bedfordshire, UK) is an affinity-purified, anti-human, MUC1 mucin monoclonal antibody (25). The minimum amino acid sequence required to form a reactive epitope is PDTR, which is contained within the ectodomain of MUC1 (26). Studies are consistent with the interpretation that the HMFG1 determinant is found in the fully glycosylated form.

MIB-1 (Serotec, Oxford, UK) is a purified monoclonal antibody to a 1002-bp Ki-67 cDNA fragment. Ki-67 is a chromatin component and was therefore chosen as a control against non-specific surface attachment of primary antibody.

Immunogold labelling for SEM. Each of the specimens that had been fixed in 10% formalin was separately washed for 1 h with three changes of PBS. All incubations were carried out in Eppendorf tubes at room temperature unless stated otherwise. Following washing, the tissue was blocked in normal goat serum (diluted 1 in 20 in PBS diluent) for 10 min and then divided into three parts. One part was incubated overnight at 4°C with 200 μl HMFG 1 (diluted 1 in 500 in PBS diluent), the second with MIB-1 (diluted 1 in 500 in PBS diluent), and the last with PBS diluent alone. After further washing with PBS, the specimens were incubated with 200 μl 5 nm goat anti-mouse gold conjugate (British Biocell International, Cardiff, UK) (diluted 1 in 100 in PBS diluent) for 1 h and then washed in

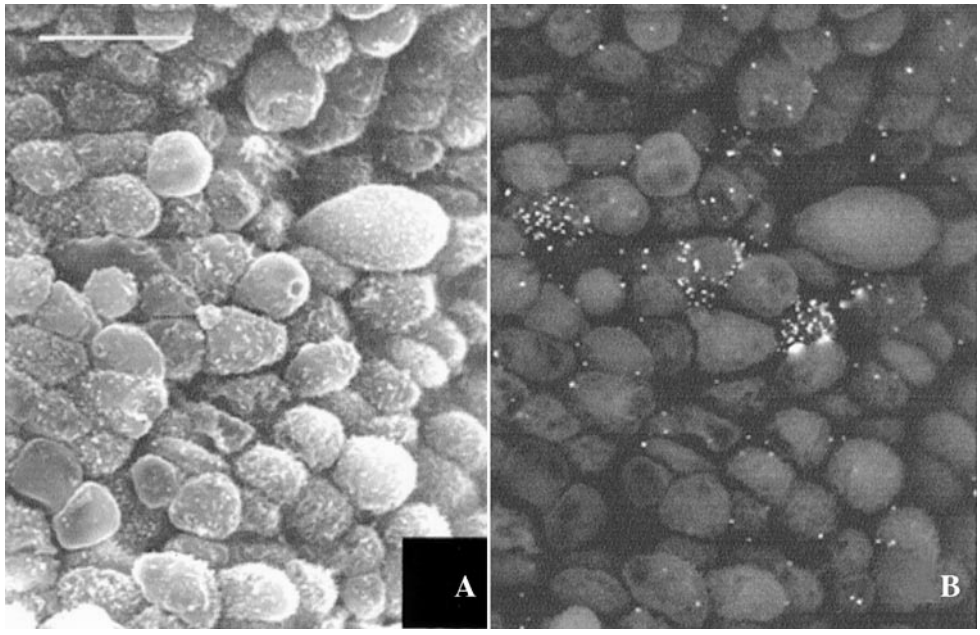

FIG. 3. Paired scanning electron microscope image of an area of pinopodes on the endometrial surface of a biopsy from the mid-secretory phase of the menstrual cycle which shows no evidence of HMFG1 labelling apart from on the surface of a few 'ciliogenic' cells. Bar, 5 μm.

several changes of distilled water (27). Silver enhancing was performed by immersion in 200 μl silver enhancing solution (British Biocell International, Cardiff, UK) for 10 min followed by further washing in distilled water (28). The tissues were fixed in neutral buffered formalin for 24 h and then dehydrated through a series of aqueous solutions of ethanol (50 to 100% v/v). The specimens were critical point dried from liquid CO_2 and mounted on carbon-coated aluminum stubs (29–31). Finally, they were coated with evaporated carbon using an Edwards 306 vacuum coating unit (32).

Scanning electron microscopy. The samples were examined using a Cambridge Stereoscan S360 electron microscope fitted with a solid-state back-scatter detector (KE Developments, UK) (33, 34). The accelerating voltage was 15 kV.

Neuraminidase digestion. Pre-digestion with neuraminidase was performed as a comparison in conjunction with the HMFG1 antibody, as this removes terminal sialic acid residues and can detect masking by glycosylation of MUC 1 (26). Following washing with PBS, the endometrial biopsies were incubated for 10 min at 37°C with 100 μl neuraminidase 1 U/ml in 0.1 M sodium acetate buffer (pH 5.5), 800 μl 0.1 M sodium acetate buffer (37°C) and 100 μl 0.1% calcium chloride solution. This was followed by immunogold labelling, using the above technique and antibodies.

RESULTS

To visualise the glycoform detected by HMFG1 in the endometrium using the scanning electron microscope, a gold-labelled secondary antibody was used and the size of the gold particles was increased from 5 nm to approximately 200 nm by silver enhancement. These particles were then visible in the back-scattered image. Throughout all specimens there was specific HMFG1 localisation to the ciliated cells and 'ciliogenic' cells (Figs. 1 and 2). The distribution of the gold was clearly restricted to the microvilli surrounding the cilia on the mature cells and absent from the non-ciliated cells. This pattern was consistent in all 10 of the biopsies. Five of the specimens examined contained pinopodes and there was no evidence of HMFG1 reactivity on their surface using the immunogold technique (Fig. 3). Following neuraminidase treatment, the intensity of the signal was increased. However, HMFG1 reactivity was still confined to the ciliated and ciliogenic cells (Fig. 4).

There was no reactivity observed on the endometrial surface using the negative control antibody MIB-1 or to specimens incubated without primary antibody in any of the specimens (data not shown).

DISCUSSION

This is the first illustration of molecular localisation to the surface ultrastructure of the human endometrial epithelium. Using immunohistochemistry at the light microscope level, it has been previously demonstrated that the MUC1 mucin glycoform detected by HMFG1 exhibits a heterogeneous distribution on the luminal endometrial epithelium during the mid-secretory phase (13, 35). However, due to the inherent resolution problems of light microscopy, its precise cellular localisation was not evident. In this study, scanning immunoelectron microscopy has allowed us to demonstrate a clear association of the HMFG1 epitope with the ciliated and ciliogenic cells of the luminal endometrial surface. Moreover, the epitope was further localised to the microvilli of the ciliated cells and was not found on

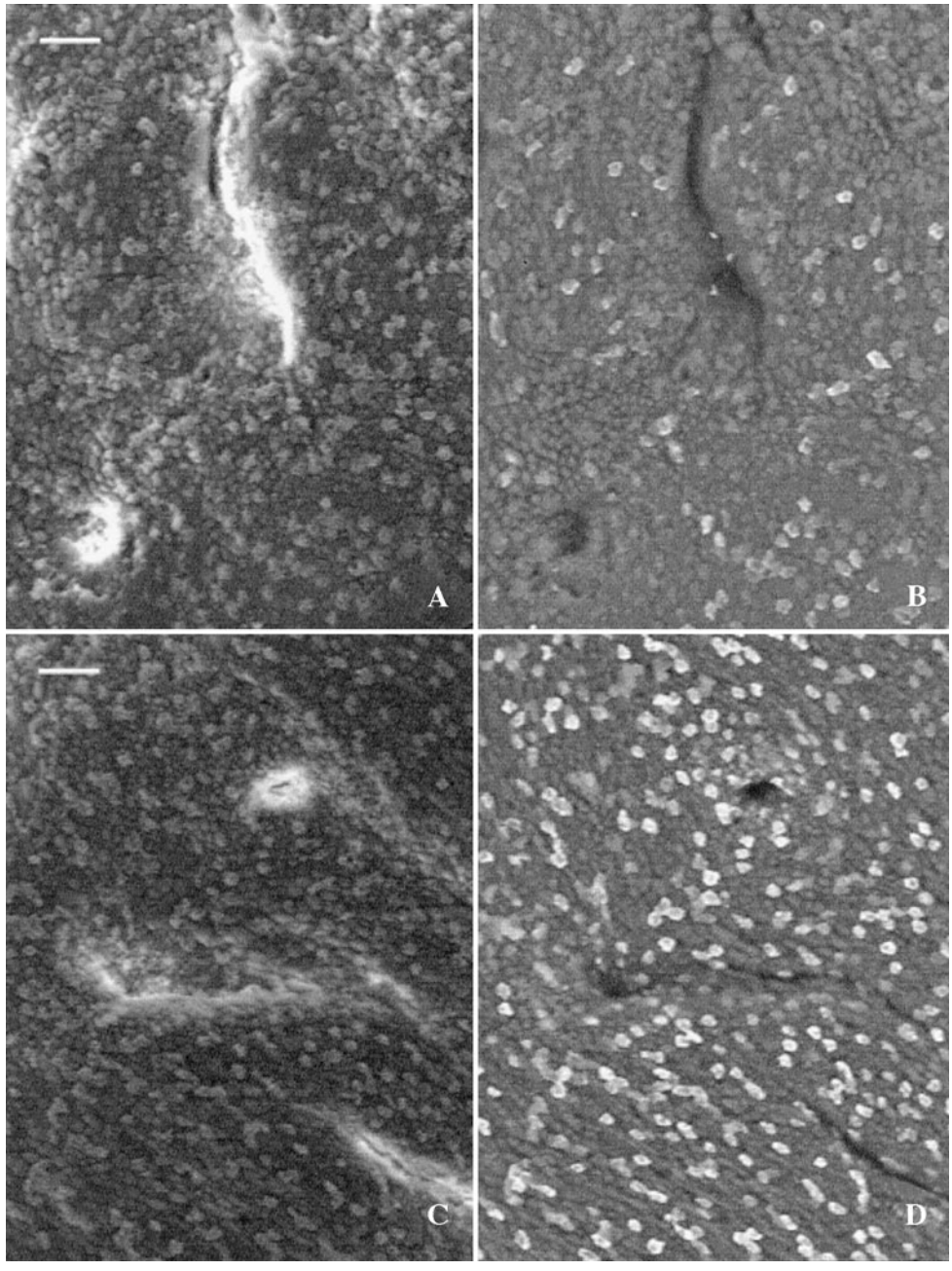

FIG. 4. Paired scanning electron microscope images of the endometrial surface of a biopsy from the mid-secretory phase of the menstrual cycle treated with neuraminidase (C and D) and untreated (A and B). Note the increased intensity of HMFG1 signal in the treated epithelium. Bars, 50 μm.

the cilia themselves. No reactivity was observed on the surface of the non-ciliated, microvillous cells and uterine pinopodes.

It is well known that the HMFG1 antibody does not bind all of the MUC1 that is present on the luminal epithelium as the epitope recognised by HMFG1 is strongly affected by glycosylation (12, 13). Consequently, terminal sialic acid residues were removed enzymatically with neuraminidase prior to immunohistochemistry to increase HMFG1 binding. Unmasking by neuraminidase increased the sensitivity of the localisation but had no effect on the selectivity.

The observation of such specific HMFG1 distribution is potentially biologically relevant. The MUC1 mucin is a key player in the complicated process of embryo implantation. Due to their large highly extended structures, MUC1 ectodomains are likely to be among the first cell surface components encountered during embryo attachment to the human endometrium (35, 36). As a consequence, it is thought that MUC1 not only

exhibits regionally restricted glycosylation during the receptive window to allow embryo adhesion, but also is subsequently down-regulated by a signal from the embryo itself (6, 12, 13, 37). Recent *in vitro* data using the monoclonal antibody BC-3 (which recognises the same peptide epitope as HMFG1 but appears to be less affected by glycosylation) suggest that MUC1 is lost from the embryo attachment site in human endometrium (14). Hence, with additional data provided from scanning immunoelectron microscopy studies performed in women of proven fertility, and using a larger panel of MUC1 antibodies, the full pattern of expression of MUC1 on the ultrastructural surface could be determined. The demonstration of genuine microheterogeneity of MUC1 glycosylation might be a possible mechanism by which some areas on the epithelium could transiently display recognition structures to the human embryo while others remain inhibitory. This finding would also be consistent with the hypothesis that there is a mutual co-operation between the maternal and embryonic cells to allow loss of the MUC1 barrier to implantation (38).

In summary, this study illustrates the value of combining SEM and immunolabelling techniques to examine the distribution of antigens on a subset of the cells forming the endometrial surface. We are confident that the further application of such a technique will increase our knowledge of embryo implantation.

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