Inducing malignant transformation of endometriosis in rats by long-term sustaining hyperestrogenemia and type II diabetes

Chang-Ting Wang, Dan-Bo Wang, Kui-Ran Liu, Yan Li, Chun-Xiao Sun, Cui-Shan Guo and Fang Ren

Department of Obstetrics and Gynecology, Shengjing Hospital, China Medical University, Shenyang, China

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Correspondence
Dan-Bo Wang, Shengjing Hospital of China Medical University, 36 Sanhao Street, Shenyang 110004, China. Tel: 86-18940251157; Fax: 86-024-83956387; E-mail: wangdb@sj-hospital.org

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Endometriosis (EMs) is a chronic estrogen-dependent gynecological disorder, which is classically defined as the presence of endometrial glands and stroma outside the uterine cavity. The rate of malignant transformation of EMs (MTOE) is estimated to be 0.7–1.5%. The most common site is the ovary, with endometrioid and clear cell carcinoma most frequently found (endometriosis-associated ovarian carcinoma, EAOC). Compared to the primary ovarian cancer, the patients are younger, diagnosed in earlier stages, have lower grade lesions, and a better survival. The extra-ovarian MTOE can be widely seen in various locations that are parallel to the frequency and distribution of EMs, with the major pathological type being endometrioid adenocarcinoma. Malignant transformation of EMs has always been the research focus due to its intriguing relationship with the leading gynecologic malignancy, ovarian cancer. However, the difficulty in obtaining samples meeting the rigorous diagnostic criteria (demonstration of both cancerous and benign endometrial tissue in the tumor; histology of the neoplasm compatible with an endometrial origin; no other primary tumor sites found) and the absence of in vitro and in vivo models retard the research on this malignancy, leaving many critical problems to be solved, such as the molecular mechanisms and the evidence-based proof for the prevention and intervention timing.

Malignant transformation of EMs has been proposed to be associated with free iron and heme-induced oxidative stress, an aberrant inflammatory milieu, and an estrogen-rich, progesterone-poor hormonal environment. Unopposed exposure to endogenous or exogenous estrogen, along with progesterone resistance, is the most widely recognized risk factor of MTOE. The estrogens, especially E2, which is accumulated in EMs lesions through excessive synthesis and decreased degradation, have been shown to result in direct cell damage with increased mitotic activity, a higher likelihood of DNA errors and somatic mutations and contribute greatly to the overgrowth and oncogenesis of EMs lesions. In particular, when unopposed estrogens and obesity were considered together, a higher risk of MTOE was found.
inactivation of tumor suppressor gene phosphatase and tensin homolog (PTEN) (located on 10q23.3) has been showed to be an early event in MTOE. (6) Loss of heterozygosity at locus 10q23.3 and mutation of PTEN could result in its inactivation and the following activation of phosphatidylinositol 3-kinase (PI3K)-protein kinase B (AKT)-mammalian target of rapamycin (mTOR) signaling pathway, which can regulate cell stress response and cell cycle. (12) The inactivation of PTEN caused by loss of heterozygosity occurs frequently in EMs, atypical EMs, and also MTOE, which might be a continuum between endometriosis and ovarian cancer. (6) Moreover, somatic mutation of the PTEN gene is frequently found in ovarian endometrioid adenocarcinoma but rarely seen in the other pathological types. (13) Therefore, PTEN may serve as a characteristic molecular alteration of MTOE into endometrioid carcinoma.

The similarity and correlation of MTOE and type I endometrial carcinoma is the new viewpoint in the pathogenesis of MTOE. (14) Both EAO (specific to endometrioid adenocarcinoma) and estrogen-dependent (type I) endometrial cancer share the same pathological procedure (from benign to atypical hyperplasia to malignancy) and carcinogenesis. Unopposed exposure to estrogen with progesterone resistance to atypical hyperplasia to malignancy) and carcinogenesis. Estrogen-dependent (type I) endometrial carcinoma is the new viewpoint in the pathogenesis of MTOE. (14) Both EAO (specific to endometrioid adenocarcinoma) and estrogen-dependent (type I) endometrial cancer share the same pathological procedure (from benign to atypical hyperplasia to malignancy) and carcinogenesis. Unopposed exposure to estrogen with progesterone resistance is the risk factor for both. Many of the same genes, such as p16 and PTEN, have been shown to be mutated in both diseases, suggesting a shared molecular pathogenesis. (13) In addition, an association between type II diabetes (the independent risk factor of endometrial cancer) and MTOE is biologically plausible through perturbations in insulin, insulin-like growth factors, gonadotropin, and steroid hormone metabolism, which could affect cell proliferation. (15) Hyperglycemia indirectly stimulates the expression of estrogen receptor so as to promote the stimulatory function of estrogens. (16) Also hyperglycemia causes the reduction of tumor suppressor genes like p16 and provides a nutrient-rich microenvironment for rapidly dividing cancer cells. (16, 17) Moreover, hyperinsulinemia induces proliferative tissue abnormalities because insulin and the cross-activation of the insulin-like growth factor-I receptor family can stimulate DNA synthesis and cell proliferation. (18)

A rat model of surgically induced EMs involves auto-transplantation of biopsies of uterus in the abdomen (19) which is widely used in the research of EMs. The EMs lesions of rats bear clear similarities to those found in humans: the progress of ectopic growth, the response to steroids, the abnormal levels of cytokines in the site of EMs lesion and peritoneal fluid, (20) and clinical presentations. (21) In addition, the combination of high carbohydrate-and-fat feed (HCF-feed) and low-dose streptozotocin (STZ)-treated rat serves as an alternative animal model for type II diabetes replicating the natural history and metabolic characteristics of human disease. (18) The mutagenic and cytotoxic effects of STZ are selective and confined to liver, kidney, and pancreas, and rare evidence could be found about its association with MTOE. (22)

Accordingly, we induced MTOE with hyperestrogenemia in a rat EMs and type II diabetes model and evaluated the similarity of this rat MTOE with human disease through detecting the histological appearance and biological behavior and the expressions of PTEN, phosphorylated (p-) AKT, and p-mTOR. This study might be a pioneer of establishing standardized animal models for this malignancy and offering new clues for research into the pathogenesis of MTOE.

Materials and Methods

Animals. Ninety adult female Sprague–Dawley rats (age, 8–12 weeks; weight, 250–300 g), were provided by the Experimental Animal Center of Shengjing Hospital of China Medical University (Shenyang, China). The animals were fed standard feed and housed in a controlled environment (22 ± 2°C) with 12:12 h light:dark cycles. This study was approved by the Ethics Committee of Medical Scientific Research and Technology, Shengjing Hospital of China Medical University (2013PS140K).

Surgical procedures. The rats were randomized into four groups: (i) the Es group (n = 55), autologous endometrium transplantation; (19) (ii) the control group (n = 25), autologous endometrium transplantation; (iii) the negative control group (n = 5), autologous epilpoon transplantation; and (iv) the blank control group (n = 5), “open–close” surgery. Details are given in Document S1.

Induction of MTOE. After surgery, the rats of the Es group were treated with 17β-estradiol (5 mg/kg, three times/week) i.p. and provided with HCF-feed (10% fat, 20% glucose, and 70% standard feed). One month after surgery, a single dose of STZ (25 mg/kg) was injected i.p. Three days later, fasting blood glucose measurement higher than 250 mg/dL was diagnosed as diabetes. The rats in the control, negative control, and blank control groups were treated with only vehicle saline and provided with standard feed. All the i.p. injections were carried out on the opposite side of the EMs foci to minimize direct simulation.

Sample collection. The rats were killed randomly by cervical dislocation 2 months (Es group, n = 5; control group, n = 6; negative control group, n = 5; blank control group, n = 5), 4 months (Es group, n = 20; control group, n = 7), and 8 months (Es group, n = 25; control group, n = 7) after the surgeries. Details are given in Document S1.

Histomorphological evaluation. For H&E staining, paraffin-embedded samples were sectioned at 5-μm thickness, stained with H&E, and examined under a light microscope. The EMs lesion was determined by the histological confirmation of glandular epithelial and stromal cells. For electron microscopic analysis, Epon 812 epoxy resin-embedded samples were cut into slices of 50-nm thickness, and then the histological ultrastructure of endometrial cells was observed with a transmission electron microscope. Details are given in Document S1.

Proliferation and apoptosis analysis. Cell proliferation was assessed by detection of proliferating cell nuclear antigen (PCNA; Keygen Biotech Company, Nanjing, China). Apoptosis was evaluated with a TUNEL detection kit (Keygen Biotech Company, Nanjing, China). Both detections were carried out following the manufacturer’s instructions. The level of apoptosis was established by counting the number of positively stained cells (brown in color).

Immunohistochemistry. For details of the procedure, see Document S1. The reagents and dilution of the primary antibody were as follows: PTEN (1:100, Cell Signaling Technology, Danvers, MA, USA), p-AKT (1:50; Santa Cruz Biotechnology, St Cruz, CA, USA), and p-mTOR (1:50; Cell Signaling Technology). The secondary antibody and 3,3'-diaminobenzidine-tetrachloride kit were purchased from Zhongshan Goldenbridge Biotechnology (Beijing, China).

Results assessment. The assessment criteria for results of the proliferation analysis, apoptosis analysis, and immunohistochemistry were as follows. Five view fields under a high power lens of the microscope (400×) were randomly chosen
to observe all the cells in the view fields. The H-score scoring method was used (details provided in Doc. S1)\(^{(23)}\).

**Statistical analysis.** SPSS version 16.0 software (Armonk, NY, USA) was used. The weight, volume, and H-score value were described by mean ± SEM. The t-test for two independent samples was used to calculate the intergroup difference. The χ²-test and Fisher’s exact probability test were adopted for comparative analysis of the count data. \(P < 0.05\) was considered significantly different.

**Results**

**Macroscopic analyses.** Of the 90 rats, 10 (five from the Es group and five from the control group) died and were excluded from the experiment. Of these 10 rats, it was difficult to identify the lesions and the causes of death in four due to severe pelvic adherence; three died of severe intestinal obstruction, and the remaining three died 3 days after the injection of STZ. The EMs lesions were survived in all cases of the Es group and the control group. In most cases, EMs of the Es group grew with time, whereas the ones in the control group showed worsening atrophy with time. The average weights of the EMs lesions in the Es group 2, 4, and 8 months after surgery (424.6 ± 1.9, 453.9 ± 21.6, 523.6 ± 23.2 mg, respectively) were significantly greater than those of the control group in the corresponding months (402.2 ± 2.3, 298.9 ± 39.7, 220.0 ± 27.5 mg, respectively, \(P < 0.05\); Table 1). Compared with the volumes of the EMs lesions in the control group 2, 4, and 8 months after surgery (71.5 ± 6.8, 36.2 ± 6.9, 24.4 ± 7.1 mm\(^3\), respectively), those in the Es group were significantly greater in the corresponding months (88.7 ± 16.9, 210.8 ± 21.8, 394.8 ± 131.9 mm\(^3\), respectively, \(P < 0.01\); Table 1).

Two cases of EMs in the Es group (8 months after surgery) were extraordinarily enlarged (Fig. 1a). Grossly, the two were cystic, consisted of thickened capsule wall with papillary hyperplasia and sanguinopurulent fluid (H&E stain revealed malignancy). Endometriosis of the control group (Fig. 1b) had the appearance of unilocular cystic structures filled with clear fluid. The uteri of the Es group showed severe edema, with no papillary hyperplasia, whereas uteri of the control group showed no obvious abnormality.

**Histologic analyses.** Histologically, the two distinctly enlarged EMs of the Es group (4.0%) were diagnosed as low-grade endometrioid adenocarcinoma, which showed a widespread cribriform arrangement with no invasion of the adjacent peritoneal muscular tissue. The corresponding eutopic endometria showed atypical hyperplasia. Three cases of EMs (6.0%) showed atypical hyperplasia with an increased number of endometrial glands, compactly arranged multiple layers, reduced cytoplasm, and enlarged and moderately irregular cell nuclei. The corresponding eutopic endometria showed simple hyperplasia. The malignant progression of the eutopic endometria was slower than that of the corresponding EMs, which was not synchronized. Endometriosis of the control group showed a similar appearance of the endometrium, consisting of cystically dilated glands with epithelium surrounded by stromal cells. Two cases of eutopic endometria showed diffuse squamous epithelium without columnar glandular epithelium (see Fig. S1). The results are shown in Table 2 and Figure 1c–g.

Transmission electron microscopy indicated that the malignant EMs showed remarkable abnormalities in cytomorphology and organelles including swollen mitochondria and vacuole changes (Fig. 1h). In addition, compared to the simple hyperplastic EMs of month 4, the ectopic endometrial cells of month 8 with the same pathological change showed more active nuclei, and obviously enlarged and well-defined nuclei with reduced cytoplasm (Fig. 1i). The ultrastructure of different pathological changes is shown in Figure S2.

**Analysis of proliferation and apoptosis.** The atrophy and simple hyperplastic EMs were excluded from the proliferation and apoptosis analysis. In the Es group, the immunostaining of PCNA protein increased (\(P < 0.01\)), and the level of TUNEL positivity decreased (\(P < 0.01\)) progressively in the order of EMs, atypical EMs, and malignant EMs. The immunostaining of PCNA in EMs of the Es group was stronger than that of the control group (\(P < 0.05\)), and the staining intensity of apoptosis in the Es group was lower than that of the control group (\(P < 0.05\) (Fig. 2).

**Immunohistochemical analysis.** The samples with simple hyperplasia and atrophy were excluded. The staining of PTEN was weakened, and stainings of p-AKT and p-mTOR were intensified progressively in the order of EMs, atypical EMs, and malignant EMs (\(P < 0.05\)). The samples with the same pathological changes showed no significant difference in the abovementioned stainings, regardless of the treatment group or whether the samples were eutopic or ectopic (\(P > 0.05\)). The differences in the H-score for PTEN, p-AKT, and p-mTOR among groups are shown in Figure 3 and Table 3.

**Discussion**

The present study explored a feasible way of inducing malignant transformation of rat EMs in combination with

| Table 1. Mean weight, mean volume, and macroscopic features of lesions in two groups of rats with surgically induced endometriosis (EMs) treated with estradiol and streptozotocin and fed a high carbohydrate-and-fat diet (Es group) or treated with placebo saline and fed a standard diet (control group) |
|---|
| **n** | **Lesion weight, mg, mean ± SEM** | **Lesion volume, mm³, mean ± SEM** | **Lesion atrophy, n (%)** | **Visible angiogenesis, n (%)** | **Papilliform hyperplasia of cyst wall, n (%)** |
| Es group | 50 | 485.8 ± 15.3\(^{†}\) | 290.6 ± 67.7\(^{§}\) | 3 (6.0) | 28 (56.0) | 2 (4.0) |
| 2 months | 5 | 424.6 ± 1.9\(^{†}\) | 88.7 ± 16.9\(^{§}\) | 0 (0.0) | 3 (60.0) | 0 (0.0) |
| 4 months | 20 | 453.9 ± 21.6\(^{†}\) | 210.8 ± 21.8\(^{§}\) | 2 (10.0) | 11 (55.0) | 0 (0.0) |
| 8 months | 25 | 523.6 ± 23.2\(^{†}\) | 394.8 ± 131.9\(^{§}\) | 1 (4.0) | 14 (56.0) | 2 (8.0) |
| Control group | 20 | 326.7 ± 21.0 | 42.7 ± 5.9 | 10 (50.0)\(^{†}\) | 5 (25.0) | 0 (0.0) |
| 2 months | 6 | 402.2 ± 2.3 | 71.5 ± 6.8 | 0 (0.0) | 2 (33.3) | 0 (0.0) |
| 4 months | 7 | 298.9 ± 39.7 | 36.2 ± 6.9 | 4 (57.1)\(^{†}\) | 2 (28.6) | 0 (0.0) |
| 8 months | 7 | 220.0 ± 27.5 | 24.4 ± 7.1 | 6 (85.7)\(^{†}\) | 1 (14.3) | 0 (0.0) |

\(^{†}\)Compared with the net weight of the EMs lesions in the control group, \(P < 0.05\). \(^{§}\)Compared with the volume of the EMs lesions in the control group, \(P < 0.01\). \(^{‡}\)Compared with the atrophy rate of the EMs lesions in the Es group, \(P < 0.05\).
hyperestrogenemia and type II diabetes. The results indicated that three cases of ectopic endometria had atypical hyperplasia (two cases in 4 months and one case in 8 months), and two cases had endometrioid adenocarcinoma (both in 8 months), which were similar to the pathological appearances of human disease. The process of pathological changes, from benign to atypical and then to malignant change, is consistent with that of the human disease, especially the occurrence of atypical EMs (3/50). Ogawa et al. (24) found atypical EMs in 78% EAOC, and it can act as a premalignant lesion. Atypical EMs was also reported to have spatial and chronological association with MTOE. (25,26) It may represent the transition from benign EMs to malignant carcinoma. In the Es group, most EMs lesions grew along with time, the ectopic endometrial cells had rigorous hyperplasia, and the organelles were active. In the control group without intervention, atrophy worsened along with time, indicating estrogen dependency and the significant role of hyperestrogenism in this study. In our previous study, we injected once only with estradiol, without STZ and HCF-feed. After 8 months, only 9 of 14 cases of EMs presented with simple hyperplasia, with no malignant or atypical cases (unpublished results, 2014). This illustrated the indispensable

Table 2. Histological features of lesions and eutopic endometria in rats with surgically induced endometriosis (EMs) treated with estradiol and streptozotocin and fed a high carbohydrate-and-fat diet (Es group) or treated with placebo saline and fed a standard diet (control group), n (%)

|        | n | EMs lesions | Eutopic endometria† |
|--------|---|-------------|-------------------|
|        |   | EMs | Atrophy | Simple hyperplasia | Atypical hyperplasia | Malignant transformation | Normal | Atrophy | Simple hyperplasia | Atypical hyperplasia | Malignant transformation |
| Es group | 50 | 13 (26.0) | 3 (6.0) | 29 (58.0) | 3 (6.0) | 2 (4.0) | 25 (50.0) | 0 (0.0) | 21 (42.0) | 2 (4.0) | 0 (0.0) |
| 2 months | 5 | 3 (60.0) | 0 (0.0) | 2 (40.0) | 0 (0.0) | 0 (0.0) | 5 (100.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| 4 months | 20 | 5 (25.0) | 2 (10.0) | 11 (55.0) | 2 (10.0) | 0 (0.0) | 11 (55.0) | 0 (0.0) | 9 (45.0) | 0 (0.0) | 0 (0.0) |
| 8 months | 25 | 5 (20.0) | 1 (4.0) | 16 (64.0) | 1 (4.0) | 2 (8.0) | 9 (36.0) | 0 (0.0) | 12 (48.0) | 2 (8.0) | 0 (0.0) |
| Control | 20 | 10 (50.0) | 10 (50.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 20 (100.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| 2 months | 6 | 6 (100.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 6 (100.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| 4 months | 7 | 3 (42.9) | 4 (57.1) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 7 (100.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| 8 months | 7 | 1 (14.3) | 6 (85.7) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 7 (100.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |

†In the Es group, another two cases of eutopic endometria (8%) showed squamous epithelium at 8 months. ‡Compared with the atrophy rate of EMs of the control group, P < 0.05.
The role of type II diabetes in this study, and the combination of both treatments was more effective and feasible. Although STZ and estradiol were both given i.p., special attention was paid during the surgery and post-surgery treatment to assure that both the eutopic and ectopic endometrial sides were not directly exposed to the peritoneal cavity and carcinogenetic stimulations. So the route of administration was not supposed to take an influential part in the hyperplastic progression of disease. Rare reports have been published regarding the correlation between STZ and MTOE, but indeed the possibility cannot be ruled out that STZ might be an unknown interference, which may be worthy of further investigation.

The inactivation of the \( \text{PTEN} \) gene and the following activation of the PI3K–AKT–mTOR pathway were reported to be associated with MTOE. \(^{(27)}\) Silencing \( \text{PTEN} \) could lead to the abnormal activation of PI3K and the phosphorylation of AKT, a serine/threonine kinase involved in cell growth and survival. Activated AKT (or p-AKT) could further activate mTOR by phosphorylation, which could regulate cell stress response and cell cycle. \(^{(12)}\) As \( \text{PTEN} \) mutations are seen early in endometrioid ovarian carcinoma and endometrioid ovarian cancer is thought to arise from EMs, it has been proposed that somatic genetic alterations in the \( \text{PTEN} \) gene may be an early event in MTOE. \(^{(6)}\) The ovarian endometrioid adenocarcinoma with an activated PI3K–AKT–mTOR signaling pathway is more likely to be of a low grade instead of a high grade. \(^{(28)}\) In our present research, \( \text{PTEN} \) expression was progressively reduced in the order of EMs, atypical EMs, and malignant EMs. The most remarkable abnormality was seen in malignant cases, with conspicuously decreased \( \text{PTEN} \) and increased p-AKT and p-mTOR. These results implied a possible role of the loss of \( \text{PTEN} \) and the following activation of the PI3K–AKT–mTOR pathway in the development of the present rat model of MTOE and a shared molecular alternation of the present rat model of MTOE with human low-grade endometrioid adenocarcinoma.

Recently, there is growing evidence pointing to hereditary predisposition and biological characteristics of the eutopic endometrium as the determinative factors in the pathogenesis of MTOE. \(^{(29)}\) Vigano et al. \(^{(30)}\) proposed that the malignant transformation of eutopic and ectopic endometrias shared similar incidence and pathological type, and that abnormal gene expressions in the eutopic endometrium could be related to MTOE. Clinically, it was reported that patients with ovarian tumor arising from EMs suffered higher risks of endometrial pathology (endometrial adenocarcinoma, hyperplasia, and polyps). \(^{(14)}\) Studies have also shown that women with EAOC will also have a simultaneous endometrial adenocarcinoma, and almost 90% of synchronous tumors identified in the ovary and in the endometrium were of the endometrioid cell type. \(^{(31,32)}\) In the present study, we found that the pathological grade of the eutopic endometria corresponding with malignant EMs was higher than that with atypical EMs. The former was atypical hyperplasia, whereas the latter was simple hyperplasia. It is assumed that rats with malignant EMs might have abnormal gene expressions in their eutopic endometria. Such endometrium and its EMs cysts may be more sensitive to carcinogenic stimulations and thus more susceptible to malignant transformation. Moreover, the EMs lesions and the corresponding eutopic endometria in the Es group showed the same progressively reduced tendency with regard to \( \text{PTEN} \), p-AKT, and p-mTOR expressions (no malignant case in the eutopic ones). But in a certain pathological grade of either eutopic or ectopic endometria, the expressions of the above proteins showed no statistical
differences. These results contributed to imply the similarity and correlation between malignant transformation of EMs and endometrium.

However, as two independent disorders, MTOE and endometrial carcinoma must have many distinctions. Malignant transformation of EMs commonly occurs at reproductive age and seems to be associated with oxidative stress induced by free iron and heme accumulated during periodic and repeated bleeding, and inflammation. (6) In contrast, type I endometrial carcinoma often occurs in premenopausal and younger

Table 3. Statistical comparison of the expressions of phosphatase and tensin homolog (PTEN), phosphorylated protein kinase B (p-AKT), and phosphorylated mammalian target of rapamycin (p-mTOR) protein by an immunohistochemical method, P-values

| Comparison                        | PTEN  | p-AKT  | p-mTOR |
|-----------------------------------|-------|--------|--------|
| Es group versus control group     |       |        |        |
| EMs                               | 0.1030| 0.7900 | 0.8730 |
| Normal eutopic endometria         | 0.4800| 0.7600 | 0.8320 |
| Comparison within the Es group    |       |        |        |
| EMs versus eutopic endometria     | 0.7540| 0.7730 | 0.5920 |
| Atypical EMs versus atypical endometria | 0.9840| 0.8970 | 0.7910 |
| Ectopic endometria                |       |        |        |
| EMs versus atypical EMs           | 0.0210*| 0.0260*| 0.0001**|
| Atypical EMs versus malignant EMs  | 0.0230*| 0.0280*| 0.0320*|
| EMs versus malignant EMs          | 0.0001**| 0.0001**| 0.0001**|
| Eutopic endometria                |       |        |        |
| Normal endometria versus atypical hyperplasia | 0.0050**| 0.0310*| 0.0220*|
| Comparison within the control group |       |        |        |
| EMs versus eutopic endometria     | 0.6410| 0.8630 | 0.8960 |

*P < 0.05, statistical difference; **P < 0.01, significant statistical difference. Control group, rats with surgically induced endometriosis (EMs) treated with placebo saline and fed a standard diet; Es group, rats with surgically induced EMs treated with estradiol and streptozotocin and fed a high carbohydrate-and-fat diet.
postmenopausal women, is strongly associated with the estrogen-related pathway, and arises in association with unopposed estrogen stimulation. In the present study, we found that the progression of hyperplasia of EMs was more advanced than the corresponding eutopic endometria, indicating a higher risk of malignization of EMs. This phenomenon may be attributed to the specific local microenvironment and enhanced sensitivity to carcinogenic risk factors of EMs. Studies on humans and rats both propose that more activated inflammatory cells and cytokines in the site of EMs than the corresponding eutopic endometria, indicating an inflammatory microenvironment of EMs. Inflammatory cells and their secreted cytokines can stimulate angiogenesis and cell proliferation, inhibit cell apoptosis, facilitate invasion and metastasis, and produce active oxygen capable of inducing DNA damage and mutation. So inflammation can prompt growth and invasion of EMs, which constitutes a link between EMs and ovarian cancer. In addition, the electron microscopic appearance of the malignant EMs lesions might support that the EMs lesions might grow in a hypoxic microenvironment and implied a possible role of the hypoxic damage to mitochondria in the relatively higher hypoxic microenvironment and implied a possible role of the hypoxic damage to mitochondria in the relatively higher malignant transformation risk of the endometriotic lesions. Apart from these, eutopic survival and growth might induce a higher sensitivity to various stimulations. Wu et al. found that ectopic endometrium was at least 100 times more sensitive to interleukin-1β, compared with eutopic endometrium. Therefore, the hypersensitive EMs lesions might exaggerate the carcinogenic simulations and trigger the cascade chain reaction of oncogenesis.

In summary, the present study found that the combination of hyperestrogenemia with type II diabetes in a rat EMs model can induce MTOE, which showed similar morphological changes and molecular abnormalities to human disease. The method of induction is easy and feasible, and provides a good foundation for establishing a animal model of MTOE. Although eutopic endometrium and EMs showed similar responses and pathological changes to the carcinogenic simulations, EMs had higher susceptibility and risk for oncogenesis. Therefore, malignant transformation of EMs and eutopic endometrium may share hereditary correlations, which are worthy of further investigation. Future studies might also focus on raising the malignant transformation rate by improving the induction method and exploring the genetic molecular mechanism of MTOE in more depth.

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Disclosure Statement
The authors have no conflict of interest.

References
1. Giudice LC, Kao LC. Endometriosis. Lancet 2004; 364: 1789–99.
2. Ness RB. Endometriosis and ovarian cancer: thoughts on shared pathophysiology. Am J Obstet Gynecol 2003; 189: 280–94.
3. Erzen M, Rakar S, Klanicnik B, Syrjanen K. Endometriosis-associated ovarian carcinoma (EAOc): an entity distinct from other ovarian carcinomas as suggested by a nested case–control study. Gynecol Oncol 2001; 83: 100–8.
4. Modesit SC, Tortolero-Luna G, Robinson JB, Gershenson DM, Wolf JK. Ovarian and extrangiotic endometriosis-associated cancer. Obstet Gynecol 2002; 100: 788–95.
5. Benoit L, Arnauld L, Cheynel N et al. Malignant extrangiotic endometriosis: a review. Eur J Surg Oncol 2006; 32: 6–11.
6. Munksgaard PS, Blaakaer J. The association between endometriosis and ovarian cancer: a review of histological, genetic and molecular alterations. Gynecol Oncol 2002; 2012: 124: 164–9.
7. Oxlholm D, Knudsen UB, Kryger-Baggesen N, Ravn P. Postmenopausal endometriosis. Acta Obstet Gynecol Scand 2004; 1: 4–7.
8. Zeiton K, Takayama K, Sasano H et al. Deficient 17β-hydroxysteroid dehydrogenase type 2 expression in endometriosis: failure to metabolize 17β-estradiol. J Clin Endocrinol Metab 1998; 83: 4474–80.
9. Turbiner J, Moreno-Bueno G, Daihya S et al. Clinicopathological and molecular analysis of endometrial carcinoma associated with tamoxifen. Mod Pathol 2008; 21: 925–36.
10. Heaps IM, Nieberg RK, Berek JS. Malignant neoplasms arising in endometriosis. Obstet Gynecol 1990; 75: 1023–8.
11. Zanetta GM, Webb MJ, Li H, Keeny GL. Hyperestrogenism: a relevant risk factor for the development of cancer from endometriosis. Gynecol Oncol 2000; 79: 18–22.
12. Masaki M, Yamaguchi K, Matsumura N, Tsukasa B, Ikao K. Ovarian cancer in endometriosis: molecular biology, pathology, and clinical management. Int J Clin Oncol 2009; 14: 383–91.
13. Kurman RJ, Shih I-M. Molecular pathogenesis and extrangiotic origin of epithelial ovarian cancer. Shifting the Paradigm. Hum Pathol 2011; 42: 418–31.
14. Van Gorp GT, Aman F, Neven P, Vergote I, Moerman P. Endometriosis and the development of malignant tumours of the pelvis. A review of literature. Best Pract Res Clin Obstet Gynaecol 2004; 18: 349–71.
15. Soliman PT et al. Association between adiponectin, insulin resistance, and endometrial cancer. Cancer 2006; 106: 2376–81.
16. Al-Jarrah M, Matalka I, Aseri HA et al. Exercise training prevents endometrial hyperplasia and biomarkers for endometrial cancer in rat model of type I diabetes. J Clin Med Res 2010; 2: 207–14.
17. Warburg O, Wind F, Negelein E. The metabolism of tumors in the body. J Gen Physiol 1927; 8: 519–30.
18. Zaafar DK, Zaitone SA, Moustafa YM. Role of mitomycin in suppressing 1,2-dimethylhydrazine-induced colon cancer in diabetic and non-diabetic mice: effect on tumor angiogenesis and cell proliferation. Plzis ONE 2014; 9: e00562.
19. Vernon MW, Wilson EA. Studies on the surgical induction of endometriosis in the rat. Fertil Steril 1985; 44: 684–94.
20. Sharpe-Timms KL. Using rats as a research model for the study of endometriosis. Ann N Y Acad Sci 2002; 985: 318–27.
21. Cason A, Samuelsen C, Berkley K. Estrous changes in vaginal nociception in a rat model of endometriosis. Horm Behav 2003; 44: 123–31.
22. Vinerean HV, Gazda LS, Hall RD, Smith BH. Streptozotocin is responsible for the induction and progression of renal tumorigenesis in diabetic wistar-furth rats treated with insulin or transplanted with agarose encapsulated porcine islets. Islets 2011; 3: 196–203.
23. Peng Y, Biliang C, Yanhong H, Xiaoyan X. Long-term regression of experimental endometriosis in a rat model treated with local application of levonorgestrel-loaded biodegradable microspheres. Hum Reprod 2012; 27: 2089–95.
24. Ogawa S, Kaku T, Amada S et al. Ovarian endometriosis associated with ovarian carcinoma: a clinicopathological and immunohistochemical study. Gynecol Oncol 2000; 77: 298–304.
25. LaGrenade A, Silverberg SG. Ovarian tumors associated with atypical endometriosis. Hum Pathol 1988; 19: 1080–4.
26. Moll UM, Chumas JC, Chalas E, Mann WJ. Ovarian carcinoma arising in endometrioid adenocarcinoma based on somatic defects in the Wnt/β-catenin and PTEN signaling pathways. J Pathol 2014; 13: 337–45.
27. Kurman RJ, Shih I-M. Molecular pathogenesis and extrangiotic origin of epithelial ovarian cancer. Shifting the Paradigm. Hum Pathol 2011; 42: 418–31.
28. van Gorp GT, Aman F, Neven P, Vergote I, Moerman P. Endometriosis and the development of malignant tumours of the pelvis. A review of literature. Best Pract Res Clin Obstet Gynaecol 2004; 18: 349–71.
29. Soliman PT et al. Association between adiponectin, insulin resistance, and endometrial cancer. Cancer 2006; 106: 2376–81.
Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. Hematoxylin–eosin staining of eutopic endometria with squamous epithelium in a rat model of surgically induced endometriosis.

Fig. S2. Ultrastructure of different pathological changes in a rat model of surgically induced endometriosis.

Doc S1. Methods of surgery, electron microscopic analysis, immunohistochemistry, and the H-score.