Estrogen is essential but not sufficient to induce endometriosis

Mosami Galvankar, Neha Singh and Deepak Modi*

Molecular and Cellular Biology Laboratory, National Institute for Research in Reproductive Health, Mumbai 400 012, India

**Corresponding author (Email, deepaknmodi@yahoo.com)*

Endometriosis is a common gynaecological disorder of unknown aetiology. Among the several factors, estrogen has been implicated as a causative factor in endometriosis. In the present study using mouse model, we assessed the role of estrogen in the initial implantation and growth of endometrium in ectopic locations. Uterine tissues from green fluorescent protein (GFP) mice were transplanted in to the peritoneum of wild type mice in presence and absence of estrogen. As compared to untreated controls, the implantation of uterine tissue at ectopic locations was higher when estrogen was administered to both host and donor animals. However, this effect was not sustained as lesions regressed within 14 days of treatment. Irrespective of the treatment, peritoneal adipose was the most preferred site of lesion establishment. The lesions did not have typical features of the endometriosis (presence of glands and stroma) even after estrogen treatment and the ectopic tissue underwent regression by apoptosis irrespective of treatment. Since estrogen promotes implantation of endometrial tissue to ectopic locations but failure of these ectopic lesions to grow and sustain even in high estrogenic environment we propose that estrogen is necessary but not sufficient to sustain endometriosis.

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1. Introduction

Endometriosis is a common gynaecological disorder associated with multiple morbidities including dysmenorrhea, dyspareunia, and infertility. Observed in 10–12% of women in reproductive age; almost 35–50% in women with pelvic pain and/or infertility, experience endometriosis (Giudice and Kao 2004; Bulletti *et al.* 2010). At present the cause of endometriosis is unknown; it is believed that retrograde flow of viable endometrial tissue through the fallopian tube into the peritoneal cavity which allows the endometrial fragments to attach and invade ectopically in to organs within the peritoneum causing endometriosis (Vercellini *et al.* 2014). However, nearly 90% of women experience retrograde menstruation but ectopic endometriotic lesions are observed in 10–12% of women suggesting that additional elements must impact its aetiology (Osuga 2010).

Amongst the several factors, estrogen has been implicated as a causative factor in endometriosis. In comparison to healthy women, the levels of estradiol are higher in menstrual blood of endometriosis patients (Rizner 2009; Huhtinen *et al.* 2012). This increase is due to an aberrant activation of local estrogen biosynthesis in the ectopic tissue. While the normal endometrium is devoid of the ability to biosynthesize steroids, there is an ectopic expression of several estrogen-metabolizing enzymes including aromatase (Zeitoun and Bulun 1999). This uncharacteristic expression of aromatase is due to gain in expression of the transcription factor SF-1 that increases aromatase transcription (Zeitoun and Bulun 1999). This results in conversion of androstenedione to estrone leading to high estrogen biosynthesis. The inactivation of estradiol requires 17β-hydroxysteroid dehydrogenase 2 which normally present in endometrial glandular cells; however, the glandular cells in the ectopic endometrium lack this enzyme thereby impairing estrogen inactivation (Rizner 2009). Such increased local estradiol synthesis with reduced ability for its inactivation would logically promote to local estrogen excess result in proliferation of ectopic endometrium promoting endometriosis (reviewed in Huhtinen *et al.* 2012; Rizner 2009). The observations that aromatase inhibitors which prevent the local estrogen biosynthesis can successfully treat

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endometriosis in a subset of patients supports the estrogen dependency of endometriosis (Bedaiwy *et al.* 2017).

Beyond estrogen, the levels of Estrogen Receptors (ER) are also altered in the ectopic endometrium of women with endometriosis (Shao *et al.* 2014; Simmen and Kelley 2016). The levels of ERβ are much higher in endometriotic tissues than in the normal endometrium (Huhtinen *et al.* 2012; Shao *et al.* 2014; Han *et al.* 2015). ERα levels are lower in endometriotic tissues compared with the normal endometrium (Shao *et al.* 2014). Analysis of the ERα and ERβ knockout mice revealed that the ERα gene is required for normal uterine growth and for ectopic lesion growth; in addition, the ERβ gene is involved in inflammation in induced ectopic lesions (Burns *et al.* 2012; Han *et al.* 2015). However, the numbers and size of ectopic lesions are smaller in ERα knockout mice than those detected in ERβ knockouts (Burns *et al.* 2012). These observations suggest that estrogen signalling is favourable for endometriosis progression. The fact that selective ER modulators are helpful in treatment of endometriosis at least in a subset of patients (Bedaiwy *et al.* 2017) further imply that estrogen and ER signalling might be essential in endometriosis.

While there are strong evidences to suggest the involvement of estrogen in endometriosis, limited studies exist regarding the role of estrogen in the initial endometrial-peritoneal attachment and its invasion at ectopic locations for the development of endometriosis. Using syngeneic immunocompetent ER-knockout mice it has been shown that both ERα and ERβ are essential in development of endometriosis lesions (Burns *et al.* 2012; Han *et al.* 2015). However, in all these studies, mice lack estrogen receptors developmentally and inherently have an abnormal uterine immunologic milieu (Hamilton *et al.* 2011). Thus it is unclear if the failure to induce endometriosis in these mice is due to lack of estrogen actions or due to a developmentally incompetent uterus.

In the present study, we aimed at assessing the direct role of estrogen in the initial implantation and growth of endometrium in ectopic locations leading to endometriosis. We performed transplantation of uterine tissue in intact mice and studied the progression of endometriosis in response to estrogen. Our results show that estrogen is essential but not sufficient to induce endometriosis in mouse model.

2. Materials and methods

2.1 *Animals*

All procedures were performed according to Institutional Animal Ethics Committee (IAEC) norms. The IAEC approval number for this project is 15/12.

Eight to ten weeks old C57BL/6 female mice were used as recipients; mice transgenic for green fluorescent protein (GFP) were used as donors. The GFP mice were originally obtained from National Institute for Immunology (NII), bred and maintained in the Experimental Animal Facility at NIRRH. Mice were housed in environmentally controlled conditions and the estrous cycle was checked regularly by vaginal smears.

2.2 *Estrogen treatment*

Mice were randomly allocated in three groups as described in figure 1. Group 1 was control where the tissues from donor animals (GFP transgenic) were directly injected in to recipients. In second group the GFP donors were treated with estrogen (1 μg/injection) for five days consecutively prior to sacrifice followed by the injection in to untreated control recipients. In the third group, both recipients and GFP donors were treated with estrogen as described in figure 1. Estrogen (Sigma, USA) was prepared as a stock in ethanol and then diluted in sterile phosphate buffered saline (PBS) at a final concentration of 1 μg/100 μL. Each animal was injected intra-peritoneally with 1 μg of estrogen as per schedule described in figure 1.

2.3 *Collection of donor tissue*

After appropriate treatments, the donor GFP mice were euthanized and the uterine horns were excised. The tissue was cut longitudinally exposing the endometrium and then minced into fragments using a sterile razor. Preliminary experiments done to determine if mincing or mild protease digestion was required for dissociation of the tissue revealed that minced tissue gave more consistent results (data not shown). Thus all further experiments were done using minced tissue resuspended in sterile PBS.

2.4 *Injection in to recipients*

The donor tissue was kept warm at 37°C in sterile PBS and the fragments were injected intraperitoneally (200 μL) with 18G needle, into recipient wild-type mice. Fragments from three donors were injected into six recipient mice. It was ensured that both the donor and recipients were in estrus stage at the time of injections.

2.5 *Imaging*

After 3, 7 and 14 days of tissue transplantation, the recipients were euthanized and the abdomen was opened. The animal was kept under a stereomicroscope with fluorescence attachment (Olympus, Japan). The abdominal area was

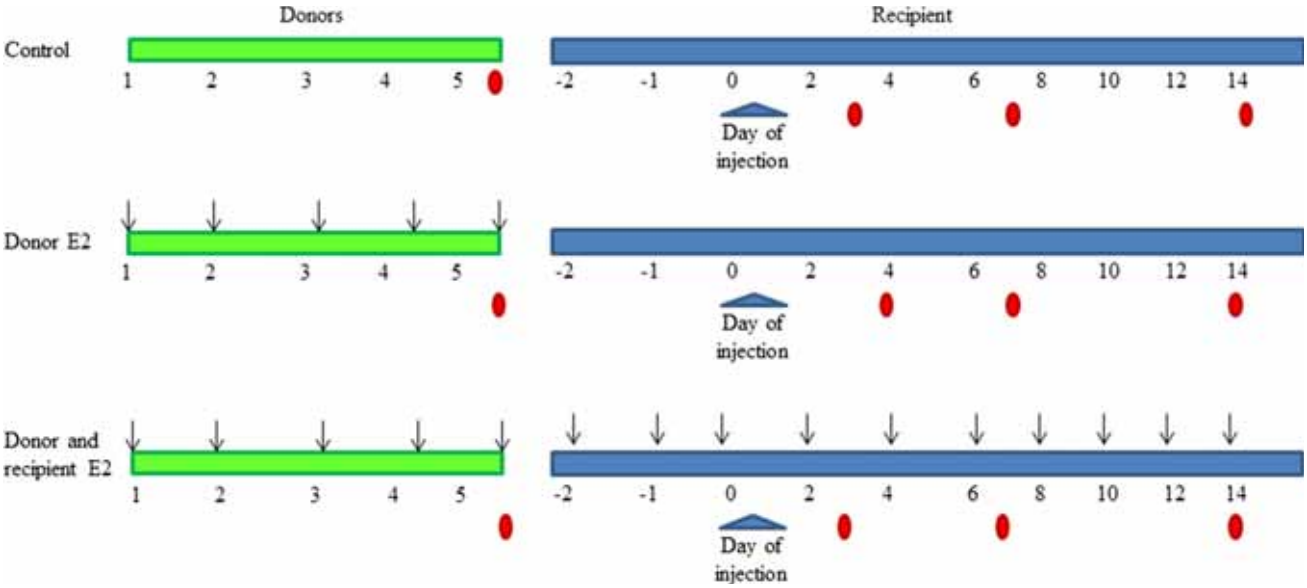

Figure 1. Schematic representation of the experimental design. Donors were mice transgenic for GFP and recipient mice were wild type. Black arrows are day of estrogen (E2) injection (1 μg intraperitoneally). The day of injection of uterine tissue is considered as day 0. Red marks are days on which the animals were scarified.

screened for GFP positive fragments at various magnifications. The number of fragments per animal were estimated, the tissue on which the fragments were found were recorded.

2.6 *Tissue processing*

Tissue was carefully excised with fine tweezers and immediately fixed in 4% paraformaldehyde. After overnight fixation, the tissue was processed for routine paraffin embedding and sectioning. 5 μm thick sections were collected on to poly-L-lysine coated glass slides and dried overnight. The tissue sections were deparaffinized and stained using Haematoxylin and eosin (HiMedia, India) according to standard protocol.

2.7 *Immunohistochemistry*

The tissue sections were processed for immunohistochemical localization for vimentin and cytokeratin as detailed previously (Godbole *et al.* 2007; Abid *et al.* 2008). Briefly, sections were deparaffinized and endogenous peroxidase was blocked by incubation in 0.3% H_2O_2 in absolute alcohol for 30 min. The slides were blocked in 1% Bovine Serum Albumin (Sigma, USA) in PBS followed by an overnight incubation with anti-vimentin (1:100 dilution; ab92547, Affinity Bioreagents, USA), anti-estrogen receptor alpha (1:50, ab37438) and anti-cytokeratin 8 (1:250, ab154301). Next day the slides were washed in PBS incubated in horseradish peroxidase conjugated secondary antibody (Dako, Denmark). The slides were washed and treated with 0.05% diaminobenzidine (Sigma, USA) in PBS with 0.06% H_2O_2. After appropriate colour development, the sections were counterstained with 1% haematoxylin, dehydrated, cleared and mounted in DPX.

2.8 *RNA extraction, reverse transcription and real time PCR*

To measure the amounts of *Esr-1* mRNA (that encodes for ERα protein) in response to estrogen treatment, real time PCR using SYBR green chemistry was done. Total RNA was isolated from the uteri of control and estrogen treated animals using the TRIZOL reagent (Gibco BRL, USA) as per manufacturer's instructions. Reverse transcription and real time PCR was done as detailed previously (Godbole and Modi 2010). Briefly, 1 μg of RNA was reverse transcribed using random hexamer primers and MMLV reverse transcriptase (Clontech, Japan). PCR was done in triplicates in a 25 μL reaction using SYBR green master mix (BioRad, USA) for four biological replicates in each group. The primers to amplify *Esr-1*and *18S* rRNA were commercially synthesized (Sigma, USA) The sequences for Esr-1 primers are Esr-1 forward 5′-CATAACAGCCTCGGAACGGA-3′, Esr-1 reverse 5′-GGGCCACCTGCTTGAGAAGA-3′. The primer sequences for 18S were forward 5′-

GGAGAGGGAGCCTGAGAAAC-3′R and reverse 5-CCTCCAATGGATCCTCGTTA′-3′. Both the primers had an optimized annealing temperature of 63°C. The expected product sizes were 156 bp for *Esr-1* and 180 bp for 18S.

2.9 *Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay*

Apoptosis in tissue sections was determined using TUNEL as detailed previously (Modi *et al.* 2003) with minor modifications. The sections were deparaffinized and the tissues were digested with 0.01 mg/ml Proteinase K (Gibco, BRL USA) followed by TUNEL using a commercial kit (Roche, Germany). Briefly, the digested sections were incubated in the reaction cocktail containing the buffer, cobalt chloride ($CoCl_2$), digoxigenin-labelled UTP, dATP and 125 IU of terminal transferase enzyme. The incubation was carried out for 30 min at 37°C followed by blocking and incubating in horseradish peroxidase conjugated anti-digoxigenin antibody (diluted 1:500 in the blocking solution; Roche). Next day the slides were washed and the colour was developed as above. Sections were then counterstained with 1% haematoxylin, dehydrated, cleared and mounted in DPX. For negative controls the slides were treated identically as above except that terminal transferase was omitted from the TUNEL reaction mixture. As positive controls, the sections were pre-incubated in 10 U in DNAse (Bangalore Genei, India) for 30 min prior to addition of the TUNEL mixture. All samples were analysed under a light microscope on a blinded basis and in duplicates. The percentage of TUNEL positive cells were quantified as detailed earlier (Modi *et al.* 2003). The percentage of TUNEL positive cells was estimated as number brown nuclei/total cells counted X 100.

2.10 *Data analysis*

All the tissue sections were photographed under an upright microscope (Olympus BX 60, Japan). Image analysis was done using the NIS-Elements Microscope Imaging Software (Nikon, Japan). Data was analysed by Student's *t*-test and chi-square test wherever appropriate and $p<0.05$ was considered statistically significant.

3. Results

3.1 *Effect of estrogen treatment on expression of estrogen receptors in the endometrium*

To test if the time and dose of estrogen treatment in donors has any effects on expression of estrogen receptors, we performed immunohistochemistry for ERα in endometrial sections from control (estrus stage) and estrogen-treated animals. ERα was detected mainly in the glands and to an extent in the stroma in estrus stage endometrium from control animals (figure 2A). In comparison, the expression of ERα was higher in estrogen treated animals (figure 2B). No staining was detected in negative controls demonstrating the specificity of staining (figure 2C). The intensity of the staining was quantified using image analysis software. As compared to estrus controls, approximately 1.3-fold increase in expression of ERα was observed in uteri of estrogen treated mice (figure 2D). This increase was statistically significant ($p<0.0001$)

The levels of *Esr-1* mRNA (that encodes for ERα protein) was quantified by real time PCR. A single melt peak was observed for both ERα and 18S rRNA indicating the homogeneity of the amplified products (figure 2E). A single band of expected size was also observed upon agarose gel electrophoresis and bands were not detected in no reverse transcriptase controls (not shown) indicate the specificity of amplification. As compared to untreated controls, the levels of *Esr-1* mRNA were found to be two folds higher in uteri of estrogen treated mice (figure 2F). This increase is statistically significant ($p<0.001$).

3.2 *Effects of estrogen on lesion establishment and tissue morphology*

We aimed to determine if endometrial tissue fragments from GFP transgenic mice could form endometriosis like lesions when injected intraperitoneally. GFP-expressing endometrium fragments were injected into the peritoneal cavity of non-transgenic mice. Under a light microscope, the lesions consisted of white to light yellow ellipsoid nodules; all the lesions also emitted green fluorescence as expected (figure 3A and C). The lesions were seen on tissues covered with peritoneal epithelium; they could also be detected behind the uterus and also embedded in the adipose tissue. Initially (day 1–2 after injections) the GFP tissue seem to just attach the organ with the host tissue covering it with its peritoneal membrane (figure 3A and B). By day 3, the donor tissue was observed to be embedded and green cells were invading the host tissue (figure 3C and D). At this time the lesions displayed a patchy surface and were soft and spongy (figure 3E).

Macroscopically, in the control and donor estrogen group, the GFP expressing tissues were small dot like and most often in the peritoneal adipose tissue. In the group where both recipient and donors were treated with estrogen, GFP lesions were larger and found on multiple locations including the intestine and the bladder (figure 4A). Figure 4B gives the number of animals that had detectable lesions

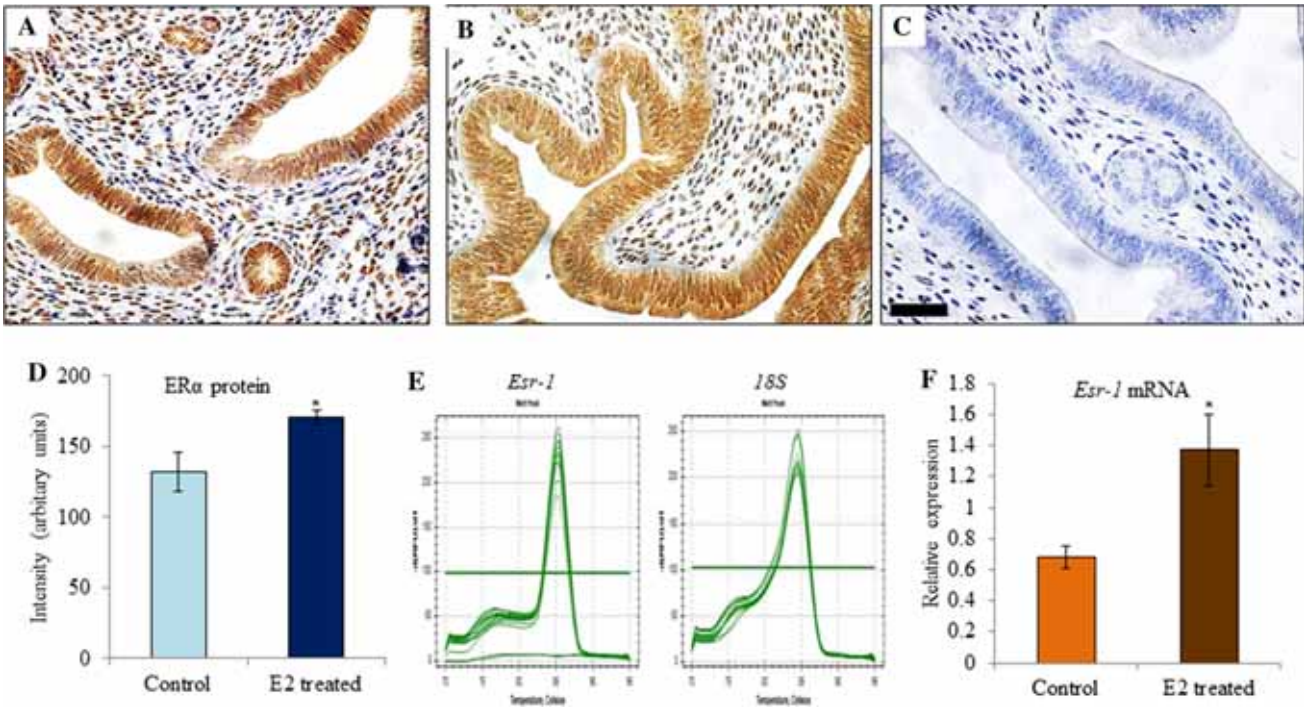

Figure 2. Effect of estrogen treatment on expression of Estrogen Receptor α (ERα). Mice were treated with estrogen (E2) for 5 days (see figure 1) and the uteri sections were immunostained for ERα. (**A**) Control animal in estrus stage. (**B**) Uteri section from E2 treated animal. (**C**) Negative control incubated with rabbit serum in place of primary antibody. Brown staining is for ERα, and blue is nuclear counterstain. Bar in C is 50 μm. (**D**) Mean ± SD of intensity values for ERα obtained by image analysis of the immmunostained sections (n = 3 animals) in each group. (**E**) Melt curves for *Esr-1* mRNA and *18S* rRNA to demonstrate the homogenity of the amplified products. X axis is temperature and Y axis is negative ratio of dF (relative fluorescence units)/dT. Each green line represents one sample, the horizontal bar represents the cut-off used for calculations. (**F**) Levels of *Esr-1* mRNA (as quantitated by qPCR, normalized to housekeeping *18S*) in uteri of control and E2 treated animals. Values are mean ± SD for four biological replicates in each group. * values significantly different as compared to controls.

(under GFP) on different days post injection. In all the three groups, on day 3 post transplantation, the GFP expression tissue was detected in all the animals. However, in the control group, on day 7 post transplantation the lesions were detected only in the 30% of animals, no lesions were detected in any of the animals by day 14. In the estrogen treated donors group, GFP expressing lesions were detected in all the animals on day 3, only 60% of animals had the lesion by day 7. No GFP expressing tissue was detected by day 14 in any of the animals. In the third group where both donor and recipient received estrogen, almost 80% of mice had the lesions by day 7, by day 14 the lesions were detected only in 10% of animals.

3.3 *Effect of estrogen on lesion numbers at different time points*

On day 3, in the control group, only 1–2 lesions were detected. The mean number of lesions increased slightly (but not significantly) when the donors were treated with estrogen. The number of lesions were significantly higher ($p<0.05$) when both donor and recipients were treated with estrogen (figure 4C).

On day 7 the number of lesions per animal reduced in all the three groups, the mean number of lesions was yet higher in the group where both donor and recipients received estrogen as compared to other two groups (figure 4C). On day 14, only the group where both donor and recipients received estrogen had lesion, the numbers reduced further as compared to day 7 in this group (figure 4C).

3.4 *Effects of estrogen treatment on tissue distribution of the lesions*

In the control group on day 3 and day 7 the lesions were mostly found to the pelvic adipose tissue; in some animals on day 3 it was found on the bladder (figure 5A). In the donor estrogen group, on day 3, the lesions were found in

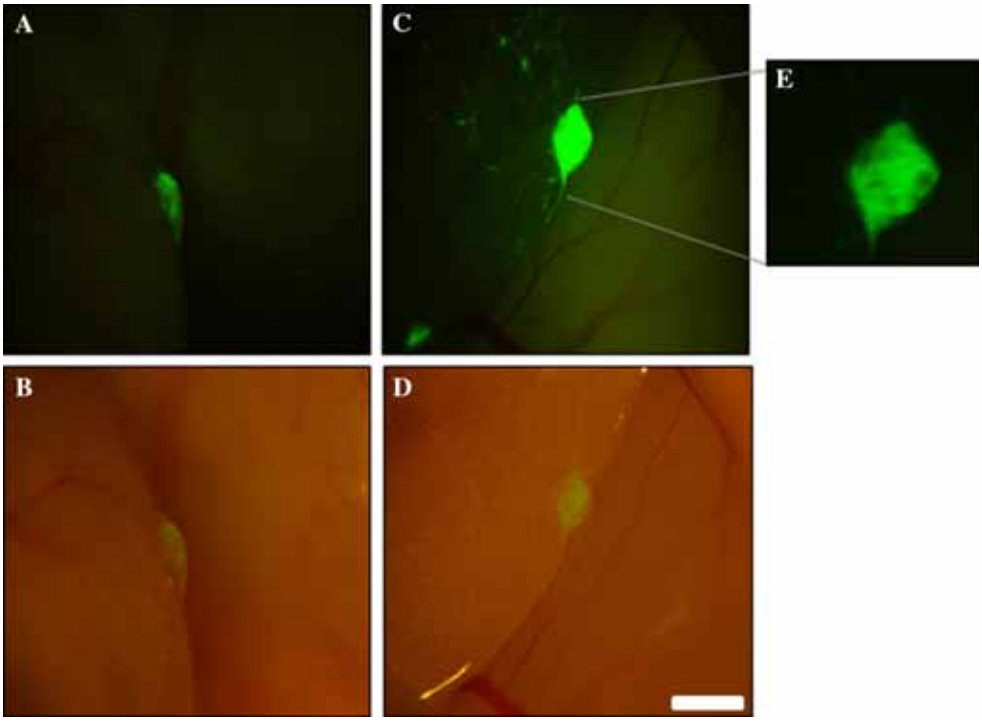

Figure 3. Visualization of GFP positive endometrial fragments in donor mice. Endometrial fragments from mice transgenic for GFP were injected in the peritonium of wild type recipients. Three (**A** and **B**) and 7 (**C** and **D**) days after injection, the animals were euthanized and the abdomen was opened to visualize the internal organs under fluorescence. **A** and **C** are images under fluorescence; **B** and **D** are images in bright field overlaid with fluorescence. **E** is magnified version of the fragment in **C** to show tissue contours. White bar in **D** is 50 μm.

the pelvic adipose tissue in all the animals; in 10% of animals it was found on the bladder (figure 5B). However, no lesions were found on the bladder on day 7 and the lesions were found only on the fatpad/adipose tissue (figure 5B).

In the group where both recipients and donors received estrogen, on day 3 and day 7 GFP lesions were found on multiple tissues. While the lesion on the pelvic adipose tissue/fatpad was common in all the animals, in some animals the lesion was seen on the liver, intestine, bladder and occasionally found loosely attached to other organs like kidney or uterus (figure 5A). However, by day 7 the lesions were not detected on any other tissue in any of the animals, except in a small number of small tissue fragments were detected on the intestine. By day 14, the lesions were detected only in the pelvic adipose tissue and not on any of the organs (figure 5B).

3.5 *Histological changes in the endometriotic lesions on different days post transplantation*

To determine whether the transplanted GFP fragments displayed histological characteristics of endometriotic-like structure, paraffin sections of the lesions were stained with haematoxylin-eosin (figure 6). In the control and donor estrogen groups, the lesions did not exhibit any characteristics of an endometrial tissue. In the control group, on day 3 and day 7 the tissues did not contain any well-defined epithelium or gland like structures. The tissue stroma had cells spread sparsely in the tissue embedded in the surrounding fat (figure 6A and B). In the estrogen treated donor group, the dysmorphic gland like structures were seen with poorly preserved epithelium, by day 7 these structures were not seen and only cells spread sparsely in the tissue embedded in the fat (figure 6C and D).

In the group where both the donors and recipients received estrogen, on day 3 well defined epithelium with gland like morphology was observed in some of the tissue sections the stromal cells were seen surrounding it (figure 6E). However, by day 7 and on day 14 the tissue organization was lost. The entire structure appeared compact (figure 6F and G). The normal endometrial tissue histology is shown in figure 6H for comparison.

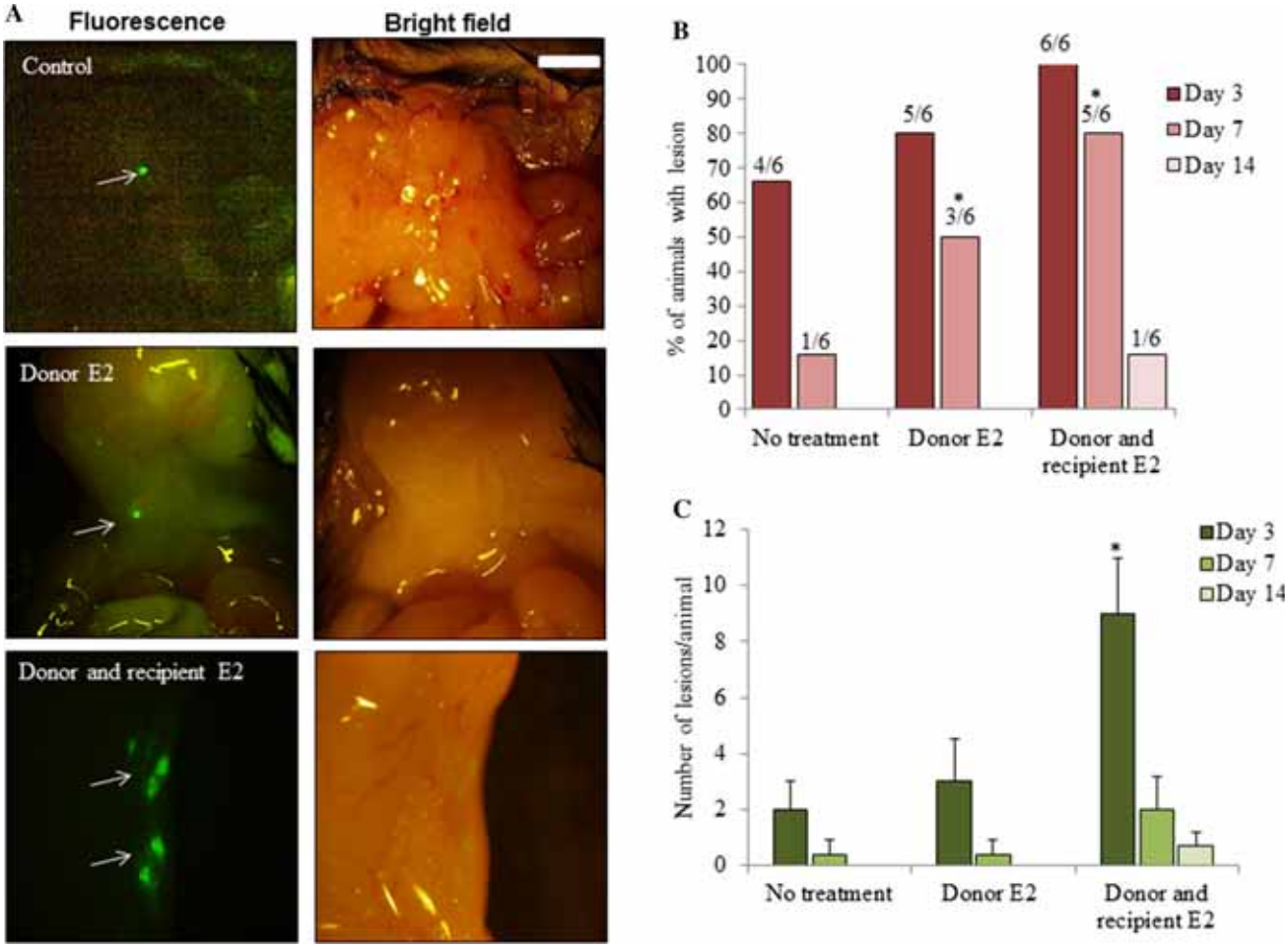

Figure 4. Effect of estrogen (E2) treatment on establishment of endometrial tissue at ectopic locations. Mice were injected with uterine fragments from GFP transgenic mice and the numbers of implanted endometrial fragments (green) were counted on day 3, 7 and 14. (**A**) Representative images of the abdominal region after 7 days of transplantation under fluorescence (green signals) and right panel are bright field images of the same. White bar is 50 μm. (**B**) Percentage of animals with GFP fragments in different groups on day 3, day 7 and day 14 after injections. The numbers on each bar are the number of animals with lesions (n = 6). (**C**) The mean number of lesions per animal in the three groups on day 3, day 7 and day 14 post injections. Values are mean ± SD for six animals in each group. * value significantly different as compared to untreated controls.

3.6 *Immunohistochemical staining for cytokeratin and vimentin in the endometriotic lesions on different days post transplantation*

To study the changes in the organization of glands and stroma in the ectopic endometrium, we stained the paraffin sections for the epithelial marker cytokeratin and stromal cell marker vimentin. As expected, the cytokeratin antibody stained only the glandular epithelium and the vimentin antibody was specific to stroma as revealed by its staining patterns in the normal endometrium (figure 7A and E). In the control group (figure 7B), a few weakly stained epithelial cells were observed; in the estrogen treated donor group the number of cytokeratin positive cells were higher but were randomly interspersed with negative cells (figure 7C). In the donor and recipient estrogen treated group, a small cluster of cytokeratin positive epithelial cell resembling a gland was observed, most cells were randomly distributed (figure 7D).

Similar to epithelial cells, the vimentin positive stromal cells were seen randomly dispersed throughout the tissue with no well-organized structures (figure 7F–H). No staining was detected in the negative control indicative of the specificity of the reaction (inset in figure 7E).

3.7 *Detection of apoptosis in endometriotic lesions*

We performed TUNEL on paraffin embedded tissue to study apoptosis in the ectopic endometrial tissue isolated on day 7 after transplantation. Apoptotic (TUNEL positive) cells were

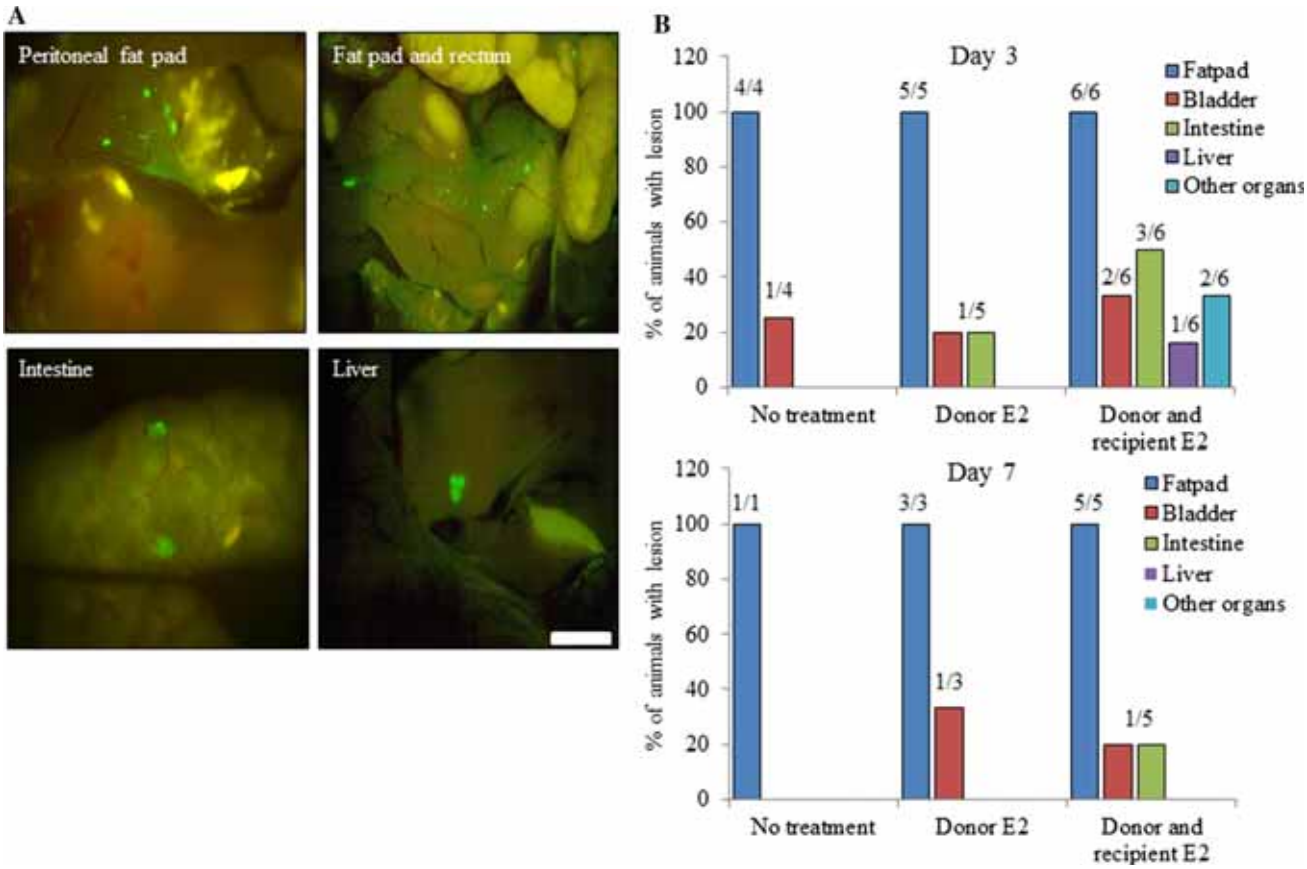

Figure 5. Effect of estrogen (E2) treatment on establishment of lesions in different tissues. Endometrial fragments from GFP transgenic mice were injected in wild type recipients and number of green fragments were estimated on day 3, day 7 and day 14 on each tissues. (**A**) Representative images of the peritoneal region after 7 days of transplantation from the donor and recipient E2 group. GFP (green fluorescence) on different tissue are shown. White bar is 50 µm. (**B**) Percentage of animals with GFP fragments on different tissues on day 3, day 7 post injection. Values above each bar are the actual number of animals with lesion on that tissue/total number of animals with lesion.

seen in all the tissue sections. In all the three groups, a number of cells were found to be TUNEL positive which were interspersed with negative cells (figure 8A−C). No differences were seen in the number of positive cells within the three groups (figure 7G). In the control eutopic endometrium from mice in late diestrus, a few TUNEL positive cells were detected as expected (figure 8D). The reaction was specific as TUNEL positive cells were not detected when the enzyme terminal transferase was omitted from reaction mixture (negative controls); all the cells were positive when the tissue section was pre-incubated with DNAse prior to addition of the reaction mixture (figure 8E and F).

4. Discussion

Endometriosis is a multifactorial disease characterised by a complex pathophysiology. An involvement of the estrogen in endometriosis has long been suspected, but exact details still remain enigmatic, therefore requiring intensive investigations to gain deeper insights into the pathophysiology of endometriosis. Estrogen is central for uterine homeostasis and aberrant estrogen biosynthesis or its actions lead do disruptions in uterine functions and failure of pregnancy (Bulun 2009). Abundant quantities of estradiol are detected in endometriotic tissue (Huhtinen *et al.* 2012). They also contain higher levels of intracellular ERβ. Loss of ERβ results in reduction of endometriotic lesion volume and decreased cellular proliferation while promoting cell death (Burns *et al.* 2012; Han *et al.* 2015). Overexpression of ERβ increases endometriotic lesion volume and proliferation while decreasing cellular death (Han *et al.* 2015). Similarly, in mice that lack ERα the number and size of endometriotic lesions are fewer (Burns *et al.* 2012) suggesting that estrogen actions are essential for progression of endometriosis. The question yet remains whether estrogen is essential for establishing the lesion or is necessary for its survival.

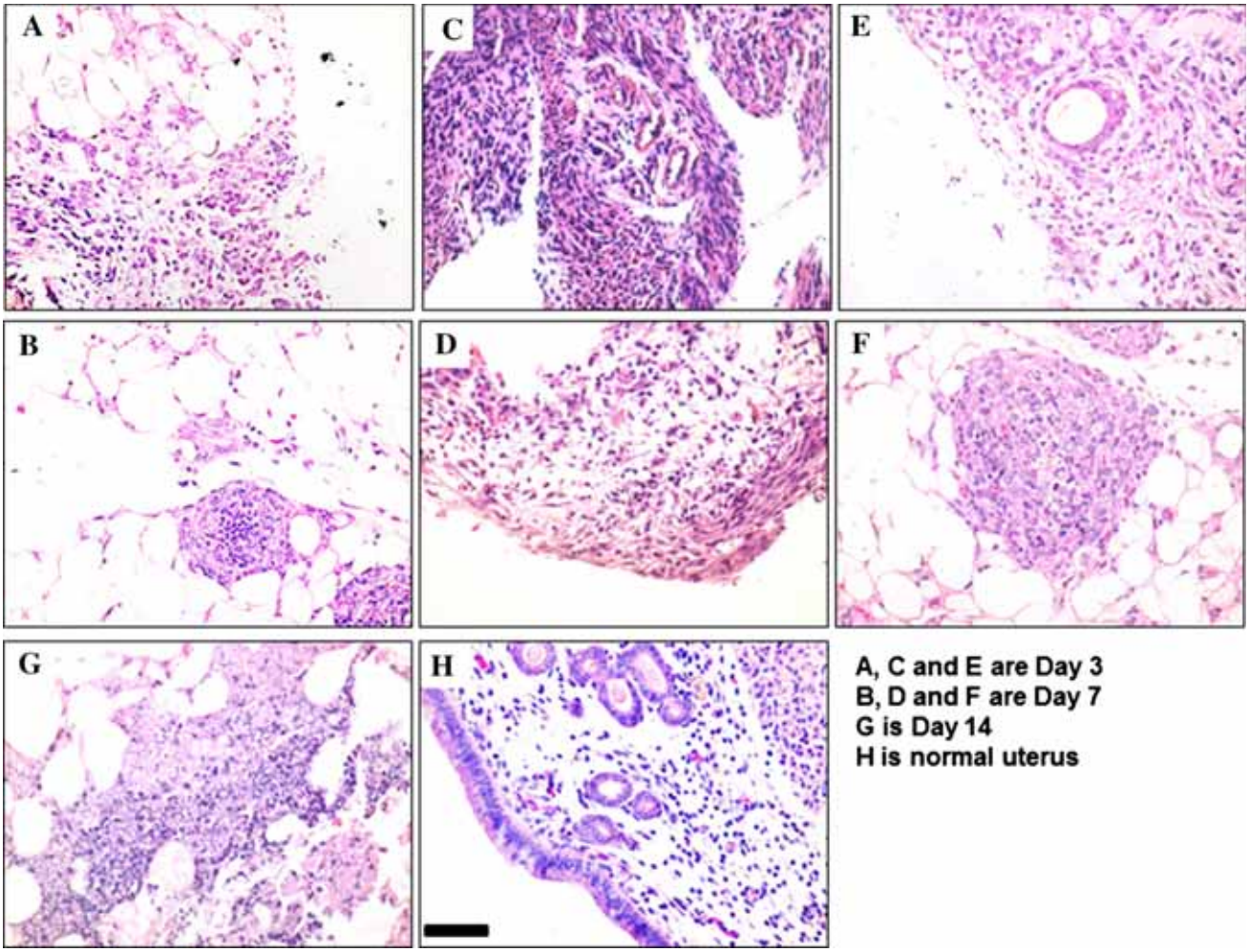

Figure 6. Histology of the ectopic tissue obtained from different groups on day 3, day 7 and day 14 after injection. Wild-type mice were injected with endometrial fragments from GFP transgenic mice and the tissues sections were stained with haematoxylin and eosin. Representative images from control group (**A–B**); donor estrogen (E2) (**C–D**); donor and recipient E2 group (**E–G**). Six animals were tested in each group. H is normal uterus as. Bar in **H** is 50 μm.

In the present study we used uterine tissue from mice transgenic for GFP as donors whereas the recipients were intact wild type mice. This method mimics retrograde menstruation and the fragments are allowed to establish freely in the peritoneal cavity (Hirata *et al.* 2005). Moreover, the GFP-expressing endometrium enables detection of small and hidden lesions or non-attached debris under fluorescent light. In accordance with the earlier observations (Hirata *et al.* 2005; Wilkosz *et al.* 2011), small GFP tissue fragments were consistently detected in recipient mice 3-7 days after transplantation. However, only 30% of animals had these fragments that lodged in the peritoneal adipose tissue; these fragments were not observed after 14 days of transplantation. The lesions were also not highly invasive and composed of several smaller pieces. Histologically, none of the lesions had well defined epithelium or gland like structures; staining with cytokeratin and vimentin revealed absence of any endometrium like structure. By day 7, most cells were found to undergo apoptosis. These observations imply that although the endometrial tissue fragments implant ectopically in the peritoneum in a subset of mice, the environment is not conducive for its sustained growth.

It has been previously reported that estrogen is essential for cellular proliferation in the uterus (Cunha and Lung 1979); in the endometriosis tissue estrogen has been shown to promote cell proliferation, invasion and angiogenesis (Xiong *et al.* 2015; Rudzitis-Auth *et al.* 2016). In the present study, the donor mice were estrogen supplemented and the endometrial tissue was injected into untreated recipients. We suspected that the failure to induce ectopic growth of

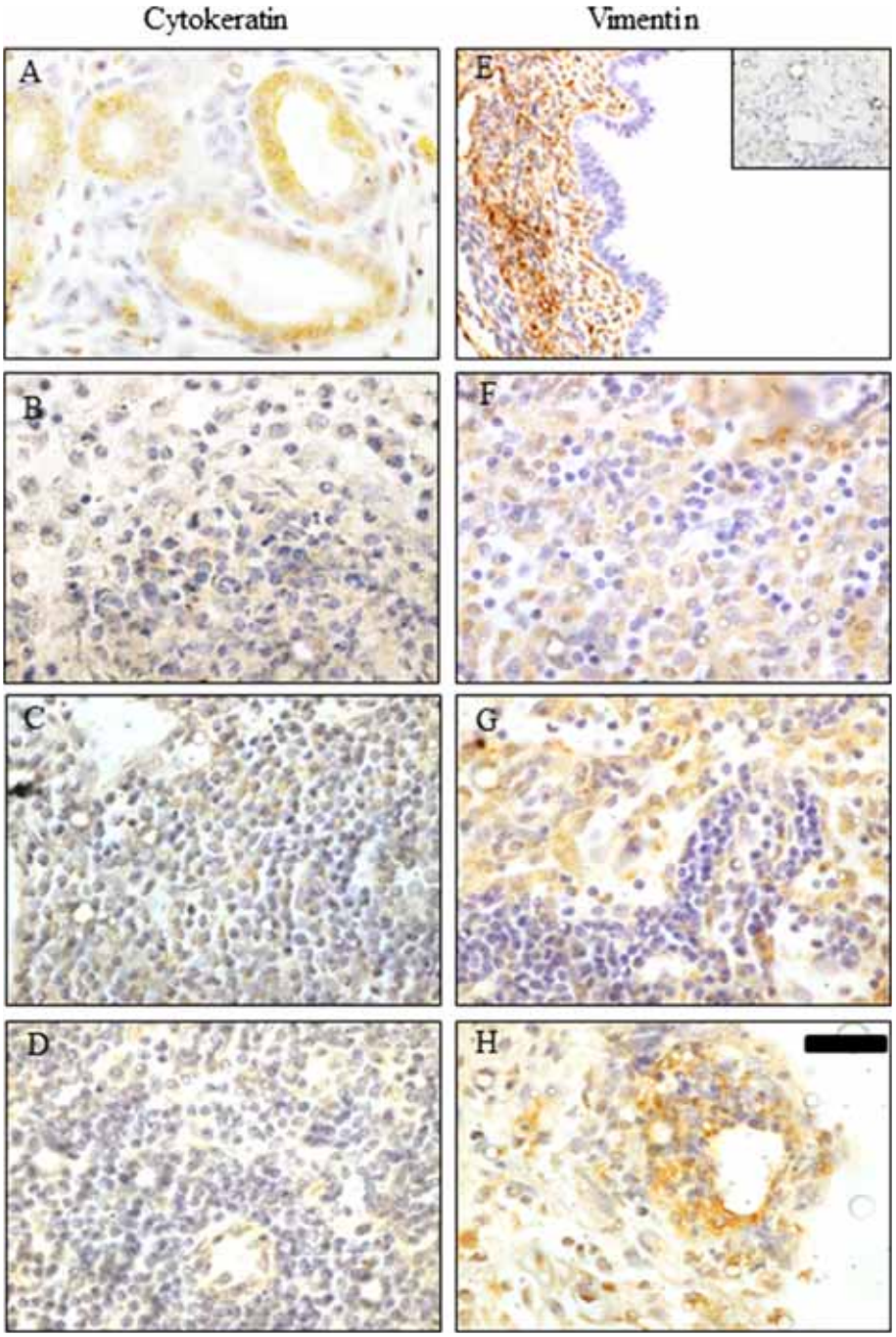

Figure 7. Immunohistochemical localization for epithelial cells marker (Cytokeratin) and stromal cell marker (vimentin) in the ectopic tissue. Mice were injected with uterine fragments from GFP transgenic mice and the tissues sections were stained for vimentin and cytokeratin (brown staining) using specific antibodies. Blue staining is nuclear counterstain. Representative images of uterus in estrus stage (**A** and **E**), control group (**B** and **F**), donor estrogen (E2) group (**C** and **G**), and donor and recipient E2 group (**D** and **H**). Bar in **H** is 50 μm. Negative control is shown in inset in **E**.

endometriosis in the recipients could be due to an insufficiently primed uterus from the donors. The results revealed that, although the numbers of GFP expressing endometrial fragments were higher in recipients that received tissues from estrogen treated donors, the lesions reduced in numbers and were not detected after a few days. Histologically, gland like structures were initially visible in a few lesions, subsequently the tissue lost its organization as cytokeratin and

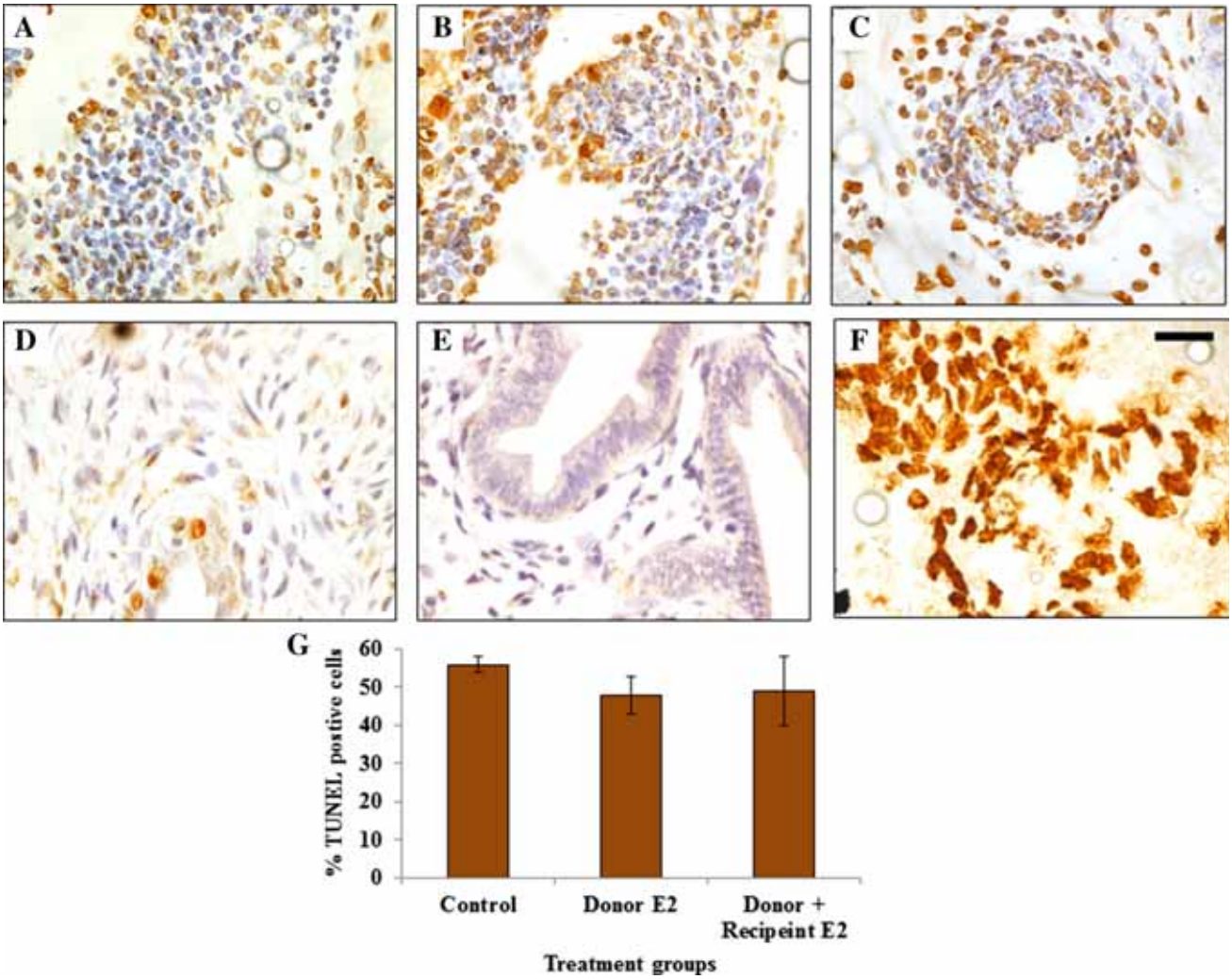

Figure 8. Detection of apoptosis in the ectopic tissue. Wild type mice were injected with uterine fragments from GFP transgenic mice and the tissues were stained for DNA fragmentation by TUNEL (brown staining). Blue staining is nuclear counterstain. Representative images of tissues obtained on day 7 from (**A**) control group, (**B**) donor estrogen (E2) group and (**C**) donor and recipient E2 group. **D** is uterus in estrus stage, **E** is negative control and **F** is DNAse treated positive control. Bar in **F** is 50 μm. **G** is mean ± SD (n = 3) of percentage of TUNEL positive cells in the three groups.

vimentin positive cells were seen scattered with no defined organization. Furthermore, most cells were found to be undergoing apoptosis. These observations imply that pre-priming of uterus by estrogen may aid in initial lesion attachment, the beneficial effects if any on sustaining these lesions are minimal.

In order to synchronise the animals, several authors used ovariectomized mice and supplemented them with estrogen pellets (Cummings and Metcalf 1995; Somigliana *et al.* 1999; Harris *et al.* 2005; Lin *et al.* 2006; Becker *et al.* 2008; Greaves *et al.* 2014). Moreover, as estrogen is known to influence the immune system (Straub 2007) it was thought that estrogen treatment to the recipients might be beneficial in sustained growth of endometriotic lesions. Thus we tested the effects of estrogen treatment in both donor and recipient. We observed that as compare to control and only donor estrogen group, estrogen treatment to the recipients increased the number and sites of GFP expressing fragments. Multiple GFP expressing uterine fragments were seen attached to several peritoneal organs initially; the number of lesions per animal reduced dramatically as time progressed. Morphologically, well defined cytokeratin positive epithelium was detected initially, the organization was impaired and the tissue appeared regressing. In this group, although, cell death was delayed, most cells were TUNEL positive after 14 days of injection of the tissues despite continuous

estrogen supplementation. These observations, in part support the previous data (Burns *et al.* 2012) regarding an essential role of estrogen on initial implantation of endometrium at ectopic lesion; however, the failure of these ectopic lesions to grow and sustain even in presence of high estrogen tempt us to suggest that estrogen is not sufficient to sustain endometriosis.

In the present study, GFP expressing endometrial fragments were used to facilitate the detection of lesions that are not otherwise traceable. In accordance to previous studies (Hirata *et al.* 2005) the tissue adhered spontaneously onto specific locations like the adipose and the other tissues in the peritoneum. However, a frequent site of lesion is the peritoneal adipose, mainly around the uterus. A similar preference of lesions on fatty tissues is also described by others (Somigliana *et al.* 1999; Hirata *et al.* 2005). Adipose tissues produce estrogen and pro-angiogenic factors (Park *et al.* 2011) that may facilitate lesion growth. Thus, the role of peritoneal adipose tissue as a favoured attachment site is an interesting finding and seems to be important in the light of estrogen production. Indeed, we did get attachment to multiple tissues when estrogen was continuously supplemented in the recipients. However, adipose tissues are not preferential attachment sites for endometriotic lesions in humans (Menni *et al.* 2016). Hence, it will be of relevance to identify the reasons of adipose tissue preference for endometrial fragments in the mouse but not humans, to understand the role of host tissue biology in occurrence of endometriosis.

Finally, an unusual observation in our study was the failure to sustain the growth of endometriotic lesions despite excessive estrogen supplementation. These results are in contrast with earlier reports (Hirata *et al.* 2005; Burns *et al.* 2012; Greaves *et al.* 2014) where endometriosis like lesions were detected in mice even after two weeks of uterine transplantation; herein the number of GFP fragments progressively declined. To first test if the failure to sustain lesions in response to estrogen treatment is not due to loss of responsiveness of the donor tissue to estrogen, we stained the sections for estrogen receptor ERα. In all the estrogen treated mice the uterine ERα expression was higher as compared to controls. This indicates that the tissues are responsive to estrogen and their failure to sustain even in presence of excessive estrogen is not at least in part due to loss of ER. A major difference between our study and earlier reports is removal of the ovaries in the host. In all the previous studies, the authors ovariectomized the recipients two weeks prior to transplantation; whereas, in this study we have used intact animals as recipients to maintain a physiological state. It is possible that in earlier studies, along with the global deficiency of steroids, there could be loss of other molecules due to ovariectomy. This might alter some changes in basal homeostasis that promoted endometriosis. This implies that under physiological conditions, although estrogen is a promotor of endometriosis, it is plausible that other ovarian factors might be protective thereby not allowing growth of tissue for longer periods at ectopic locations. This although speculative, is a possible explanation of why endometriosis does not develop in most women despite retrograde menstruation. It will be of interest to identify ovarian factors that might be protective against endometriosis.

5. Conclusion

The results of the present study demonstrate that in intact animals, estrogen may be necessary for initial induction but is not sufficient for growth of endometriosis. These findings will have important implication in expanding our understanding of factors that cause endometriosis and designing rational therapeutic modalities for the treatment of this common disease.

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