

Characterization of Cell Fusion in an Experimental Mouse Model of Endometriosis

Running title: Cell Fusion in Experimental Endometriosis

Summary sentence: Cells of the endometriotic lesion fuse with cells of the host animal; bone marrow-derived cells recruited to the endometriosis lesion contribute to this process.

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Conflict of interests: The authors have no conflict of interests to declare.

Grant support: Institutional support

Abstract

Cell fusion is involved in the development of some adult organs, is implicated in the pathogenesis of specific types of cancer, and is known to participate in repair/regeneration processes mediated by bone-marrow derived cells (BMDCs). Endometriosis is a disease characterized by growth of functional endometrial tissue outside of the uterine cavity.

Endometriosis shares some molecular properties with cancer and BMDCs home to endometriosis lesions in a mouse model. Our objective was to determine if cell fusion can occur in endometriosis and establish whether bone marrow derived cells participate in cell fusion events in lesions. We employed a Cre-Lox system to identify cell fusion events in a mouse model of endometriosis. Fused cells were detected in endometriotic lesions, albeit at a low frequency (~1 in 400 cells), localized to the stromal compartment, and displayed restricted proliferation. Using 5-fluorouracil-based non-gonadotoxic bone marrow transplantation model, we demonstrate that bone marrow cells represent a principal cell source for fusion events in lesions. Cell fusion progeny uniformly lacked expression of selected markers of hematopoietic, endothelial, and epithelial markers, though they expressed the mesenchymal/stromal markers Sca-1 and CD29. This study is first to describe the phenomenon of cell fusion in endometriosis and points to a mesenchymal population derived from cell fusion events with limited proliferative activity, properties previously attributed to endometrial stem cells. Their putative role in the pathogenesis of the disease remains to be elucidated.

Keywords

Endometriosis; cell fusion; bone marrow-derived cells.

Introduction

Cell fusion is a highly controlled process critical for mammalian development. It occurs as early as the merging of gametes during sexual reproduction and is implicated in the formation of diverse tissues and organs, including muscle fibers (myoblast fusion), bone (macrophage fusion), eye lens (fiber cell fusion), liver (hepatocyte fusion) and placenta [1, 2]. The latter process involves formation of a multinucleated syncytiotrophoblast layer through which most of the materno-fetal exchanges take place [3], and is catalyzed by syncytin - a proviral protein encoded

by the human endogenous retrovirus envelope gene (HERV-W, rev. [4]). Cell fusion also occurs in post-developmental homeostatic processes of wound repair, tissue regeneration and the inflammatory response. Whereas some of these processes require phagocyte fusion to form osteoclasts or giant cells, others involve fusion of stem cells with cells in the damaged tissue [5]. Cell fusion was also suggested to contribute to the pathogenesis of some cancers [6, 7], and the phenomenon has been shown to occur in bone-marrow transplant (BMT) recipients [8, 9].

Endometriosis is a chronic, recurrent and progressive disease characterized by the presence and growth of functional endometrial tissue, glands, and stroma outside of the uterine cavity. Its manifestations, including acute or chronic pelvic pain and infertility, occur in approximately 10% of reproductive age women (rev.[10]). Although it is a benign condition, it has molecular and cellular features in common with malignancy, including increased cell proliferation, re-expression of the pluripotent transcription factor OCT4 [11], epithelial-to-mesenchymal transition [12], acquisition of migratory phenotype [11, 13], development of distant foci, cell adhesion, invasion (rev. [14]), angiogenesis, and at times, treatment resistance [15]. These features raise the possibility that cell fusion may also be a shared phenomenon.

In support of this, the fusagen syncytin, which is highly expressed only in placental trophoblasts and in some cancers (including endometrial carcinoma [16, 17]), is induced in endometriosis lesions. Specifically, syncytin-1 expression is absent in the eutopic endometrium of endometriosis patients (concomitant with hypermethylation of the LTR promoter region of the gene), whereas in ectopic endometriotic lesions, syncytin-1 mRNA and protein are detected, concurrent with hypomethylation of the LTR region [18]. Furthermore, our group demonstrated homing of bone-marrow derived cells (BMDCs) to the endometrium and endometriosis lesions [19-22]. Bone-marrow derived cells were shown to fuse with cells in various organs they are

recruited to, including cardiomyocytes, hepatocytes, intestinal cells, and Purkinje neurons. [8, 9, 23-32]. However, it is unknown whether fusion occurs in endometriosis and whether BMDCs may contribute to this process.

To investigate whether cell fusion can occur in endometriosis, we used an established mouse model of the disease together with a Cre-Lox system that enables detection of fusion events upon access of Cre recombinase to a floxed STOP cassette upstream of an enhanced green fluorescent protein (EGFP) reporter of the fusion partner. We utilized two experimental models; the first involved suturing of uterine explants from loxP-STOP-loxP-EGFP donors into the peritoneum of Cre recipients. The second employed non-gonadotoxic bone marrow transplantation from loxP-STOP-loxP-EGFP donors into WT recipients, followed by endometriosis induction (EI) using Cre donor uterine explants, to explore whether cell fusion in the endometriosis lesion can originate from BM cells.

Methods

Animals

Mature (7-8-weeks old) C57BL/6J (wild type) and homozygous Ai6(RCL-ZsGreen) were purchased from Jackson Laboratories. Ai6 contains a targeted mutation of the *Gt(ROSA)26Sor* locus with a loxP-STOP-loxP-ZsGreen1 cassette, preventing transcription of EGFP. ZsGreen1 is expressed following Cre-mediated recombination. Mature β -actin-cre mice (expressing Cre recombinase directed by the human beta actin gene promoter) were obtained from the Yale Genome Editing Center. The following primers were used to screen for mice carrying the Cre transgene: 5'-GCG GTC TGG CAG TAA AAA CTA TC-3', 5'-GTG AAA CAG CAT TGC TGT CAC TT-3' (transgene forward and reverse, respectively), 5'-

CTA GGC CAC AGA ATT GAA AGA TCT-3', 5'-GTA GGT GGA AAT TCT AGC ATC ATC C-3' (internal control forward and reverse, respectively). Both Ai6(RCL-ZsGreen) and β -actin-cre are isogenic with C57BL/6J.

Mice were maintained in environmentally controlled facilities in the Animal Facility at Yale School of Medicine in a room with a 12-hour light, 12-hour dark cycle (7 am to 7 pm) with ad libitum access to food and water. All animal procedures were performed according to an approved Yale University Institutional Animal Care and Use Committee protocol.

Endometriosis induction

Endometriosis was induced as previously described [21] with modifications. Diestrous uteri from 7-8 weeks old cycling females were isolated, horns longitudinally dissected and the lumen exposed. Four 3-mm² pieces were sutured onto the peritoneum (two on each side) of isogenic females, with the luminal side facing the peritoneum. 1 mg/kg meloxicam (Boehringer Ingelheim) was administered subcutaneously immediately following surgery. The diestrous stage was chosen for tissue harvesting as it is comparable to the human secretory phase preceding menstruation, and may therefore more closely resemble the endometrial cell population that is shed and deposited in the peritoneal cavity during retrograde menstruation. Females were kept cycling for 4 weeks by changing the cages' bedding 3 times per week with fresh male bedding, and estrous cyclicity was verified by vaginal cytology. At 4 weeks post-surgery, endometriosis-like mouse lesions demonstrating increase in size and cyst formation (Fig. 1, bottom panel) were isolated and analyzed by flow cytometry, immunohistochemistry, and immunofluorescence.

Bone marrow transplant

Non-gonadotoxic BMT was performed as described [33]. In brief, 125 mg/kg 5-fluorouracil (5-FU) was filtered and injected intraperitoneally on days -6 and -1 prior to BMT, and 75 µg/kg stem cell factor (SCF) was injected intraperitoneally at 21 and 9 hours before the second 5-FU dose. Donor whole BM was obtained by flushing the femur and tibia of 8-10 weeks-old ZsGreen1 male mice with cold sterile Dulbecco's modified eagle medium: nutrient mixture F-12 (DMEM-F12, Gibco, Thermo Fisher Scientific). The cell suspension was filtered through a 70-µm sterile nylon mesh cell strainer (BD Biosciences) and 3×10^7 unfractionated BMCs in 100 µL PBS were injected retro-orbitally into 8-9-weeks old female C57BL/6J recipients.

Mouse models of cell fusion in endometriosis

To study cell fusion in endometriosis, we employed two models. The first examines cell fusion between uterine cells of the endometriosis lesion and cells of the host. For this whole body fusion model, uteri from ZsGreen1 homozygous females were sutured in the peritoneal cavity of transgenic Cre females (Fig. 1, scheme A). In this model, only fused cells should express GFP. Control animals had a similar configuration, with the exception that the host for the ZsGreen1-uterine implants was a wild-type C57BL/6J female mouse. This controlled for potential leaky EGFP expression in the mouse lesion from the ZsGreen1 locus.

The second model tested specifically for cell fusion occurring between cells of the endometriosis lesion and circulating BMDCs. In this BMDC fusion model, whole BM was isolated from ZsGreen1 homozygous males and transplanted into age-matched cycling C57BL/6J female recipients that underwent non-gonadotoxic sub-myeloablation. This protocol was chosen over whole body irradiation as it was shown to preserve ovarian function, estrus cyclicity and fertility

[33]. Since development of endometriosis-like lesions is estrogen-dependent, non-gonadotoxic sub-myeloablation allowed for more physiological modeling of the contribution of BM by avoiding the requirement of exogenous estrogen to support lesion growth. Five weeks post-BMT (allowing adequate time for recovery), EI was performed as before, with the donor uterus derived from cycling age-matched Cre females (Fig. 1, scheme B). Control animals were wild-type, received BMT from ZsGreen1 males and were subsequently the recipients of uterine implants derived from a wild type mouse. This design controlled for potential leaky EGFP expression originating in the BM.

Flow cytometry

Flow cytometry of mouse endometriosis lesions was performed as previously described [33]. In brief, mouse endometriosis lesions were finely minced and digested in a solution of Hanks' balanced salt solution (HBSS) containing 25 mM HEPES (Life Technologies), 1 mg/mL collagenase B (Roche Diagnostics) and 0.1 mg/mL deoxyribonuclease I (Sigma-Aldrich) for 45 minutes at 37°C with periodic pipetting. Samples were filtered using 70- μ m mesh, centrifuged at 2000 rpm at 4°C for 8 minutes and resuspended in PBS. After a washing step, flow cytometry was performed on FACS MoFlo (Beckman Coulter). Gates were applied to forward-scatter/side-scatter dot plots to exclude nonviable cells and cell debris. Data were analyzed using FlowJo V10.

Immunostaining

Mouse endometriosis lesions were fixed in 4% paraformaldehyde, paraffin-embedded and cut into 5- μ m thin sections. Antigen retrieval was accomplished by boiling in sodium citrate (pH 6) for 10 minutes. For immunohistochemistry (IHC), blocking was performed by incubating

sections in PBS containing 0.3% Triton X-100 (Sigma) and 5% normal rabbit serum at room temperature for 30 min. Sections were then incubated with goat anti-GFP antibody (1 µg/mL; Abcam (ab6673) overnight at 4 °C. Secondary antibody and detection reagents were supplied by the Vectastain Elite ABC HRP kit (Peroxidase, goat IgG) and ImmPACT DAB (Vector Laboratories), and carried out according to manufacturer's instructions. Tissue sections were counterstained with hematoxylin (Sigma-Aldrich). Images of stained sections were captured using Nikon Eclipse 80i microscope (Nikon).

For immunofluorescence (IF), blocking was performed with 10% donkey serum (Vector Laboratories) for 1-hour. Sections were then incubated with the following primary antibodies (Abcam) at 4 °C overnight: goat anti-GFP antibody (2.5 µg/mL; ab6673), rat anti-CD45 (2.5 µg/mL; ab25386), rabbit anti-CD31 (4.5 µg/mL; ab28634), rabbit anti-vimentin (0.6 µg/mL; ab92547), rabbit anti-pan cytokeratin (2.5 µg/mL; ab9377), rabbit anti-PCNA (2.5 µg/mL; ab18197), rabbit anti-Sca-1 (0.5 µg/mL; ab109211), rabbit anti-CD29 (0.6 µg/mL; ab179471). The secondary antibodies (ThermoFisher Scientific): Alexa Fluor 568-conjugated donkey anti-goat (A-11057), Alexa Fluor 488-conjugated donkey anti-rabbit (A-21206), or Alexa Fluor 488-conjugated donkey anti-rat (A-21208), were all used at a concentration of 10 µg/mL. Sections were DAPI stained and cover-slipped using Vectashield fluorescent mounting media with DAPI (Vector Laboratories). Sections were imaged using laser scanning confocal microscope (LSM 710; Zeiss) and captured using ZEN software (Carl Zeiss).

Quantification of GFP+ cells in lesions and statistical analysis

The frequency of fusion-derived (GFP+) cells in immunohistochemical sections was assessed by counting cells in three high power (20X) fields imaged using NIS-Elements D 3.1 software. At least 3000 cells were counted in each uterine section, and three sections were counted per mouse

uterus. Uteri from a total of 10 mice were analyzed by cell counting. Data were analyzed using GraphPad Prism 6 software (GraphPad Software). An unpaired t-test was used to compare cell fusion frequency in the whole- and BMT-models.

Results

Cell fusion between cells of the endometriosis lesion and host cells is a rare event restricted to the stromal compartment of the lesion

To quantitate cell fusion between endometrial cells of the endometriosis-like lesion and host cells from the whole body model, mouse endometriosis lesions were subjected to flow-cytometry. The incidence of GFP⁺ events was approximately 2.5 in 1000, with a mean of $0.26\% \pm 0.07\%$ ($n=10$ lesions) (Figure 2A-2C). These GFP⁺ events represent cells arising from fusion between cells of the ZsGreen1-derived uterine implant and cells in the β -actin-cre host. Immunohistochemistry using GFP antibody demonstrated GFP-positive fused cells in the β -actin-cre host but not in wild-type control host mice (Figure 2D and 2E). Immunohistochemistry further revealed that GFP⁺ cells localized specifically to the stromal compartment of the mouse lesion and appeared invariably mononucleated (Fig. 2E). Quantification of the GFP⁺ cells in the endometriosis tissue by cell counting showed that the frequency of GFP⁺ cells was $0.29\% \pm 0.09\%$ within the lesion ($n=6$) (Fig. 2F, left column), consistent with our flow cytometry data.

BM-derived cells participate in fusion events within endometriosis lesions

Our published observations of BMDCs homing to endometriosis lesions following BMT [19-21] led us to investigate whether BM cells recruited to the lesion can contribute to the observed cell fusion events. BMT was performed as described above. In this model, GFP⁺ mononucleated

fusion-derived cells were again detected in the stromal compartment of the endometriosis-like lesion four weeks following EI (Fig. 3B), albeit at about one-third the frequency observed in the whole body model (mean $0.11\% \pm 0.02\%$, $n=12$, $p=0.02$) (Fig. 2F, right column). Fusion-derived cells in this model were also clustered more closely together, at times appearing binucleated by immunohistochemistry (Fig. 3B, right image). No GFP⁺ cells were detected in control mice (Fig. 3A).

Phenotype of fusion-derived cells in the endometriosis-like lesion

We next sought to characterize fused cells using selected markers commonly found in endometriosis-like lesions. In both whole animal and BMT-fusion models, CD45 (pan-hematopoietic), CD31 (endothelial), cytokeratin (epithelial) and vimentin (stromal) were uniformly absent in fusion-derived cells in all sections/lesions examined. GFP⁺ cells were consistently positive for both mesenchymal/stromal markers Sca-1 and CD29. Fig. 4 shows representative images from the whole-animal fusion model.

To study the proliferative nature of the fused cells, we used PCNA as a marker of proliferation. In the whole-animal fusion model, none of the fusion-derived cells expressed the proliferation marker PCNA (Fig. 5), though in the BMT model these cells were PCNA positive when they were in very close proximity (Fig. 5, bottom panel), potentially representing earlier stages of the physical separation of the original fused cell.

Discussion

We describe herein a novel phenomenon in a mouse model of endometriosis, namely, cell fusion. The relatively rare frequency of the fusion events we observed is consistent with that reported for fusion between BMDCs and diseased pneumocytes [32] and for fusion between BMDCs and

non-injured hepatocytes [34] in BM-transplanted mice. We show that fusion occurs between endometriosis lesion cells and host cells, some of which are of BM origin.

In this study, we used two models to investigate the occurrence of cell fusion in endometriosis: a whole body model and a BMT model. In both models, we employed a genetic Cre-Lox approach in which Cre-mediated recombination only occurs in hybrid cells, thus minimizing the risk of false-positives. Still, some limitations of the system may mask expression of the gene encoded by ZsGreen1, including inadequate Cre expression, inaccessibility of the transgene to Cre recombinase, and loss of genomic DNA containing the transgene in the process of fusion [34]. These inherent limitations may underestimate fusion rates. Importantly, both models used in this study had intact ovarian function. For the BMT model, we used our previously described 5-fluorouracil-based non-gonadotoxic submyeloablation [33] in order to preserve ovarian function and estrus cyclicity, as estrogen is well-known to be a driving force of endometriosis.

The models we employed to study cell fusion between cells originating in the BM and transplanted tissue demonstrate that BMDCs recruited to the endometriosis lesion can fuse with uterine cells as they do in processes of tissue regeneration and cancer [8, 9, 24, 26, 28, 29]. As 50% BM chimerism is typical in the sub-myeloablation protocol we employed [33], the adjusted number of fused cells detected in the BM transplant model is similar to the number of cells obtained in the whole animal Cre host model. This suggests that the BMDCs are likely the principal contributors to fusion events in this endometriosis model, identifying a novel mechanism by which BMDCs potentially contribute to this disease.

Multinucleated hybrids are often chromosomally unstable and eliminate supernumerary nuclei by extrusion (i.e., nuclear shedding) or reductive-division [23, 35]. This may explain the

difficulty to detect numerous multinucleated GFP⁺ cells. Furthermore, the limited proliferation evident in fusion-derived cells point to a quiescent state. Preferential proliferation of the surrounding stroma may effectively disperse these cells in lesion space. Limited proliferation was previously attributed to endometrial stem cells [36, 37]. In ovarian carcinoma, fusion-derived quiescent cells were suggested to play a role in maintaining stem-cell like phenotypes [13]. We were limited in our capacity to expand fused cells *in vitro* due to their scarcity and restricted proliferation, precluding a more comprehensive phenotyping such as evaluation of the stem-cell potential of fused cells.

The fusion-derived cells detected in the lesions were negative for pan-hematopoietic (CD45), endothelial (CD31), epithelial (CK8), and stromal (vimentin) markers. These cells were, however, positive for Sca-1 and CD29, which are known to be expressed on mesenchymal/stromal cells [38, 39]. Taken together, this suggests that fusion occurs between host/BM-derived non-hematopoietic/non-endothelial cells and a resident endometrial mesenchymal/stromal/stem cell in the implanted uterine tissue. Alternatively, as loss of expression of hematopoietic markers was often reported in various adult cell types in mouse studies employing BMT [24, 26, 28, 29], fusion with BMDCs in our endometriosis model may similarly be associated with epigenetic reprogramming that silences genes originally expressed in the fusion donors. The lack of vimentin staining in fusion-derived cells in our model is interesting in light of the reduced vimentin staining reported in ectopic endometrium of women with endometriosis, attributed to lower degree of cell differentiation [40].

Endometriosis is frequently resistant to standard progestin-based therapies and ovarian endometriosis is known to be associated with development of malignancy [41-43]. Additionally, the reduced proliferation rate of cancer-stem cells (CSCs) was proposed to confer drug resistance

and to result from stem cell fusion [45]. The fused cells we observed in endometriosis lesions in our experimental model may be related to the pathogenesis of both these phenomena: Fusion-derived cells may represent quiescent cells with the potential to become treatment resistant or malignant under selective pressure or in the presence of mutations in *kras* and *p53* [44]. However, these possibilities were not explored in the current study and some limitations of our study prevent extrapolating into the causative role of fusion events in endometriosis. These limitations include their low numbers and proliferative potential, as well as the lack of markers usually found in lesions. Moreover, the occurrence of cell fusion was not yet investigated in human lesions, and the evidence gathered so far is circumstantial (i.e., expression of syncytin-1). With the advent of mouse models of endometriosis progression, the fate and role of cells derived from cell fusion events in the pathogenesis of this disease may be further investigated.

In conclusion, we describe the phenomenon of cell fusion occurring in an experimental mouse model of endometriosis. Our data points to a mesenchymal population derived from cell fusion events with limited proliferative activity in lesions, and that many of these cells may originate in the bone marrow. Their putative role in the pathogenesis of the disease remains to be elucidated.

Author contributions statement

A.T. conceived and designed the study, performed the experiments, analyzed the data and drafted the manuscript. R.T. helped perform experiments, analyze the data and revise the manuscript. S.S. and S.G. helped perform the experiments. R.M. helped perform the experiments and revise the final manuscript. H.S.T. contributed to study design and revised the final manuscript.

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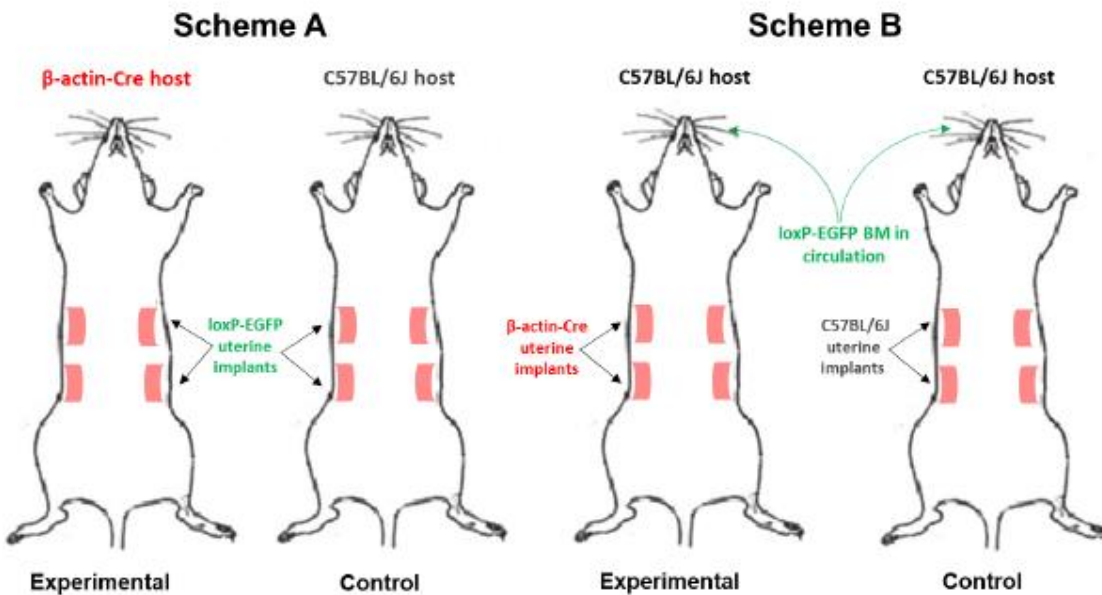


Figure 1. Experimental design examining the occurrence of cell fusion in a mouse model of endometriosis. (A) A schematic of the model used to assess fusion between cells of the endometriosis-like lesion and cells of the host. (B) A schematic of the model used to assess fusion between cells of the endometriosis-like lesion and circulating BMDCs recruited to the lesion. Hosts in this model underwent BMT five weeks prior to EI. Bottom: Development of mouse endometriosis lesions in vivo. Two adjacent vascularized endometriosis-like lesions (labeled with white arrows) forming ~4-5 mm cyst-like structures in the peritoneum of female mice that underwent endometriosis induction four weeks earlier.

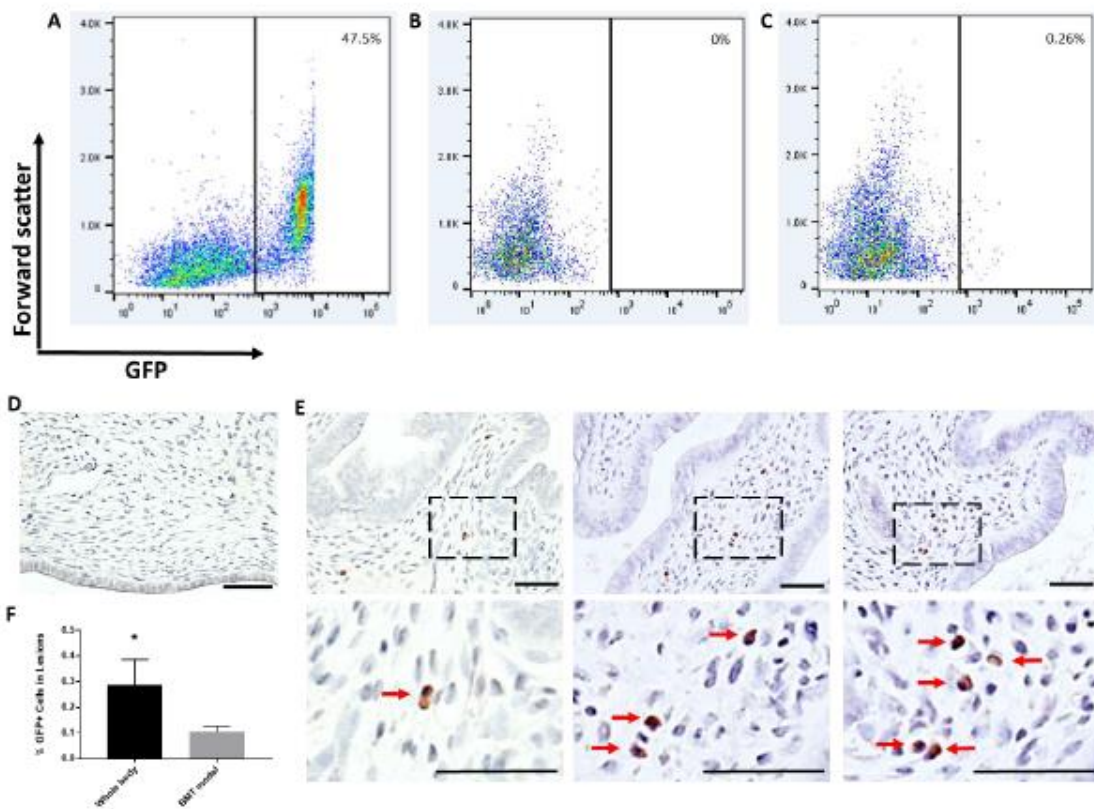


Figure 2. Cell fusion in endometriosis-like lesions in the whole-body model. (A-C) Flow-cytometric analysis of GFP⁺ cells (A) in the uterus of a female offspring resulting from the cross β -actin-Cre X ZsGreen1 (positive control), (B) in ZsGreen1 lesions implanted within a wild-type mouse (negative control) at 4 weeks post EI, and in (C) ZsGreen1 lesions implanted within β -actin-Cre mouse at 4 weeks post EI. n=10 mice in each group. (D) Representative image of GFP immunostaining in control endometriosis lesions at 4-weeks post EI. (E) Representative images of GFP immunostaining in experimental lesions showing GFP⁺ cells (brown) at 4 weeks post EI. Bottom panel: higher magnification images of dashed areas. Red arrows point to fusion-derived cells. n=6-12 mice in each group. *p<0.02. Scale bar=50 μ m. (F) Quantification of GFP⁺ cells in mouse lesions in immunohistochemical sections from the whole body model (left column) and BMT model (right column). n=5 mice in each group.

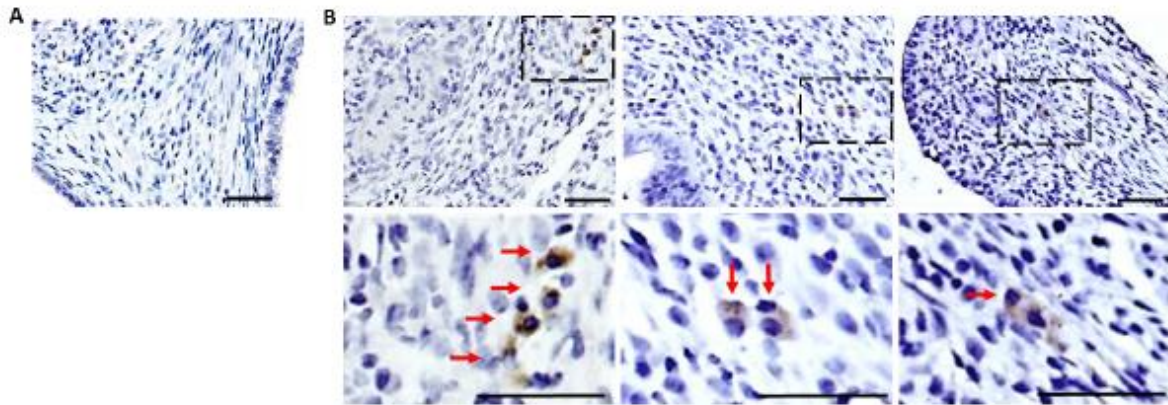


Figure 3. Cell fusion in endometriosis-like lesions in the BMT model. Immunohistochemistry was performed using GFP antibody to detect fused cells. (A) Representative image of GFP immunostaining in control endometriosis lesions (Scheme B) at 4 weeks post EI. (B) Representative images of GFP staining in experimental lesions, showing GFP+ cells (brown) at 4 weeks post EI. Bottom panel: higher magnification images of the dashed areas. Red arrows point to fusion-derived cells. Scale bar=50 μm . n=12.

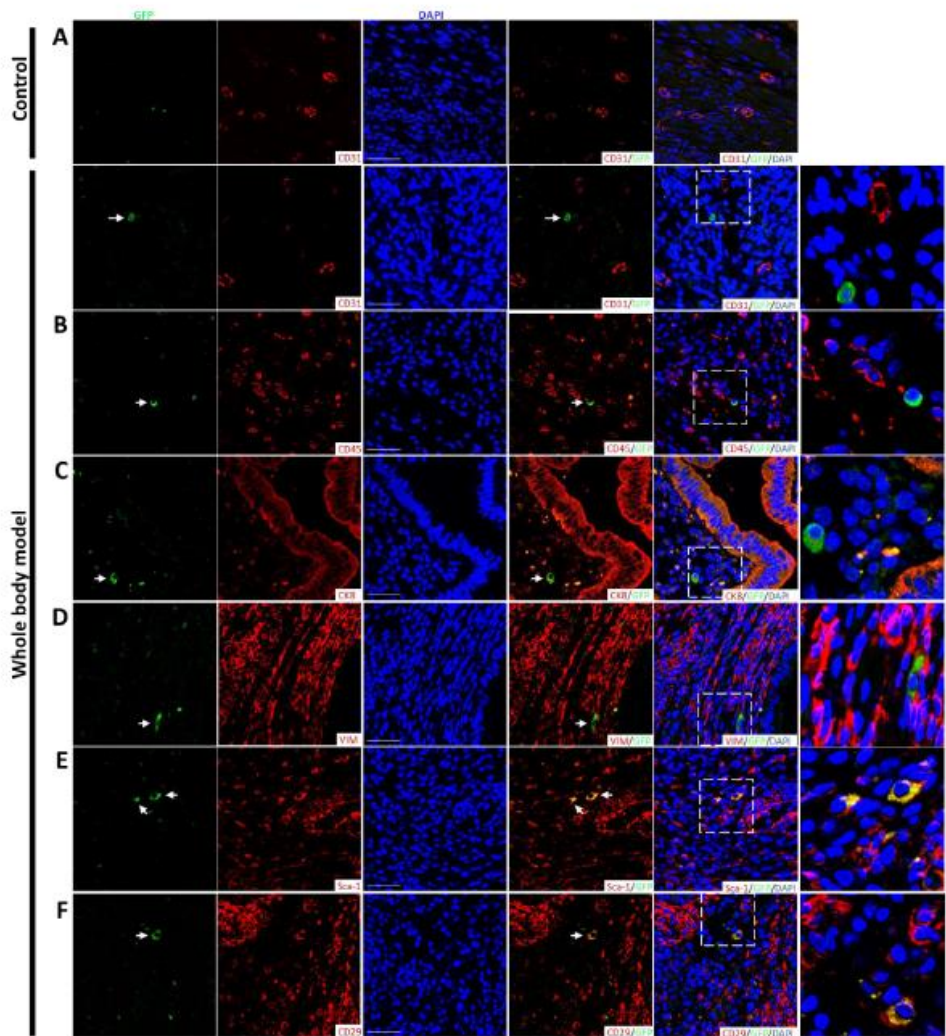


Figure 4. Characterization of fusion-derived cells in mouse endometriosis lesions. Single-stain and merged immunofluorescent photomicrographs of endometriosis lesions 4 weeks post EI. Sections from control and experimental lesions from the whole body fusion model are shown. Co-staining of GFP-positive fusion-derived cells (green) with (A) CD31, (B) CD45, (C) CK8, (D) Vimentin, (E) Sca-1, and (F) CD29 (red). Sections were counterstained with DAPI showing nuclei (blue). Arrowheads point to GFP+ fusion-derived cells. Right column: higher magnification images of dashed areas. Scale bar=50 μ m. n=12.

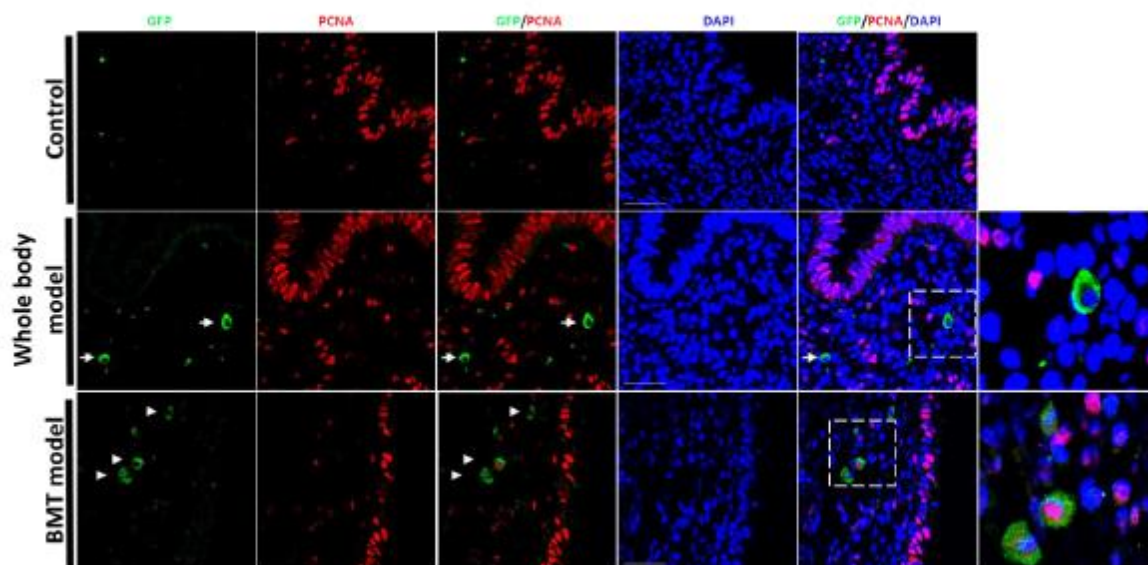


Figure 5. Proliferation status of fusion-derived cells in mouse endometriosis lesions. PCNA immunostaining of endometriosis lesion sections from control (upper panel), whole body model (middle panel), and BMT model (lower panel). Immunofluorescence photomicrographs demonstrate co-staining of GFP-positive fusion-derived cells (green) with PCNA (red). Sections were counterstained with DAPI showing nuclei (blue). White arrows point to GFP-positive cells. Right column: higher magnification of the dashed areas showing GFP-positive cells. Scale bar=50 μm . n=12.