Identification of uterine telocytes and their architecture in leiomyoma

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Abstract: Introduction: Uterine leiomyoma is the most widespread benign tumor affecting women of childbearing age. There are still gaps in the understanding of its pathogenesiss. Telocytes are unique cells described in greater than 50 different locations inside the human body. The functional relationship of cells could clarify the pathogenesis of leiomyomata. In the current study, we focused on the identification of telocytes in all regions of the human uterus to explain their involvement in leiomyoma development.

Materials and Methods: Tissue samples from a healthy and myomatous uterus were stained for c-kit, tryptase, CD34 and PDGFRα to identify telocytes. Routine histology was performed to analyze tissue morphology and collagen deposits.

Results: Telocytes were detected in the cervix, corpus of the uterus and leiomyoma. The density of telocytes in fibroid foci was reduced compared with normal myometrium.

Conclusions: Our results demonstrated the existence of telocytes in all parts of the human body affected and unaffected by leiomyoma of the uterus. In addition, telocytes were also present in leiomyoma foci. Our results suggest that the reduced density of telocytes is important for the patho-mechanisms of myometrial growth, demonstrating its value as a main component of the myomatous architecture.

Key words: telocytes, uterus, extracellular matrix (ECM), stem cells, collagen, CD34, PDGFRα.

Introduction

The uterus is a unique myometrial organ that undergoes structural and functional remodeling. Successful implantation plays a crucial role in female reproductive processes. Rich vascularization, a network of autonomic innervation, sensitivity to hormonal regulation, and fluctuation in growth factors and cytokines, forms a physiological background of myometrial tissue contractility and growth. Unfortunately, the human uterus is commonly affected by leiomyomas and adenomyosis in childbearing age that lead to severe medical and social problems, such as infertility [1].

The most widespread benign monoclonal tumor of the female reproductive system is uterine leiomyoma (UL) originating from the Müllerian duct [2–4]. Despite a long history of discovery even in the time of Hypocrates and numerous explanations for thousands of years, the nature of fibroids is still not well understood [3]. Uterine fibroids exhibit heterogeneous cellular phenotypes but are consistently characterized by excessive production of extracellular matrix (ECM) that abnormally forms in fibroid foci [5–8]. The ECM consists of fibroblasts that are often termed myofibroblasts [9]. These cells produce collagen and other components of the matrix, but their inappropriate function causes fibrosis [10]. Most collagen deposits include type I [5] and type III collagen [11]. A similar trend is noted for the expression of its messenger ribonucleic acid (mRNA) [12]. Clear detailed observations of cell-cell interactions inside fibroids and adjacent myometrium reveal the pathomechanisms of ECM composition.

For the past twenty years, the scientific community has been intently discussing a new type of cells — interstitial Cajal-like cells (ICLC), which are also referred to as fibroblast-like cells or PDGFRα-positive cells. Since 2010, these cells have been identified as telocytes (TCs) in nomenclature with unique features of identification [13–15]. TCs exhibit an oval-shaped body with several long extensions, called telopodes, which represent a cell form and exhibit variability in homo and heterocellular contacts [16, 17]. Their thick and thin parts (podoms and podomers, respectively) are variable in size in pregnant and nonpregnant myometrium (Table 1) [13, 15, 18–21].

TCs are completely different from fibroblasts and mesenchymal stem cells in a variety of features, including phenotype. Their gene profile contains thousands of up- and downregulated genes compared with other cells. Some of these genes are involved in tissue remodeling. Collagen type IV is upregulated in cultured TCs [22]. These cells were described in several diseases [23–25]. Systematic sclerosis (SSc) was accompanied by ultrastructural alterations (swollen mitochondria, cytoplasmic vacuolization and presence of lipofuscinic bodies) of TCs that are reduced in the skin, correlating with disease subsets and stages. Moreover, the same changes were

described in the gastric wall (submucosa and muscle layers), the myocardium and the lung [26]. Manetti *et al.* observed limited and diffuse cutaneous SSc in early and advanced stages and concluded that the damage and loss of TCs might be caused by an ischemic injury as TCs appear to be more sensitive to ischemia compared with other stromal cell types, such as fibroblasts, myofibroblasts and mast cells [27]. A reduction in TCs in organs affected by SSc might be a cause of uncontrolled fibroblast/myofibroblast activity [26, 27].

Table 1. Differences in TC morphology in pregnant and nonpregnant myometrium.

	Pregnant myometrium	Nonpregnant myometrium
Length of telopodes (Tps)	Normal	Longer
Podomers of telocytes	Thinner (75.53 ± 1.81 nm)	Thicker (81.94 ± 1.77 nm)
Podoms of telocytes	Thicker (316.38 ± 17.56 nm)	Thinner (268.60 ± 8.27 nm)
Evidence of exosomes/shedding microvesicles (SMVs)	Normal	Lower
Diameter of extracellular vesicles measured in the myometrial interstitium	58–405 nm	65–362 nm
*Median value	151 nm	170 nm
Exosomes: SMVs	20 vs. 168	26 vs. 89
Mean diameter of exosomes/SMVs	No difference	No difference

The aim of our study was to determine the location of TCs in different parts of the human uterus (exo- and endocervix, corpus and focus of fibroid) and to clarify their possible role in the architecture of leiomyomata.

Material and Methods

Subjects

Nineteen patients with symptomatic UL were scheduled for elective surgery (laparoscopic hysterectomy) and selected for the study group (19 women, mean age 59.5 ± 14.6 years). Patients with UL exhibited detectable tumors in the uterus during gynecological examination before the operation. They presented with mild, recurrent episodes of vaginal bleeding and pain. The control group consisted of 15 patients (15 women, mean age 57.6 ± 12.8 years) who underwent elective surgery for other

reasons and had no pre- or intraoperative signs of uterine fibroids. Hysterectomy was performed according to the standard procedure. Postsurgery histological examination did not reveal any signs of UL. Tissue samples from the foci of fibrosis and adjacent myometrium were obtained from the study group for further observation. Samples of unaffected myometrium were also prepared from the control group. All patients were surgically treated at the Institute of Gynecology and Obstetrics at the Jagiellonian University Medical College in 2018.

Ethical approval

The study was conducted in accordance with the moral, ethical, regulatory and scientific principles governing clinical research. All surgical samples were retrieved with the approval of the Jagiellonian University Bioethical Committee using procedures that conformed to the Declaration of Helsinki guidelines (protocol number 122.6120.40.2016).

Tissue processing

Fresh hysterectomy specimens were collected and rinsed thoroughly with PBS (phosphate-buffered saline, 0.01 M, pH = 7.4), fixed in 4% phosphate-buffered paraformaldehyde, and routinely processed and embedded in paraffin. Serial sections were cut and mounted on poly-L-lysine-coated glass slides.

Routine histology

The sections were deparaffinized, rehydrated and stained with either hematoxylin–eosin (H&E) to evaluate the gross tissue organization or Masson trichrome staining to detect collagen deposits.

Immunofluorescence

Indirect double immunofluorescence after heat-induced epitope retrieval was used for simultaneous visualization of two antigens. After deparaffinization and rehydration, the slides were incubated for 30 min in PBS with appropriate normal serum at room temperature followed by overnight incubation at 4°C in a solution of PBS with appropriate normal serum containing primary antibody (or mixture of primary antibodies) and 0.3% Triton X-100 (Sigma, USA). After 5 washes (10 min each) in PBS, the specimens were then incubated for 1 h at room temperature with secondary antibody (or mixture of secondary antibodies) diluted in PBS. Finally, the slides were washed twice in PBS (10 min each) and cover-slipped

with Fluorescence Mounting Medium (Dako, Denmark). Labeled specimens were analyzed immediately. The primary antisera and secondary antibodies used are listed in Table 2.

Table 2. Type, sources and dilution of antibodies.

Antibody	Catalog number and company	Dilution
Primary antibodies		
Polyclonal rabbit anti-c-kit	A4502, Dako	1 : 100
Monoclonal mouse anti-CD34	M7165, Dako	1 : 100
Polyclonal goat anti-PDGFR alpha	AF-307-NA, R&D Systems	1 : 100
Monoclonal Mouse anti-tryptase	M7052, Dako	1 : 100
Secondary antibodies		
Alexa Fluor 488 Goat Anti-Mouse	115-545-146, Jackson ImmunoResearch	1 : 400
Alexa Fluor 594 Goat Anti-Rabbit	111-585-144, Jackson ImmunoResearch	1 : 400
Alexa Fluor 594 Donkey Anti-Goat	705-585-003, Jackson ImmunoResearch	1 : 400
Alexa Fluor 488 Rabbit Anti-Mouse	315-545-045, Jackson ImmunoResearch	1 : 400

Microscopic examination

Slides were examined using an MN800FL epifluorescence microscope (OptaTech, Warszawa, Poland) equipped with a Jenoptik Progress C15Plus color camera. Digital images were collected at either 200× or 400× magnification. Qualitative analysis of cells was provided in 10 consecutive high-power fields of vision (400×) using the computer-based image analysis system Multiscan 18.03 software (CSS, Warszawa, Poland). All samples were assessed by two independent specialists (each blinded to the other) without any knowledge of the clinical parameters or other prognostic factors to avoid bias. The use of mast cell tryptase staining enabled c-kit-positive mast cells to be distinguished from c-kit-positive TCs. TCs were considered cells that were c-kit positive and tryptase negative concurrently with the characteristic morphology in tissue samples. Additionally, cells double positive for CD34 and PDGFRα with characteristic morphology and localization were also recognized as TCs. In all sections, the immunoreactive cells identified were evaluated with respect to the relative frequency (arbitrarily graded as very few = (+), few = +, moderate density = + +, multiply density = + + +). The percentage of collagen deposits and muscle tissue were analyzed in specimens stained with Masson trichrome. The collagen and muscle fiber volume ratio was assessed in ten different fields of each sample.

Results

The histopathological observation of the human myomatous and unaffected uterus using hematoxylin and eosin and Masson's trichrome staining was performed (Fig. 1, 2). Corpus of myomatous uterus presented as foci of UL and adjacent myometrium. Immunofluorescent labeling was also used for all samples: the corpus and uterine cervix (exo- and endocervix) as well as leiomyoma foci. We assessed
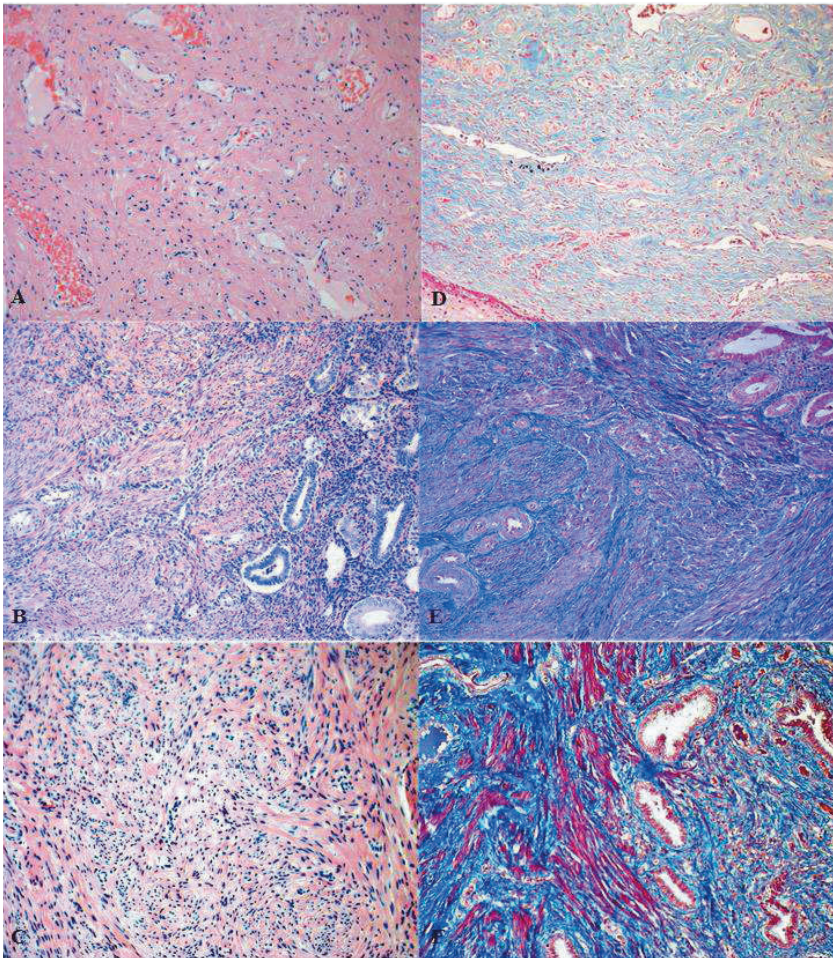

Fig. 1. Hematoxylin-eosin and Masson's trichrome stained sections of human uterus unaffected by leiomyoma. The sections from exocervix (A, D) endocervix (B, E) and myometrium from the uterus body (C, F). On Masson's trichrome staining, collagen deposits appear as blue, and muscle fibers appear as red. Total magnification: 200×.
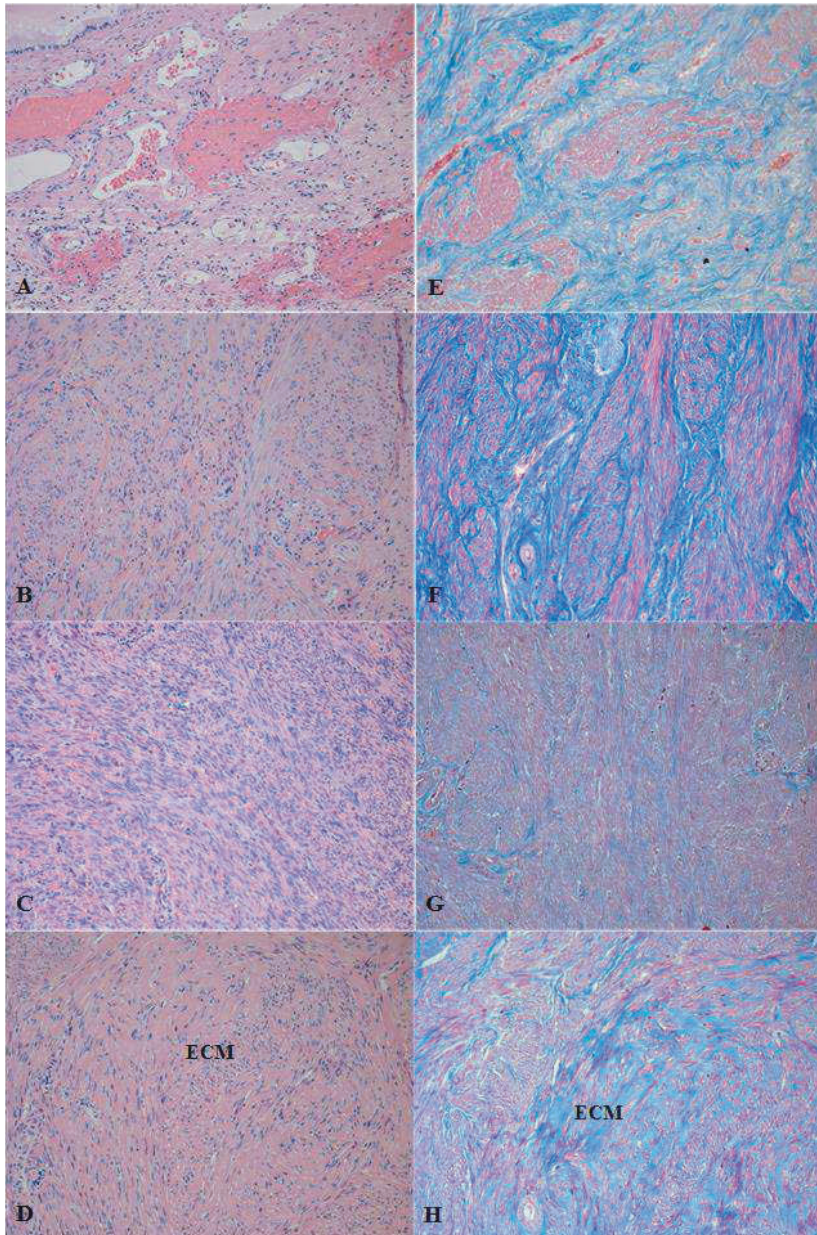
Fig. 2. Hematoxylin-eosin and Masson's trichrome stained sections of human myomatous uterus. Sections from the exocervix (A, E) endocervix (B, F), leiomyoma foci (D, H) and adjacent myometrium from the same uterus (C, G). On Masson's trichrome staining, collagen deposits appear as blue, and muscle fibers appear as red. Fragments of disordered smooth-muscle cells separated by abundant extracellular matrix (ECM). Total magnification: 200×.

mostly currently proven markers: CD34, PDGFRα and canonic c-kit (Fig. 3, 4). Double immunolabeling for c-kit and tryptase was used for the identification of mast cells and subsequent signs of inflammation. The c-kit-positive/mast cell tryptase-negative cells were considered TCs. CD34-positive and PDGFRα-positive cells were detected as uterine TCs.
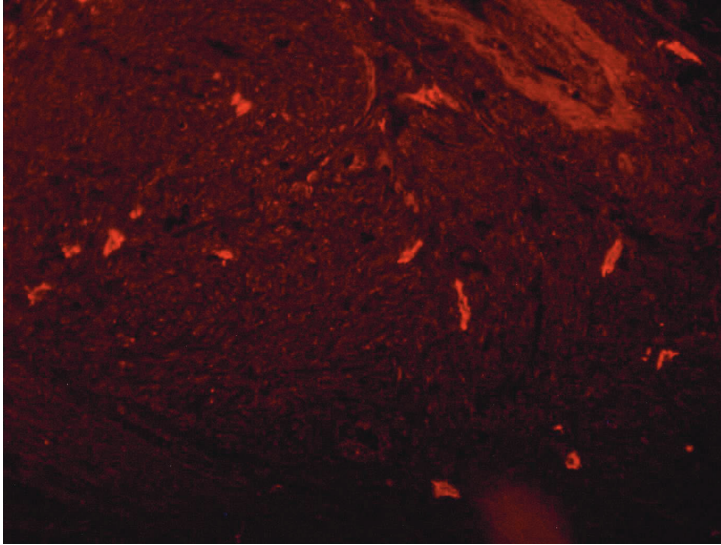

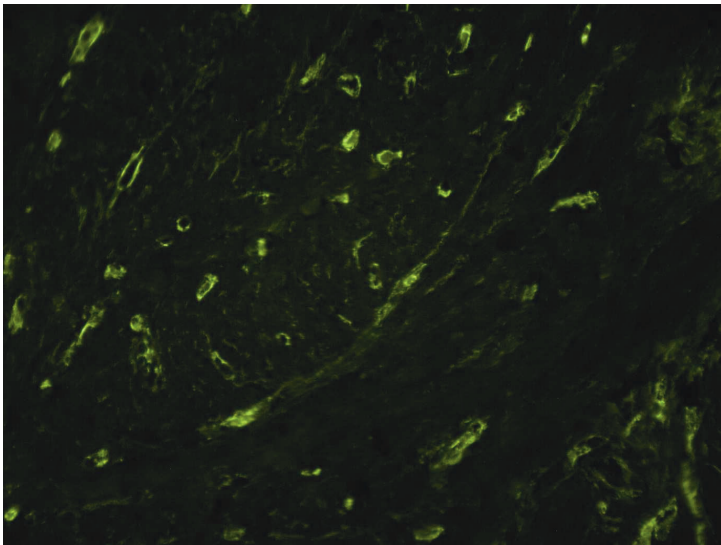**Fig. 3.** Sample from the a leiomyoma focus stained for c-kit (red, Alexa Fluor 594). Total magnification: 400×.

Fig. 4. Sample from a leiomyoma focus stained for CD34 (green, Alexa Fluor 488). Total magnification: 400×.

Hematoxylin and eosin staining demonstrated than UL were mainly composed of smooth muscle cells and fibrous connective tissue. Smooth muscle cells exhibited a uniform spindle size and shape with rhabditiform nuclei. The adjacent myometrium and fibroid foci were cytologically identical, but the latter exhibited circumscription, nodularity and denser cellularity. Masson's trichrome staining revealed the prevalence of collagen deposits in UL compared with all other observed samples.

We found that cells with the characteristic morphology and immunopositivity were located in all parts of the human uterus. These cells exhibit a triangular or spindle body with long, slender, moniliform cytoplasmic extensions. The endocervix contains more c-kit-positive, CD34-positive and PDGFRα-immunopositive cells compared with the exocervix. These cells formed bundles mainly located longitudinally (parallel to the cervical canal). No differences in TC density were noted in all parts of the uterine cervix between myomatous and healthy uteruse.

In the corpus of the uterus, TCs were located in close vicinity to blood vessels and inside muscle bundles. The general pattern of their localization resembled parallel eccentric lines in UL. We stressed that CD34-immunopositive and PDGFRα-immunopositive cells were observed in leiomyoma foci as well as in adjacent and control myometrium (Fig. 5). In all sections, immunoreactive cells were evaluated with respect to the relative frequency (arbitrarily graded as very few = (+), few = +, moderate density = + +, multiply density = + + +) (Table 3). Subjective qualitative analyses exhibited a reduction in TC density in fibroids compare with both types of unaffected myometrium (adjacent and from healthy uterus).

Table 3. Relative frequency of c-kit-positive/tryptase-negative, CD34-positive and PRGFRα-positive cells in different parts of human uterus that was not affected or affected by leiomyoma. 0 = absence of telocytes, (+) = very few, + = few, + + = moderate density, + + + = multiply density.

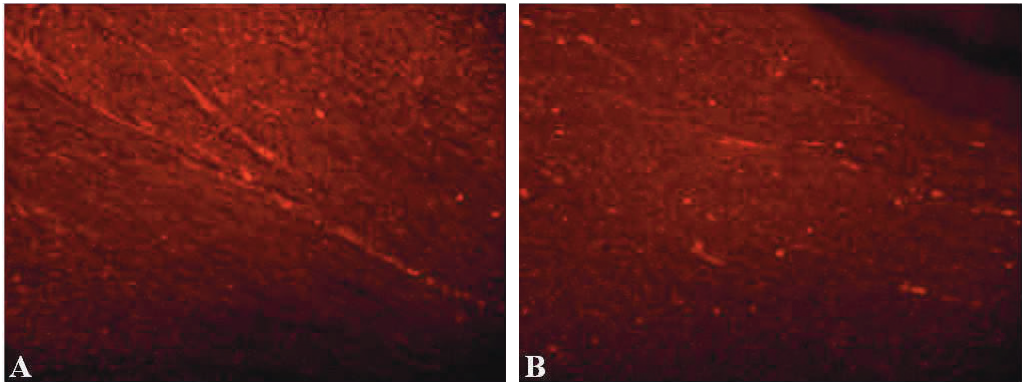|	c-kit+/trypatse–	CD34+/ PDGFRα+
Normal Uterus		
Exocervix	(+)	(+)
Endocervix	+	+
Corpus	+++	+++
Myomatous Uterus		
Exocervix	(+)	(+)
Endocervix	+	+
Corpus	++	++
Fibroid	+	+

Fig. 5. Uterine samples stained for PDGFRα (red, Alexa Fluor 594) from uterus unaffected by leiomyoma myometrium (A) and fibroid focus (B). Telocytes with their longitudinal extensions are mostly located among the intertwined myometrial fibers and in close vicinity to blood vessels. Total magnification: 400×.

Discussion

This study presents evidence for the presence of TCs in different parts of the uterus, including exocervix, endocervix, and corpus in human healthy uterus, as well as fibroid foci in myomatous uterus. Identification was based on morphological and immunocytochemical criteria in fluorescence microscopy. We assumed that CD34- and PDGFRα-positive cells are TCs. In addition, c-kit-positive cells and trypatase-negative cells were also recognized as TCs.

Homo and heterocellular contacts between TCs and smooth muscle cells, nerves, immunocytes (macrophages, mast cells and lymphocytes), stem cells, melanocytes, erythrocytes and Schwann cells highlight their involvement in creating of 3D structure of tissue and facilitating muscle contractions and immune responses. The crucial role of these cells in the physiology and pathophysiology of organs has been described and hypothetically focused on their involvement in pathomechanisms of various diseases. Uterine TCs could represent a key cell type in the uterine leiomyoma that exhibits its own architecture despite its monoclonal origin [28–30].

The main feature of each uterine fibroid is excessive production of ECM that could play a role in the storage of cytokines, chemokines, growth factors, angiogenic and inflammatory response mediators that subsequently stimulate cell growth and differentiation [9]. Uterine smooth muscle cells and fibroblasts produce several growth factors that are present in different amounts in fibroids and adjacent myometrium. The ECM of leiomyomas demonstrates the focal localization of basic fibroblast growth factor (FGF)-2 and insulin-like growth factor (IGF)-I. The amount of epidermal growth factor (EGF) is significantly reduced in UL compared

with normal myometrium. Dixon *et al.* illustrated a hormonal regulation of growth factor production based on the suggestion that EGF secretion could be regulated by progesterone (and not estrogen) [31].

Richter *et al.* assessed the correlation between collagen type I deposits and TC distribution in heart muscle [32]. Fibroblasts produced collagen type I upon stimulation by growth factors. In the normal human heart, TCs were identified in close vicinity to thin collagen fibrils. In contrast, in heart failure, some parts of myocardium have been replaced by focuses of fibrosis that are grossly characterized by excessive amounts of type I collagen. In these areas of the heart, no TCs were identified. Zhao *et al.* stressed that TCs in the myocardium are important for maintenance of the physiological integrity of heart muscle [33]. Of note, a direct correlation was observed between collagen type I deposits and the presence of TCs. Moreover, the number of TCs and Tps was positively correlated with degraded collagen type I [32]. Tps were characterized by shrinkage and shortening in areas of abundant ECM. We observed the same results in the myometrium by comparing the density of uterus TCs in fibroid foci characterized by excessive amounts of ECM and normal myometrium. In UL, an increased ECM density correlates with rare cell observations that represent typical morphological and immunohistochemical features of TCs.

Another important feature of leiomyoma is the origination of the cell population. The myometrium itself has a regenerative capacity. Ono *et al.* observed two groups of leiomyoma-derived cell populations: side and main populations. The first population was undifferentiated and rarely expressed steroid hormone receptors and smooth muscle cell markers. After some time, these cells naturally express all receptors and become similar to the main population, which is common for fibroids. Ono *et al.* stressed the importance of paracrine factor-mediated signals from steroid receptor-positive cells adjacent to leiomyoma-derived side population cells [34]. We suggest that TCs dominate among adjacent cells. TCs exhibit place-dependent specificity for estrogen and progesterone receptors. TCs in the gallbladder are negative for both types of receptors [35] but positive in myometrium, Fallopian tubes or human urinary bladder [36–38]. TCs could play the role of effector cells in paracrine cooperation between steroid hormones and side population cells, as reported by the Ono scientific group [34]. We also want to emphasize that undifferentiated cells in UL are an important component of fibroid cell architecture and appear to be somatic stem cells.

Telocytes were detected in the stem cell niche of different organs, such as the heart, lung, skeletal muscle, and skin [39–43]. Heterocellular contacts between these two types of cells explain the possible involvement of TCs in tissue regeneration and repair. The first explanation of microenvironment-controlled stem cell activity, which is referred to as the niche, was provided 30 years ago [44]. Since that time,

numerous studies observed interplay between stem cell behavior and the surrounding tissue. Stem cells not only respond to multiple stimuli but also have an impact on the organism; it is therefore important to consider each stem cell interaction in both directions [45]. Gherghiceanu *et al.* focused on TC involvement in cardiac stem cell homeostasis [43]. Popescu *et al.* observed TCs-stem cells complexes in subepithelial niches of the bronchiolar tree [42]. Perlea *et al.* discussed possible detection of TCs in dental pulp stem niches [40], whereas Ye *et al.* assessed the genetic profile of murine lung TCs and their functional role in the stem cell niche [41]. We hypothesized that the bilateral interaction between uterine TCs and stem cells could represent a step in the pathogenesis of leiomyoma.

Our study proved the existence of telocytes in different parts of the human uterus (cervix, corpus, focuses of fibroids) — both affected and not affected by leiomyoma. Qualitative analysis revealed the reduction of TCs in fibroid foci, whereas the prevalence of collagen deposits was detected using routine histology. We attempted to explain the place of TCs in myoma architecture and focused on basic connecting points. We intend to continue our research to clarify the versatility of myometrial TCs and their fascinating properties.

Conflict of interest

None declared.

Author contribution

Veronika Aleksandrovych, Krzysztof Gil: study concept and design, acquisition of data, analysis and interpretation of data, drafting of the manuscript, critical revision of the manuscript for important intellectual content, statistical analysis, study supervision, and final approval of the manuscript. Magdalena Białas: histology, analysis and interpretation of data, critical revision of the manuscript for important intellectual content, and final approval of the manuscript. Artur Pasternak: searching bibliographic databases, editing and revising of the manuscript, analysis and interpretation of data, and final acceptance of the manuscript. Jerzy Walocha, Tomasz Bereza and Marek Sajewicz: analysis and interpretation of data and final acceptance of the manuscript.

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