Original Article

Novel Technique of Vaginoplasty Developing Normal Vagina, Role of Stemness Markers and Translational Genes

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Abstract

Aims and Objectives: To study development of neo-vagina by metaplastic conversion of peritoneum, To identify translational Stemness markers using NANOG/OCT4/SOX2 from serial neo-vaginal mRNA, cDNA and to study role of WNT and HOXA genes in patients undergoing vaginoplasty. **Material and Methods:** 75 MRKH Syndrome women underwent laparoscopic peritoneal vaginoplasty (LPV). Two patients underwent serial neo-vaginal biopsies on day 0, 7-9, 12-14, 21 and 33. Fifteen MRKHS and twelve controls were subjected for neo-vaginal biopsy to detect genes upregulation. Remaining patients were evaluated anatomically and functionally. **Results:** The translational stemness markers NANOG, OCT4 and SOX2 responsible for neo-vaginal formation were identified. Their appearance, concentration at different stages of conversion were demonstrated. The neo-vagina has shown up-regulation of these translational stemness markers. The study demonstrates expression of the specific genes (WNT4, WNT5A and WNT7A) and their role in formation of the neo-vagina. In the subjects stemness markers (NANOG, OCT4 and SOX2) appeared from day 9 to 14 of the neo-vaginal biopsies and after achieving the peak declined later. Genetic analysis showed low values in HOXA 9,10,11,13 and up-regulation of WNT 4A,5A,7 genes in neo-vagina. **Conclusions:** Study shows peritoneal metaplastic conversion to normal vagina, identified the translational stemness markers and genes responsible. The neo-vagina has shown up-regulation of these genes. The study demonstrates expression of the specific genes (WNT4, WNT5A and WNT7A) and their role in formation of the neo-vagina. Furthering this research, activating these genes may lead to treatment of developmental defects of Mullerian duct, obviating the need of transplant.

Keywords: *Absent vagina, genetics, Mayer–Rokitansky–Kustner–Hauser, progenitor cell, vaginoplasty*

Introduction

Mayer–Rokitansky–Kustner–Hauser syndrome (MRKH) poses a multitude of problems to the clinician, with the main being creation of neo vagina. Many surgical and nonsurgical techniques have been described, however none results in the formation of normal vagina. These surgical techniques are difficult, are multistage, and associated with morbidity.[1-7] The nonsurgical techniques being lengthy, difficult to practice with low patient compliance. The use of peritoneum was first described by Davidov.[8] Many modifications of Davidov procedure have been described.[1,9,10] The present study describes laparoscopic peritoneal vaginoplasty (LPV), a modification of original Davidov procedure.

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The peritoneum has shown metaplastic properties and is known to transform into a variety of tissues including vaginal epithelium.[11,12] These changes are very complex and result due to progenitor cell, activation of translational Stemness markers, and specific genomes. This hypothesis formed the basis of the present study. The modified LPV technique has resulted in the formation of normal vagina and the progenitor cell responsible for this metaplastic conversion has been identified.[11,13]

Peter Muller described the Mullerian duct in 1730, almost 290 years ago; however, the genetic information regarding its normal and anomalous development is still ill understood. Previously, it was assumed that MRKH syndrome has sporadic genetic etiology, but familial cases have been reported in the literature, suggesting autosomal dominant inheritance.[14-16]

The genes involved in the development of the Mullerian tract include Lim homeobox 1 (Lim1),[17] wingless-type Mouse mammary tumor virus (MMTV) integration site family, member 4 (WNT4),[17] Paired box 2 (Pax2),[18] Empty spiracles homeobox 2,[19] POU domain-containing transcription factor 2,[20] large homolog 1,[21] dachshund homolog 1 and 2,[22] Hoxa13,[23] Wnt5a, Wnt7a,[24] Catenin (cadherin-associated protein), beta 1 (Ctnnb1),[25] and forkhead box A2.[26]

Three types of MRKH syndrome have been described in the literature. The first being pure MRKH syndrome; the second being associated with renal, somatic, and cardiac anomalies; and the third type is association with hyper-androgenism. No mutations in anti-Mullerian hormone or activating mutation of its associated receptor, WT1, PBX1, PAX2, Hoxa10, Hoxa11, a cofactor of HOX genes, have been linked to MRKH syndrome.[27-32]

The third type of MRKH syndrome which is associated with hyper-androgenism and is implicated to have WNT4 mutation.[33-35] WNT4 mutation confirms that this signaling molecule is involved in Mullerian development and androgen repression in the ovary.[36] WNT4, HNF1B, and LHX1 have been studied in genetic analysis of MRKH syndrome.[37-40] A study using whole-genome expression was done to identify etiology of MRKH syndrome, and it suggested either increased expression of estrogen receptors 1 Wilms Tumor 1, and GATA binding protein 4 might lead to abnormal development of the female reproductive tract.[41] Ectopic expression of certain HOXA genes may also be implicated.[27,41] There were three microdeletions at 16p11.2, 17q12, and 22q11.2 that were found in syndromic Müllerian aplasia case population was compared to the control population.[42] Ledig *et al*.[43] found recurrent deletions affecting TBX6, HNF1 β, and LHX1 in their cohort of MRKH patients. Duplication in the SHOX gene has recently been identified in two daughters with MRKH type I,[44] miRNAs may have an important role in the development and function of the female reproductive tract.[45]

The literature remains inconclusive on genetic etiology of MRKH syndrome. Having identified the progenitor cell an attempt is made to identify the specific translational stemness markers and the activation of specific genomes in the neo vagina. In the previous study, presence of stemness markers was shown by staining technique using immuno histochemistry. This was not specific with the DNA material hence RNA latter was prepared from the neo vaginal tissue and specific cDNA was obtained. The serial expression and concentration of translational stemness markers (NANOG, OCT4, and SOX2) were identified by using the conventional polymerase chain reaction (PCR). The activation of various genes (HOXA9, HOXA10A, HOXA11, HOX13, WNT4, WNT5A, and WNT7A) and their concentration was identified in neo-vaginal tissue.

Materials and Methods

The present study design is a controlled clinical study. A total of 75 individuals with congenital absence of vagina (MRKH syndrome) were treated with laparoscopic peritoneal pull through technique from 2013 to 2019. The stemness marker and gene expression study were conducted in the National Institute. All study patients were counseled and offered different treatment procedures for neo-vagina creation. Written informed consent was obtained from all patients before performing LPV. Institutional authority approved our study after departmental peer review and ethical consideration. The metaplastic conversion of the peritoneum to normal vagina and identification of the progenitor cell has already been published.[13]

In all the 75 participants, the neo-vagina was subjected to anatomical and physiological parameters confirmation. These were the histology, pH, cytology, Doppler blood

Table 1: Translational Stemness Markers with their primer sequence

Gene name	Primer Sequence (5'-3')
Sox2	Forward primer-ACT TTT GTC GGA GAC GGA GA
	Reverse primer-GTT CAT GTG CGC GTA ACT GT
Oct4	Forward primer-GGA GAA GCT GGA GCA AAA CC
	Reverse primer-TGG CTG AAT ACC TTC CCA AA
Nanog	Forward primer-GAT TTG TGG GCC TGA AGA AA
	Reverse primer-CTT TGG GAC TGG TGG AAG AA
GAPDH	Forward primer-TGT TGC CAT CAA TGA CCC CTT
	Reverse primer-CTC CAC GAC GTA CTC AGC G

flow, and 3-D ultrasound examination. All the parameters confirmed normal vaginal development by metaplasia of peritoneum.

Out of the 75 participants, subjects (T15 and T9) were selected. These two participants underwent serial neo-vaginal biopsies for the quantification of translational stemness markers. Participant T15 underwent biopsies on day: 0, 7, 12, 21, and 33, while in participant T9, neo-vaginal biopsies samples were collected on day: 0, 9, 14, and 21 and subjected for RNA isolation and cDNA synthesis. The presence of translational stemness markers (NANOG, OCT4 and SOX2) expression was identified by using the conventional PCR. The quantitative expression of stemness markers was done by using real-time PCR (standard curve method). The standard curve was run using pooled cDNA from all the samples in equal quantity. The absolute quantification for translational stemness markers [enlisted in Table 1 with their primer sequence] was performed by qRT-PCR using Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix (Agilent Technologies) and were normalized with GAPDH. The thermal cycling conditions were: initial denaturation at 94°C for 10 min, 40 cycles of 94°C for 10 s, annealing at 62°C for 30 s. The absolute translational stemness markers expression of SOX2, OCT4, and NANOG was determined by standard curve method.

Out of the 75 participants 15 participants of MRKH (T1–T15) operated by modified LPV along with 12 normal controls were selected and subjected to vaginal biopsy to check the expression pattern of HOXA and WNT genes involved in Mullerian development process. The patient samples were collected 3 months post surgery. Total RNA was extracted by TrizolTM method and cDNA was prepared using Superscript III (Invitrogen) Reverse transcriptase enzyme. The expression of candidate genes (viz., HOXA9, HOXA10A, HOXA11, HOX13, WNT4, WNT5A, and WNT7A) was done using Taqman chemistry. The data were normalized using 18s rRNA as a reference housekeeping gene. The delta-delta ct was calculated against the expression level of control.

Table 2: The identity document for each gene

Gene name	Product ID
18s	Hs03928989_g1
HOXA9	Hs04931836_s1
HOXA10	Hs00172012_m1
HOXA11	Hs00194149_m1
HOXA13	Hs00426284_m1
WNT4	Hs01573505_m1
WNT5A	Hs00998537_m1
WNT7A	Hs01114990_m1

Quantitative gene expression method

Total RNA from the neo-vaginal tissue sample was extracted using the guanidinium-phenol-chloroform method. Tissue sample was homogenized in TRIzol® reagent and was further extracted in chloroform and Isopropanol. The RNA pellet was washed in 75% ethanol and reconstituted in Diethyl pyrocarbonate (DEPC)-treated water and stored in −80°C till use. The RNA purity and concentration were determined on Nano spectrophotometer (BioTek, USA). About 2 µg of RNA was used to synthesize cDNA using SuperScript™ VILO™ cDNASynthesis Kit as per manufacture's protocol. The qRT-PCR was performed in AriaMx RT-PCR system (Agilent Technologies) using TaqMan® Universal Master Mix II, withUNG (Applied Biosystems). The relative expression of the target genes, viz., HOXA9, HOXA10, HOXA11, HOXA13, Wnt4, Wnt5A, and Wnt7A was determined using TaqMan® Gene Expression Arrays normalized to 18s rRNA. The identity document for each gene is listed in Table 2. The thermal cycling conditions were set as: with Uracil-DNA glycosylases (UDG) incubation at 50°C for 2 min; polymerase activation at 95°C for 10 min followed by 40 cycles of 95°C for 0.15 s, and annealing at 60°C for 1 min. The amplification reaction was run in triplicates with a no-template control. The relative gene expression variations were evaluated in term of fold change respect to the control samples using 2-ΔΔCt method. Briefly, the Ct values obtained for the target genes were normalized against the endogenous control 18s rRNA to obtain relative gene expression.

Results

Out of 75 participants, two (T15 and T9) were selected. These two underwent serial neo-vaginal biopsies for the quantification of translational stemness markers. Participant T15 underwent biopsies on day: 0, 7, 12,

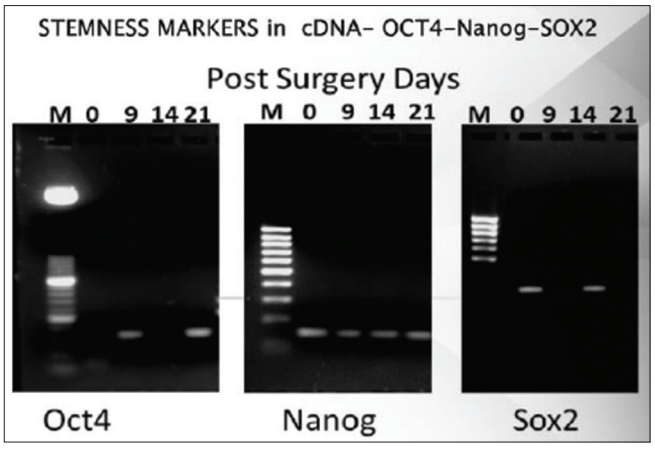

Figure 1: Translational stemness markers (NANOG, OCT4, and SOX2) expression

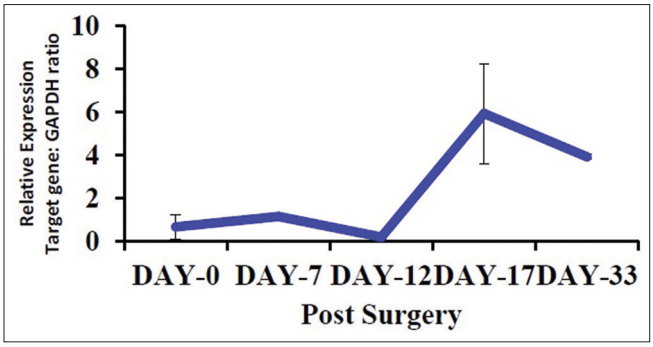

Figure 2: Expression of stemness Marker-Nanog participant T15

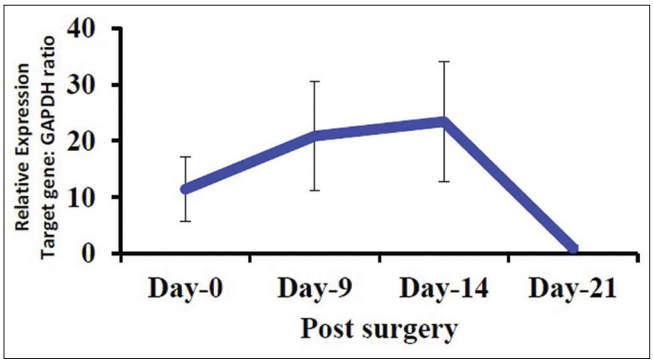

Figure 4: Expression of stemness Marker-OCT4 participant T9

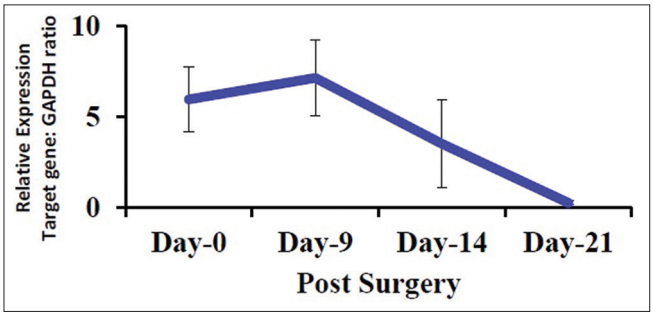

Figure 6: Expression of stemness Marker-Sox2 participant T9

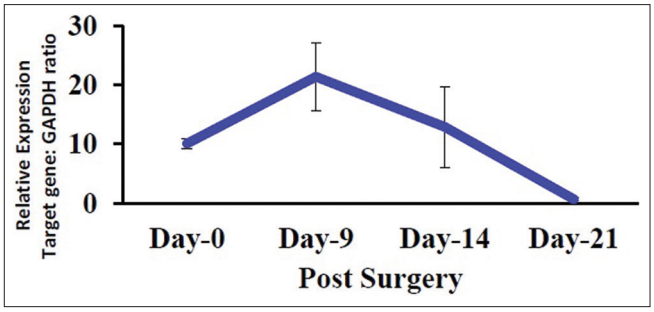

Figure 3: Expression of stemness Marker-Nanog participant T9

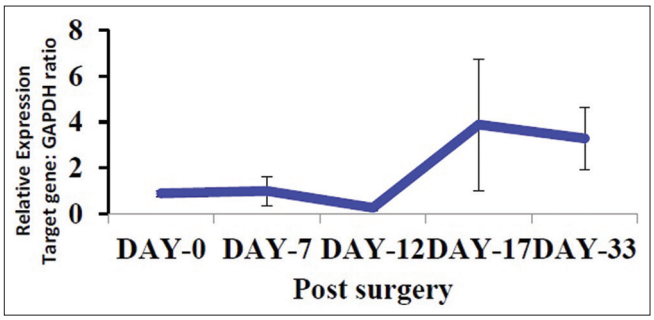

Figure 5: Expression of stemness Marker-OCT4 participant T15

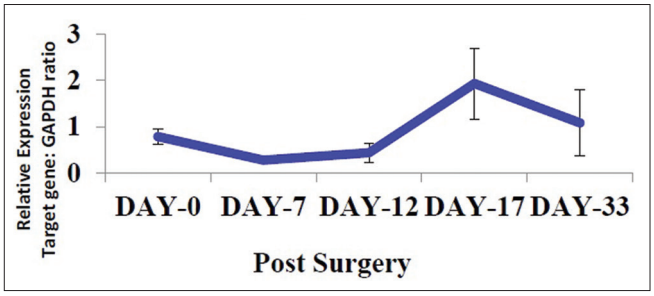

Figure 7: Expression of stemness Marker-Sox2 participant T15

21, and 33, while in participant T9 neo-vaginal biopsies samples were collected on day: 0, 9, 14, and 21 and subjected for RNA isolation and cDNA synthesis.

The presence of translational stemness markers (NANOG, OCT4, and SOX2) expression was shown by using the conventional PCR [Figure 1].

In both the participants, the expression of NANOG, OCT4, and SOX2 was found to be at basal level on day 0 postsurgery. As the days advanced, the expression of these translational stemness markers was rising and was at peak on day 9 and day 17, respectively. Subsequently, the expression of these translational stemness markers had gone down to the basal level, as the differentiation process gets completed. They act as translational factors to activate specific genomes to initiate the process of perotineal metaplasia and converting it into vaginal epithelium. The results are shown in Figures 2-7.

Fifteen participants of MRKH operated by modified LPV and 12 normal subjects were subjected to vaginal biopsy to check the expression pattern of HOXA and WNT genes involved in Mullerian development process. The expression of candidate genes (viz., HOXA9, HOXA10A, HOXA11, HOX13, WNT4, WNT5A, and WNT7A) is shown in Figures 8 and 9. The expression of developmental genes namely HOXA9, HOXA10A, HOXA11, and HOX13 in 14 participants were found to be lesser than the control samples as expected. The expression of WNT4, WNT5A, and WNT7A was found to be increased compared to the control

However, participant no. T5 showed altogether different results. Almost all the developmental genes namely HOXA9, HOXA10A, HOXA11, HOX13, WNT4, WNT5A, and WNT7A were expressed in very high

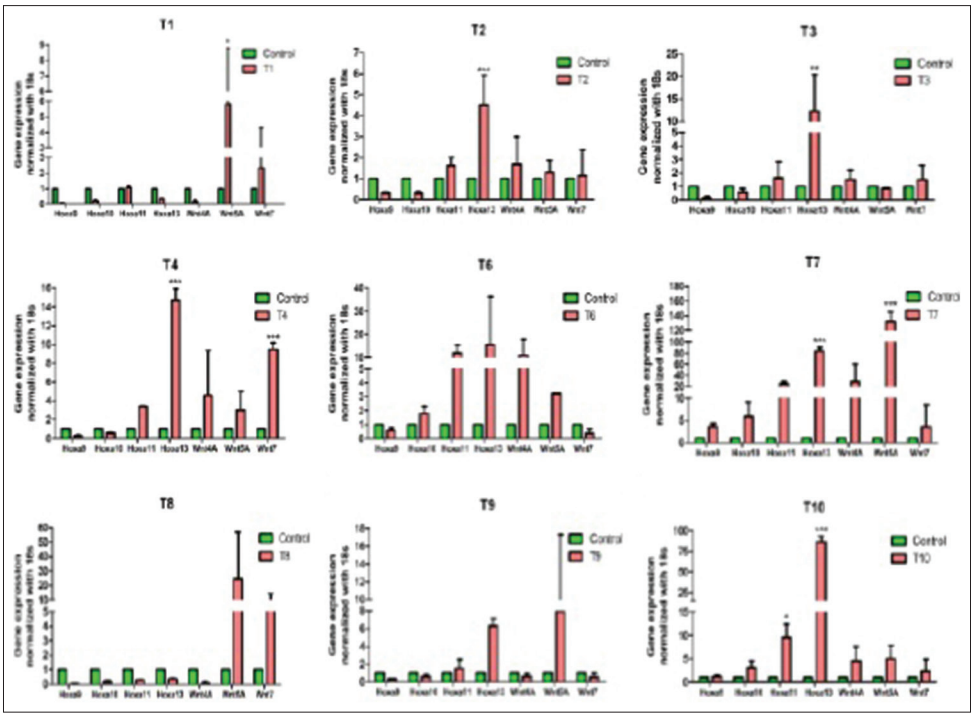

Figure 8: The expression of candidate genes (viz., HOXA9, HOXA10A, HOXA11, HOX13, WNT4, WNT5A, and WNT7A) in participants T 1–4 and T6–10

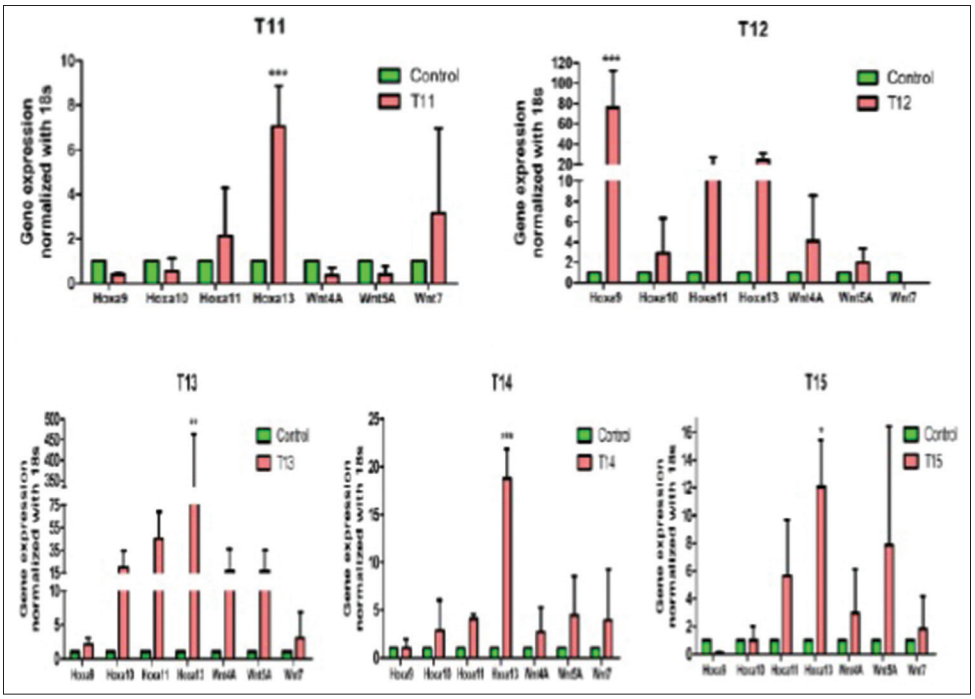

Figure 9: The expression of candidate genes (viz., HOXA9, HOXA10A, HOXA11, HOX13, WNT4, WNT5A, and WNT7A) in participants T 11-14

quantum [Figure 10]. These results were reconfirmed to avoid procedural error. All the results were statistically significant. The average conversion time of peritoneum to vaginal epithelium takes about 2–3 months by then it achieves all the anatomical and physiological characters.[13] The only clinical difference in participant no. T5 was a rapid metaplastic conversion of peritoneum to vagina. In participant no. T5, this conversion time was 1 month. These high values of all the developmental genes in participant T5 is difficult to explain and remains an enigma.

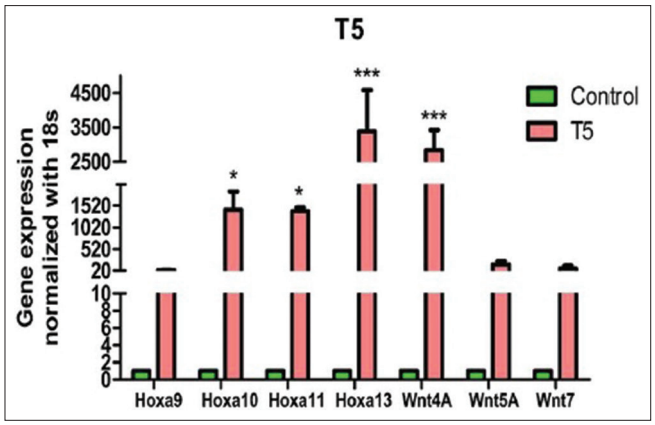

Figure 10: The expression of candidate genes (viz., HOXA9, HOXA10A, HOXA11, HOX13, WNT4, WNT5A, and WNT7A) in participant T5

Discussion

1. Principal findings – The study shows peritoneal metaplastic conversion to normal vagina by LPV. The translational stemness markers (NANOG, OCT4, and SOX2) and expression of the specific genes (WNT4, WNT5A, and WNT7A) responsible for the neo-vaginal formation were identified
2. Results – The translational stemness markers (NANOG, OCT4, and SOX2) appearance and concentration at different stages of conversion were demonstrated. The neo-vagina has shown upregulation of these translational stemness markers. The expression of developmental genes namely HOXA9, HOXA10A, HOXA11, and HOX13 was found to be lesser than the control samples as expected. The expression of WNT4, WNT5A, and WNT7A was found to be increased compared to the control
3. Clinical implications – The study confirms the normal vaginal development by peritoneal metaplasia. This technique should replace the current treatment of absent vagina
4. Research implications – Furthering this research, manipulating and activating these genes by genetic engineering may lead to the treatment of developmental defects of Mullerian duct
5. Strength and limitation – The role of stemness markers and specific genes in formation of neo vagina was confirmed. However, one participant showed altogether different results. Almost all the developmental genes were expressed in very high quantum. These high values of all the developmental genes in this participant is difficult to explain and remains an enigma.

Conclusions

The study shows peritoneal metaplastic conversion to normal vagina by LPV. The progenitor cell responsible for the metaplastic conversion was identified in the peritoneum and different stages of neo-vaginal development.[23] The translational stemness markers NANOG, OCT4, and SOX2 responsible for the neovaginal formation were identified. Their appearance and concentration at different stages of conversion were demonstrated. The neo-vagina has shown upregulation of these translational stemness markers. The study demonstrates the expression of the specific genes (WNT4, WNT5A, and WNT7A) and their role in the formation of the neo-vagina. Furthering this research, manipulating and activating these genes by genetic engineering may lead to the treatment of developmental defects of Mullerian duct and developing the rudimentary uterus to normal size, obviating the need of transplant. To the best of our knowledge, this study is the first to show the metaplastic conversion of peritoneum to normal vagina, identifying the progenitor cell, expression of translational stemness markers, and upregulation of specific genes.

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Conflicts of interest

There are no conflicts of interest.

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