Role of progenitor cell producing normal vagina by metaplasia in laparoscopic peritoneal vaginoplasty

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ABSTRACT

CONTEXT: Host of vaginoplasty techniques have been described. None has been successful in developing normal vagina. Laparoscopic peritoneal vaginoplasty (LPV) is performed in Mayer–Rokitansky–Küster–Hauser syndrome (MRKHS) culminating in normal vagina. **AIMS:** This study aims to confirm normal development of neovagina by anatomical and functional parameters of histology, cytology, and ultrasonography (USG) in LPV. To identify peritoneal progenitor cell by OCT4/SOX2 markers. To demonstrate the metaplastic conversion of peritoneum to neovagina and the progenitor cell concentration, distribution pattern. **SETTINGS AND DESIGN:** This is prospective experimental study, conducted at teaching hospital and private hospital. **SUBJECTS AND METHODS:** Fifteen women of MRKHS underwent LPV followed by histology, cytology, two-/three-dimensional USG of neovagina. Four women underwent peritoneal biopsy for identification of progenitor cells with OCT4/SOX2 markers. One patient underwent serial biopsies for 4 weeks for histology and progenitor cell immunohistochemistry. **RESULTS:** Normal vaginal histology and cytology were apparent. USG of neovagina showed normal appearance and blood flow. Two peritoneal samples confirmed the presence of progenitor cells. Serial biopsies demonstrated the epithelial change from single to multilayer with stromal compaction and neoangiogenesis. The progenitor cells concentration and different distribution patterns were described using SOX2/OCT4 markers. **CONCLUSIONS:** We have shown successful peritoneal metaplastic conversion to normal vagina in LPV. The progenitor cell was identified in normal peritoneum using SOX2/OCT4 markers. The progenitor cell concentration and pattern were demonstrated at various stages of neovaginal development.

KEY WORDS: Absent vagina, Mayer–Rokitansky–Küster–Hauser, peritoneal metaplasia, primary amenorrhea, stem cell, vaginoplasty

INTRODUCTION

The partial or total absence of Mullerian development results in absent vagina. This is classically described as Mayer–Rokitansky–Küster–Hauser syndrome (MRKHS). Patients presenting with the syndrome, however, have normal secondary sexual development. The estimated prevalence is about 1:4,000–5,000 women.[1,2] Almost 30%–40% of these patients have associated renal anomalies.[3]

There are several surgical procedures described for the neovaginal creation.[4] McIndoe first described the creation of neovaginal space.[5] Free skin graft,[4] artificial dermis,[6] skin allograft,[4,7] intestinal[8] or sigmoid vaginoplasty,[9] amnion graft,[4,10] and pelvic peritoneum graft[4,11] have been used. Their disadvantages include stenosis, poor lubrication, scarring, and contracture, leading to dyspareunia, and the need for laparotomy. Besides this, the transformation to squamous cell carcinoma[12] from free skin graft and adenocarcinoma from sigmoid has been reported. The amnion graft can also transmit hepatitis or human immunodeficiency virus, although the use of freeze-dried amnion prevents such a transmission.[4] Recently,

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there are reports regarding the creation of neovagina through endoscopic assistance[4,13] based on Vecchietti technique.[14] The procedures are complex, time-consuming, and fraught with complications. Techniques using cultured vaginal tissue, buccal mucosa,[15] artificial material like cellulose and vaginal substitutes have been described.[16-18] The use of peritoneum in vaginoplasty was first described in Russian literature. Davydov[19] popularized the peritoneal vaginoplasty method. Many modifications of Davydov procedure have been described.[4,20,21]

The peritoneum undergoes metaplasia and transforms into a variety of tissues,[22] like endometrium, cartilage. The peritoneal metaplasia is not from the simple multiplication of low cuboidal epithelium but complex phenomenon passing through activation followed by suppression of mesothelial progenitor cells. This hypothesis formed the basis of the present study.

The objective of this study was to confirm normal development of neovagina by anatomical and functional parameters of histology, cytology, and ultrasonography (USG) in laparoscopic peritoneal vaginoplasty (LPV), to identify peritoneal progenitor cell by OCT4 and SOX2 markers and to demonstrate the metaplastic conversion of peritoneum to normal vagina by serial biopsies and the progenitor cell concentration, distribution pattern.

SUBJECTS AND METHODS

The present study design is prospective experimental cohort study. A total of forty-five patients with congenital absence of vagina (MRKH syndrome) were treated with laparoscopic peritoneal pull through technique of Dr. Mhatre at, Department of Obstetrics and Gynaecology of N. Wadia Maternity Hospital and Seth G.S.Medical College, Mumbai, and Kedar Hospital, a private obstetrics and gynecology facility, Mumbai between 2003 and 2015. All study patients were counseled and offered nonsurgical and surgical procedures for neovagina creation. Written informed consent was obtained in all patients before performing LVP.

Fifteen patients out of 45 consented for vaginal biopsy on follow-up visits. Institutional authority approved our study after departmental peer review and ethical consideration. All 15 women had a weekly follow-up for 3 months and monthly thereafter for 1 year.

They underwent detailed clinical examination, cytology and vaginoscopy at monthly interval. Trans-perineal USG evaluation of neovagina was performed using two and three dimensional probes (2D/3D USG). The vaginoscopy revealed a near normal vagina in all the subjects at 9 months postoperative period. The structural imaging along with Doppler flow analysis of neovagina was evaluated at the same time. This USG image of neovagina was compared with normal vaginal images, in both sexually active and inactive women.

Vaginoscopy was performed in outpatient department (OPD) to determine the neovaginal appearance. The vaginal biopsies were performed over a period of 2½–9 months to document the transformation. The timing of the biopsy was determined depending on the vaginoscopy showing near normal appearance. The biopsies were performed in OPD under sedation using tooth forceps and scissor and sent for histological staining by eosin and hematoxylin. Four out of 15 women consented for peritoneal biopsy to identify the stem cells and one gave consent for serial weekly biopsies of the neovagina. These four peritoneal samples were taken during the LPV operation and sent for histology and immunohistochemistry (IHC) staining for OCT4 and SOX2 as progenitor cell markers. One patient underwent serial vaginoscopy and vaginal biopsy at weekly intervals for 4 weeks as an OPD procedure under sedation. These biopsies were subjected for histology to document the different stages of transformation. The vaginal biopsies were collected in neutral buffer solution and subjected to IHC staining using OCT4 and SOX2 stem cell markers.

The immunohistochemical staining and localization of stem cell markers OCT4 and SOX2 was conducted at the National Institute of Research in Reproductive Health (NIRRH).

Immunohistochemistry

Neovagina sections of 5 mm thickness were deparaffinized and rehydrated using descending grades of methanol. Endogenous peroxidase activity was quenched by incubating the sections in 0.3% hydrogen peroxide for 30 min. This was followed by microwave treatment of the sections for 10 min in antigen unmasking solution (Vector Laboratories, Burlingame, CA, USA). Sections were blocked with 2% goat serum (for the section to be incubated with polyclonal antibodies against all antigens) in phosphate buffered solution for 1 h at room temperature. Sections were then incubated with the respective primary antibodies for 16 h at 4°C–8°C. The negative control sections were incubated with rabbit or mouse IgG in place of primary antibodies. This was followed by incubations of the sections with fluorescent labeled secondary antibodies raised in goat (Vector Laboratories, Burlingame, CA, USA) for 1 h at 37°C. Sections were then counterstained with hematoxylin and gradually dehydrated, cleared in xylene, and mounted. The staining intensities for immunoreactive antigens were determined using confocal microscope (Zeiss, Germany). At least ten areas from each section were randomly selected for measuring the integrated optical density (IOD). The IOD values of the respective negative control (without primary antibody) were subtracted from the IOD values for the sections stained with primary antibody.

RESULTS

All 15 women were followed monthly for 1 year. There was no subject lost to follow-up. On each visit every subject was clinically evaluated. The cytology smears depicted the presence of exfoliated superficial and intermediate vaginal cells by the end of 6 months.

The biopsies were performed in two subjects at 2½ months, two subjects at 5 months, 6 subjects at 6 months whereas remaining five subjects underwent biopsy at 9 months postprocedure [Figure 1].

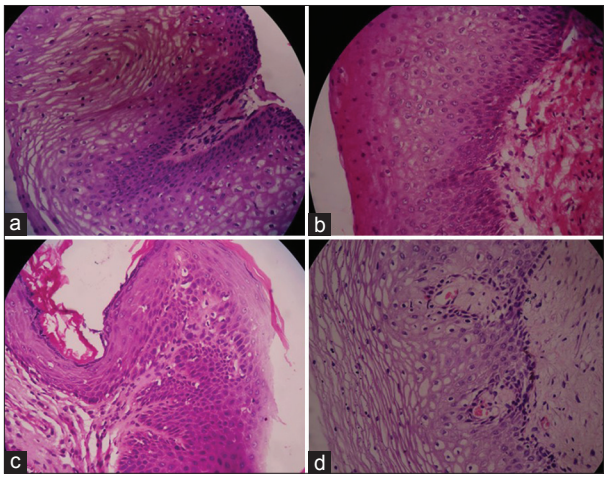

Figure 1: Neovaginal biopsies (×40) at 2 months and 15 days of surgery (a), 2 months and 15 days of surgery (b), 5 months of surgery (c), 9 months of surgery (d)

The biopsies done at <5 months of duration postprocedure showed multilayer squamous epithelium without keratinization. All biopsies done post 5 months showed multilayer squamous epithelium with keratinization, similar to normal vaginal epithelium. The average transformation time for multilayer squamous epithelium development was 6 months.

Both 2D and 3D USG images are almost comparable with normal vagina, except the fact that the vaginal cavity remains an open space and not a potentially closed space as in normal subjects. The neovagina remains open in upper part while lower vagina closes due to pelvic floor muscles. In the normal vagina of a sexually inactive woman, the vagina has an appearance of a transverse slit while sexual activity converts the potential space into a concavity. The neovagina in a similar situation of sexual activity displays a larger open space as compared with sexual inactivity [Figure 2].

IHC tests were performed on four peritoneal biopsy samples. Two samples demonstrated presences of progenitor cells by OCT4 and SOX2 as markers. Immunostaining with stem cell marker OCT4 is seen in the nucleus in green; whereas DAPI (4′, 6-diamidino-2-phenylindole fluorescent stain) is used as nuclear stain in blue. The immunostained images have been overlaid with the differential interference contrast image (DIC is the bright field contrast image of the unstained sample) to show the localization of OCT4. Immunostaining with stem cell marker SOX2 is seen in the nucleus as green; whereas DAPI is used as nuclear stain in

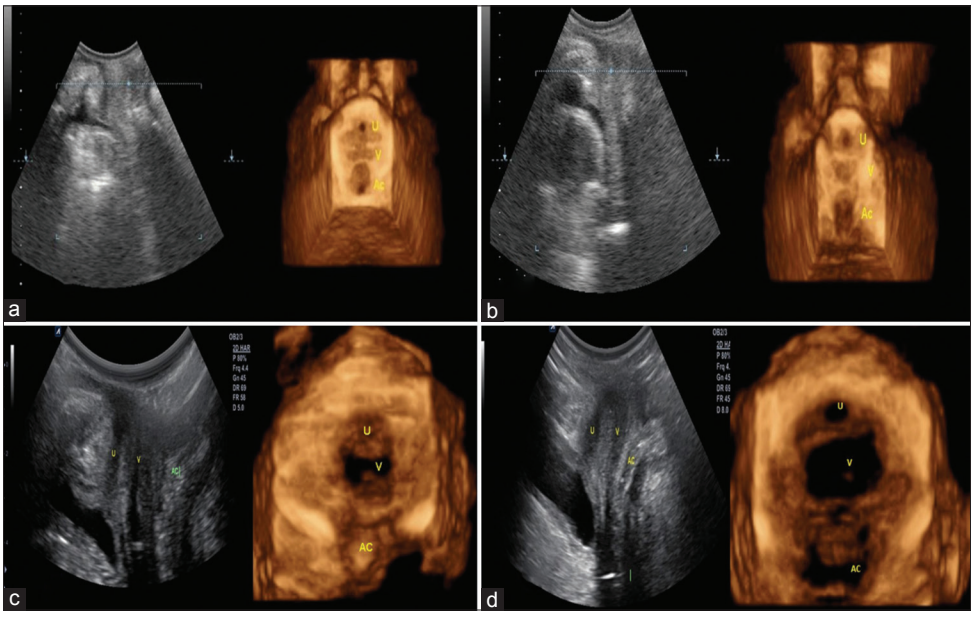

Figure 2: Ultrasonography features of normal vagina and neovagina. (a) Two-/three-dimensional images of sexually inactive normal vagina. (b) Two-/three-dimensional images of sexually active normal vagina. (c) Two-/three-dimensional image of neovagina in a sexually inactive vaginoplasty patient at 9 months postoperative period. (d) Two-/three-dimensional image of neovagina in a sexually active vaginoplasty patient at 9-month postoperative period

blue. The immunostained images have been overlaid with the DIC image to show the localization of SOX2 [Figure 3].

The serial changes leading to the transformation of peritoneum to normal vagina were demonstrated by weekly biopsy in one subject.

Biopsy (×20) from day 7 of neovagina showed single layer epithelium with loose adventitial layer below it. Immunostaining with stem cell marker SOX2 is seen in the nucleus as green, whereas DAPI is used as blue nuclear stain. The immunostained images have been overlaid with the DIC image to show the localization of SOX2. The expression is scattered in the tissue and in some areas it is seen in parallel distribution. Vaginoscopy of neovagina at day 7 showed pink color lining with healthy look. The day 7 neovaginal biopsy for OCT4 was lost due to staining procedure error [Figure 4].

Biopsy (×20) from day 14 of neovagina showed polymorph and mononuclear infiltrate along with vascular endothelium. Adventitial layer is dense as compared to day 7. The

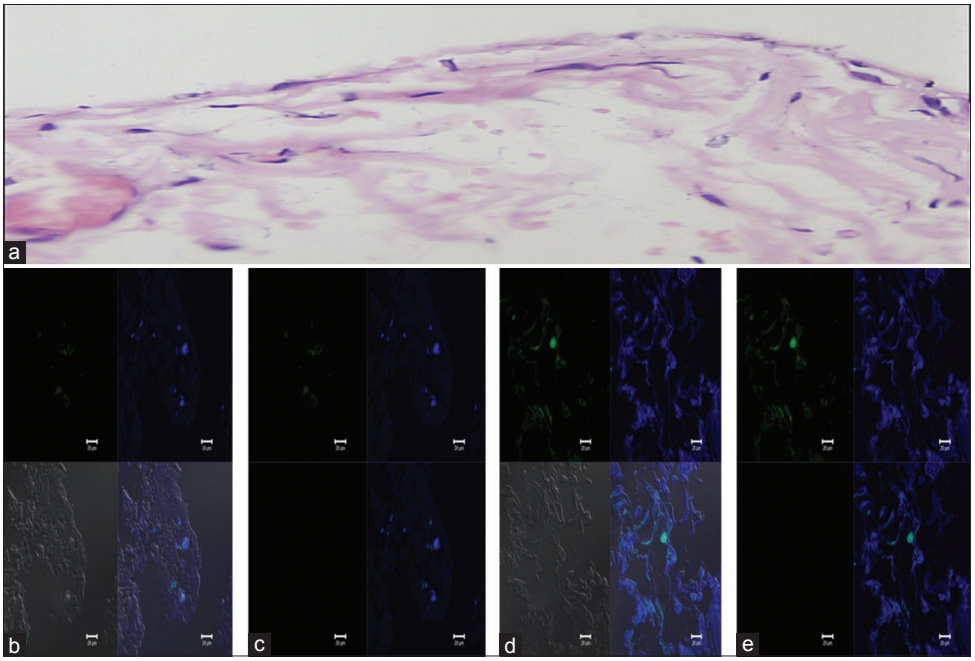

Figure 3: (a) Shows slide (×40) of biopsy from normal peritoneum. (b and c) Confocal images (×20) of OCT4 of normal peritoneum. (d and e) Confocal image (×20) of SOX2 in of normal peritoneum

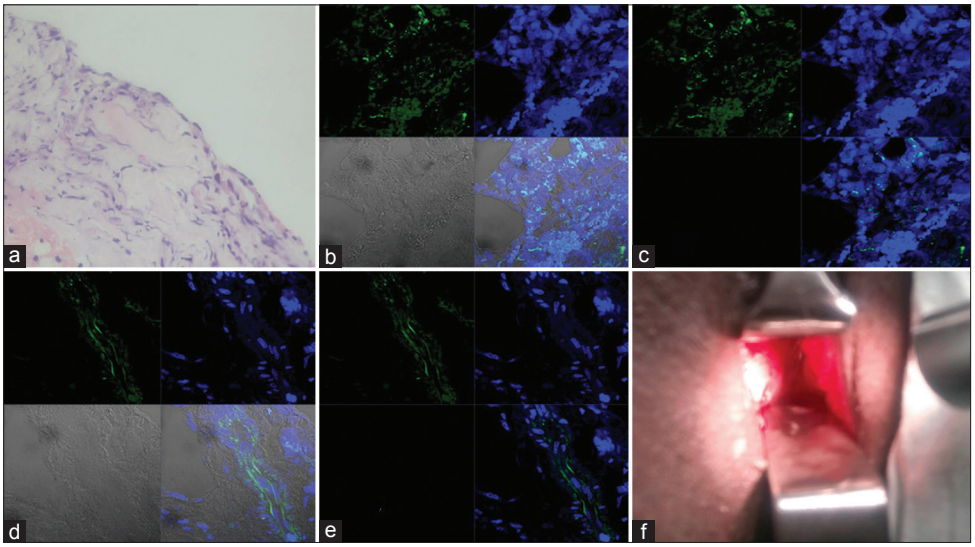

Figure 4: (a) Slide (×40) of biopsy from day 7 of neovagina (b and c) confocal image (×20) of SOX2 in day 7 of neovagina (d and e) expression is seen in parallel distribution. (f) Photograph of vaginoscopy of neovagina of day 7

expression of OCT4 is scattered in the tissue. The expression of SOX2 is increased as compared to day 7. The expression is seen in two distribution patterns tubular and circular. Day 14 vaginoscopy showed pale neovaginal mucosa [Figure 5].

Biopsy (×20) from day 21 of neovagina showed increased polymorph and mononuclear infiltrate with angiogenesis. Adventitial layer is dense and compact as compared to day 14. Comparing with day 14, OCT4 expressions is reduced on day 21, and the SOX2 expression is increased with wide distribution. Day 21 vaginoscopy showed metaplastic white areas with normal looking pink posterior wall [Figure 6].

Biopsy (×20) from day 28 of neovagina showed increased polymorph and mononuclear infiltrate with well-organized capillaries and edematous adventitial layer. Expression of OCT4 and SOX2 is very low in the day 28 vaginal biopsy. Day 28 vaginoscopy showed white lesions on vaginal wall representing metaplastic activity [Figure 7].

DISCUSSION

This surgical technique is comparatively simple with no morbidity.[21] It maintains self-respect and dignity of the patient, as it has no marks of surgery to declare and results in formation of normal vaginal lining. Peritoneal lining having the same parentage of Mullerian duct undergoes metaplasia and transforms itself into stratified squamous epithelium of normal vagina.

Various stages of metaplastic changes in the peritoneum are demonstrated over a 4-week period changing itself to normal vagina. These findings confirm the theory of Mullerianosis. The cytological and histology findings over the period of 9 months showed evidence of formation of mature multilayered squamous epithelium. The normal vagina offers patients good coital function with natural lubrication and pleasure, a function, which is otherwise denied by nature in the context of their earlier quandary.

The ability of normal vagina to be a potentially closed space is due to elastic tissue present in the substratum. The LPV procedure results in normal vaginal cavity and lining, but still fails to produce the vaginal substratum containing elastic tissue. This is evident in 2D/3D USG examination. The progenitor cell responsible for the metaplastic conversion is documented. The progenitor cell pattern and population concentration over a 4-week period is demonstrated.

We hypothesize that the probable stimulus for the peritoneal conversion is exposure to the external environment and the friction stimulus offered by the vaginal dilatation.

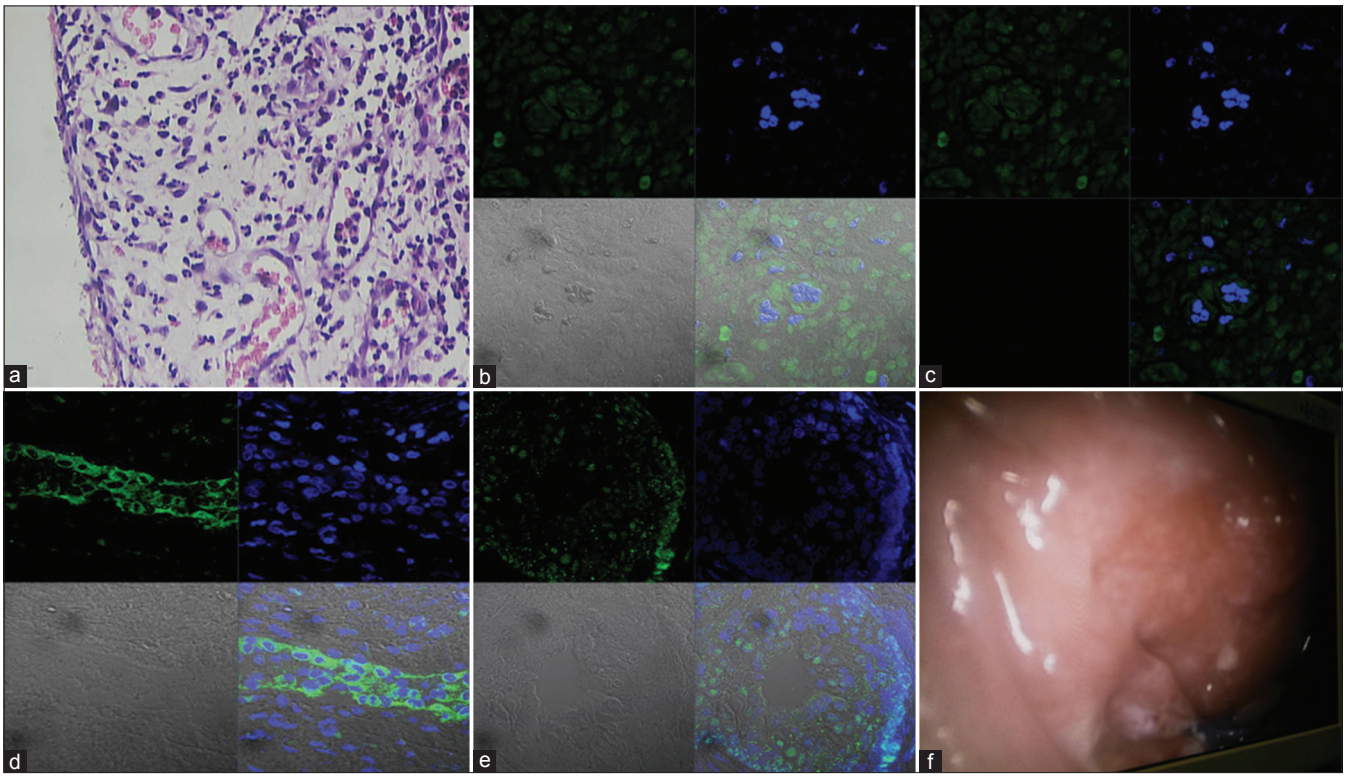

Figure 5: (a) Slide (×40) of biopsy from day 14 of neovagina (b and c) confocal images (×20) of OCT4 in day 14 of neovagina. (d and e) Confocal image (×20) of SOX2 in day 14 of neovagina with negative control (f) vaginoscopy of neovagina of day 14

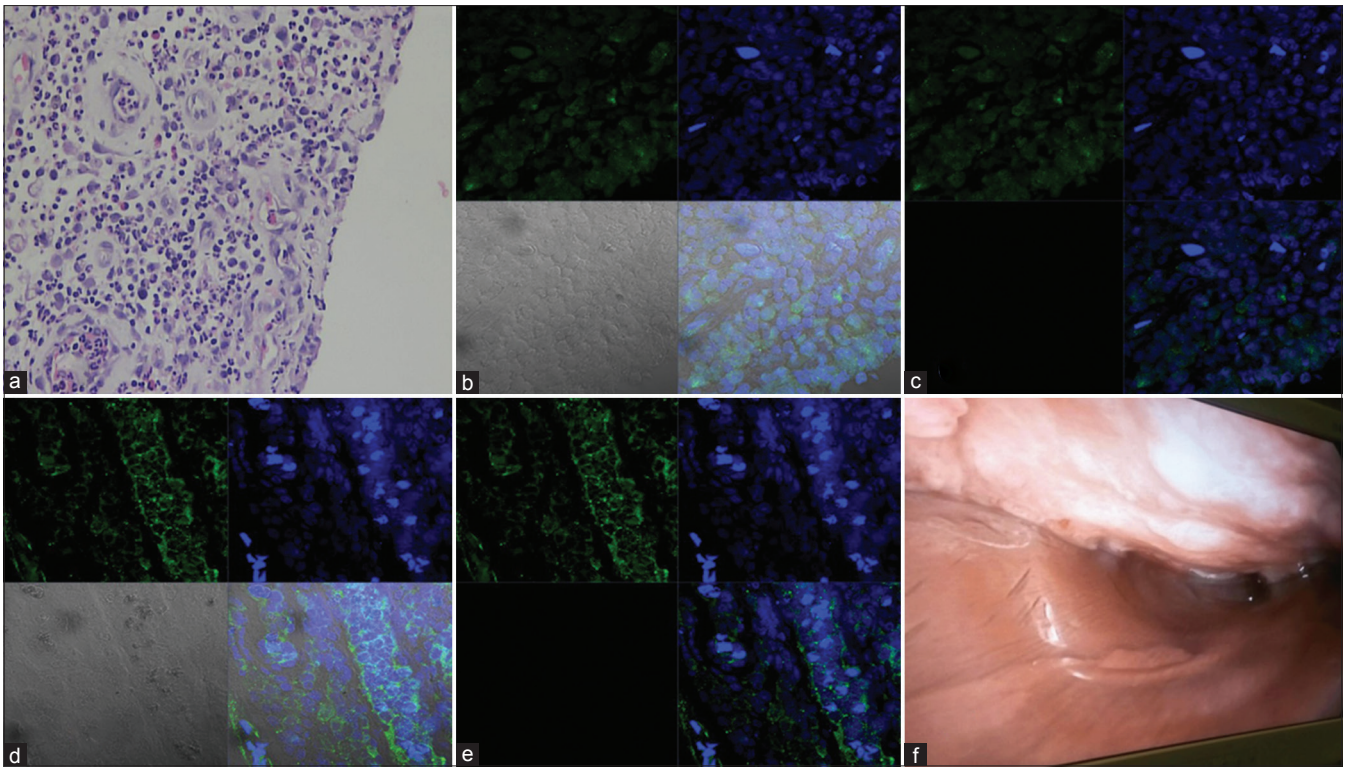

Figure 6: (a) Slide (×20) of biopsy from day 21 of neovagina. (b and c) Confocal images (×20) of OCT4 in day 21 of neovagina. (d and e) Confocal image (×20) of SOX2 in day 21 of neovagina (f) vaginoscopy of neovagina of day 21

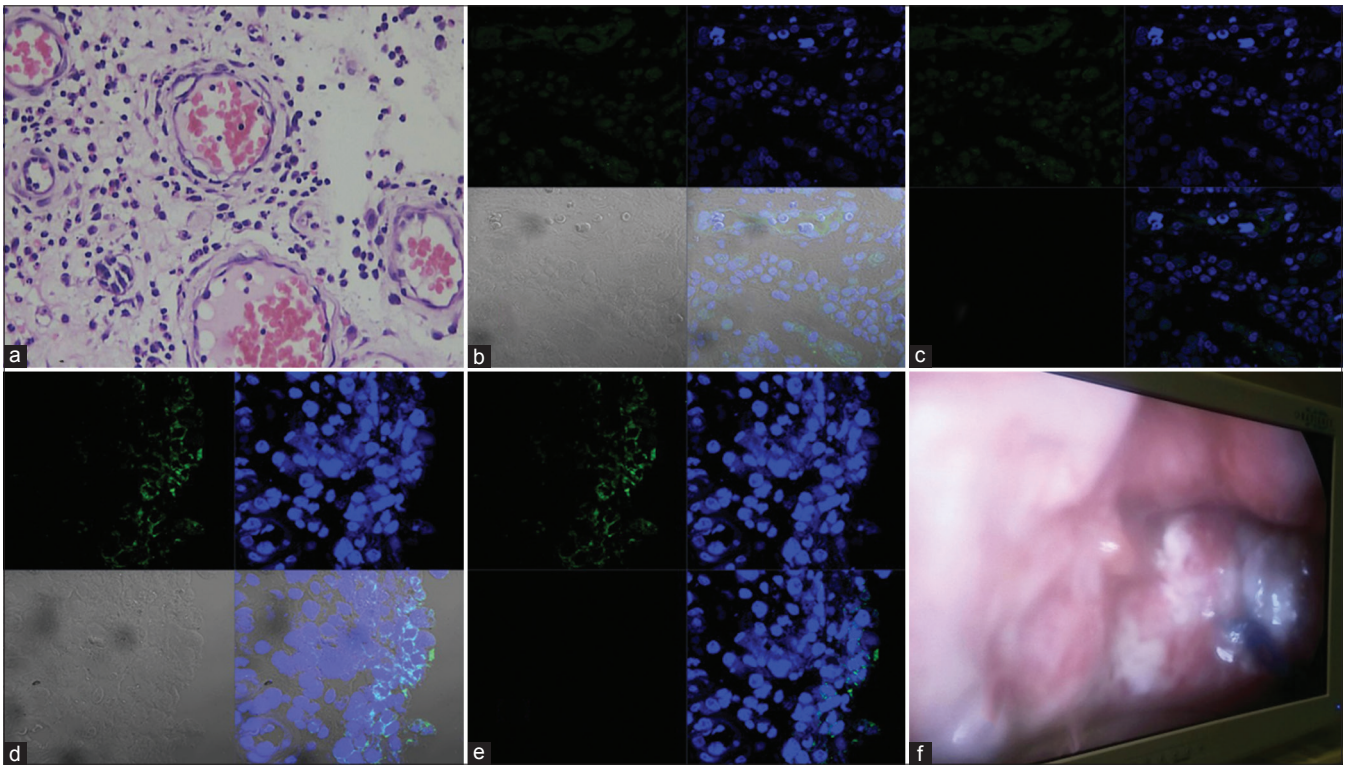

Figure 7: (a) Slide (×20) of biopsy from day 28 of neovagina. (b and c) Confocal images (×20) of OCT4 in day 28 of neovagina. (d and e) Confocal image (×20) of SOX2 in day 28 of neovagina with negative control. (f) Vaginoscopy of neovagina

This study may lead us to future research of these progenitor cells, its role in metaplastic conversion and iatrogenic intervention to modify these cells to the desired outcome.

The major contribution of this study clearly demonstrates the gradual transition from peritoneal mesothelium to stratified squamous epithelium of neovagina in LPV. There is marked paucity of data on such metaplastic conversion. Previously available evidence of peritoneal metaplasia is due to certain irritation factors at its normal anatomical position. Our study identified progenitor cells in peritoneum and its pattern of distribution during metaplasia in sequential manner. Anatomical appearance of the neovagina is also shown of nearly similar features as a normal vagina on ultrasonographic evaluation. There is no study in literature depicting the presence of progenitor cells in neovaginal tissue.

Although the importance of distribution pattern of progenitor cells cannot be ascertained at this point of time, these findings raises question of where and why this pattern localized. The study highlights the importance of constant efforts to understand the tissue remodeling.

The surgical procedure of LPV, vaginal biopsy was performed by the same surgical team. The clinical evaluation was done by single observer. Similarly, the ultrasound evaluation was done by the same sonologist. The staining of IHC was done in NIRRH by the same researcher.

Due to limited available resources, it was not possible to perform progenitor cell identification in more subjects. Similarly, repeated follow-ups precluded the subjects to enroll for the study. The distribution pattern of progenitor cells at higher magnification would have probably provided more information.

To the best of our knowledge, this study is the first to show the metaplastic changes in the peritoneum changing it to normal vagina and identifying the progenitor cell responsible for this change. The appearance, concentration, and phasing out of progenitor cell in 1st first month of neovaginal development was identified. The different patterns of progenitor cells as demonstrated by OCT4 and SOX2 markers remains an enigma. As the ovary became accessible per vaginum, three patients underwent ovum retrieval and pregnancy using surrogate mothers, making this a fertility-enhancing procedure.[21] We have demonstrated that peritoneal metaplasia is not from the simple multiplication of low cuboidal epithelium of peritoneum but complex phenomenon passing through activation followed by suppression of mesothelial progenitor cells.

The progenitor cell population timing and distribution pattern will help us in understanding the metaplastic conversion of peritoneum to Mullerian structures leading to the development of uterus and cervix artificially in near future. Developing these progenitor cell populations will help us in developing the neovaginal elastic substratum. The research in this direction is already in progress. We are developing mesothelial stem cells for the clinical application in vaginoplasty patients (Unpublished data).

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Conflicts of interest

There are no conflicts of interest.

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