

Decidualized and pre-decidualized normal endometrial stromal cells produce more O-linked N-acetylglucosamine containing epitope H than non-decidualized normal endometrial stromal cells

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Summary. The epitope H contains an O-linked N-acetylglucosamine residue in a specific conformation and/or environment recognized by the monoclonal antibody H (mAbH). mAbH stains two bands with Mr $\times 10^{-3}$ of 209 and 62 in lysates of cultured rat astrocytes. In addition, in extracts of cultured MCF-7 breast carcinoma cell line cells it stains cytokeratin 8 and five polypeptides originating from Triton X-100-soluble (Mr $\times 10^{-3}$ of 232, 67 and 37) and from the Triton X-100-insoluble (Mr $\times 10^{-3}$ of 51 and 50) fractions, respectively. In our previous studies we used the mAbH to investigate by immunostaining the expression of the epitope H in normal human brains, human brains with a variety of lesions, astrocytic tumors, infiltrating ductal breast carcinomas, fibroadenomas, and mitochondria-rich normal, metaplastic and neoplastic cells. In order to gain further insight into the expression patterns of the epitope H in human tissues we used the mAbH to investigate the immunohistochemical expression of the epitope H in normal human endometrium, including 30 cases of proliferative endometrium, 30 cases of early secretory endometrium, 30 cases of mid secretory endometrium, 30 cases of late secretory endometrium and 30 cases of decidual tissues. The main results were the following: 1) The decidual stromal cells presented in all cases high cytoplasmic expression of the epitope H; 2) The pre-decidual stromal cells presented in all cases of late secretory endometrium significant cytoplasmic expression of the epitope H ranging from moderate to

high expression; 3) The non pre-decidual stromal cells of the functional endometrial layer presented in all cases insignificant cytoplasmic expression of the epitope H ranging from null to low expression; 4) The stromal cells of the basal layer of the endometrium and decidua did not express the epitope H in any case; 5) The endometrial stromal granulocytes did not express the epitope H in any case and 6) The blood vessel wall cells (endothelial and smooth muscle) of the endometrium through the whole duration of the menstrual cycle and of the decidua presented high cytoplasmic expression of the epitope H. It is concluded that decidualized and pre-decidualized human normal endometrial stromal cells show increased expression of the O-linked N-acetylglucosamine containing epitope H compared to non-decidualized endometrial stromal cells. These findings suggest that the expression of the epitope H may be under positive progesterone control in normal human endometrium. Further investigation of the antigens bearing the epitope H might help to gain further insight into the histophysiology and the pathology of human endometrium.

Key words: Epitope H, O-linked N-acetylglucosamine, Endometrium

Introduction

The IgM monoclonal antibody mAbH was recently described by Arvanitis et al 2001. The mAbH stains two bands with Mr $\times 10^{-3}$ of 209 and 62 in lysates of cultured rat astrocytes and recognizes the epitope H consisting of an O-linked N-acetylglucosamine (O-GlcNAc) and

neighboring amino acids (Arvanitis et al., 2001). In addition, in extracts of cultured MCF-7 breast carcinoma cell line cells it stains cytokeratin 8 and five polypeptides originating from Triton X-100-soluble ($M_r \times 10^{-3}$ of 232, 67 and 37) and from the Triton X-100-insoluble ($M_r \times 10^{-3}$ of 51 and 50) fractions, respectively (Arvanitis et al., 1995). Modification of Ser and Thr residues by the attachment of O-linked N-acetylglucosamine [Ser (Thr)-O-GlcNAcylation] to nuclear and cytosolic proteins is a dynamic process and possibly as abundant as Ser (Thr) phosphorylation (Hart, 1997; Zachara and Hart, 2002, 2004). O-GlcNAc modification appears to have a reciprocal relationship with protein phosphorylation; in this respect, O-GlcNAc could change the pattern of available hydroxyl residues which can be phosphorylated in response to signal transduction events (Miller et al., 1999; Zachara and Hart, 2004). The rapid and dynamic change in O-GlcNAc levels in response to mitogens, growth factors, morphogens and the cell cycle suggests an important role of O-GlcNAc in signal transduction pathways (Van den Steen et al., 1998; Zachara and Hart, 2002, 2004). Changes in O-GlcNAc levels have been shown to alter the behavior of specific proteins by modulating 1) enzyme activity or regulation, 2) protein-protein interaction, 3) DNA-binding, 4) subcellular localization and 5) the half-life and proteolytic processing of proteins (Van den Steen et al., 1998; Miller et al., 1999; Zachara and Hart, 2002, 2004). Recently, O-GlcNAc has been implicated in the regulation of stress response pathways, in the regulation of the proteasome and in the etiology of Type II diabetes (Zachara and Hart, 2004). A large number of nuclear and cytoplasmic proteins are now known to be modified by O-GlcNAc. Indeed, O-GlcNAcylated proteins include cytoskeletal proteins (e.g., keratins 8, 13, and 18, Neurofilaments, Talin, Vinculin), transcription factors (e.g., Sp1, AP-1, RNA polymerase II, NF-kappa B, Estrogen receptors, β -catenin), heat-shock proteins (e.g., HSP70, HSP90), tumor suppressor proteins (e.g., p53), oncoproteins (e.g., v-erbA and c-myc), nuclear pore proteins (e.g., p62, Nup 153, 214, 358) and many chromatin proteins (e.g., chromatin-associated proteins) (Jackson and Tjian, 1989; Privalsky, 1990; Chou et al., 1992; Chou and Omary, 1993; Shaw et al., 1996; Haltiwagner et al., 1997; Hart, 1997; Kreppel et al., 1997; Van den Steen et al., 1998; Miller et al., 1999; Reuter and Gabius, 2000; Chen and Hart, 2001; Chou and Hart, 2001; Zachara and Hart, 2002, 2004; Guinez et al., 2005). In addition, O-GlcNAc is involved in apoptosis pathways (Boehmelt et al., 2000; Vosseler et al., 2002; Wells et al., 2002, 2003) and cell cycle progression (Fang and Miller, 2001; Lefebvre et al., 2004). Furthermore, a working model is emerging that O-GlcNAc may be a nutrient metabolic sensor that attenuates a cell's response to extracellular stimuli based on the energy state of the cell (Wells et al., 2003).

The widespread distribution of O-linked N-acetylglucosamine on proteins and its regulatory function on basic biological processes, suggests that it

may play a role in the normal histophysiology as well as in the pathology of many diseases, including cancer (Chou and Hart, 2001; Zachara and Hart, 2004). In this respect, we have used the mAbH to investigate by immunostaining the expression of the epitope H in normal human brains, human brains with a variety of lesions, astrocytic tumors, infiltrating ductal breast carcinomas, fibroadenomas, and mitochondria-rich normal, metaplastic and neoplastic cells (Arvanitis et al., 1995, 2001, 2005a,b; Havaki et al., 2003). In normal human brains the epitope H was absent from the overwhelming majority of normal astrocytes and only sparse reactivity was observed, confined mostly to fibrous astrocytes (Arvanitis et al., 2001). Upregulation of the expression of the epitope H was found in reactive astrocytes observed in pathological specimens from a variety of brain lesions, including anisomorphic and isomorphic gliosis (Arvanitis et al., 2001). The expression of the epitope H was higher in astrocytomas compared to anaplastic astrocytomas and glioblastomas (Arvanitis et al., 2005b). The intensity of mAbH ultrastructural immunostaining over the mitochondria, the nucleoli and the cytoplasmic vesicles appeared to be decreased in infiltrating ductal breast carcinomas when compared to fibroadenomas (Havaki et al., 2003). Mitochondria-rich normal, metaplastic and neoplastic cells showed overexpression of the epitope H (Arvanitis et al., 2005a). The above mentioned fluctuations of the expression of the epitope H in various normal and pathological human tissues prompted us to investigate the expression pattern of the epitope H in human normal endometrium. Therefore, in the present study we used the mAbH to investigate the immunohistochemical expression of the epitope H in 150 cases of normal human endometrium (proliferative, early secretory endometrium, mid secretory, and late secretory endometrium and normal decidual tissues).

Materials and methods

The material of the present study was composed of 30 cases of human normal proliferative endometrium, 30 cases of human normal early secretory endometrium, 30 cases of human normal mid secretory endometrium, 30 cases of human normal late secretory endometrium and 30 cases of human normal decidual tissues. The specimens were obtained from uteri removed for cervical carcinomas, endometrial curettings which did not show inflammation or necrosis and deciduas from cases of legal abortions. All the patients studied did not receive any hormonal medications. For the immunodetection of the epitope H, the indirect immunoperoxidase procedure was applied as described in details previously (Arvanitis et al., 2001). Briefly, tissue sections about $4\mu\text{m}$ were cut from formalin fixed paraffin-embedded tissue blocks. After deparaffinization and blocking of endogenous peroxidase activity by immersing the sections in 3% H_2O_2 in Tris-Saline buffer pH 7.6, the sections were incubated in 10% normal

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rabbit serum in buffer for 30 minutes in order to inhibit the non-specific binding of antibodies. Then the sections were incubated in undiluted supernatant containing the mouse monoclonal antibody H (Arvanitis et al., 2001) for 2 hours at room temperature. After washing 3x10 minutes in buffer the sections were incubated with peroxidase conjugated rabbit anti-mouse antibody diluted 1:50 in buffer for 1 hour. After washing 3x10 minutes the color was developed by incubating the sections in DAB-H₂O₂ in buffer for 8 minutes, then after washing, counter staining in Hematoxylin, and dehydrating, the sections were covered with permount. In the negative control sections the primary antibody was omitted and instead of it a non-immune isotype-matched mouse Ig at the same concentration as the primary antibody was applied. The staining pattern was graded as negative (-), when no stained cells were present, low (+), when less than 30% of the cells were

stained, moderate (++) when 30-75% of the cells were stained and high (+++) when 75-100% of the cells were stained.

Results

The main results were the following: 1) The decidual stromal cells presented in all cases high cytoplasmic expression of the epitope H (Fig. 1A,F,G.); 2) The pre-decidual stromal cells presented in all cases of late secretory endometrium moderate cytoplasmic expression of the epitope H (Fig. 1B); 3) The non pre-decidual stromal cells of the functional endometrial layer presented in all cases insignificant cytoplasmic expression of the epitope H ranging from negative (40%) (Fig. 1C) to low (60%) expression in proliferative endometrium, negative (20%) to low (80%) (Fig. 1D) in early secretory endometrium and low (100%) in mid

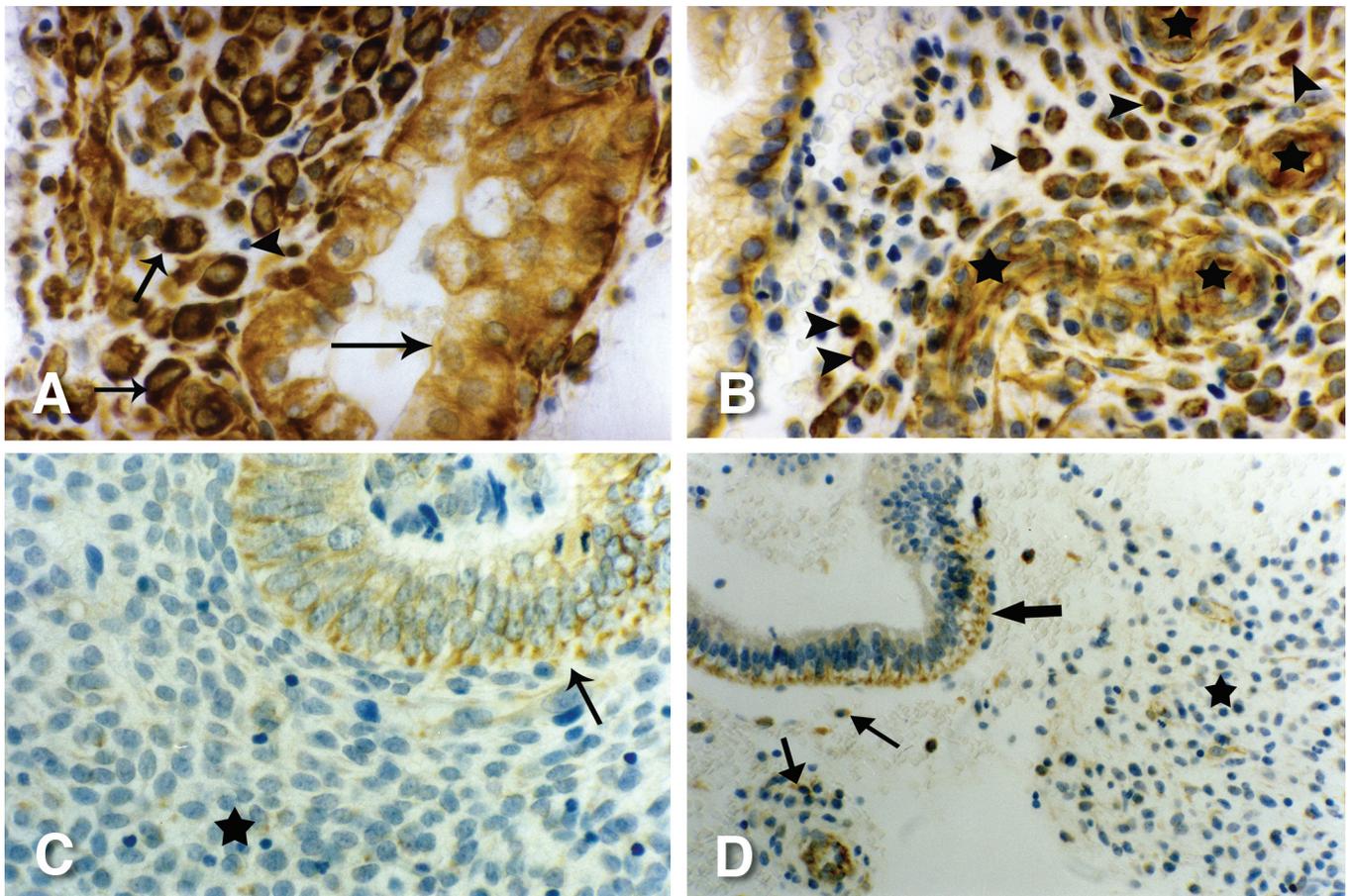


Fig. 1. **A.** Decidua showing intense cytoplasmic staining of stromal cells (short arrows) and moderate cytoplasmic staining of epithelial cells (long arrow). The stromal granulocytes cells are negative (arrow head). x 450. **B.** Late secretory endometrium showing intensely stained predecidual cells (arrowheads) around blood vessels. The blood vessel wall cells are also intensely stained (asterisks). x 400. **C.** Proliferative endometrium showing unstained stromal cells (asterisk), and moderately stained epithelial glandular cells (arrow). x 400. **D.** Early secretory endometrium showing moderately stained epithelial glandular cells with subnuclear vacuoles (thick arrow). The overwhelming majority of stromal cells remain unstained (asterisk). Occasional stromal cells are intensely stained (thin arrows). x 250

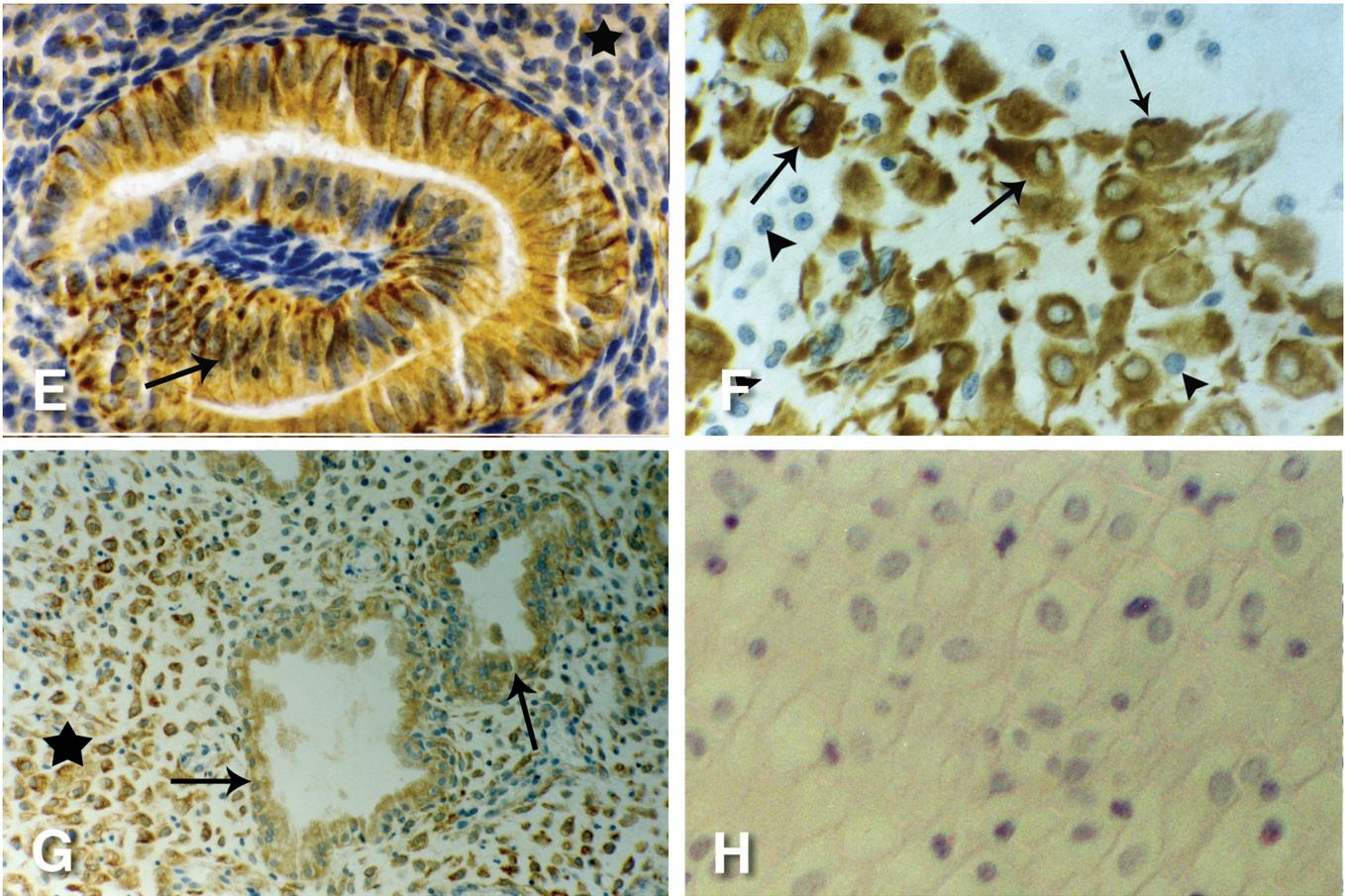


Fig. 1. **E.** Endometrial basal layer, showing moderately stained epithelial glandular cells (arrow) and unstained stromal cells (asterisk) x 400. **F.** Decidual stromal cells show intense cytoplasmic staining (arrows) and stromal granulocytes remain unstained (arrow heads) x 450. **G.** Decidua showing stained epithelial glandular cells (arrows) and stained stromal cells (asterisk) for the epitope H. x 250. **H.** Negative control figure showing all stromal cells (decidual and granulocytes) to appear unstained. The section used as a negative control is from the same tissue block as the positive figure F. x 400

secretory endometrium; 4) The stromal cells of the basal layer of the endometrium (Fig. 1E) and decidua did not express the epitope H in any case; 5) The endometrial stromal granulocytes did not express the epitope H in any case (Fig. 1A,F) and 6) The blood vessel wall cells (endothelial and smooth muscle) of the endometrium (Fig. 1B) through the whole duration of the menstrual cycle and of the decidua presented high cytoplasmic expression of the epitope H.

Discussion

The present study shows that the endometrial stromal cells of the functional layer of the normal human endometrium produce a protein or proteins which carry the O-linked N-acetylglucosamine containing epitope H. It was found that decidualized and pre-decidualized human normal endometrial stromal cells display increased expression of the epitope H compared to non-

decidualized endometrial stromal cells. It is noteworthy that the fluctuations of the expression of the epitope H are observed in the stromal endometrial cells which are under hormonal influence in normal human endometrium while the stromal cells of the basal layer of the endometrium and decidua, which are not under hormonal influence, did not express the epitope H. These findings suggest that the expression of the epitope H may be under hormonal influence in normal human endometrium. Moreover, the findings that the decidualized and pre-decidualized stromal cells display increased expression of the epitope H compared to non-decidualized stromal cells, suggest that the expression of the O-linked N-acetylglucosamine containing epitope H may be under positive progesterone control in normal human endometrium.

Taken together, our present and previous results indicate that the expression of the epitope H fluctuates in various normal and neoplastic cells and tissues including

astrocytes, astrocytomas, breast fibroadenomas infiltrating ductal breast carcinomas and mitochondria-rich normal, metaplastic and neoplastic cells (Arvanitis et al., 1995, 2001, 2005a,b; Havaki et al., 2003). Since the epitope H contains an O-linked N-acetylglucosamine residue (Arvanitis et al., 2001), it is possible that the fluctuations of the expression of the epitope H reflect differences in the expression of O-GlcNAc glycosylated cellular protein or proteins. These fluctuations may be of interest for gaining insight into the histophysiology and the pathology of endometrium since O-GlcNAc glycosylation may modify proteins involved in important biological functions such as cytoskeletal proteins, transcription factors, heat-shock proteins, chromatin proteins, tumor suppressor proteins and oncoproteins (Jackson and Tjian, 1989; Privalsky, 1990; Chou et al., 1995; Shaw et al., 1996; Haltiwagner et al., 1997; Hart, 1997; Kreppel et al., 1997; Van den Steen et al., 1998; Miller et al., 1999; Chou and Hart, 2001; Wells et al., 2003; Zachara and Hart, 2002, 2004).

It is noteworthy that O-GlcNAc is involved in apoptosis pathways (Boehmelt et al., 2000; Vosseler et al., 2002; Wells et al., 2002, 2003). Indeed, O-GlcNAcase is cleaved by caspase 3 and elevation of O-GlcNAc levels inhibits/enhances activation of the anti-apoptotic AKT (Boehmelt et al., 2000; Vosseler et al., 2002; Wells et al., 2002, 2003). In addition, recent observations suggest that O-GlcNAc is important for cell cycle progression (Fang and Miller 2001; Hiromura et al., 2003; Lefebvre et al., 2004). For example, O-GlcNAc glycosylation might have a profound effect on cell cycle transitions that regulate the YY1-Rb heterodimerization and promote the activity of the YY1 zinc finger DNA-binding transcription factor (Hiromura et al., 2003). Since the epitope H contains an O-linked N-acetylglucosamine residue (Arvanitis et al., 2001), it could be suggested that the fluctuations of the expression of the epitope H reflect differences in the expression of O-GlcNAc glycosylated cellular proteins involved in the cell cycle and apoptosis regulation in normal human endometrium.

A working model is emerging that O-GlcNAc may be a nutrient metabolic sensor that attenuates a cell's response to extracellular stimuli based on the energy state of the cell (Wells et al., 2003). The model proposes that cells are not blindly responding to extracellular stimuli but instead are taking into account their own energy stores (Wells et al., 2003). In this respect, O-GlcNAc, which appears to be a metabolic sensor highly responsive to nutrient state, modifies signalling components, cytoskeletal components and the transcriptional and translational machinery (Wells et al., 2003). Since the epitope H contains an O-linked N-acetylglucosamine residue (Arvanitis et al., 2001), it could be hypothesized that the fluctuations of the expression of the epitope H reflect differences in the expression of O-GlcNAc glycosylated cellular proteins involved in the metabolic regulation of the normal human endometrium.

There is an open question whether the epitope H in the endometrial stromal cells is present in one or more polypeptides containing O-linked N-acetylglucosamine. The increased expression of the epitope H in decidualized and pre-decidualized human normal endometrial stromal cells may be due to the increased expression of an already existing protein or to the expression of a newly formed protein or proteins containing an O-linked N-acetylglucosamine residue. Further studies using biochemical methods and immunoelectron microscopy will be helpful to clarify the nature of the polypeptide(s) bearing the epitope H and their accurate localization upon organelles of normal endometrial cells.

It is concluded that decidualized and pre-decidualized human normal endometrial stromal cells show increased expression of the O-linked N-acetylglucosamine containing epitope H compared to non-decidualized endometrial stromal cells. These findings suggest that the expression of the epitope H may be under positive progesteronic control in normal human endometrium. Further investigation of the antigens bearing the epitope H might help to gain further insight into the histophysiology and the pathology of human endometrium.

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