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Establishment and Characterization of Vaginal Tissue Primary Culture: Feasibility of Cell Therapy for Mayer-Rokitansky-Kuster-Hauser Syndrome (MRKHS) Patient Treatment

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Abstract

Title: Establishment and Characterization of Vaginal Tissue Primary Culture: Feasibility of Cell Therapy Usage in Mayer-Rokitansky-Kuster-Hauser Syndrome (MRKHS) Patient´s Treatment.

Background: The uterine and vaginal agenesis, Mayer-Rokitansky-Küster-Hauser Syndrome (SMRKH), is a congenital malformation that implies in the impairment of sexual activity and reproductive life. The first line therapy is nonsurgical (dilatation method) but surgical methods may be necessary to create a functional neovagina. In the surgical procedure, various types of graft materials have been used to line the neovagina, and recently, alternative grafts have been studied to minimize adverse effects. Our aim was to establish vaginal primary cultures in order to prove the feasibility of cell therapy for the reconstruction of the vaginal lining.

Methods and findings: Nine small biopsies were processed according to explant technique in order to isolate vaginal cells (VC). Different cell types were obtained, including fibroblast and epithelial cells, according to optical microscopy, flow cytometry and immunofluorescence assessment. These cells could not differentiate into mesenchymal lineages (adipogenic, chondrogenic and osteogenic) but are able to form tissue-like cell aggregates. The expression of telomeres and telomerase genes were similar to the endometrial fibroblast.

Conclusion: We are able to obtain and characterize vagina-derived cells in primary cultures. The isolated cells seem to be good candidates to cell therapy usage in MRKHS as they have phenotypic and physiological characteristics suitable to the reconstruction of vaginal lining.

Keywords Autologous transplantation; Mayer-Rokitansky-Küster-Hauser syndrome; Vaginal agenesis; Vaginoplasty

Abbreviations

EF: Endometrial Fibroblasts; Emsc: Endometrial Mesenchymal Stem Cells; MF: Mammary Fibroblasts; MRKHS: Mayer-Rokitansky-Kuster-Hauser Syndrome; VC: Vaginal Cells

Introduction

Mayer-Rokitansky-Küster-Hauser syndrome (MRKHS) is characterized by congenital aplasia of the uterus and the upper two-thirds of the vagina in women with normal secondary sexual characteristics. MRKHS may be associated with vaginal and uterine abnormalities, but other associated malformation can be found, such as renal or skeletal malformation [1]. MRKHS has an incidence of 1:1500 to 1:4000 female births [2], and it is the second most common cause of primary amenorrhea. The syndrome has a significant influence on both physical and psychosocial women's health, due to sexual and reproductive impairment.

The goal of the treatment, either clinical or surgical, has been to create an anatomically and functionally vagina, adequate for a satisfactory sexual intercourse and covered by an epithelium with the same characteristics of the original as to appearance, lubrication and protective barrier against infectious diseases. In addition, the procedure ideally should be able to be permanent and require minimal use of dilators.

In order to contribute to this goal our study proposed to establish the use of autologous vaginal cells for the canal lining in patients with MRKHS subjected to vaginoplasty.

Several techniques have been reported for the creation of neovagina. Noninvasive methods are based in progressive dilation of the vaginal rudiment [3], whereas, in the invasive methods, the aim is the surgical creation of a neovagina. The Abeè-McIndoe vaginoplasty is the most common procedure and consists in the creation of a canal between the bladder and the rectum, which is covered with a skin graft.

The use of materials other than the skin graft avoids the presence of visible scars and leads to the formation of a vaginal canal lined by epithelium similar to the original. In the last decades modifications of the original technique, changing the tissue adopted for the canal lining have been attempted, and several materials have been used, including amniotic membrane [4], cellulose [5], peritoneum [6] and acellular grafts [7,8].

The vaginal organ is composed of several layers, including epithelia, muscle and a matrix with various structural proteins such as collagen, elastin and microfibrils [9]. After the vaginoplasty, some patients might have the potential to develop chronic stenosis or graft contracture, or both, possibly requiring long-term periodic dilation. These complications can be explained by the fact of the grafts used may promote adequate epithelial function and regeneration, but this tissue does not present all the vaginal layers of the original tissue [10]. Besides that, the epithelization occurs from vaginal vestibule and usually requires several months.

Recently the use of autologous tissue has been proposed. In 2007 the first case of in vitro autologous vaginal tissue transplantation was described [11] and in 2015 the same authors showed, a series of 23 women who underwent neovaginoplasty, with safe and feasible [12].

Raya-Rivera et al. in turn, proposed the use of engineered autologous tissue and showed that vaginal organs could be created using autologous muscle and vaginal mucosal cells in a pilot cohort study with 4 patients [10].

More recently, Orabi et al. using the self-assembly technique created, after in vivo animal implantation, vaginal tissues that formed epithelium, basement membrane and stroma comparable to native vaginal tissues [13].

We aimed in this study to establish vaginal primary cultures to test the feasibility of cell therapy usage for autologous transplantation. We hypothesize that a mixture of original tissue cells will be better to patient's recovery. In order to attain these objectives we isolated through explant technic vaginal cells and characterize the obtained cells phenotype by flow cytometry, immunofluorescence and differentiation assay. We also perform telomeres and telomerase gene expression, in order to identify the proliferation ability of these cells. The cells were assayed in low-attachment cultures demonstrating ability to form tissue-like structures endorsing the feasibility of these cells usage in MRKHS women treatment **(Figure 1)**.

Methods

Human tissues and cell culture

Vaginal tissue was obtained from patients who had indication for surgical correction of genital prolapse. Excess tissue of the lower third of the vagina, which is usually discarded in the surgery, were sampled in 1cm^2 pieces and immediately transferred into a sterile tube containing sterile phosphate-buffered saline (PBS 1X) with 400 µ/mL of penicillin and 400 µg/mL streptomycin and stored at 4ºC and processed within 2 hours. After several washes in PBS with 200 µ/mL of penicillin and 200 µ/mL streptomycin, the tissue was minced into small pieces which were put in culture plates with DMEM/F-12 (Dulbecco's Modified Eagle Medium: Nutriente Misture F-12 - (GIBCO, CA, EUA) supplemented with 20% of fetal bovine serum (GIBCO, CA, EUA) and 100 µ/mL of penicillin and 100 µg/mL streptomycin. This medium allowed the cellular migration from the explants to the plate.

Study flow-chart

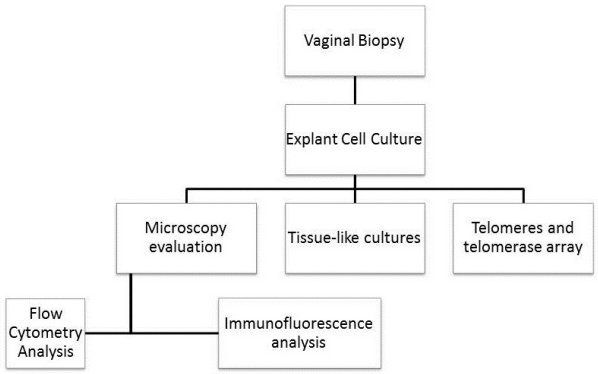

Figure 1: Study flow-chart.

The culture plates were maintained in a 5% CO_2 incubator at 37°C in a humidified atmosphere. The medium was changed twice a week. After cells reached 70% of confluence, the explants were removed and the cells grew into the remaining spaces. After an average of 48 hours, when reaching 80-90% confluence, vaginal cells (VC) were detached with Trypsin -EDTA 0.25% (GIBCO, CA, EUA) for 5 min at 37°C.

This procedure was monitored using the optical microscopy, after trypsin inactivation (DMEM: F12 10%FBS) the cells are transferred to culture flasks. Cells were stored in liquid nitrogen.

Table 1: Surface and Cytoplasmatic markers fluorochrome conjugated antibodies.

Primary Antibodies	Isotype	Clone	Fluorochrome*	Source

CD9	Mouse IgG1, κ	M-L13	PE	BD Bioscience
CD29	Mouse IgG1, κ	MAR4	PE	BD PharmigenTM
CD34	Mouse IgG1, κ	581	PE-CyTM7	BD PharmigenTM
CD73	Mouse IgG1, κ	AD2	PE	BD PharmigenTM
CD90	Mouse IgG1, κ	5E10	FITC	BD PharmigenTM
CD105	Mouse IgG1, κ	266	PerCP-CyTM5.5	BD PharmigenTM
PDGFR-Rβ	Mouse IgG1, κ	J105-412	Alexa Fluor® 647	BD PharmigenTM
CD1d	Mouse IgG1, κ	CD1d42	PE	BD PharmigenTM
Vimentin	Mouse IgG1	RV202	FITC	BD PharmigenTM
CD227	Mouse IgG1, κ	HMPV	FITC	BD PharmigenTM
Cytokeratin	Mouse IgG2a, κ	CAM 5.2	PE	BD PharmigenTM
*FITC- Fluorosceinisothiocyanate, PE- Phycoerythrin, PerCP- Peridininchlorophyll, APC- allophycocyanin.				

Flow cytometry

VC were incubated with directly conjugated antibodies for surface and cytoplasmic markers for 1h at 4°C and analyzed by flow cytometry using a FACS CANTO II - 6-colour flow-Cytometer (BD Bioscience).

The experiment control are performed using isotype controls conjugated with the same fluorochromes used (BD Bioscience). The data were analyzed in FlowJo X 10.0.7 (Tree Star Inc.) **(Table 1).**

Immunofluorescence assay

VC was cultured in 12 well plates until 70% of confluence. The cells were fixed with 4% paraformaldehyde (PFA), permeabilized with PBS 0.1% of Triton X-100, blocked with PBS 2% BSA (bovine serum albumin) and then stained with anti-vimentin - Alexa 488 (BD Pharmigen) and anti-cytokeratin – PE (BD Pharmigen). The nuclei were stained with DAPI (Sigma-Aldrich). IgG1 conjugated with Alexa 488 and PE antibodies were used as control.The images were captured using a Zeiss Observer I Fluorescence Microscope and the images were analyzed in AxioVision SE64 4.9.1 (Carl Zeiss Microscopy GmbH 2011).

In vitro differentiation

VC were expanded in DMEM:F12 with 10% FBS were seeded in 24-well plates (5000 cell/cm^2) and cultured in specific differentiation-induction media **(Table 2)** for 5 weeks (adipogenic), 3 weeks (osteogenic) and 2 weeks (chondrogenic). Media were changed every 2-3 days.

Undifferentiated control cells were cultured concurrently in low serum medium (1% FBS) for the same incubation time. Endometrial mesenchymal stem cells were concurrently assayed as positive control in the same differentiation-induction media. Cells were fixed with 4% paraformaldehyde and stained by histochemical methods **(Table 2)**.

Table 2: Differentiation media and staining.

Lineage	Media	Serum	Supplementation	Staining
Control	DMEM pH7.4	FBS 1%	None	All
Adipogenic	DMEM pH7.4	FBS 10%	0.5 µmol/L Isobutyl-methylxanthine	Oil Red O (1%)
			1 µmol/L Dexamethasone	
			200 µmol/L Indomethacin	
			10 µmol/L Insulin	
Osteogenic	DMEM pH7.4	FBS 10%	10 µmol/L 1 α-25-dihydroxyvitamin-D3	Alizarin Red S (2%)
			50 µmol/L Ascorbic acid-2-phosphate	
			10 µmol/L B-glycerophosphate	
Chondrogenic	DMEM pH7.4	FBS 1%	6.25 µg/mL Insulin	Acidified Alcian Blue (1%)
			10 µg/mL Transforming growth factor-β1 (TGFβ1)	
			50 µmol/L Ascorbic acid-2-phosphate	

Cell aggregates assay

To obtain the VC aggregates 10,000 cells (suspended in DMEM: F12 10% FBS) were seeded in 24-well ultra-low adhesion plates and incubated at 37°C, 5% CO_2. The VC aggregates were detected by microscopy and reported in the day 0, 2, 4, 7, 10, 15 and 20 of culture. Fresh media were added every 2-3 days.

Polymerase chain reaction array

The total RNA from each sample was extracted using the TRIzol Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), following the manufacturer's instructions. The RNA concentration and quality (260/280 ratio) were assessed using a Nano Drop instrument (Thermo Scientific, Wilmington, DE, USA). Reverse transcription was performed with 0.5 µg of total RNA to generate double-stranded complementary DNA (cDNA) using an RT2 First Strand Kit (SA Biosciences, Qiagen).

Real-time quantitative polymerase chain reaction (RT^2 qPCR) assays were performed to evaluate gene expression. We used PCR arrays for Human Telomerase and Telomeres (PAHS-010Z, RT^2 Profiler™ PCR Array, and SA Biosciences/Qiagen). The 96-well PCR array plates were assayed using a Step One plus Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA). We obtained cycle threshold (Ct) values for the genes under investigation, and the ΔΔCt method was utilized for gene expression analysis using the PCR Array Data Analysis Web Portal (SA Biosciences). Were assayed three (3) VC samples and the mean results compared to endometrial fibroblasts (EF), mammary fibroblasts (MF) and endometrial mesenchymal stem cells (eMSC). Genes that presented expression levels with a fold change of at least ≥ 2 were considered to be differentially expressed. The differentially expressed genes are discussed based on their function, as determined using an online database.

Ethical approval

The study protocol was approved by São Paulo Federal University ethical committee (CEP nº17451) and informed written consent was obtained from all nine patients before the enrollment. The procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional or regional) and with the Helsinki Declaration of 1975, as revised in 1983.

Results

Vaginal cell isolation and characterization

The explant technique demonstrated to be a good strategy in order to obtain different primary vaginal derived cells. **Figure 2** demonstrated these cells and highlights the morphological different cells obtained. Also, is possible to observe the layering of the cells, probably to feed cell types that do not have the ability to attach to culture flasks. All samples presented at least 85% of viability in the first passage.

The VC obtained was characterized by flow cytometry indicating that the fibroblasts are the main population. **Figure 3** demonstrates the expression of membrane and cytoplasm markers detected. The main population (58.87%, SD=6.91) of the cells expresses the markers CD90, CD73, CD105; as expected for fibroblast vimentin (80.37%, SD = 3.71) and CD29 (73.13%, SD = 19.20) were highly expressed. The VC have low expression of CD34 (4.61%, SD = 1.55) and no expression of PDGFR-Rβ (0.50%, SD = 0.31). The epithelial markers CD9 (74.73%, SD = 4.96) and CD227 (80.30%, SD 8.65) were highly expressed and CD1d (5.73%, SD 2.28) and cytokeratin (5.36%, SD 5.86) were low expressed. The vimentin and cytokeratin co-expression were also low, 5.09% (SD 5.96). Almost all the cytokeratin positive cells expresse vimentin which could indicate an epithelial to mesenchymal cell transition. Concurrently the immunofluorescence assay demonstrated the co-expression of cytokeratin and vimentin, with the majority of the cells labeled with anti-cytokeratin antibody **(Figure 4).**

The VC primary cells are unable to differentiate into mesenchymal lineages (adipogenic, chondrogenic and osteogenic) as reported in the **Figure 5.**

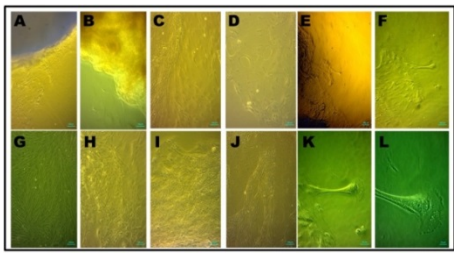

Figure 2: Vaginal Cells Morphology. Legend: A and B, cells migrating from explants after 7 days of culture. C, D, I and J images from different samples after 14 days of culture. Cell layers supporting the growth of morphologically different cells. E, F, K and L shows the same sample after 14 days of culture, in L it is possible to observe cytoplasma prolongations characteristic of the cell migratory process. G and H after 28 days, cultures showed cells with different morphologies.

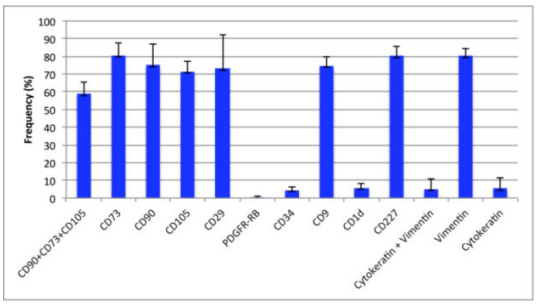

Figure 3: Flow Cytometry results. Chart indicating the mean expression of each cell marker expression detected by flow cytometry: CD1d, CD9, CD29, CD34, CD73, CD105, CD227, PDGFR-Rβ, Vimentin and Cytokeratin. The x axis indicates each marker and the y axis represents frequency % of each marker expression. The bars represent standard deviation.

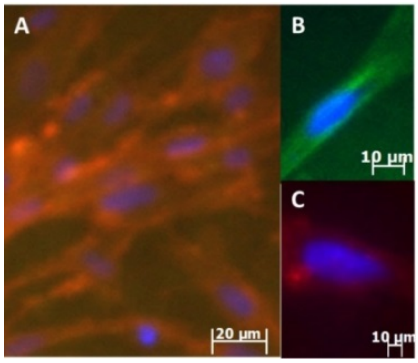

Figure 4: Immunofluorescence images for vimentin-Alexa488 and cytokeratin-PE stained vagina derived cells. Legend: A-Fluorescence detected in the UV, green and red channels merged, nuclei stained with DAPI, vimentin filaments stained with anti-vimentin conjugated with Alexa-488 fluorochrome and cytokeratin filaments stained with anti-cytokeratin conjugated with PE. B – Fluorescence detected in the UV and green channels merged image – nuclei stained with DAPI and vimentin filaments stained with anti-vimentin conjugated with alexa-488 fluorochrome. C-Fluorescence detected in the UV and red channels merged image-nuclei stained with DAPI and cytokeratin filaments stained with anti-cytokeratin conjugated with PE fluorochrome. Controls were made using IgG1 isotyping control-Alexa 488 and PE (data not shown). (A)Bars=20 µm, (B) and (C) Bars=10 µm. The images are representative for all samples.

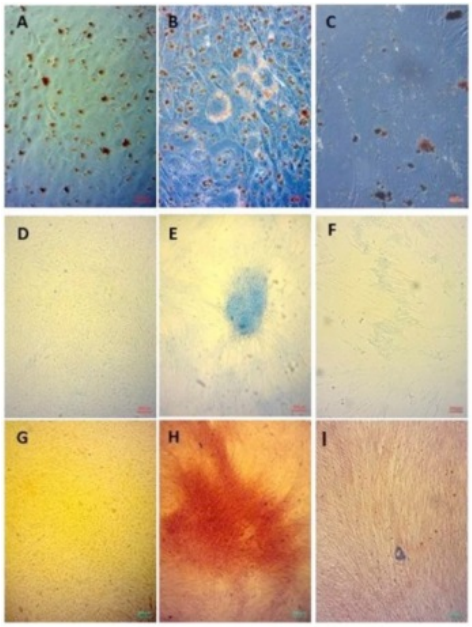

Figure 5: Multilineage differentiation of Vagina-derived cells. Non induced controls stained with Oil Red O (A), Alcian blue (D) and Alizarin Red S (E). Differentiated eMSC: (B) Adipogenic differentiation visualized by Oil Red O staining of lipid droplets, (E) Chondrogenic differentiation visualized by Alcian blue staining of glycosaminoglycans and proteoglycans typical of chondrogenic tissues and (H) Osteogenic differentiation visualized by Alizarin Red S staining of calcium deposits characteristic of bone tissues. Induced VC demonstrating no Adipogenic (C), Chondrogenic (F) or Osteogenic (I) differentiation. Showed results are representative of all patients.

Tissue like VC *in vitro* assembly

The VC primary cells isolated were able to assemble a tissue-like structure in ultra-low attachment culture system. The **Figure 6** shows that the initial plated cells are not in contact with each other or cluttered. In the day 2 we could observe the primary clusterization. In the images that follows, the cells still dividing and a tissue-like structure is assembled. In the day 20, the structure is large enough to be naked eye detected.

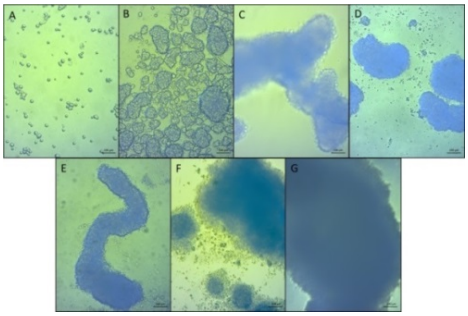

Figure 6: Tissue-like aggregates assembled from vagina-derived cells. (A) Image of cells seeded (Day 0). (B) Initial clusterization of the VC (Day 2). (C) VC aggregates formation (Day 4). (D) VC aggregates (Day 7). Tissue-like structures: (E) - Day 10, (F) – Day 15 and (G) – Day 20. Bars=100 µm. Showed results are representative of all patients.

Telomeres and telomerase genes expression

In order to identify the proliferative capability as well as the stem potential of the cells we perform the Real-time quantitative polymerase chain reaction (RT^2 qPCR) using the Human Telomerase and Telomeres (PAHS-010Z, RT^2 Profiler™ PCR Array, SA Biosciences/Qiagen). We choose endometrial fibroblasts (EF), endometrial mesenchymal stem cells (eMSC) and mammary fibroblasts (MF) to compare with VC as these cells have similar molecular phenotype, are in a contiguous tissue (EF and eMSC) or are also obtained from a gynaecological tissue. The VC appears to be similar to EF and eMSC. VC have less 2.23 fold Heterogeneous nuclear ribonucleoprotein A2/B1 (HNRNPA2B1) expression when compared to EF and 2.28 fold less expression compared to eMSC. As reported in the **Figure 7**, the genes HSP90AA1 (Heat Shock Protein 90 Alpha Family Class A Member 1) and PPP2R1A (Protein Phosphatase 2 Scaffold Subunit Alpha) were upper expressed in the VC (HSP90AA1 = 2.28 fold; PPP2R1A = 2.85 fold). The VC presented more differences with MF having three genes down-expressed: TGFβ1 = 2.84 fold, TP53 = 2.22 fold, TPP = 2.24 fold and one upper-expressed: XRCC5 = 2.24 fold.

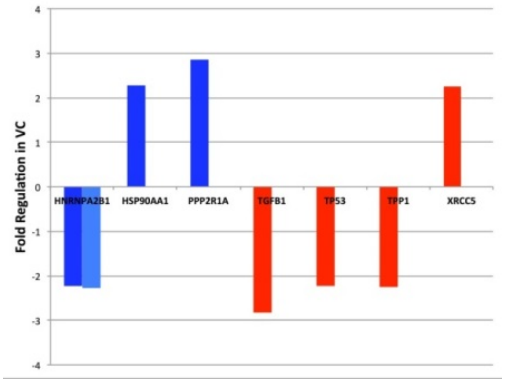

Figure 7: Differentially expressed genes between VC and EF, eMSC and MF. Significant fold changes in the VC compared with EF (light blue), eMSC (dark blue) and MF (red). qRT-PCR array analysis of the Telomeres and telomerase gene expression profile in the VC, EF and eMSC. The x axis indicates each gene and the y axis represents fold regulation of each gene.

Discussion

Explants technique is a well-established methodology to obtain primary cell cultures [14-16]. We established vaginal cultures, which showed heterogeneous morphology. After passaging, we observed that culture was becoming more homogeneous, possibly due to selection made by the medium used. Therefore, to prioritize enriched cultures with more than one cell type, we choose to freeze cells at low passages.

Previous publications [17-19] showed that the explant method has been successfully used to isolate stem cells from different tissues, being a relatively simple, easy and fast method. Ishige et al. [19,20], demonstrated that the explant method was better when compared to the enzymatic method for the isolation of mesenchymal stem cells from umbilical cord blood. We found at least three subpopulations, putative mesenchymal stem cells, fibroblasts and epithelial cells. Mesenchymal stem cells have been identified in several human tissues, including heart, liver, dental pulp, adipose tissue, umbilical cord, endometrial, and amniotic fluid [21-25]. The mesenchymal stem cells were characterized by the expression of CD90, CD105 and CD73 markers and absence expression of CD34 [26]; as we found in our samples. In contrast, Sidney et al. in 2014, showed that CD34 expression may be a marker for other strains that not only haemopoietic and corresponds to naïve cells [27]. Thus, in our findings we reported low expression of this marker meaning the presence of stromal cells (also confirmed by CD9 presence) no contamination with hematopoietic cells and absence of stem-like cells. That was confirmed by the inability of these cells to differentiate into mesenchymal lineages. In your study, we obtained more than 65% positive for vimentin marker, indicating that the main population isolated was fiblastoid. In previous study Skala et al. [28] isolated only fibroblast vaginal tissue by enzymatic method, to be used as lining for reconstructive surgery meshes. According to this and the ability of VC to form

tissue-like structures we assume that our model is also feasible to vaginal lining reconstruction.

MUC1 is distributed in various tissues of the human mucosa, including the female reproductive tract, and has been reported to high expression of mucin in the vaginal epithelial tissue [29,30] which would explain the high expression of CD227 marker (MUC1) found in our study. Kawana et al. showed different levels of CD1d expression in epithelial cells in the lower reproductive tract, ranging from high expression in the vagina, ectocervix and penile urethra and very low expression in endocervix and endometrium [31]. We found different results: low CD1d expression (ranging from 4% to 8%) in material obtained from patients with genital prolapse.

The mesenchymal epithelial transition (MET) is defined based on the change of morphology and organization of epithelial to mesenchymal cells, or motility based on molecular exchange. Typically E-cadherinae is acquired and mesenchymal markers such as N-cadherin and vimentin are lost [32]. The co-expression of vimentin and cytokeratin detected by immunofluorescence and flow cytometry indicate that possible, in culture environment, the VC are in MET. In spite of these, the cells are able to form tissue-like structures and do not have highly proliferative phenotype, confirmed by the absence of cell differentiation as well as expression of telomeres and telomerase genes similar to endometrial fibroblast cells, indicating the safeness of this cells for cellular therapy.

Conclusion

In the present study we were able to establish primary cultures of vaginal cells and their phenotypic characterization was favorable to their possible use for vaginal reconstruction surgery. Autologous vaginal tissue cultured in vitro seems to combine the best features to be used in reconstructive surgery. Further studies will be necessary to characterize the biology of these cells in order to certify the feasibility of this approach for patient's usage.

Future Directions

To confirm the feasibility of the explant obtained vaginal cells for MRKHS cell therapy must be assayed in support structures and therefore in animal models.

Funding

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Competing and Conflicting Interests

The authors declare no conflicts of interest.

Author´s Contributions

ALI performed the flow cytometry, differentiation and ultra-low attachment experiments, helped with culture experiments, conceived and revised the manuscript.

PBR conceived and revised the manuscript and performed culture experiments.

TPV performed culture experiments and drafted the manuscript.

CTN collected the samples and revised the manuscript.

MJBCG conceived and revised the manuscript.

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