Immunohistochemical and ultrastructural identification of telocytes in the lamina propria of human vaginal mucosa

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ARTICLE INFO

ABSTRACT

Since their relatively recent discovery, telocytes (TCs) have been described as peculiar cells strategically positioned in the stromal tissue component of multiple organ systems of the mammalian body including female reproductive organs (i.e., ovary, uterine tube, and uterus). Nevertheless, current knowledge of TCs in the vagina is very limited. The present study was therefore undertaken to investigate the existence and characteristics of TCs in the stromal tissue of human vaginal mucosa by means of immunohistochemistry, immunofluorescence confocal microscopy, and transmission electron microscopy. In the vaginal lamina propria, TCs were first identified by CD34 immunohistochemistry that revealed the presence of CD34^{+} stromal cells arranged in networks, especially around blood vessels. Double immunofluorescence confocal microscopy allowed to precisely distinguish the perivascular networks of CD34^{+} stromal cells lacking CD31 immunoreactivity from adjacent CD31^{+} microvessels. All the perivascular networks of TCs/CD34^{+} stromal cells situated in the vaginal lamina propria coexpressed platelet-derived growth factor receptor α, which strengthened their identification as TCs. Instead, vaginal mucosal TCs were immunophenotypically negative for c-kit/CD117. The ultrastructural examination confirmed the presence of TCs, namely stromal cells with characteristic cytoplasmic processes (i.e., telopodes) forming labyrinthine networks around blood vessels and releasing extracellular vesicles. Together, our morphological findings provide the first comprehensive demonstration that TCs reside in the human vaginal lamina propria, thus paving the way for further investigation of their putative functions in vaginal mucosal homeostasis and pathophysiology.

1. Introduction

Over the past decade, growing attention has been placed on telocytes (TCs) as cells characterized by a distinctive ultrastructural morphology – a small cell body giving rise to very long and slender prolongations (i.e., telopodes) with a characteristic moniliform/varicose shape conferred by the alternation of extremely thin segments (i.e., podomers) and small cistern-like dilated portions (i.e., podoms) – which are strategically positioned in the stromal compartment of numerous organs throughout the body (Cretoiu and Popescu, 2014; Kondo and Kaestner, 2019; Popescu and Faussone-Pellegrini, 2010). Indeed, TCs are finely organized, with their telopodes that build intricate interstitial networks bordering parenchymal cell structures, vessels, nerves, and muscle cells, and often establish physical intercellular contacts with other cells of the stromal microenvironment, especially tissue-resident stem/progenitor cells and cells of both the innate and adaptive immune systems (Kondo and Kaestner, 2019; Rosa et al., 2021). Besides such direct cell-to-cell communications, TCs are currently even more on the focus for their unique proneness to deliver molecular signals to neighboring cells through the release of different kinds of extracellular vesicles, which led to propose their implication in the regulation of tissue morphogenesis and homeostasis, as well as their participation to a variety of

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Table 1
List of primary antibodies, their sources and working dilutions.

Primary antibody	Host species	Catalog no.	Producer	Dilution
anti-CD34	Mouse	M7165	Dako, Glostrup, Denmark	1:50 (IHC, IF)
anti-CD31/ PECAM-1	Rabbit	ab28364	Abcam, Cambridge, UK	1:50 (IHC, IF)
anti-α-SMA	Rabbit	ab5694	Abcam, Cambridge, UK	1:100 (IF)
anti-PDGFRα	Goat	AF-307-NA	R&D Systems, Minneapolis, MN, USA	1:100 (IF)
anti-c-kit/ CD117	Rabbit	A4502	Dako, Glostrup, Denmark	1:200 (IF)

PECAM-1, platelet-endothelial cell adhesion molecule-1; α-SMA, α-smooth muscle actin; PDGFRα, platelet-derived growth factor receptor α; IHC, immunohistochemistry; IF, immunofluorescence.

regenerative or pathological processes (Bei et al., 2015; Cretoiu et al., 2016, 2020; Díaz-Flores et al., 2016, 2020, 2021; Kondo and Kaestner, 2019; Ibba-Manneschi et al., 2016; Marini et al., 2017; Shoshkes-Carmel et al., 2018).

Despite current challenges of studying TCs as a distinct stromal cell type are mainly related to the lack of specific markers such as cluster of differentiation (CD) antigens, it is now broadly recognized that they are CD34$^+$ and, indeed, are often referred to as TCs/CD34$^+$ stromal cells (Cretoiu et al., 2017, 2020; Díaz-Flores et al., 2014, 2020; Rosa et al., 2021). More precisely, the CD31$^-$/CD34$^+$ immunophenotype of TCs has proven useful to determine their distribution in many organs by distinguishing them from the frequently adjacent endothelial cells of blood microvessels that are CD31$^+$/CD34$^+$ (Manetti et al., 2019; Marini et al., 2018a, 2018b; Rosa et al., 2021, 2022). The coexpression of CD34 and platelet-derived growth factor receptor α (PDGFRα) also appears to be a
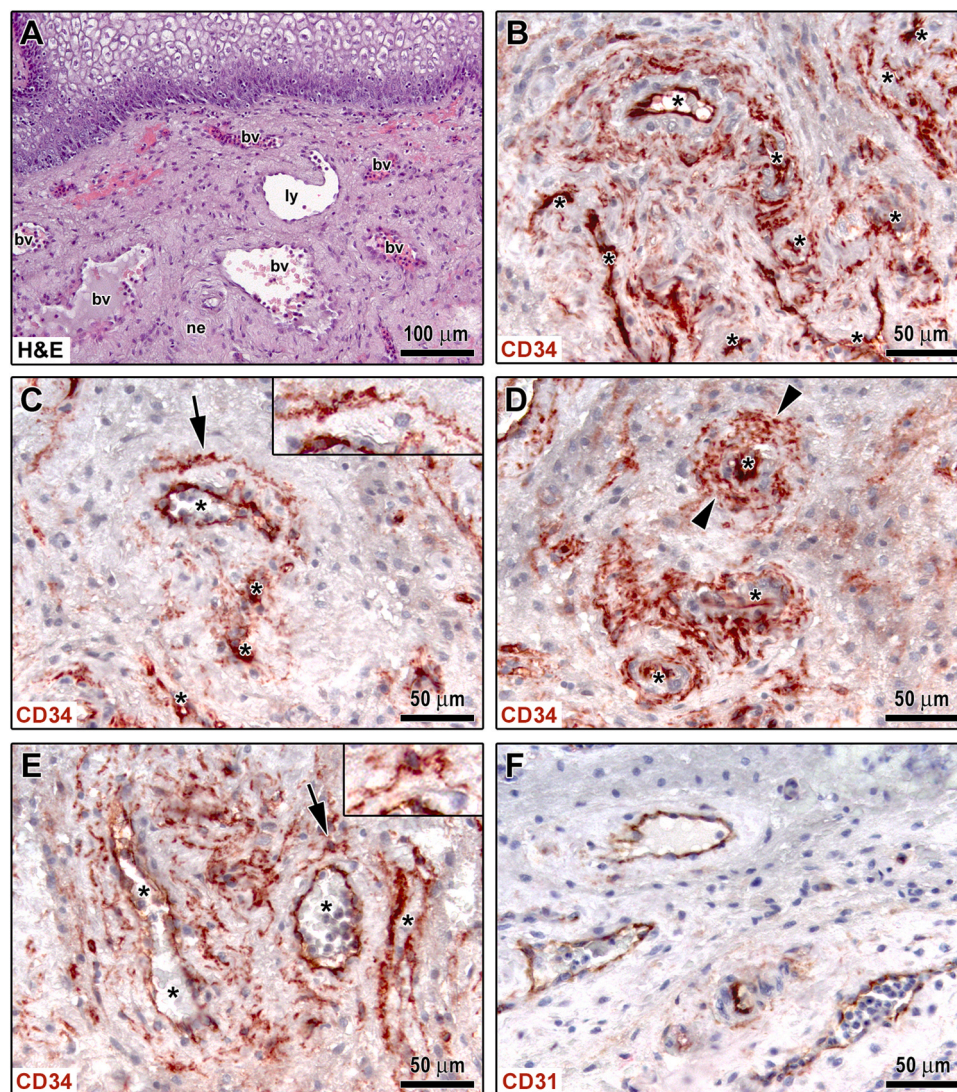

Fig. 1. Immunohistochemical identification of telocytes (TCs)/CD34$^+$ stromal cells in human vaginal mucosal tissue sections. (A) Hematoxylin and eosin (H&E) staining testifies the normal appearance of the vaginal mucosa consisting of a glycogenated stratified squamous epithelium standing above a dense fibrovascular connective tissue (i.e., lamina propria) containing abundant extracellular matrix fibers and blood vessels (bv), along with lymphatics (ly), nerves (ne), stromal cells, and mononuclear cells. (B–E) CD34 immunohistochemistry with hematoxylin nuclear counterstain shows the presence of networks of CD34$^+$ stromal cells, especially around blood vessels, in the vaginal lamina propria; CD34 immunoreactivity is found also in endothelial cells of blood vessels (asterisks). Insets in (C,E) represent higher magnifications of the tissue areas pointed by arrows; CD34$^+$ stromal cells display the typical TC morphology, that is spindle-shaped cells with long moniliform and sinuous processes. CD34$^+$ TCs often form multilayered labyrinthine networks around blood vessels (D, arrowheads). Representative photomicrographs of CD34 stained sections from three different vaginal mucosal samples are shown. (F) CD31/pan-endothelial marker immunohistochemistry with hematoxylin nuclear counterstain; CD31 immunoreactivity is detectable in endothelial cells forming the inner lining of vessels, but not in stromal cell networks. Scale bar: 100 µm (A), 50 µm (B–F).
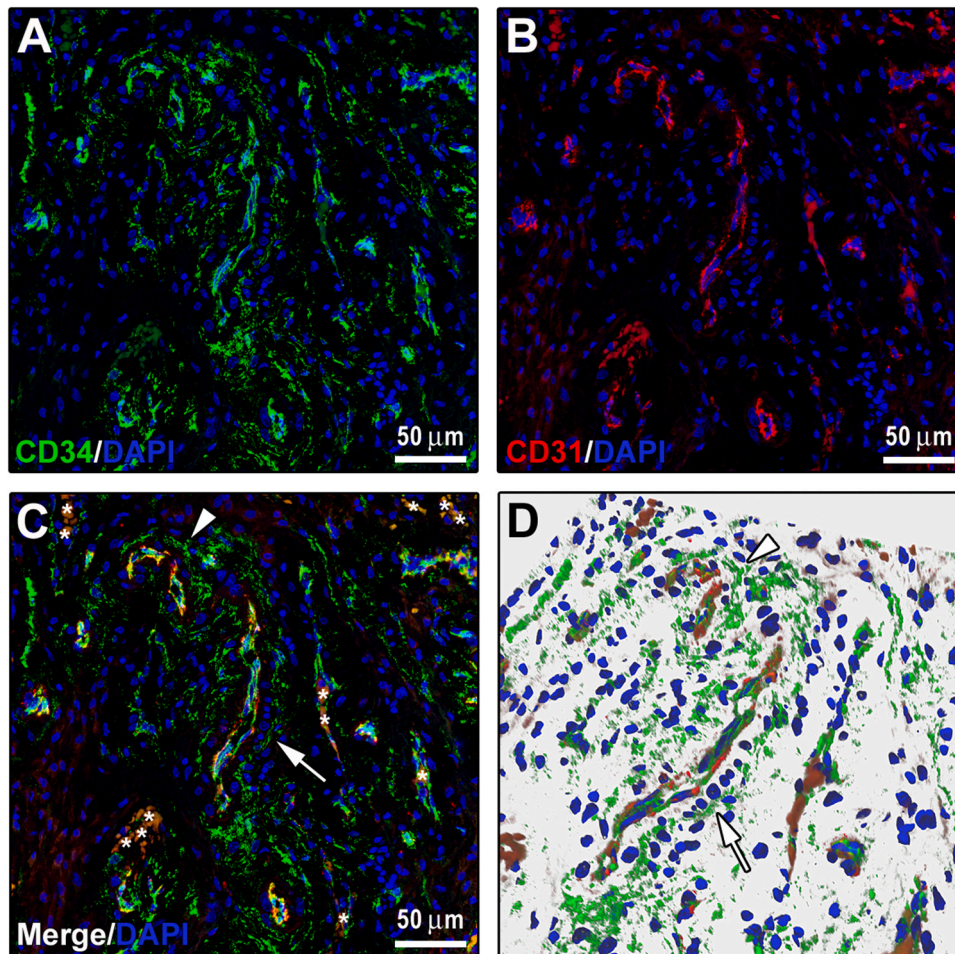
Fig. 2. Double immunofluorescence confocal microscopy allows to precisely distinguish the perivascular networks of telocytes (TCs) from adjacent microvessels in the lamina propria of human vaginal mucosa. (A–C) Maximum projection of z-stack confocal microscopy images of double staining for CD34 (green) and CD31/pan-endothelial marker (red) with DAPI (blue) counterstain for nuclei. The networks of TCs/CD34$^+$ stromal cells lack CD31 immunoreactivity and extend around CD31$^+$ microvessels; endothelial cells of blood vessels are CD31$^+$/CD34$^+$ giving rise to orange/yellow staining (C). Autofluorescent erythrocytes are marked by asterisks in (C). Scale bar: 50 µm (A–C). (D) Three-dimensional volume rendering of the CD34/CD31/DAPI merge image; the arrow and arrowhead point to the same tissue structures indicated in (C).

feature shared by TCs at multiple anatomical sites, while either the presence or the lack of c-kit/CD117 expression has been reported for the TCs in different organs (Cretoiu et al., 2017; Kondo and Kaestner, 2019; Rosa et al., 2021; Vannucchi et al., 2013, 2014). Additional immuno-phenotypes uncovered in the most recent years include the Foxl1$^+$ TCs residing in the intestinal stem cell niches and the Lgr5$^+$ TCs located at the villus tip (Bahar Halpern et al., 2020; Bernier-Latmani et al., 2022; Kaestner, 2019; Kondo and Kaestner, 2019; Pomerleau et al., 2023; Shoshkes-Carmel et al., 2018).

Focusing on the organs of the female reproductive system, TCs were extensively identified so far in the ovary, uterine tube, and uterus, but their functions have not been fully elucidated yet (Aleksandrovych et al., 2016; Condrat et al., 2021; Cretoiu and Cretoiu, 2016; Cretoiu et al., 2013; Janas et al., 2018; Klein et al., 2022). Furthermore, they have been found in the placenta and mammary gland, which are strictly related to the female reproductive function (Cretoiu and Cretoiu, 2016; Janas et al., 2018; Klein et al., 2022; Petre et al., 2016; Suciu et al., 2010). The main role presumed for the TCs in the female reproductive organs is the regulation of local microenvironment, such as myogenic contractile mechanisms and bioelectrical signaling in the myometrium, immunomodulation, and regulation of blood flow (Banciu et al., 2018; Condrat et al., 2021; Cretoiu and Cretoiu, 2016; Cretoiu et al., 2013; Crețoiu et al., 2012; Janas et al., 2018; Klein et al., 2022; Zhu et al., 2023). Increasing evidence also suggests that TCs might act as sensors of

sex hormone levels and be related to pregnancy maintenance and outcome, as testified by changes in their morphology and number in pre-eclampsia, endometriosis, and ovarian failure (Janas et al., 2018; Klein et al., 2022; Liu et al., 2016; Nizyaeva et al., 2018; Xu et al., 2023). Hence, there is a chance that TCs might prove useful to develop novel therapies for the abovementioned conditions, as well as innovative approaches in the field of regenerative medicine of the female reproductive system, especially taking advantage of their impact on stem cell fate and angiogenesis (Aleksandrovych et al., 2022; Bei et al., 2015; Díaz-Flores et al., 2022, 2023; Janas et al., 2018; Rosa et al., 2021; Sanches et al., 2020; Soliman, 2021). Nonetheless, current knowledge of TCs in the vaginal wall is very limited (Janas et al., 2018; Klein et al., 2022). Therefore, we undertook the present research to gain insights into the presence and characteristics of TCs in the human vaginal mucosa.

2. Materials and methods

2.1. Tissue samples

Archival paraffin wax-embedded and epoxy resin-embedded samples of human anterior vaginal mucosa from three postmenopausal women (aged 52–59 years) who underwent transvaginal hysterectomy for uterine fibroids (leiomyomas) were retrieved at the Section of Anatomy and Histology, Department of Experimental and Clinical Medicine,
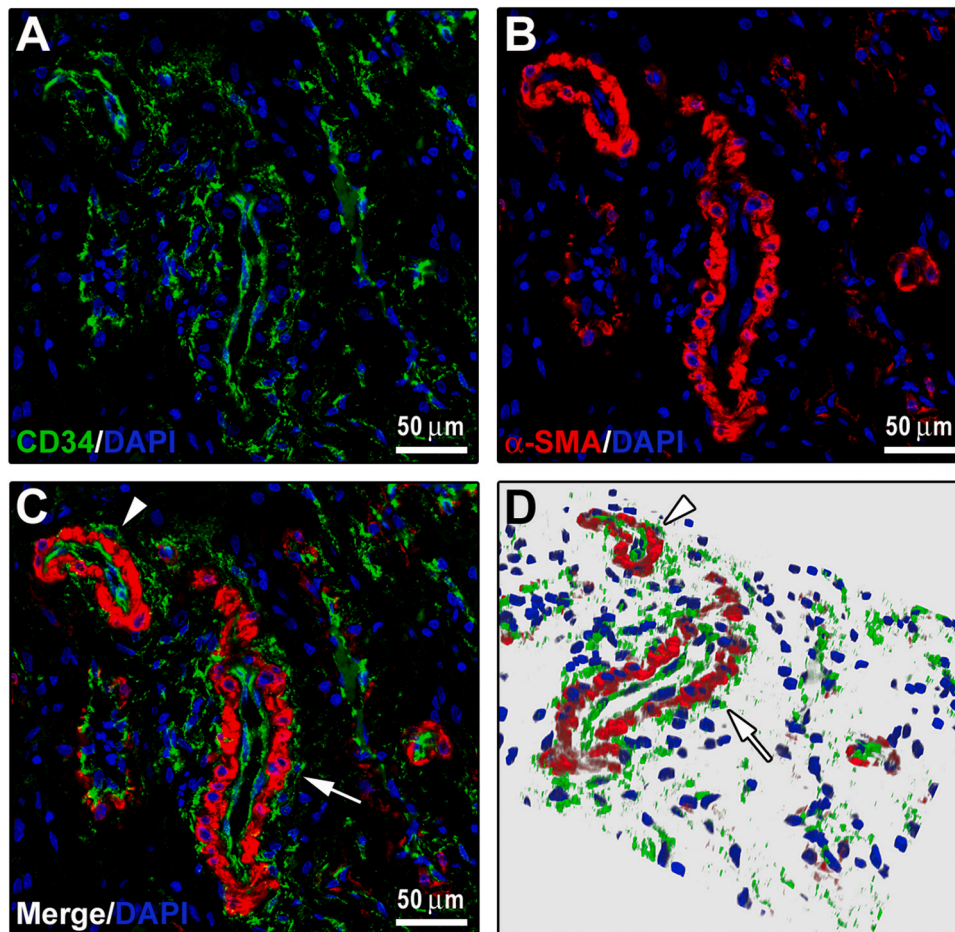
Fig. 3. The perivascular networks of telocytes (TCs)/CD34^{+} stromal cells are intimately arranged outside vascular smooth muscle cells in the lamina propria of human vaginal mucosa. (A–C) Maximum projection of z-stack confocal microscopy images of double staining for CD34 (green) and α-smooth muscle actin (α-SMA; red) with DAPI (blue) counterstain for nuclei. CD34^{+} TCs do not express α-SMA; CD34^{+} TC networks externally surround α-SMA^{+} vascular smooth muscle cells (C). Scale bar: 50 μm (A–C). (D) Three-dimensional volume rendering of the CD34/α-SMA/DAPI merge image; the arrow and arrowhead point to the same tissue structures indicated in (C).

University of Florence. All cases did not report clinical symptoms of vaginal atrophy, such as vaginal dryness, burning, itching, dysuria, and dyspareunia (i.e., painful sexual intercourse). The investigation was conducted in accordance with the ethical principles for medical research involving human subjects of the Declaration of Helsinki, and the use of human tissue biopsies for research purposes was approved by the local Institutional Review Board of the Careggi University Hospital, Florence, Italy (protocol no. 6783–04).

2.2. Histochemistry and immunohistochemistry

Vaginal mucosal tissue sections (5 μm thick) from each formalin-fixed, paraffin wax-embedded sample were deparaffinized with xylene, rehydrated, and routinely stained with hematoxylin and eosin to confirm the normal tissue appearance (i.e., absence of any obvious histopathological sign). For immunoperoxidase-based immunohisto-chemistry, tissue sections (5 μm thick) were deparaffinized and sub-jected to heat-mediated antigen retrieval in sodium citrate buffer (10 mM, pH 6.0; Sigma-Aldrich, St. Louis, MO, USA) followed by inactiva-tion of endogenous peroxidases in 3% hydrogen peroxide solution for 15 min at room temperature. Tissue slides then underwent blockade of non-specific antibody binding sites with Ultra V block (catalog no. TA-125-UB; Lab Vision, Fremont, CA, USA) for 10 min at room tempera-ture, and subsequently incubated overnight at 4 °C with primary anti-bodies against human CD34 or CD31/platelet-endothelial cell adhesion

molecule-1 (PECAM-1). Detailed information on the antibody sources and dilutions is provided in Table 1. Negative controls were performed by overnight incubation of serial sections with isotype-matched and concentration-matched irrelevant IgG (Sigma-Aldrich). Antigen-antibody complexes were revealed by sequentially applying to tissue sections biotinylated secondary antibodies (catalog no. TP-125-BN; Lab Vision) and streptavidin peroxidase reagent (catalog no. TS-125-HR; Lab Vision), both for 10 min at room temperature, followed by 3-amino-9-ethylcarbazole chromogenic solution (catalog no. TA-125-SA; Lab Vision). After counterstaining with Mayer's hematoxylin (Bio-Optica, Milan, Italy), tissue slides were mounted with VectaMount AQ aqueous mounting medium (catalog no. H-5501; Vector Laboratories, Burlin-game, CA, USA) and observed under a Leica DM4000 B microscope equipped with a Leica DFC310 FX 1.4-megapixel digital color camera and the LAS V3.8 software (Leica Microsystems, Mannheim, Germany).

2.3. Immunofluorescence and confocal laser scanning microscopy

Tissue sections (5 μm thick) were deparaffinized, rehydrated, unmasked in sodium citrate buffer (10 mM, pH 6.0; Sigma-Aldrich), and treated with a glycine solution (2 mg/mL) to quench autofluorescence signals before starting the immunofluorescence staining protocol. After blockade of non-specific antibody binding sites by applying a solution of 1% bovine serum albumin (BSA; Sigma-Aldrich) in phosphate-buffered saline (PBS; Euroclone, Milan, Italy) for 1 h at room temperature,
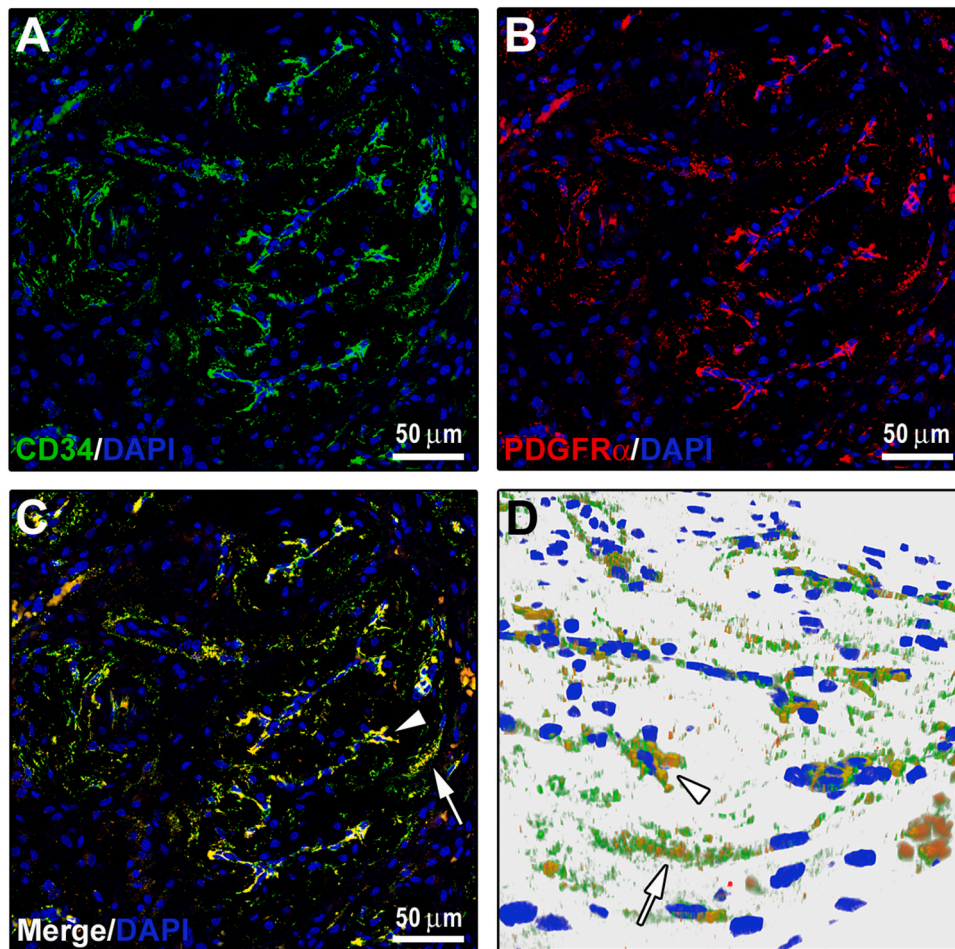
Fig. 4. Telocytes (TCs)/CD34^{+} stromal cells display immunoreactivity for platelet-derived growth factor receptor α (PDGFRα) in the lamina propria of human vaginal mucosa. (A–C) Maximum projection of z-stack confocal microscopy images of double staining for CD34 (green) and PDGFRα (red) with DAPI (blue) counterstain for nuclei. All the networks of TCs/CD34^{+} stromal cells located in the vaginal lamina propria are PDGFRα^{+} as testified by the orange/yellow staining (C). Scale bar: 50 μm (A–C). (D) Three-dimensional volume rendering of the CD34/PDGFRα/DAPI merge image; the arrow and arrowhead point to the same tissue structures indicated in (C).

tissue sections were incubated overnight at 4 °C with a mixture of mouse and rabbit or goat primary antibodies diluted in PBS with 1% BSA. Details on primary antibody sources and dilutions are shown in Table 1. Irrelevant isotype-matched and concentration-matched mouse, rabbit and goat IgG (Sigma-Aldrich) were applied in parallel to serial tissue sections as negative controls. The day after, the immunofluorescence reactions were revealed by using the following secondary antibodies diluted 1:200 in PBS with 1% BSA: Alexa Fluor-488-conjugated donkey anti-mouse IgG for CD34, Rhodamine Red-X-conjugated goat anti-rabbit IgG for CD31/PECAM-1, α-smooth muscle actin (α-SMA) and c-kit/CD117, and Alexa Fluor-568-conjugated donkey anti-goat IgG for PDGFRα (Invitrogen, San Diego, CA, USA). After nuclear counterstaining with 4′,6-diamidino-2-phenylindole (DAPI; Chemicon International, Temecula, CA, USA), tissue slides were mounted with Fluoro-Gel antifade aqueous mounting medium (catalog no. 17985–10; Electron Microscopy Sciences, Hatfield, PA, USA). The immunolabeled slides were finally observed with a Leica Stellaris 5 confocal laser scanning microscope equipped with the LAS X software (Leica Microsystems) using a Plan-Apo 63 × /1.4NA oil immersion objective. Each image was reconstructed using 16 fields of view obtained by a mosaic acquisition system. For each field, a z-stack of 16 slices with step interval of 300 nm was obtained and superimposed to create a single composite image (i.e., maximum projection).

2.4. Transmission electron microscopy

Small vaginal mucosal tissue fragments fixed with Karnovsky's fixative, post-fixed in 1% OsO$_4$ (Electron Microscopy Sciences), and embedded in Epon 812 resin (Sigma-Aldrich) were cut with an RMC MT-X ultramicrotome (EMME3, Milan, Italy). Semithin sections (2 μm thick) were stained with a toluidine blue solution in borate buffer (0.1 M) and observed under a light microscope to identify tissue areas suitable for transmission electron microscopy. Ultrathin sections (~70 nm thick) were stained with UranyLess (Electron Microscopy Sciences) and alkaline bismuth subnitrate solutions, and then examined under a JEM-1010 electron microscope (Jeol, Tokyo, Japan) at 80 Kv. Photomicrographs were acquired with a MegaView III high-resolution digital camera equipped with AnalySIS imaging software (Soft Imaging System, Muenster, Germany). TCs, telopodes and their extracellular vesicles detected in electron photomicrographs were digitally colored in blue using Adobe Photoshop CS6 software (Adobe Systems, San Jose, CA, USA).
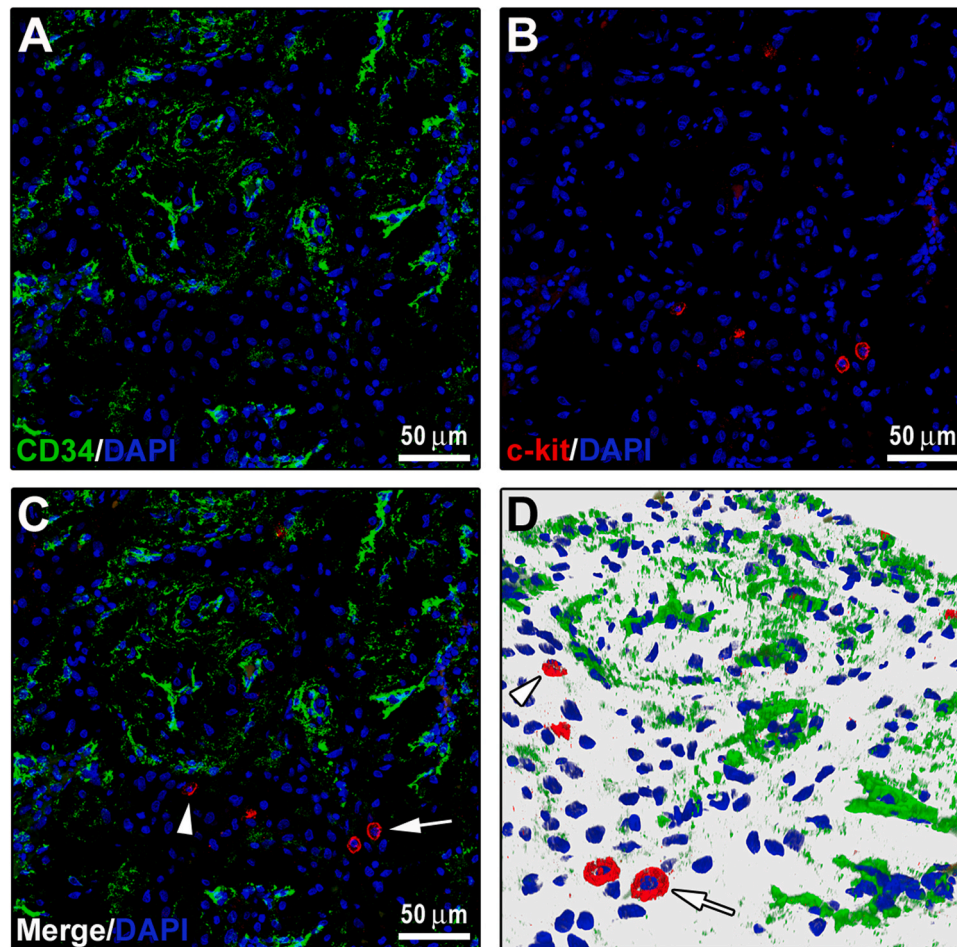
Fig. 5. Lack of c-kit/CD117 expression in the telocytes (TCs)/CD34$^+$ stromal cells residing in the lamina propria of human vaginal mucosa. (A–C) Maximum projection of z-stack confocal microscopy images of double staining for CD34 (green) and c-kit (red) with DAPI (blue) counterstain for nuclei. The perivascular CD34$^+$ TC networks are immunophenotypically negative for the c-kit marker; c-kit expression is detectable only in oval/round-shaped mast cells (C). Scale bar: 50 μm (A–C). (D) Three-dimensional volume rendering of the CD34/c-kit/DAPI merge image; the arrow and arrowhead point to the same tissue structures indicated in (C).

3. Results

3.1. Identification of telocytes in the connective tissue of human vaginal mucosa by immunohistochemistry and immunofluorescence confocal microscopy

The analysis of hematoxylin and eosin-stained tissue sections allowed to exclude any obvious histopathological sign of vaginal atrophy, as well as to select vaginal mucosal sections with an optimally preserved connective tissue (i.e., lamina propria) and, hence, appropriate for the immunohistochemical identification of TCs (Fig. 1A).

Immunohistochemistry for the CD34 antigen revealed the presence of CD34$^+$ interstitial cells displaying the characteristic TC morphology (i.e., TCs/CD34$^+$ stromal cells) in the vaginal lamina propria (Fig. 1B–E). Indeed, these stromal cells were spindle-shaped and had very long and moniliform cytoplasmic processes that often exhibited a sinuous trajectory (Fig. 1C,E, insets). In particular, TCs/CD34$^+$ stromal cells were concentrated around blood vessels, where they were often arranged in multilayered labyrinthine networks (Fig. 1B–E). No noticeable difference in the arrangement of perivascular TCs was found depending on the location of the vessels within the vaginal mucosa, that is between subepithelial vessels and those positioned in the deeper lamina propria.

Since CD34 immunostaining was also detected in the endothelium of blood microvessels which form an extensive plexus in the vaginal lamina propria and can appear as elongated profiles depending on the section,

the same tissue specimens were subjected to immunohistochemistry for the pan-endothelial cell marker CD31/PECAM-1. As shown in Fig. 1F, stromal CD31 immunoreactivity did not appear distributed as networks and was found exclusively in endothelial cells lining the lumen of vessels.

In addition, we employed double immunofluorescence confocal microscopy to simultaneously detect the CD34 and CD31 antigens (Fig. 2A–D). This analysis confirmed the presence of multiple interstitial networks of CD31$^-$/CD34$^+$ TCs surrounding CD31$^+$/CD34$^+$ microvessels within the connective tissue of the vaginal mucosa (Fig. 2A–D).

Considering that a 'myoid' subtype of TCs expressing α-SMA has been described in some organs (Rusu et al., 2018; Vannucchi et al., 2014), human vaginal mucosal sections were further subjected to CD34/α-SMA double immunofluorescence. As displayed in Fig. 3A–D, the CD34$^+$ TCs detectable in the vaginal lamina propria lacked α-SMA expression. Of note, this analysis further confirmed the preferential perivascular location of CD34$^+$ TCs, which constituted the adventitial cell layer of arterioles and venules immediately outside the α-SMA$^+$ vascular smooth muscle cells (Fig. 3A–D).

Since the coexpression of CD34 and PDGFRα has been consistently reported as an immunophenotypical feature of the TCs located in a variety of organs (Cretoiu et al., 2017, 2020; Cretoiu and Popescu, 2014; Kondo and Kaestner, 2019; Marini et al., 2018b; Rosa et al., 2021; Vannucchi et al., 2013, 2014), we next carried out CD34/PDGFRα double immunofluorescence staining. This analysis revealed that the CD34$^+$ stromal cells of the human vaginal mucosa were also PDGFRα$^+$,
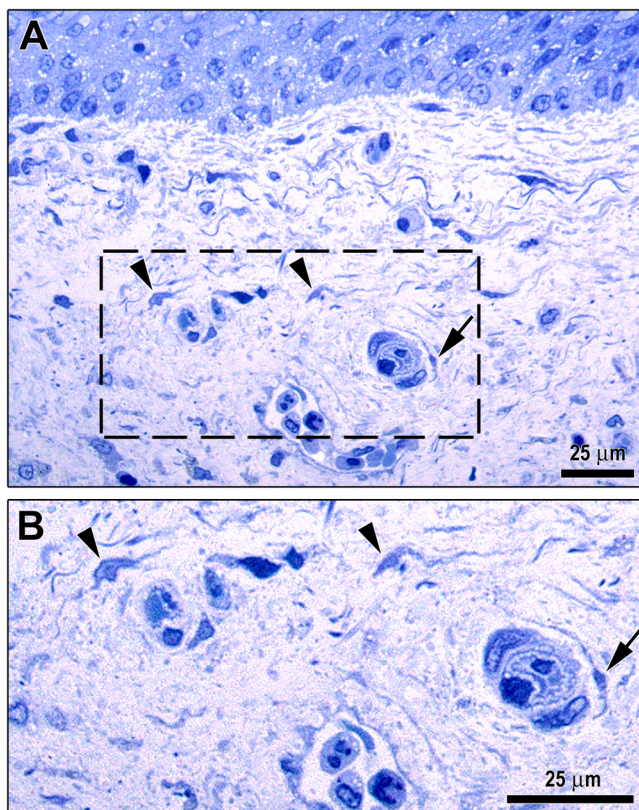
Fig. 6. Light microscopy observation of semithin sections of human vaginal mucosa reveals the presence of stromal cells with morphological features of telocytes (TCs). (A,B) Toluidine blue staining. In the vaginal lamina propria, TCs are identifiable as spindle-shaped cells with long and thin moniliform cytoplasmic processes; they can be found both in neutral position (arrowheads) and closely around microvessels (arrow). (B) Higher magnification of the boxed area in (A). Scale bar: 25 μm (A,B).

thus further supporting their identification as TCs (Fig. 4A–D).

Finally, we wondered whether vaginal mucosal TCs expressed the receptor tyrosine kinase c-kit/CD117, a marker previously found in TCs from other organs of the female genital system, such as the uterine tubes and the uterus (Cretoiu, 2016; Kondo and Kaestner, 2019; Rosa et al., 2021). However, CD34/c-kit double immunostaining of vaginal mucosal tissue sections showed that the CD34$^+$ TCs were immunonegative for c-kit (Fig. 5A–D). Moreover, no c-kit$^+$ spindle-shaped stromal cells could be identified (Fig. 5A–D). Indeed, c-kit immunoreactivity was detected exclusively in oval/round-shaped mast cells (Fig. 5A–D).

3.2. Identification of telocytes in the connective tissue of human vaginal mucosa by transmission electron microscopy

Following the aforedescribed immunohistochemical and immunofluorescence findings, we performed toluidine blue staining on semithin sections and transmission electron microscopy analysis on ultrathin sections obtained from epoxy resin-embedded human vaginal mucosal specimens to confirm the existence of TCs according to relevant ultrastructural identification criteria (Cretoiu et al., 2020; Popescu and Faussone-Pellegrini, 2010). In toluidine blue-stained vaginal mucosal semithin sections, spindle-shaped stromal cells with long and thin cytoplasmic processes displaying a moniliform silhouette were observed throughout the lamina propria both in neutral and perivascular positions (Fig. 6A,B).

With respect to transmission electron microscopy, the ultrastructural hallmark of TCs located in the vaginal mucosal connective tissue was that they exhibited very long, slender and moniliform telopodes with a

narrow emergence from the cell body, which was spindle-shaped, oval, or piriform and contained a large nucleus surrounded by a small amount of cytoplasm (Fig. 7A–E and Fig. 8A–D). TCs and telopodes appeared embedded in the extracellular matrix (ECM) and interconnected (Fig. 7A, inset), and were often found in close relationship with blood microvessels (Fig. 7B). The telopodes of the TCs concentrated around blood vessels were typically convoluted and formed labyrinth-like networks establishing intercellular contacts with perivascular mononuclear cells (Fig. 7C,D and Fig. 8A,C). Moreover, numerous extracellular vesicles of different sizes were detected nearby telopodes (Fig. 7E and Fig. 8B,D).

4. Discussion

The results of this study provide a significant reappraisal of the microscopic anatomical structure of the human vagina, as they demonstrate for the first time that the vaginal mucosa harbors stromal cells with the distinctive morphological and immunophenotypical features of TCs. Indeed, the stromal cells we uncovered in the vaginal lamina propria fulfill the main criteria for the histological diagnosis of TCs, namely they exhibit peculiar ultrastructural features – very long distinctive cytoplasmic processes (i.e., telopodes) abruptly emerging from the cell body and looking moniliform due to the alternation of thin podomers and small dilated podoms – and a dual immunopositivity for CD34 and PDGFRα. Of note, transmission electron microscopy is currently considered the gold standard methodology for the identification of TCs but, owing to their well-documented CD34$^+$ immunophenotype, it appears that they may have previously been referred to as CD34$^+$ stromal fibroblastic/fibrocytic or dendritic cells in several organs. Nevertheless, to our knowledge, the presence of CD34$^+$ stromal cells or fibroblasts has never been noticed before within the connective compartment of the healthy vaginal wall. In addition, double immunostaining for CD34 and CD31/PECAM-1 proved to be essential not only to differentiate CD31$^-$/CD34$^+$ TCs from CD31$^+$/CD34$^+$ endothelial cells of adjacent blood vessels (Manetti et al., 2019; Marini et al., 2018a, 2018b; Romano et al., 2020; Rosa et al., 2021), but also to unveil that TCs are preferentially organized as extensive labyrinth-like perivascular networks in the vaginal lamina propria – a peculiar arrangement that was further confirmed by transmission electron microscopy. CD34/α-SMA double immunofluorescence was also useful to corroborate the perivascular location of TCs, which are indeed blood vessel adventitial cells immediately outside the α-SMA$^+$ smooth muscle cell layer, as well as to exclude the presence of α-SMA$^+$ myoid TCs that have instead been observed in some organs (Rusu et al., 2018; Vannucchi et al., 2014).

Though further functional studies will be fundamental to extend our morphological data, the unique features and tissue distribution of the TCs found in the vaginal mucosa suggest that these stromal cells might play important roles. In keeping with the variety of functions that have been proposed or demonstrated for the TCs in other organs (Condrat et al., 2021; Cretoiu et al., 2017, 2020; Cretoiu and Popescu, 2014; Klein et al., 2022; Kondo and Kaestner, 2019; Rosa et al., 2021), the highly intricate perivascular networks shaped by telopodes within the vaginal lamina propria might have mechanical supportive properties and/or mediate local molecular trafficking and intercellular signaling, thus contributing to the homeostasis of the whole vaginal mucosa.

First, as structural components of the supportive connective tissue of the vaginal lamina propria, the TCs could condition the ECM composition and the biomechanical properties of the vaginal wall helping maintain its integrity, essential to keep pelvic organs in place and avoid pelvic organ prolapse (De Landsheere et al., 2013). Hence, it can be postulated that further knowledge about the vaginal TCs could arise from an investigation of vaginal tissues from patients with pelvic organ prolapse, which are known to be stiffer and to have a different ECM composition than healthy vaginal tissues (De Landsheere et al., 2013). Indeed, both ECM stiffness and composition have been demonstrated to regulate the differentiation of vaginal fibroblasts into α-SMA$^+$
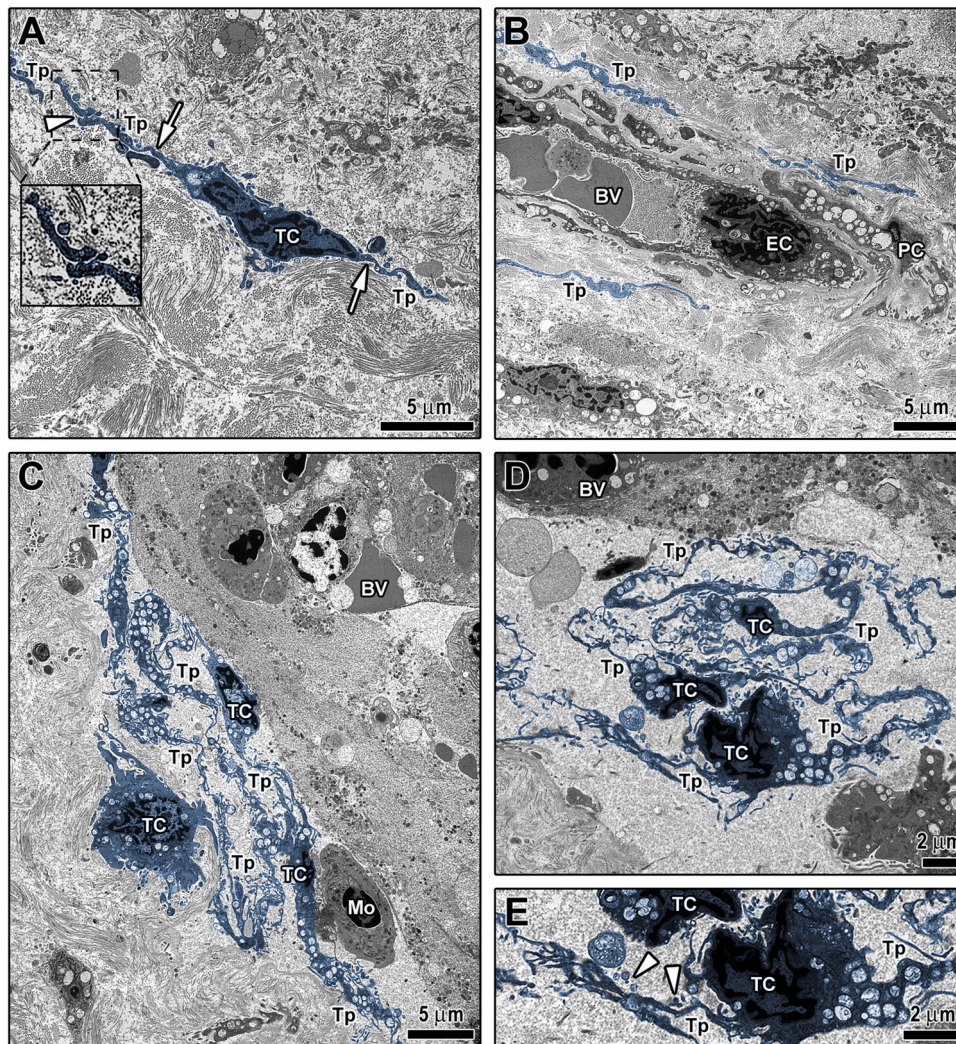
Fig. 7. Identification of telocytes (TCs) in ultrathin sections of human vaginal mucosa by transmission electron microscopy. (A–E) UranyLess and bismuth subnitrate solution staining. In the vaginal lamina propria, TCs are ultrastructurally identifiable as stromal cells with a cell body mostly occupied by the nucleus and characteristic long cytoplasmic projections (telopodes). (A) A bipolar TC embedded in the extracellular matrix displays two telopodes with a narrow emergence (arrows) from the spindle-shaped cell body; telopodes have a moniliform silhouette, due to the alternation of thin segments (podomers) and expanded portions (podoms), and are often interconnecting (arrowhead). Inset in (A) is a higher magnification view of the boxed area showing a gap junction with apposition of the contiguous plasma membranes of two telopodes. (B) Note the presence of telopodes around a capillary blood vessel of the vaginal lamina propria. (C–E) By their convolute telopodes, TCs form labyrinthine networks around blood vessels and are in close relationship with perivascular mononuclear cells. Numerous extracellular vesicles of different sizes are present nearby telopodes (E, arrowheads). TCs, telopodes and their extracellular vesicles have been digitally colored in blue. Scale bar: 5 μm (A–C), 2 μm (D, E). BV, blood vessel; EC, endothelial cell; Mo, mononuclear cell; PC, pericyte; TC, telocyte; Tp, telopode.

myofibroblasts able to contract and synthesize elevated amounts of collagen (Ruiz-Zapata et al., 2020), and TCs/CD34$^+$ stromal cells are thought to give rise to α-SMA$^+$ myofibroblasts in a variety of pathologies (Díaz-Flores et al., 2015, 2016). Moreover, since explanted vaginal fibroblastic cells from women with pelvic organ prolapse were found to produce matrices with increased stiffness and collagen content (Ruiz--Zapata et al., 2016), further in-depth investigations are required to unveil whether, among the different subpopulations of vaginal stromal/fibroblastic cells, TCs can make a specific contribution to such a scenario.

Second, being closely associated with the mucosal blood vessels, TCs could contribute to vaginal lubrication. In fact, the vaginal wall contains no glands, and a major contributor to vaginal moisture is plasma transudate derived from the mucosal vasculature and then filtered through the intercellular junctions between vaginal epithelial cells (Goldstein et al., 2013). Because of their intimate spatial relationship with blood vessels, the labyrinthine networks formed by telopodes within the vaginal lamina propria might participate in the transfer of the small

molecular elements and water constituting the vaginal transudate that then combines at the mucosal surface with dead epithelial cell debris. Of note, this assumption is supported by the presence of numerous extracellular vesicles in the close proximity of the telopodes surrounding the vaginal mucosal microvessels. Either by the release of extracellular vesicles or by establishing intercellular contacts through specialized junctions, TCs could also be involved in vaginal mucosal immune surveillance and in the guidance of putative tissue-resident stem/progenitor cells, as previously suggested in the mucosal compartment of other organs (Cretoiu et al., 2020; Kondo and Kaestner, 2019; Rosa et al., 2021). In addition, considering that TCs from the uterus and fallopian tubes have been shown to express progesterone and estrogen receptors and likely act as hormonal sensors (Aleksandrovych et al., 2016; Banciu et al., 2018; Cretoiu, 2016; Cretoiu and Cretoiu, 2016; Janas et al., 2018; Klein et al., 2022), further work is necessary to determine whether vaginal TCs may display similar features and, hence, have a role in the well-known responsiveness of the vaginal mucosa to sex steroid hormones (Goldstein et al., 2013).
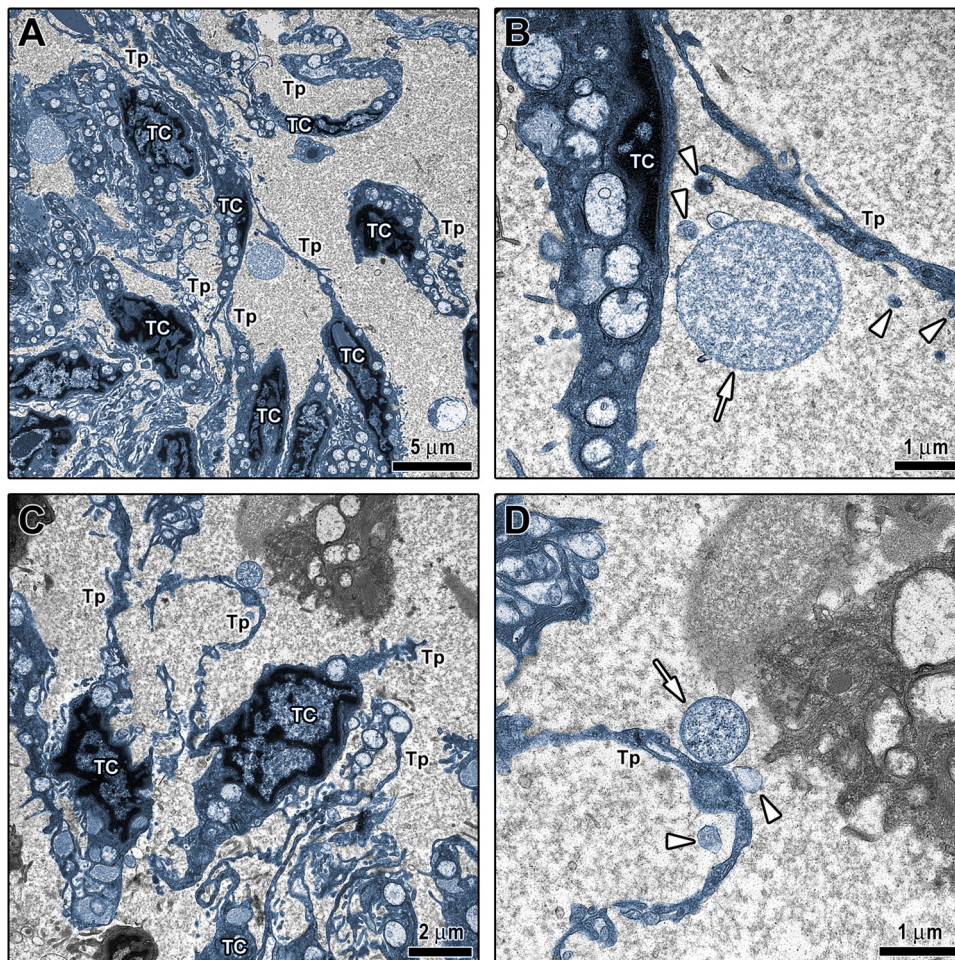
Fig. 8. Transmission electron microscopy observation of perivascular networks of telocytes (TCs) in human vaginal mucosa. (A–D) UranyLess and bismuth subnitrate solution staining. The convolute telopodes of TCs build multilayered labyrinthine networks around blood vessels of the vaginal lamina propria; both small-sized (B,D, arrowheads) and large-sized (B,D, arrows) extracellular vesicles are observed in the close proximity of telopodes. TCs, telopodes and their extracellular vesicles have been digitally colored in blue. Scale bar: 5 μm (A), 2 μm (C), 1 μm (B,D). TC, telocyte; Tp, telopode.

Although we found that vaginal mucosal TCs do not express the receptor tyrosine kinase c-kit, we should consider that c-kit$^+$ interstitial cells with long cytoplasmic processes were described by Shafik et al. in the muscular layer of the human vagina (Janas et al., 2018; Klein et al., 2022; Shafik et al., 2005). Of note, it was recently suggested that such c-kit$^+$ stromal cells might be reclassified as TCs, and that they may be potentially involved in the generation of slow waves resulting in the contractility of vaginal smooth muscle cells (Klein et al., 2022; Shafik et al., 2005). Therefore, the possibility that the different layers of the vaginal wall might harbor specialized subpopulations of TCs with diverse immunophenotypes and/or functions should be thoroughly investigated in the future.

Finally, we should consider that our study has some obvious limitations due to the small number of samples examined. Furthermore, it is to be considered that our investigation was restricted to vaginal mucosal samples from postmenopausal women, though they were selected on the basis of the absence of clinical and histopathological signs of vaginal atrophy. Therefore, further research is necessary to ascertain whether the vaginal mucosa of younger, premenopausal women may present similar or different arrangements of the TC networks. A more detailed characterization of the intercellular junctions, both homocellular and heterocellular contacts, as well as of the extracellular vesicles released by vaginal mucosal TCs is also warranted.

In summary, our work sheds light on TCs as a previously overlooked stromal cell type residing in the lamina propria of the human vagina and provides the rationale for further investigation of these peculiar cells in different vaginal disorders, such as vaginal atrophy and pelvic organ prolapse-related vaginal modifications (De Landsheere et al., 2013; Goldstein et al., 2013), as well as a variety of vulvovaginal mesenchymal lesions that are thought to arise from the specialized subepithelial stroma of the lower female genital tract extending from the cervix to the vulva (McCluggage, 2009). For instance, the very common CD34 immunoreactivity of most vulvovaginal mesenchymal lesions, such as angiomyofibroblastoma, cellular angiofibroma and superficial myofibroblastoma (McCluggage, 2009), suggests that TCs could be among the stromal elements involved in the pathogenesis of these conditions. Lastly, an in-depth investigation of TCs in vaginal diseased tissues and experimental models will also help to unveil their specific roles, which at present can only be speculated, and eventually provide new clues for their possible therapeutic implications and/or potential relevancy in the field of regenerative medicine of the female genital tract.

Funding

CRediT authorship contribution statement

Irene Rosa: Conceptualization, Methodology, Formal analysis,

Investigation, Writing – original draft preparation, Writing – review & editing. **Patrizia Nardini:** Methodology, Formal analysis, Investigation, Writing – review & editing. **Bianca Saveria Fioretto:** Methodology, Formal analysis, Investigation, Writing – review & editing. **Daniele Guasti:** Methodology, Formal analysis, Investigation, Writing – review & editing. **Eloisa Romano:** Formal analysis, Investigation, Writing – review & editing. **Eleonora Sgambati:** Formal analysis, Investigation, Writing – review & editing. **Mirca Marini:** Formal analysis, Investigation, Writing – review & editing. **Mirko Manetti:** Conceptualization, Methodology, Formal analysis, Investigation, Resources, Supervision, Writing – original draft preparation, Writing – review & editing.

Declaration of Competing Interest

The authors declare no conflict of interest.

Data Availability

All relevant data are included within the manuscript.

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