

Undifferentiated Cell Markers in the Endometrium

Huyen Pham

Department of Gynecologist, USA

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Abstract

The presence of endometrial-like tissue outside the uterus is the hallmark of endometriosis, a benign chronic illness. Although the precise pathogenic pathways are yet unknown, the aetiology of endometriosis seems to be polygenic and complex. The pathophysiology of proliferative gynaecological illnesses like endometriosis may be linked to adult stem cells, which have recently been discovered in a number of human tissues, including the endometrium. It is very difficult to identify adult stem/progenitor cells (SPCs) in tissues, however undifferentiated cell markers may be used to make inferences. Using immunohistochemistry (IHC) using a non-biotin polymer detection technique, this research examined the expression of undifferentiated markers Musashi-1, Oct-4, and c-kit in eutopic endometria of patients with and without endometriosis and endometriotic lesions. Six samples of eutopic and ectopic endometria from endometriosis patients and four tissue specimens of normal endometrium were assessed. The Kruskal-Wallis test was used to evaluate immunoreactivity scores (IRS) and the chi-square test was used to analyse categorical data. Oct-4 staining was mostly nuclear, Musashi-1 protein expression was cytoplasmic or nuclear, and c-kit protein expression was transmembrane and cytoplasmic. All examined endometrial compartments (glandular epithelium, stroma, and endothelium) showed positive immunostaining for the three markers. Patients with endometriosis had a considerably increased proportion of Musashi-1 expressing cells in their endothelium ($p < 0.05$). Cell groupings that may represent stem cell clusters were stained by Musashi-1 and c-kit. All three markers showed strong staining percentages and intensities. Stromal IRS was considerably greater in eutopic endometrial samples from endometriosis patients ($p < 0.05$), and c-kit expression was significantly higher in glandular epithelium than in the stromal compartment ($p < 0.05$). In summary, putative endometrial SPCs may contribute to the physiopathology of endometriosis by participating in neoangiogenesis as well as the development of ectopic implants.

Keywords: Surface antigens. Adult stem cells. Endometrium. Endometriosis. Physiopathology.

Introduction

The presence of glandular and/or stromal tissue that resembles the endometrium outside the uterine cavity is a characteristic of endometriosis, a benign chronic illness. Endometriosis affects 5–15% of women of reproductive age and roughly 35–50% of patients with chronic pelvic pain or infertility (GAZVANI and TEMPLETON, 2002; JENSEN et al., 2002; VINATIER et al., 2001). The incidence varies greatly depending on the research population. The pathophysiology of endometriosis is still poorly understood despite intensive research, and a number of abnormal biological processes may be implicated (BULUN, 2009). There is growing evidence that the human endometrium contains adult stem/progenitor cells (SPCs) (GARGETT, 2007). This suggests that these cells may play a role in the physiopathology of endometrial proliferative disorders like endometriosis (DU and TAYLOR, 2007; GARGETT and MASUDA, 2010; MARUYAMA et al., 2010; Oliveira et al., 2012). Research Somatic SPCs have been found in a variety of tissues and organs, including bone marrow, breast, prostate, brain, liver (LI and CLEVERS, 2002), and, more recently, endometrium (CHAN, SCHWAB, and GARGETT, 2004). SPCs have attracted a lot of attention over the past ten years because of their potential therapeutic implications (MIMEAULT and BATRA, 2008).

Cell markers unique to adult SPCs have not yet been found, despite the abundance of research on stem cells. The characterisation of adult quiescent SPCs would therefore greatly benefit from the identification of a panel of

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molecular markers. To date, however, SPCs have been identified phenotypically by the lack of markers of highly differentiated cell types and the presence of markers typical of undifferentiated progenitor cell lineages (GRITTI, VESCOVI, and GALLI, 2002; SOUZA et al., 2003). Two cell markers that are frequently employed in stem cell research are the nuclear transcription factor Oct-4 (PESCE and SCHÖLER, 2001) and the stem cell factor receptor c-kit (SOGO et al., 1997). Neural SPCs have been shown to express the RNA-binding protein Musashi-1, which is linked to progenitor cell self-renewal. This molecule may be a promising indicator of endometrial SPCs (GÖTTE et al., 2008).

By examining the expression of Oct-4, c-kit, and Musashi-1 markers in the ectopic endometria of women with endometriosis and the eutopic endometria of women with and without endometriosis, this study sought to establish a correlation between the presence of endometrial SPCs and the pathogenesis of endometriosis.

Methods

Eutopic endometrial samples from premenopausal women who had hysterectomy for a variety of medical reasons (n = 4), endometriotic tissue samples (n = 6), and eutopic endometrial samples from patients who had surgery to treat deep infiltrating endometriosis with intestinal involvement (n = 6), as confirmed by the histopathological study, were all included in this controlled cross-sectional study.

The Federal University of Minas Gerais (COEP–UFMG) Research Ethics Committee in Belo Horizonte, Brazil, accepted the study under protocol number ETIC/0628.0.203.000-09.

The study excluded patients with endometrial hyperplasia or polyps, as well as those receiving hormone suppression therapy or endometriosis treatment within the preceding six months.

Immunohistochemistry Tissue samples were embedded in paraffin blocks after being treated in 10% formalin. After a 24-hour incubation period at 56 °C, sections were deparaffinized in xylene and rehydrated in a succession of decreasing ethanol.

To obtain the best exposure of the target epitopes in ethylenediaminetetraacetic acid (EDTA) buffer (pH 8.0) for Oct-4 and c-kit and citrate buffer (pH 6.0) for Musashi-1, heat-induced antigen retrieval was carried out in a steamer (98–100 °C) for 45 minutes.

The Novolink™ nonbiotin polymer detection system kit (Novocastra®, Newcastle Upon Tyne, UK) was used for all immunohistochemistry procedures.

Peroxidase block was used to stop endogenous peroxidase activity for five minutes.

To lessen background staining, sections were treated with protein block for five minutes. Rabbit polyclonal primary antibody (Abcam, Cambridge, UK) diluted 1:50 (Oct-4 and c-kit) and 1:100 (Musashi-1) was then added to the sections.

To improve the polymer reagent's penetration, a post-primary block was applied for five minutes after the primary antibodies were incubated for sixty minutes at room temperature in a moisture chamber. 3,3'-diaminobenzidine (DAB) was used to create immunohistochemical reactions, and Harris's haematoxylin was used to counterstain the sections.

Positive controls for Musashi-1, Oct-4, and c-kit antibodies were slices of human intestinal tissue. As negative controls, two sections of eutopic endometrium from a healthy patient were incubated with rabbit normal serum (normal rabbit IgG –0.1 mg/mL; Calbiochem, Darmstadt, Germany) diluted 1:100 in one section while the primary antibody was omitted in the other.

Two separate observers looked at the slides under an Olympus CX31 light microscope (Olympus, Tokyo, Japan), and the median scores of the two observers were taken into account.

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At 40x magnification, a semiquantitative estimate of the percentage of positive cells was made. The immunoreactive score (IRS) (Remmele and Stegner, 1987), which has been validated in human endometrium for the Oct-4 antibody (BENTZ et al., 2010), was used to grade the stained sections for each marker.

The predominant pattern of cell staining (cytoplasmic, transmembrane, and/or nuclear), endometrial location (glandular epithelium, stroma, and vascular endothelium), and immunostaining of cell clusters were also evaluated.

Statistical analysis

Categorical variables were analyzed by the chi-square test and corrected by the likelihood ratio test. The immunoreactivity scores were tested using the Kruskal-Wallis test, whereas nonparametric paired samples were analyzed using the Wilcoxon test. The significance level was set at $p < 0.05$.

Results

93.7% of samples had positive Musashi-1 staining in the cytoplasm and nucleus, while none of the samples had positive staining in the cell membrane (Fig. I-A). The cell membrane showed no Oct-4 staining, the cytoplasm showed very little staining (6.7% of samples), and nuclear staining was the most common Oct-4 staining pattern (96.9%) (Fig. I-B). The membrane (43.0%) and cytoplasm (75.0%) showed c-kit staining, however the nucleus did not. Musashi-1 showed endothelial staining in 50.0% of samples and glandular and stromal staining in a significant proportion of samples (93.8%). The Oct-4 staining signal was moderate in the stroma (75.0%), robust in the glandular epithelium (93.8%), and intermediate in the vascular endothelium (46.2%). Only 6.7% of samples had a c-kit staining signal in the vascular endothelium, whereas the signal was robust in the glandular epithelium (81.3%) and moderate in the stroma (43.8%). Compared to normal and eutopic samples, musashi-1 staining was considerably greater in vascular endothelium samples from endometriosis patients ($p = 0.021$) (Table 1). On the other hand, Oct-4 and c-kit expression did not change significantly across groups ($p > 0.05$, Table 1).

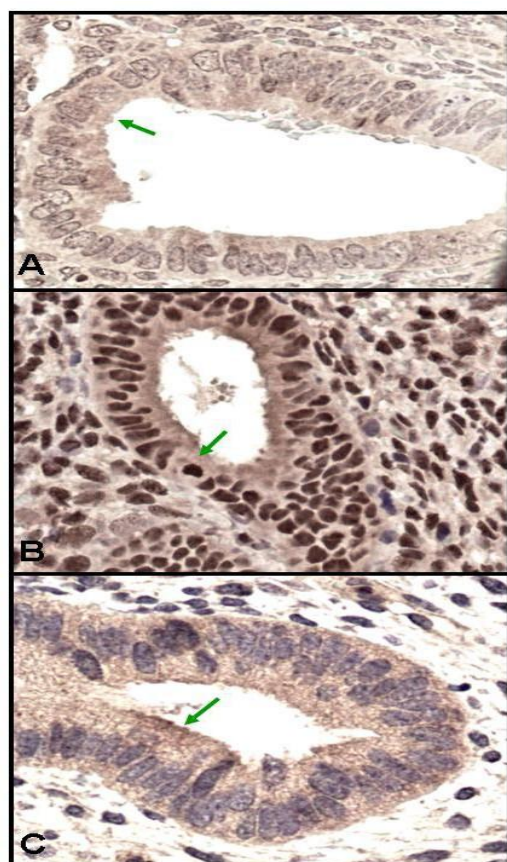


Figure I. Eutopic endometrial sample from a healthy patient (200x) showing the cell staining pattern of Musashi-1, Oct-4, and c-kit antibodies. The arrow indicates the predominant immunostaining pattern for each antibody. (A) Cytoplasmic and nuclear expression of Musashi-1 protein. (B) Predominantly nuclear expression of Oct-4. (C) Cytoplasmic and transmembrane expression of c-kit protein.

Table1. Expression of Musashi-1, Oct-4, and c-kit proteins according to endometrial location and tissue type (number of stained samples/total number of samples).

Protein/location	Normal tissue	Eutopic with endometriosis	Lesion
Musashi-1			
Glandular epithelium	4/4	5/6	6/6
Stroma	4/4	5/6	6/6
Vascular endothelium*	0/4	4/5	3/5
Oct-4			
Glandular epithelium	4/4	6/6	5/6
Stroma	4/4	4/6	4/6
Vascular endothelium	3/4	2/4	1/5
c-kit			
Glandular epithelium	4/4	4/6	5/6
Stroma	1/4	3/6	3/6
Vascular endothelium	0/4	1/5	0/6

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* $p < 0.05$, Chi-square test.

Analysis of the pooled samples for Musashi-1 staining showed a moderate percentage of stained cells and staining intensity, and no significant difference in staining intensity was observed between glandular and stromal sections. Furthermore, immunostaining percentage, intensity, and IRS for Musashi-1 did not significantly change across sample groups or tissue types.

The Oct-4 expression analysis of the pooled samples revealed a high percentage of stained cells ($> 80\%$) and high staining intensity (moderate and strong) (Fig. II), which resulted to high IRS for every sample.

Additionally, for Oct-4 (pooled samples), there was no discernible change in IRS between the glandular and stromal compartments. Similarly, immunostaining percentage, intensity, and IRS for Oct-4 did not significantly change across sample groups or tissue types.

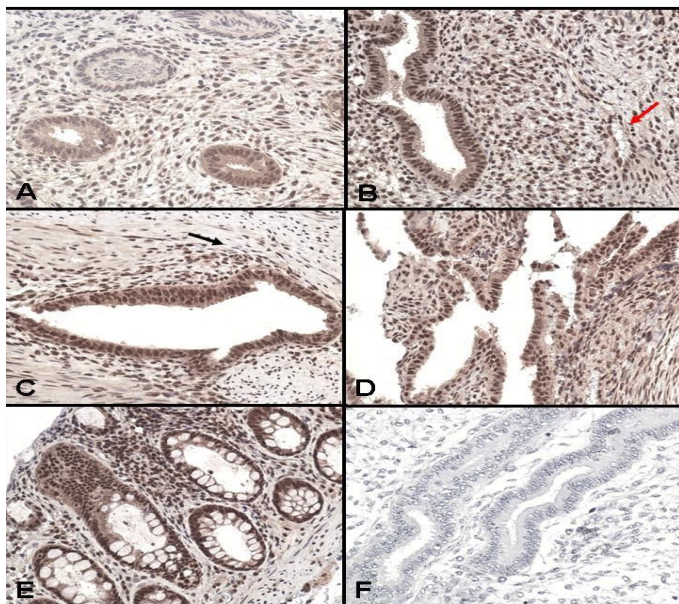


Figure II. (A) Endometrial sample from a healthy patient showing strong glandular and stromal staining. (B) Eutopic endometrial sample from a patient with endometriosis showing strong staining. Stained endothelial cells. (C and D) Endometriotic lesions with strong and moderate immunostaining. Transition zone between the endometriotic lesion and normal tissue. (E) Positive control. (F) Negative control. (200x – Oct-4).

The number of stained cells (10–50%) and staining intensity (poor to moderate) varied, according to an analysis of the pooled samples for c-kit staining. Protein expression was much greater in the epithelial compartment than in the stromal compartment, according to a comparison of the glandular and stromal compartments for c-kit staining (immunoreactive scores) (Fig III).

Furthermore, compared to normal and lesion samples, eutopic endometrial samples had considerably greater stromal IRS ($p < 0.05$). On the other hand, there were no discernible variations in glandular and total IRS for c-kit staining across sample groups and tissue types (Fig. IV).

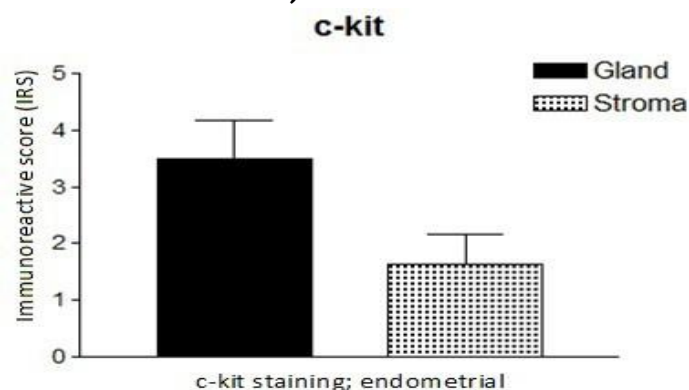


Figure III. Glandular and stromal immunoreactive scores (IRS) for c-kit staining (pooled samples, $p < 0.05$).

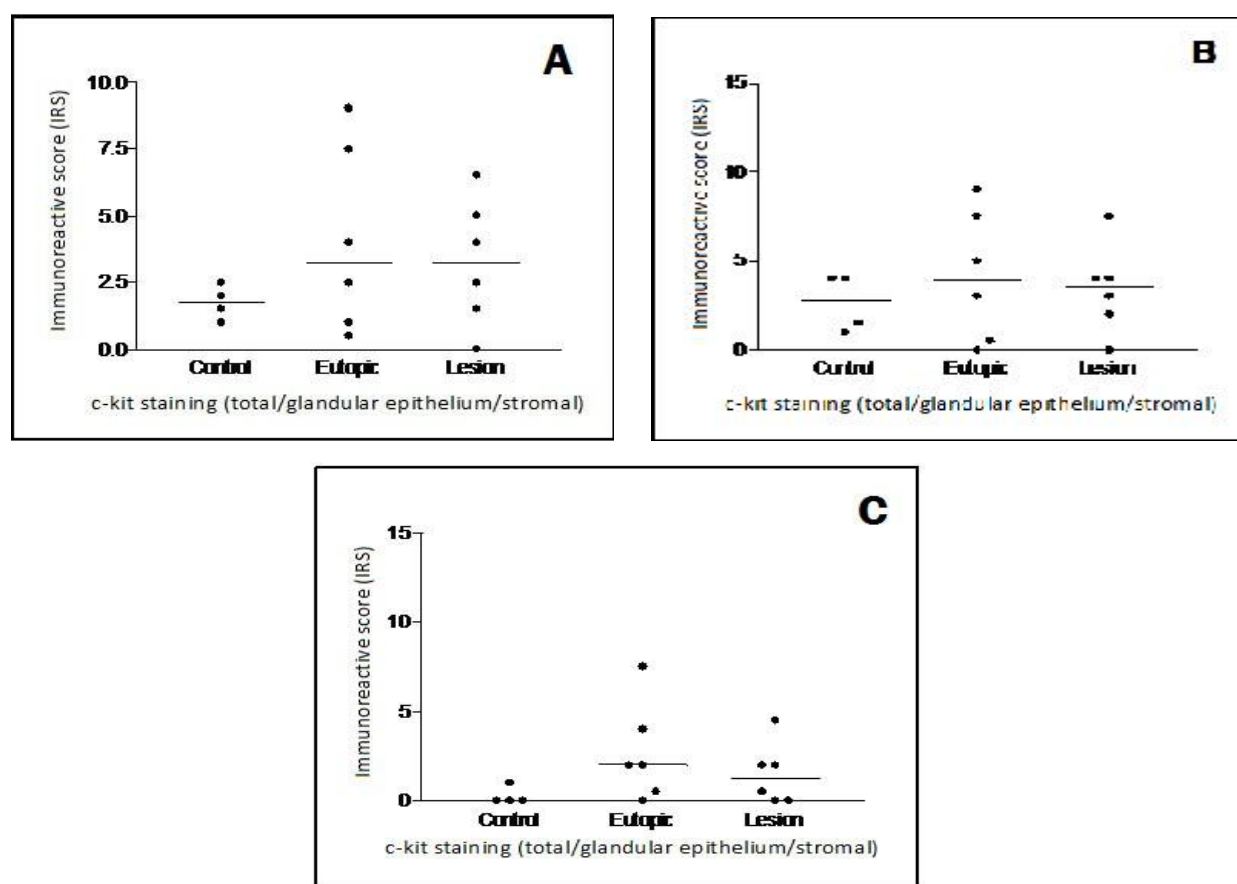


Figure IV. Immunoreactive scores (IRS) for c-kit staining between sampling groups. (A) Total IRS, (B) glandular epithelium IRS, and (C) stromal IRS.

Clustered staining was observed only for Musashi-1 and c-kit.

Discussion

In this investigation, we demonstrate that the cellular expression patterns of the three markers—Musashi-1, Oct-4, and c-kit—were somewhat in line with their known cellular locations. Musashi-1 protein expression manifested as nuclear or cytoplasmic staining, resembling the pattern reported by Götte et al. (2008). Since Oct-4 is a transcription factor, its cellular expression pattern was, as predicted, mostly nuclear (96.7% of samples). On the other hand, c-kit protein expression showed cytoplasmic and transmembrane staining, but no nuclear staining was

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seen. These findings are in line with c-kit's recognised role as a transmembrane tyrosine kinase receptor (SOGO et al., 1997). All examined endometrial compartments (glandular epithelium, stroma, and endothelium) showed positive immunostaining for the three markers. For Musashi-1 and Oct-4, glandular epithelium and endometrial stroma showed comparable staining proportions; however, c-kit staining was mostly epithelial. Musashi-1 staining positivity was greater in endometriotic lesion samples compared to eutopic endometrial samples with and without endometriosis, indicating a potential link between SPCs and neoangiogenesis. Musashi-1 expression was much greater in the stroma of endometriotic lesions than in normal endometrium, despite the fact that Götte et al. (2008) did not look at the vascular compartment. The authors of the research discovered significant staining in cell groupings, which could be SPC clusters, in line with our results. There was no difference in Oct-4 expression in endometrial compartments across sampling methods or groups. Matthai et al. (2006) found similar findings when they used reverse transcriptase polymerase chain reaction (RT-PCR) to identify Oct-4 expression in all endometrial samples, although immunohistochemistry (IHC) revealed a varied pattern of expression. Additionally, Oct-4 was expressed in both eutopic endometrial epithelial cells and ectopic epithelial cells of stage IV endometriotic lesions, with the latter showing noticeably greater protein expression (Sbracia et al., 2007). Using RT-PCR and IHC, Bentz et al. (2010) examined Oct-4 expression in the various stages of the menstrual cycle and discovered that Oct-4 was not expressed differently in the endometrium throughout the follicular and luteal phases. Although c-kit was mostly expressed in glands, it was also found in the stromal compartment and, seldom, in the vascular endothelium. Furthermore, compared to samples from normal endometrium and endometriotic lesions, c-kit expression in endometrial stroma was much greater in eutopic endometrium from individuals with endometriosis. However, there is contradictory information about c-kit expression in the endometrium. For example, c-kit expression in the endometrium was not detected by Lammie et al. (1994). On the other hand, Elmore et al. (2001) found that 79% of secretory endometria and 93% of benign proliferative glandular endometrial samples had positive c-kit immunostaining. Later, Cho et al. (2004) found that whereas c-kit was strongly expressed in the stroma and basalis glands in all previous lifetime endometrial samples, it was not seen in the foetal endometrium. SPCs may be involved in the pathophysiology of endometriosis, according to Osuga et al. (2000), who examined the expression of c-kit in the peritoneal fluid of women with and without endometriosis. Additionally, Uzan et al. (2005) proposed that the increased expression of c-kit in deep endometriosis suggests that SPCs are implicated in the pathophysiology of endometriosis; Sbracia et al. (2007) also reported comparable results. High staining intensity and percentage for potential SPC indicators were shown by the three markers assessed in this investigation. While our results for Oct-4 and c-kit expression are in line with other studies (Sbracia et al., 2007; Bentz, 2010; Elmore et al., 2001), the expected expression for Musashi-1 should be lower based on the results of other assays for SPCs, such as clonogenic assays (CHAN, SCHWAB, and GARRETT, 2004). When combined, these findings imply that Musashi-1, Oct-4, and c-kit may be expressed in cells other than SPCs and have poor selectivity for identifying SPCs. Future research must thus find new compounds with higher selectivity for undifferentiated cells that have been confirmed by further experiments. Because the percentage of marked cells with undifferentiated markers was equivalent to or higher than in specimens from endometriosis patients, our findings point to a potential involvement for putative endometrial SPCs in the pathophysiology of endometriosis. The increased Musashi-1 staining intensity in the endometriotic vascular endothelium compartment

COBELLIS et al. Notch protein members' expression patterns in both healthy and diseased endometrium. Lesions may be linked to the angiogenesis of endometriosis, and the high proportion of c-kit-expressing cells in eutopic endometrial samples from endometriosis patients may provide additional proof that reflowed SPCs contributed to the development of ectopic endometrial implants (J Anat, v. 213, n. 4, p. 464-72, Oct. 2008).

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