Histol Histopathol (2011) 26: 1127-1133

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Expression of a putative stem cell marker Musashi-1 in endometrium

Xiaoye Lu, Fangfang Lin, Huijuan Fang, Xuesong Yang and Li Qin

Department of Histology and Embryology, Medical College of Jinan University, Guangzhou, Guangdong, China

Summary. Aim: Firstly to examine the expression characteristics of Musashi (Msi)-1 in fetal endometrium, reproductive normal endometrium, endometrial hyperplasia and endometrioid adenocarcinoma, next, to focus on exploring the possibility that Msi-1 serves as a marker of the endometrial stem cells in-situ.

Methods: Immunohistochemical staining was performed to detect the expression of Msi-1 in 20 cases of fetal endometrium, 20 cases of normal endometrium, 20 cases of endometrial hyperplasia and 50 cases of endometrioid adenocarcinoma respectively.

Results: In fetal endometrium, Msi-1 positive cells were observed from the 12th week in epithelium, but the number of Msi-1 positive cells decreased with an increase in gestational age. In reproductive normal endometrium, Msi-1 expression presented as dispersed single cell and cell groups in the stroma adjacent to the myometrium. In endometrial hyperplasia and endometrioid adenocarcinoma, Msi-1 expression significantly increased and was more widely distributed.

Conclusions: Msi-1 positive cells mainly lie in the stroma of normal endometrium, and the distribution pattern is consistent with that of the speculated endometrial stem cells. The high expression of Msi-1 in fetal endometrium and endometrioid adenocarcinoma suggests that Msi-1 positive cells have several characteristics of stem cells, such as high proliferative potentiality and multipotency. Considering these factors, this makes Msi-1 potentially a promising stem cell marker.

Offprint requests to: Xiaoye Lu, Department of Histology and Embryology, Medical College of Jinan University, Guangzhou, Guangdong 510632 China. e-mail: tluxye@jnu.edu.cn

Key words: Endometrium, Endometrial hyperplasia, Endometrioid adenocarcinoma, Stem cells, Musashi-1(Msi-1)

Introduction

Endometrium is one of the tissue types with very strong self-renewal capacity; it sheds and regenerates during the menstrual cycle throughout the female reproductive age. For this reason, it has long been speculated that there might be a pool of endometrial stem cells (SC) in the endometrium (Prianishnikov, 1978). Chan et al. (2004) provided the first evidence for the existence of endometrial stem/progenitor cells by identifying clonogenic human endometrial epithelial and stromal cells. Label retaining cells (LRCs) have been identified as candidate adult stem cells *in vivo* in mouse endometrium (Chan and Gargett, 2006; Cervello et al., 2007; Szotek et al., 2007). The LRC approach identifies adult stem cells by their quiescent, slowly cycling nature. Recently epithelial side population (SP) cells were identified in human endometrial cell suspensions (Kato et al., 2007). The SP phenotype is thought to be a universal marker of adult stem cell activity (Smalley and Clarke, 2005). However, it is extremely difficult to distinguish endometrial stem cells from other cell types in situ due to the lack of specific markers. Screening of specific markers of endometrial stem cells will facilitate further studies of the precise locations, morphological and functional characteristics of endometrial stem cells, as well as favor the identification and separation of these stem cells.

In this study, immunohistochemistry was used to examine the expression of Msi-1 in the fetal endometrium, reproductive normal endometrium, endometrial hyperplasia and endometrioid

adenocarcinoma tissue, with the aim of exploring the possibility of Msi-1 serving as a marker of endometrial stem cells in-situ.

Materials and methods

Tissue samples

A range of endometrial samples were selected to represent the spectrum from fetal endometrium to endometrial neoplasia, including 20 cases of fetal endometrium from 12 to 39 weeks, 20 cases of reproductive normal endometrium from uterine fibroid in the hospital (11 cases of proliferative phase and 9 cases of secretory phase); 20 selected cases of endometrial hyperplasia over the same period (18 cases of simple type, 2 cases of complex type); 50 cases of endometrial cancer biopsy or surgical resection specimens pathologically confirmed as endometrioid adenocarcinoma. Fetal endometrium specimens were obtained from discarded human tissues following fetal loss or death. The crown rump length of fetus (CRL) was measured to determine the gestational age. Fetal samples were obtained at 12 w, 16 w, 18 w, 20 w, 24 w, 26 w, 30 w, 34 w and 39 w of gestation respectively. The endometrium was taken from the posterior walls of uterine. The patients of endometrioid adenocarcinoma had a median age of 57 years (range 37-83) and had not received chemotherapy or radiotherapy prior to surgery. All human tissues were obtained with consent from the human subjects. All human materials examined in this study were treated in accordance with the guideline of the Helsinki Declaration.

Immunohistochemistry and evaluation

The specimens were fixed in 10% formalin and paraffin-embedded. Consecutive 4 μm sections were cut from paraffin blocks and placed on poly-L-lysine-coated slides.

Immunohistochemical staining was conducted using the streptavidin -peroxidase conjugation method (S-P) according to standard procedures. Briefly, deparaffinized tissue sections were first treated with 3% H_2O_2 for 15 min to inhibit endogenous peroxidase activity and then subjected to microwave antigen retrieval. After incubation with blocking serum for 20 min, sections were incubated with goat anti-Musashi-1 polyclonal antibody (R&D systems; diluted 1:1000 in PBS) overnight at 4°C followed by incubation with biotinylated anti-goat secondary antibodies and peroxidase-labeled streptavidin (R&D systems). Between each step, sections were fully washed with phosphate-buffered saline (PBS, 0.01 mol/L, pH 7.4). The staining was developed by reaction with 3, 3-diaminobenzidine substrate-chromogen solution (DAB), followed by counterstaining with hematoxylin. For a negative control, sections were incubated with PBS instead of the primary antibodies. The specificity of the antibodies used in our study for Msi-1 was confirmed in positive control sections.

No positive staining was visible in the negative control. For Msi-1 staining, the stained particles may be distributed in the cytoplasm or nucleus. For Msi-1 positive sections, 6-10 high-powered fields (40X) were randomly chosen according to the sample size, and their percentage of positive cells (the percentage of stained cells among the total number of endometrial cells in each biopsy tissue section) were counted.

Statistics

Statistical analysis was performed using SPSS Version 13.0 software. X^2 test was used to compare the positive rate of Msi-1 in different groups; t test was conducted to compare the percentage of Msi-1 positive cells in different groups. Only values with $P<0.05$ were considered as statistically significant.

Results

Msi-1 expression in fetal endometrium

In fetal endometrium, Msi-1 positive cells were observed in both epithelium and stroma from 12th week of gestation. These Msi-1 positive cells were mainly distributed in the surface epithelium and presented as cytoplasmic staining (Fig. 1A). With the formation of endometrial glands at the 18th week of gestation, Msi-1 positive cells distributed in glandular epithelium (Fig. 1B,C). Single Msi-1 positive cells were scattered in the stroma (Fig. 1D). The number of Msi-1 positive cells decreased with an increase in gestational age (Fig. 1E).

Msi-1 expression in reproductive normal endometrium

In normal endometrial tissue, Msi-1 expression appeared principally in the stroma cells adjacent to myometrium (Fig. 2A), locating around the gland or along the blood vessels, either presenting singly or in groups (Fig. 2B,C); single large positive cells were found in the connective tissue of myometrium (Fig. 2D).

Msi-1 expression in endometrial hyperplasia and endometrioid adenocarcinoma

In endometrial hyperplasia, the Msi-1 expression pattern in the stroma was similar to that in normal endometrium, but the number of positive cells was increased (Fig. 3A,B). In endometrioid adenocarcinoma, Msi-1 diffusely expressed in the cytoplasm of local glandular cells (Fig. 3C). Single Msi-1 positive cells were observed in the stroma (Fig. 3D).

The positive rates and percentage of positive cells of Msi-1 in normal endometrium, endometrial hyperplasia and endometrioid adenocarcinoma are listed in Table 1. Statistical analysis showed that endometrioid adenocarcinoma and endometrial hyperplasia were

significantly different compared with those of normal endometrium ($P<0.05$). There was no significant difference between the endometrioid adenocarcinoma and endometrial hyperplasia group ($P>0.05$).

Discussion

Adult stem cells (ASCs) are thought to be responsible for the high regenerative capacity of the

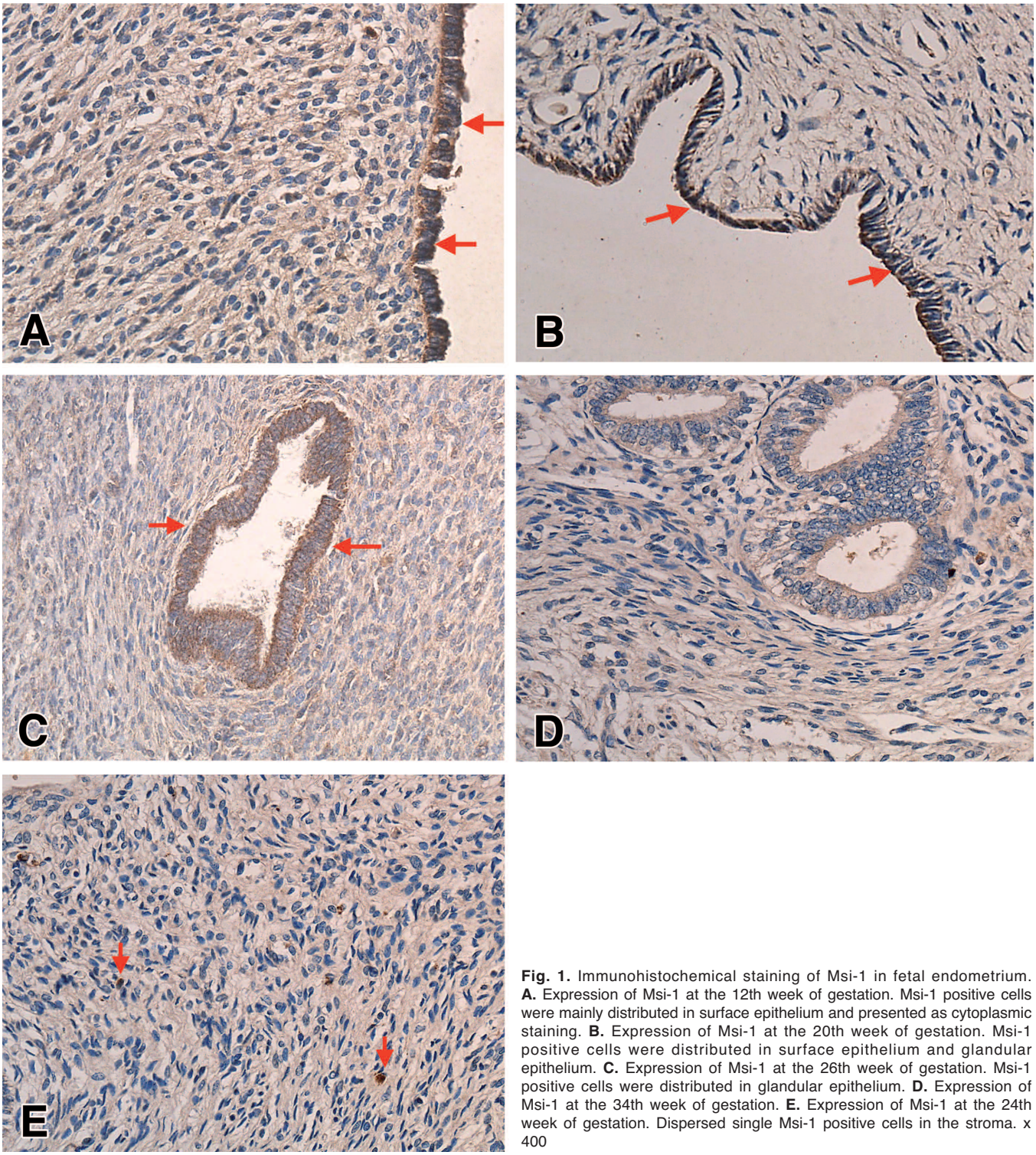

Fig. 1. Immunohistochemical staining of Msi-1 in fetal endometrium. **A.** Expression of Msi-1 at the 12th week of gestation. Msi-1 positive cells were mainly distributed in surface epithelium and presented as cytoplasmic staining. **B.** Expression of Msi-1 at the 20th week of gestation. Msi-1 positive cells were distributed in surface epithelium and glandular epithelium. **C.** Expression of Msi-1 at the 26th week of gestation. Msi-1 positive cells were distributed in glandular epithelium. **D.** Expression of Msi-1 at the 34th week of gestation. **E.** Expression of Msi-1 at the 24th week of gestation. Dispersed single Msi-1 positive cells in the stroma. x 400

human endometrium. A dysregulation of stem cell function may be involved in the pathogenesis of proliferative diseases of the endometrium, such as endometrial carcinoma, endometriosis and endometrial hyperplasia (Gotte et al., 2008). Investigating endometrial stem cell biology is crucial to understand normal endometrial physiology and to determine their roles in endometrial proliferative diseases.

Indirect evidence for the existence of adult stem/progenitor cells in the endometrium has accumulated over the years. Attempts to locate endometrial stem/progenitor cells have been undertaken using established stem cell markers, such as Oct-4, bcl-2, c-kit, P63 etc. (Cervelló et al., 2007; Cho et al., 2004; O'Connell et al., 2001). Currently, one of the most promising stem cell markers is Musashi-1. Msi-1 is an RNA-binding protein, which is likely to be associated with self-renewal and asymmetric division of neural progenitor cells, and can be used as a neural stem cell marker in the mouse nervous system (Kaneko et al.,

Table 1. The positive rates and percentage of positive cells of Msi-1 in normal endometrium, endometrial hyperplasia and endometrioid adenocarcinoma.

groups	N	positive rate (%)	percentage of positive cells (%) ($\chi \pm s$)
normal endometrium,	20	15	1.57±0.23
endometrial hyperplasia	20	65	27.78±11.47
endometrioid adenocarcinoma	50	54	40.33±7.42

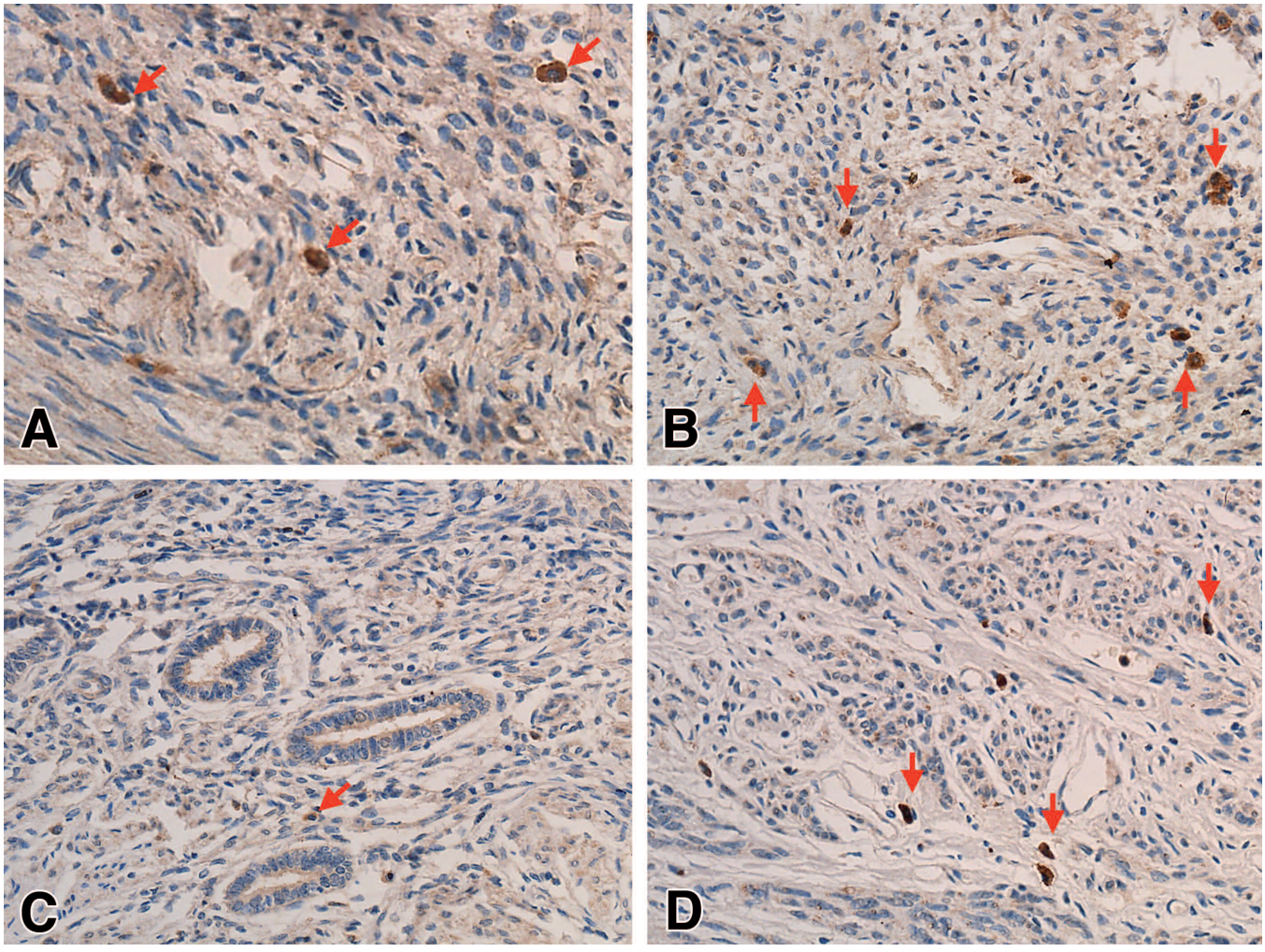

Fig. 2. Immunohistochemical staining of Msi-1 in reproductive endometrium. **A.** Expression of Msi-1 in normal endometrium. Msi-1 positive cells located near myometrium. **B.** Expression of Msi-1 in normal endometrium. Msi-1 positive cells located around the blood vessels. **C.** Expression of Msi-1 in normal endometrium. Msi-1 positive cells located around the gland. **D.** Expression of Msi-1 in normal endometrium. Msi-1 positive cells distributed in the connective tissue of myometrium. A, B, D x 400; C x 200

2000; Okano et al., 2002). In recent years, studies have shown that the expression pattern of Msi-1 is highly consistent with those of stem cells in the gastrointestinal tract and that Msi-1 is considered as a reliable gastrointestinal stem cell marker (Potten et al., 2003; Asai et al., 2005). Msi-1 as a marker of epithelial stem cells is also characteristically expressed in mammary glands and hair follicles (Clarke et al., 2005; Sugiyama-Nakagiri et al., 2006).

Recently, Gotte et al. (2008) reported that a small quantity of Msi-1 positive cells was detected in normal endometrium (increased numbers of cells expressing Msi-1 in proliferative phase endometrium compared with in secretory phase endometrium). The increased Msi-1 expression was detected in endometriosis and endometrial carcinoma. Our study showed that Msi-1 was expressed markedly in early fetal endometrium. In normal endometrial tissue, Msi-1 expression appeared mainly in the stroma cells adjacent to myometrium. In endometrial hyperplasia and endometrioid adenocarcinoma, Msi-1 expression significantly increased and was more widely distributed. These results are consistent with the report by Gotte et al. (2008), suggesting that Msi-1 positive cells possess high proliferative potentiality similar to stem cells. Furthermore, high Msi-1 expression in fetal endometrium and endometrioid adenocarcinoma was coincident with multipotency. Our results also showed that single Msi-1 positive cells were observed in connective tissue of myometrium in normal endometrium, and indicated that there are high proliferative potentiality and multipotent cells in myometrium. Quantitative analysis of Msi-1 gene expression showed a strong correlation with that of Ki-

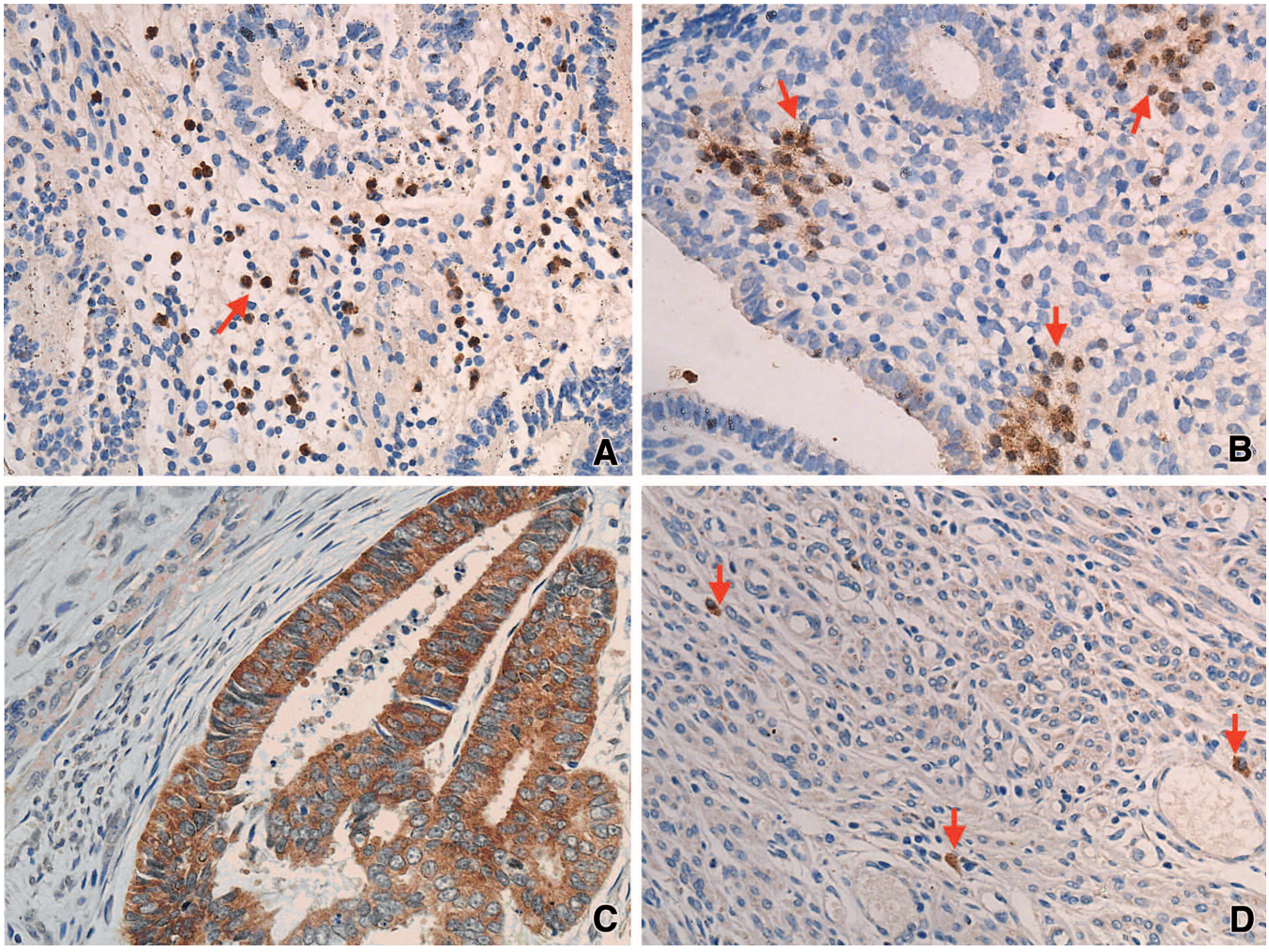

Fig. 3. Immunohistochemical staining of Msi-1 in endometrial hyperplasia and endometrioid adenocarcinoma. **A.** Expression of Msi-1 in endometrial hyperplasia. Single Msi-1 positive cells in stroma. **B.** Expression of Msi-1 in endometrial hyperplasia. Stromal Msi-1 positive cells in groups. **C.** Expression of Msi-1 in endometrioid adenocarcinoma. Diffuse cytoplasmic expression of Msi-1 in the glands. **D.** Expression of Msi-1 in endometrioid adenocarcinoma. Msi-1positive cells in the stroma. x 400

67, which is a marker of cell proliferation (Colitti and Farinacci, 2010). Immunofluorescence microscopy revealed colocalization of Msi-1 with its molecular target Noch-1 and telomerase (Gotte et al., 2008). Telomerase is a reverse transcriptase whose activity is associated with the immortality of embryonic stem cells, germ cells and cancer cells. Notch-1 signaling was seen as a means of keeping stem cells in the undifferentiated state and promoting the proliferation of stem cells (Xiao et al., 2008).

The location of endometrial stem cells is still controversial. The clonogenic experiment by Chan et al. (2004) showed the presence of epithelial and stromal cells that possess cloning activity in inactive endometrium. They proposed that stem cells in the epithelium and stroma jointly maintain endometrial proliferation and renewal. Kato reported that the sorted endometrial epithelial SP cells proliferated slowly and differentiated into CD9- and E-cadherin-expressing gland-like structures in long-term culture (Kato et al., 2007). These studies collectively suggest that two distinct types of stem/progenitor cells are present in the human endometrium. Cho et al. (2004) showed that common stem cell markers bcl-2, c-kit, CD34 and Ki-67 were expressed in the stroma of endometrial basalis from fetus period. They regard that endometrial stem cells were independent of hormonal regulation to avoid cyclic shedding. Gotte et al. (2008) detected Msi-1 positive cells in both endometrial glands and stroma. Our results showed that Msi-1 positive cells were identified mainly in the stroma in normal endometrium. It probably because there are neither sufficient sample size nor Msi-1 positive cells.

With regard to the source of adult endometrial stem cells, there are two main hypotheses. Some scholars believed that some of the fetal epithelial and mesenchymal stem cells remained in the adult endometrium so that the endometrium could renew itself in cycles; therefore the endometrial stem cells are residual fetal stem cells (Snyder and Loring, 2005). The uterus is derived from the Müllerian duct during embryo development, while the Müllerian duct is formed from the coelom epithelium (mesoderm origin). In addition to contributing to the formation of the uterus, The Müllerian duct also splits to form the fallopian tubes, cervix and vaginal fornix. Therefore, the embryonic Müllerian duct has multi-directional differentiation capabilities to form a variety of epithelia, connective tissue and smooth muscle, meaning that early embryonic cells have the property of epithelial to mesenchymal transition (EMT). Some cells in the adult uterus retain this multi-directional differentiation potential. Under certain conditions, the cells with this potential are activated and participate in endometrial cycle changes or lead to proliferative diseases under pathological conditions. Moreover, emerging evidence indicates a bone marrow contribution. The major evidence is that bone marrow stem cell marker CD34 expresses in a considerable number of endometrial stromal cells (Blau et al., 2001). In addition, studies have found that endometrial cells derived from donor bone marrow were identified in the uterus biopsy tissue from a female patient receiving bone marrow transplantation treatment (Taylor, 2004). Recently, endometrial endothelial progenitor cells have been detected as side population (SP) cells, which express several endothelial cell markers and differentiate into endometrial glandular epithelial, stromal and endothelial cells (Gargett and Masuda, 2010). Endometrial stem/progenitor cell candidates may include endogenous and exogenous stem cells, rather than a single candidate.

Most scholars seem to agree that cancer stem cells mainly originate from normal epithelial stem/progenitor cells. The cells with long-term proliferative capacity lead to tumor-genesis due to inhibition of the process of normal stem cell differentiation. Kato et al. (2010) recently found that SP cells within an endometrial carcinoma gave rise to a highly proliferative and invasive cancer cell population, to tumor stroma cells, and to the secreted matrix surrounding the tumor. This evidence suggests that endometrial stem cells are similar to early embryonic cells, having the property of EMT. The report shows that stem cells or cancer stem cells are rare; the percentage rate of cancer stem cells is 0.3%~1.6% in prostatic carcinoma (Collins et al., 2005) and less than 5% in lung cancer (Ho et al., 2007; Eramo et al., 2008). It is worth bearing in mind that the percentage of Msi-1 positive cells in endometrioid adenocarcinoma is higher than that from the other research. The discrepancy may be due to the different tumors and different checking methods; besides, the biological characteristics, such as self-renewal and de-differentiation in the tumor cells, are very similar to those of stem cells, and stem cell antigens may highly express in the tumor cells too. We therefore believe that the positive cells for Msi-1 might contain a group of cells rich in stem cells rather than all of them belonging to cancer stem cells.

In conclusion, in this study we investigated the expression of putative stem cell marker Msi-1 in a range of endometrial samples from fetal endometrium to endometrial neoplasia. The results showed that Msi-1 positive cells present mainly in the stroma and the distribution pattern was consistent with that of the speculated stem cells in the uterus. Furthermore, the high expression of Msi-1 in fetal endometrium and endometrioid adenocarcinoma suggests that Msi-1 positive cells have several characteristics of stem cells, such as high proliferative potentiality and multipotency. Lan et al. (2010) confirmed recently that Musashi-1 positive cells derived from mouse embryonic stem cells can differentiate into neural and intestinal epithelial-like cells in vivo. These results support that Msi-1 can act as a uterine stem cell marker. Further studies on the clonogenic capacity of Msi-1 positive cells both in vivo and in vitro will help to clarify their relationship with

endometrial stem cells.

Acknowledgments. The study was supported by grants from the National Natural Science Fund of China (No.30973244 and 30971493).

References

Asai R., Okano H. and Yasugi S. (2005). Correlation between Msi-1 and c-hairy-1 expression and cell proliferation activity in the developing intestine and stomach of both chicken and mouse. Dev. Growth Differ. 47, 501-510.

Blau H.M., Brazelton T.R. and Weimann J.M. (2001) The evolving concept of a stem cell: entity or function? Cell 105, 829-841.

Cervelló I., Martinez-Conejero J.A., Horcajadas J.A., Pellicer A. and Simón C. (2007). Identification, characterization and co-localization of label-retaining cell population in mouse endometrium with typical undifferentiated markers. Hum. Reprod. 22, 45-51.

Chan R.W. and Gargett C.E. (2006). Identification of label-retaining cells in mouse endometrium. Stem Cells. 24, 1529-1538.

Chan R.W., Schwab K.E. and Gargett C.E. (2004). Clonogenicity of human endometrial epithelial and stromal cells. Biol. Reprod.70, 1738-1750.

Cho N.H., Park Y.K., Kim Y.T., Yang H.and Kim S.K. (2004). Lifetime expression of stem cell markers in the uterine endometrium. Fertil Steril. 81, 403-407.

Clarke R.B., Spence K., Anderson E., Howell A., Okano H. and Potten C.S. (2005). A putative human breast stem cell population is enriched for steroid receptor-positive cells. Dev Biol.277, 443-456.

Collins A.T., Berry P.A, Hyde C., Stower M.J. and Maitland N.J. (2005). Prospective identification of tumorigenic prostate cancer stem cells. Cancer Res. 65, 10946-10951.

Colitti M. and Farinacci M. (2010). Expression of a putative stem cell marker, Musashi 1, in mammary glands of ewes. J Mol Hist. 40, 139-149.

Eramo A., Lotti F., Sette G., Pilozzi E., Biffoni M., Di Virgilio A., Conticello C., Ruco L., Peschle C. and De Maria R. (2008). Identification and expansion of the tumorigenic lung cancer stem cell population. Cell Death Differ. 15, 504-514.

Gargett C.E., Chan R.W. and Schwab K.E. (2008). Hormone and growth factor signaling in endometrial renewal: Role of stem cells. Molecular and Cellular Endocrinology. 288, 22-29.

Gargett C.E. and Masuda H. (2010). Adult Stem Cells in the Endometrium. Mol Hum Reprod. Jul 13, [Epub ahead of print]

Gotte M., Wolf M., Staebler A., Buchweitz O., Kelsch R., Schuring A.N. and Kiesel L. (2008). Increased expression of the adult stem cell marker Musashi-1 in endometriosis and endometrial carcinoma. J. Pathol. 215, 317-329.

Ho M.M., Ng A.V., Lam S. and Hung J.Y. (2007). Side population in human lung cancer stem cell lines and tumors is enriched stem-like cancer stem cells. Cancer Res. 67, 4827-4833.

O'Connell J.T., Mutter G.L., Cviko A., Nucci M., Quade B.J., Kozakewich H.P., Neffen E., Sun D., Yang A., McKeon F.D. and Crum C.P. (2001). Identification of a basal/reserve cell immuophenotype in benign and neoplastic endometrium: a study with the P53 homologue P63. Gynecologic Oncology. 80, 30-36.

Kaneko Y., Sakakibara S., Imai T., Suzuki A., Nakamura Y., Sawamoto K., Ogawa Y., Toyama Y., Miyata T. and Okano H. (2000) .Musashi1: an evolutionally conserved marker for CNS progenitor cells including neural stem cells. Dev. Neurosci. 22, 139-153.

Kato K., Yoshimoto M., Kato K., Adachi S., Yamayoshi A., Arima T., Asanoma K., Kyo S., Nakahata T. and Wake N. (2007). Characterization of side-population cells in human normal endometrium. Hum. Reprod. 22, 1214-1223.

Kato K., Takao T., Kuboyama A., Tanaka Y., Ohgami T., Yamaguchi S., Adachi S., Yoneda T., Ueoka Y., Kato K., Hayashi S., Asanoma K. and Wake N. (2010). Endometrial cancer side-population cells show prominent migration and have a potential to differentiate into the mesenchymal cell lineage. Am. J. Pathol. 176, 381-392.

Lan S.Y., Yu T., Xia Z.S., Yuan Y.H., Shi L., Lin Y., Huang K.H. and Chen Q.K. (2010). Musashi 1 positive cells derived from mouse embryonic stem cells can differentiate into neural and intestinal epithelial-like cells in vivo. Cell Biol Int. Jul 29, [Epub ahead of print]

Okano H., Imai T. and Okabe M. (2002). Musashi: a translational regulator of cell fate. J. Cell Sci. 115, 355-1359.

Potten C.S., Booth C., Tudor G.L., Booth D., Brady G., Hurley P., Ashton G., Clarke R., Sakakibara S. and Okano H. (2003). Identification of a putative intestinal stem cell and early lineage marker; musashi-1. Differentiation.71, 28-41.

Prianishnikov V.A. (1978). On the concept of stem cell and a model of functional morphological structure of the endometrium. Contraception 18, 213-233.

Smalley M.J.and Clarke R.B. (2005). The mammary gland "side population": a putative stem/progenitor cell marker? J. Mammary Gland Biol Neoplasia. 10, 37-47.

Snyder E.Y. and Loring J.F. (2005). A role for stem cell biology in the physiological and pathological aspects of aging. J. Am. Geriatr. Soc. 53(Suppl), S287-S291.

Sugiyama-Nakagiri Y., Akiyama M., Shibata S., Okano H. and Shimizu H. (2006). Expression of RNA-binding protein Musashi in hair follicle development and hair cycle progression. Am. J. Pathol. 168, 80-92.

Szotek P.P., Chang H.L., Zhang L., Preffer F., Dombkowski D., Donahoe P.K. and Teixeira J. (2007). Adult mouse myometrial label-retaining cells divide in response to gonadotropin stimulation. Stem Cells. 25, 1317-1325.

Taylor H.S. (2004). Endometrial cells derived from donor stem cells in bone marrow transplant recipients. JAMA 292, 81-85.

Xiao Y., Wang Q., Tang S.B., Huang B. and Lin S.F. (2008). Expression of Notch1 protein in induction of embryonic stem cells into nerve cells. J. Clin. Rehab. Tiss. Eng. Res. 25, 4967-4970.

Accepted January 28, 2011