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Sex steroid hormone receptors of telocytes — potential key role in leiomyoma development

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Abstract: Background: Uterine leiomyoma is the most widespread benign tumor affecting women of childbearing age. There are still gaps in the understanding of its pathogenesis. Telocytes are unique cells found in more than 50 different locations inside the human body. The functional relationship between cells could clarify the pathogenesis of leiomyomata. Examination of membrane receptors on telocytes could explain their role in fibrosis, oxidative stress, and myometrial contractility.
Aim: This research was conducted to assess the density of telocytes in terms of their putative role in leiomyoma formation by focusing on their correlation with the expression of estrogen and progesterone receptors.
Methods: For gross evaluation of uterine tissue samples from leiomyoma, routine histology of adjacent and unaffected myometrium was performed. Immunohistochemical analysis of c-kit, tryptase, CD34, PDGFRα (telocyte-specific), and ER and PRs (estrogen and progesterone receptors) was performed to examine uterine telocytes and the expression of sex steroid receptors.
Results: The decline in telocyte density in leiomyoma foci was correlated with high progesterone expression and low estrogen receptor expression. The unchanged myometrium showed the opposite correlation and balance between both steroid hormone receptors. The difference in sex steroid receptor expression is correlated with the density of uterine telocytes, which emphasizes their conductor function.
Conclusions: A reduction in telocyte density and the changes in examined marker expression demonstrate the involvement of telocytes in local homeostasis. The expression of membrane receptors explicitly indicates their functional potential in the human myometrium, focusing attention on contractility and local homeostasis.

Key words: leiomyoma, extracellular matrix, telocytes, CD34, estrogen, progesterone, fibrosis.

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Introduction

Uterine leiomyomas, also known as fibroids, represent a major public health problem and can be spontaneously diagnosed as a factor contributing to infertility among women of childbearing age. Leiomyoma is a benign, monoclonal diploid, and highly common gynecological tumor originating from uterine smooth muscle tissue [1–3]. The main pathological feature is the production of excessive quantities of extracellular matrix (ECM) and consequent fibrosis development [2]. The primacy of the transformation of a single myocyte into a leiomyoma is not clear [4]. Genetic, chromosomal, and epigenetic transformations as well as growth factors, cytokines, and steroid hormones may promote tumor growth [3, 4]. Controversy exists regarding the role of sex steroids in fibrosis development and the pathogenesis of uterine leiomyoma. Nevertheless, it is well known that a combination of estrogen and progesterone regulates most of the genes that encode growth factors [3]. Leiomyoma has a mechanical impact on myometrial contractility and induces a chronic local inflammatory reaction, making implantation almost impossible. In some cases, patients with leiomyoma have abnormal myometrial peristalsis and low levels of cytokines essential for implantation, which can also be considered contributing factors to infertility [5–7].

The majority of estrogen receptors are nuclear, whereas only up to 10% are located in the cell membrane [8–10]. Nuclear receptors are divided into the alpha (ERα) and beta (ERβ) subtypes [4], which are coexpressed in different kinds of tissue. However, the human myometrium is characterized by the prevalence of ERα receptor expression [11]. Membrane estrogen receptors have been revealed in cytoplasmic organelles (mostly in mitochondria and the endoplasmic reticulum), but their role is still unknown [9]. There is no doubt that estrogen receptors are essential for the development and growth of leiomyoma, which express both kinds of estrogen receptors. Estrogen-related aberrations are common in such patients [12, 13] because of the impact on the expression of growth factors via several signaling pathways [3]. For instance, estrogens upregulate platelet-derived growth factor (PDGF) expression in leiomyoma cells and downregulate epidermal growth factor (EGF) expression. At the same time, they upregulate the expression of epidermal growth factor receptor (EGFR) in both myometrium and leiomyoma cells [3, 14].

Progesterone receptors (PRs) are also divided into two groups, nuclear and membrane, which have rapid nonclassical action and therefore attract the attention of scientists [15, 16]. Nuclear progesterone receptors are also divided into alpha (PR-A) and beta (PR-B) subtypes, each of which has a different role. The beta subtype activates progesterone-responsive genes, while the alpha type can repress the activity of progesterone receptor beta [17]. The ratio of the two isoforms is not stable and depends on the phase of the cycle (physiological changes) and probably also on diseases. Membrane progesterone receptors reflect the nongenomic action of steroids.

They are comprised of proteins, including progesterone membrane components 1 and 2 and membrane progestin receptors (mPRs). The role of membrane receptors is often described outside of the female reproductive system. Thomas *et al.* demonstrated that these kinds of steroid receptors are expressed in the central nervous system, where they participate in a variety of nonclassical progesterone actions in neural tissues [16].

Nevertheless, progesterone and its receptors stimulate cell proliferation, the accumulation of extracellular matrix, and cellular hypertrophy, which form the background of leiomyoma development [3]. According to the literature data, the expression of PR-A and PR-B is significantly higher in leiomyoma tissue than in normal myometrium. Progesterone upregulates the expression of epidermal growth factor (which has mitogenic activity) and transforming growth factor beta-3 (TGF-β3) with bimodal action. Ciavattini *et al.* hypothesized that progesterone could stimulate leiomyoma cell growth by upregulating B-cell lymphoma 2 (Bcl-2) protein expression and downregulating tumor necrosis factor-alpha (TNF-α) expression [3, 18].

Telocytes (TCs) are a population of cells with a mesenchymal origin that have been identified in different organs in humans and animals [19, 20]. Many original articles and reviews have depicted their morphology, main features, criteria for identification, and possible roles [21–23]. We intend to emphasize the essence of their junctions and immunopositivity for sex steroid receptors in the context of the current study. These cells make homo- and heterocellular contacts with smooth muscle cells, blood vessels, nerves, immunocytes, stem cells, and other cell types. In addition, TCs are positive for both kinds of steroid hormone receptors [20, 24]. We also know that the myometrium in pregnant and nonpregnant women contains different numbers of telocytes, which reflects the correlation with the local hormonal balance. The next important point is that the density of TCs is decreased in tissue, which is affected by inflammation or fibrosis [20, 21]. All of the previously mentioned factors allow us to ask a question: "Does the immunopositivity for estrogen and progesterone receptors of uterine telocytes highlight their role in fibrosis development as conductor cells?"

Our study aimed to reveal a correlation between uterine telocytes and the expression of estrogen and progesterone receptors in leiomyoma and unchanged myometrium in women. We suggest that the immunopositivity of TCs for sex steroid hormone receptors could be an additional contributor to the pathophysiology of myometrial changes and local homeostasis.

Material and Methods

Subjects

Ten patients with symptomatic intramuscular solid UL were scheduled for elective surgery (laparoscopic hysterectomy) and selected for the study group (10 women,

mean age 58.2 ± 10.3 years). The control group consisted of 9 patients (9 women, mean age 53.5 ± 11.4 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. Samples of tissue from the fibrotic foci and adjacent myometrium were obtained from the study group for further observation. Samples of unchanged myometrium were also obtained from the control group. All patients were surgically treated at the Institute of Gynecology, Jagiellonian University Medical College, in 2019–2020.

Ethical approval

The study was conducted in accordance with the moral, ethical, regulatory and scientific principles governing clinical research. All samples were retrieved with the approval of the Jagiellonian University Bioethical Committee using procedures that conformed to the Declaration of Helsinki guidelines (protocol number — 1072.6120.48.2018).

Tissue processing

Tissue samples from 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, 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's trichrome stain to detect collagen deposits.

Immunofluorescence

After deparaffinization and rehydration, the slides were incubated for 30 min in PBS with appropriate normal serum at room temperature, which was followed by overnight incubation at 4°C in a solution of PBS with appropriate normal serum containing a mixture of primary antibodies. After 5 washes (10 min each) in PBS, the specimens were then incubated for 1 h at room temperature with a mixture of secondary antibodies diluted in PBS. Indirect double immunofluorescence for identification of telocytes and sex steroid hormone receptors in sections was performed with polyclonal rabbit anti-c-kit (1:100; code: A4502, Dako); monoclonal mouse anti-CD34 (1:100;

code: M7165, Dako); polyclonal goat anti-PDGFR alpha (1;100; code: AF-307-NA, R&D Systems); monoclonal mouse anti-tryptase (1:100; code: M7052, Dako); monoclonal mouse estrogen receptor (1:50; code: NCL-L-ER-6F11, Leica Biosystems); and monoclonal mouse progesterone receptor (1:100; code: Clone PgR636). Secondary antibodies included goat anti-mouse Alexa 488-conjugated antibody (1:400; code: 115-545-146, Jackson ImmunoResearch), goat anti-rabbit Alexa 594-conjugated antibody (1:400; code: 111-585-144, Jackson ImmunoResearch), and donkey anti-goat Alexa 594-conjugated antibody (1:400; code: 705-585-003, Jackson ImmunoResearch). Negative controls were performed that omitted the primary antibodies. Finally, the slides were washed with two changes (10 min each) of PBS, cover-slipped with fluorescence mounting medium (Dako, Denmark) and covered with Menzel-Gläser glasses. The labeled specimens were analyzed immediately.

Microscopic examination of telocytes, mast cells and collagen deposits

Slides were examined using an MN800FL epifluorescence microscope (OptaTech, Warszawa, Poland) equipped with an Olympus DP74 digital camera. Digital images were collected at either 200× or 400× magnification. The qualitative analysis of cells was performed in 10 consecutive high-power fields (400×) using the computer-based image analysis system software Multiscan 18.03 (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 that were double positive for CD34 and PDGFRα with the characteristic morphology and localization were also recognized as TCs. In all sections, the immunoreactive cells were evaluated with respect to the relative frequency (arbitrarily graded as very few = (+), few = +, moderate density = + +, high density = + + +). The same scale was used to describe estrogen and progesterone receptor expression in all tissue samples. The percentages of collagen deposits and muscle tissue were analyzed in specimens stained with Masson's trichrome stain. The collagen and muscle fiber volume ratios were determined in ten different fields for each sample.

Results

Histopathological description of tissue samples

The cellular pattern of tissue samples affected and unaffected by leiomyoma myometrium was observed under a microscope. The histopathological changes in all uterine samples in the current study were determined by hematoxylin–eosin and Masson's trichrome staining. Tissue samples from leiomyoma were characterized by islands in the extracellular matrix and divided groups of smooth muscle bundles without a regular orientation. The nuclei of spindle cells were mostly elongated (Fig. 1). In the foci of leiomyoma, muscle bundles form nodular structures. The tissue samples from the normal (unchanged) myometrium had smooth muscle bundles that maintained holistic structures. The adjacent myometrium had a less well organized structure in comparison with the unchanged tissue (from the "healthy" uterus). Light microscopy of uterine fibroids, adjacent myometrium, and normal myometrium using Masson's trichrome staining of collagen revealed collagen to be abundant in the fibroid tissue, while the myometrium had sparse, well-aligned collagen bundles adjacent to smooth muscle cells.

Expression of estrogen and progesterone receptors

The normal (unchanged) myometrium has a high prevalence of estrogen receptor expression in comparison with progesterone expression. Numerous ER-positive nuclei were found in all areas. In the samples affected by leiomyoma, the balance between both steroid hormone receptors was changed. In the leiomyoma foci, progesterone receptor expression was increased and estrogen expression was decreased (Fig. 2). In addition, we found that the expression of estrogen receptors was lower in leiomyoma than that in unchanged ("healthy") myometrium, while the expression of progesterone receptors was increased. In adjacent myometrium in areas of unchanged myometrium, which were close to a capsule of leiomyoma, expression of both types of receptors was decreased (vs that in normal and leiomyoma-affected tissue samples). However, the general pattern in both tissue samples resembled bundles of lines going in different directions (resembling fibers) (Table 1).

Description of uterine telocytes

Double-immunopositive cells for CD34 and PDGFRα were detected in all observed tissue samples of the human myometrium. Based on their morphology, immunoprofile, and localization in the tissue, we believed that they represented uterine telocytes. Some of these cells were observed close to blood vessels. The unchanged myometrium

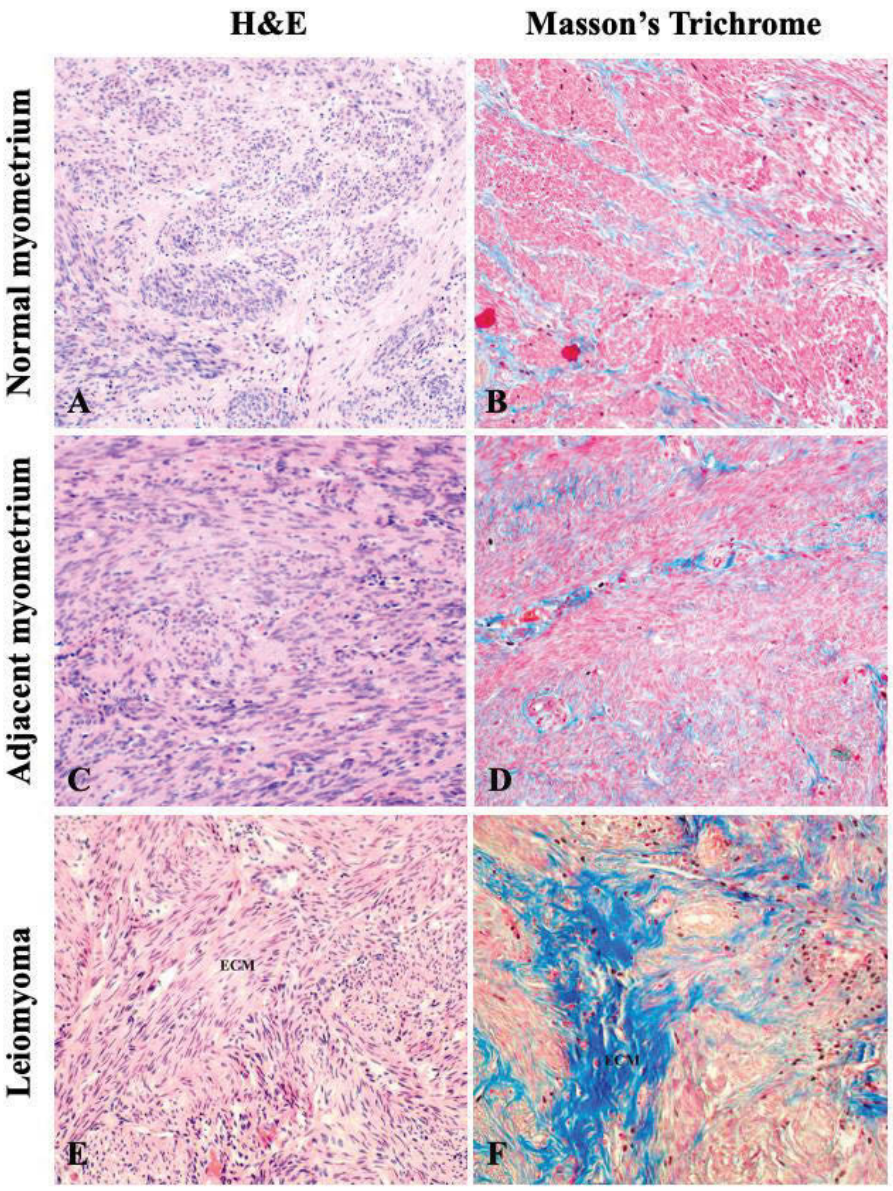

Fig. 1. Hematoxylin–eosin- and Masson's trichrome-stained sections of human myometrium. The myometrium sections from the control group (A, B) compared with the sections of foci from leiomyoma (E, F) and adjacent myometrium from the same uterus (C, D). After Masson's trichrome staining, collagen deposits were blue in color whereas muscle fibers were red in color. Fragments of disordered smooth muscle cells were separated by abundant extracellular matrix (ECM). Total magnification: 200×.

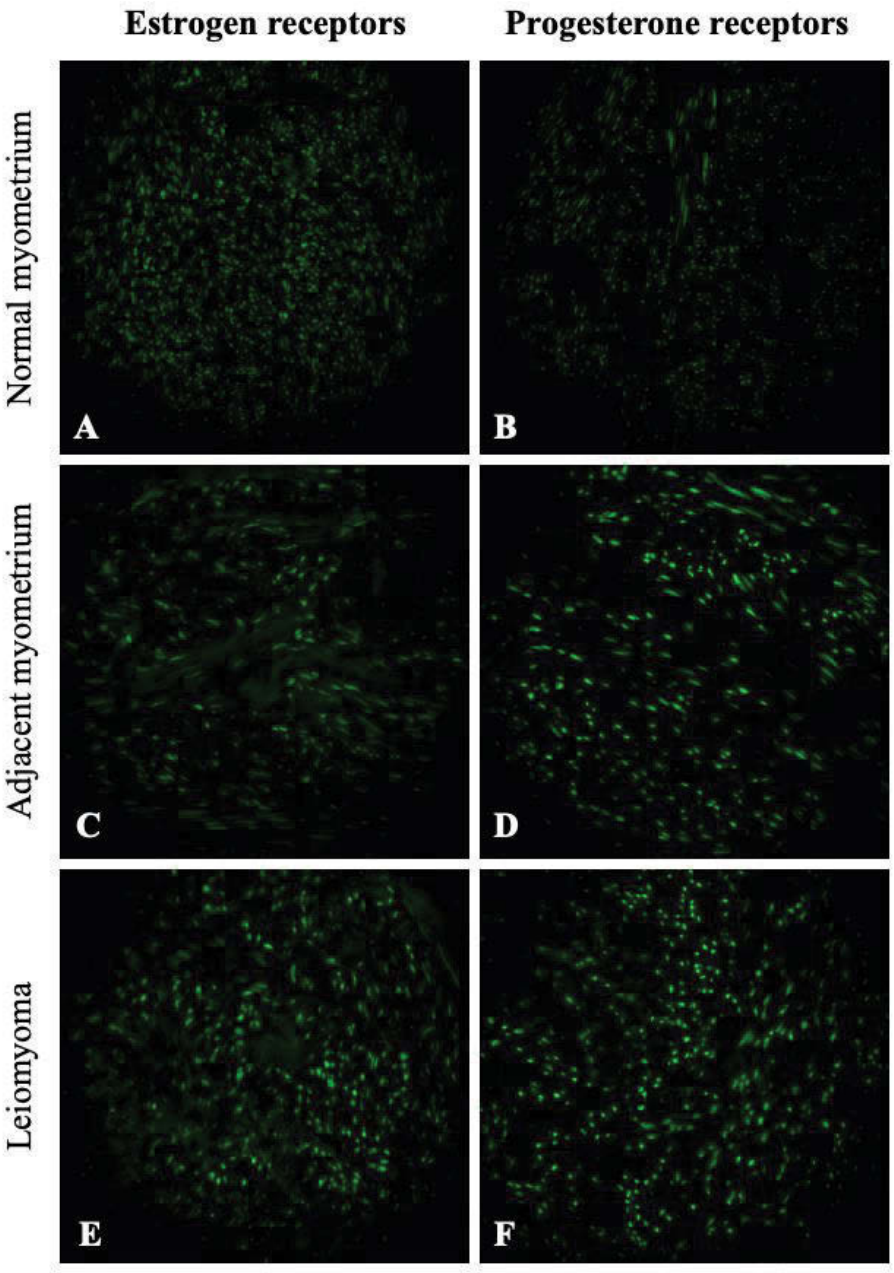

Fig. 2. Nuclear positivity for estrogen (green, Alexa Fluor 488) and progesterone (green, Alexa Fluor 488) receptors in myometrial samples in the unchanged myometrium (A, B), leiomyoma-affected myometrium (E, F) and adjacent myometrium (C, D). Total magnification: 400×.

Table 1. Relative frequency of c-kit-positive/tryptase-negative, CD34–positive and PDGFRα-positive, estrogen receptor-positive and progesterone receptor-positive cells in different parts of the unchanged human uterus and uterus affected by leiomyoma. 0 = absent, (+) = very few, + = few, + + = moderate density, + + + = high density.

		c-kit +/ tryptase –	CD34+/ PDGFRα+	ER expression	PR expression
Uterus without leiomyoma	Unchanged myometrium	+ + +	+ + +	+ + +	+ +
Uterus with leiomyoma	Adjacent myometrium	+ +	+ +	+ +	+ +
	Leiomyoma	+	+	+ +	+ + +

has more double-positive cells in comparison with that in all samples from the uterus affected by leiomyoma. In the leiomyoma foci, fewer telocytes were revealed. The scant architectonics of the cellular patterns were mostly due to the abundance of collagen. In the same results, we analyzed c-kit-positive/tryptase-negative cells (Table 1). We also performed double immunolabeling of c-kit/estrogen receptors as well as c-kit/progesterone receptors in the myometrium to analyze the role of telocytes, which were double-positive for both markers (Fig. 3, 4).

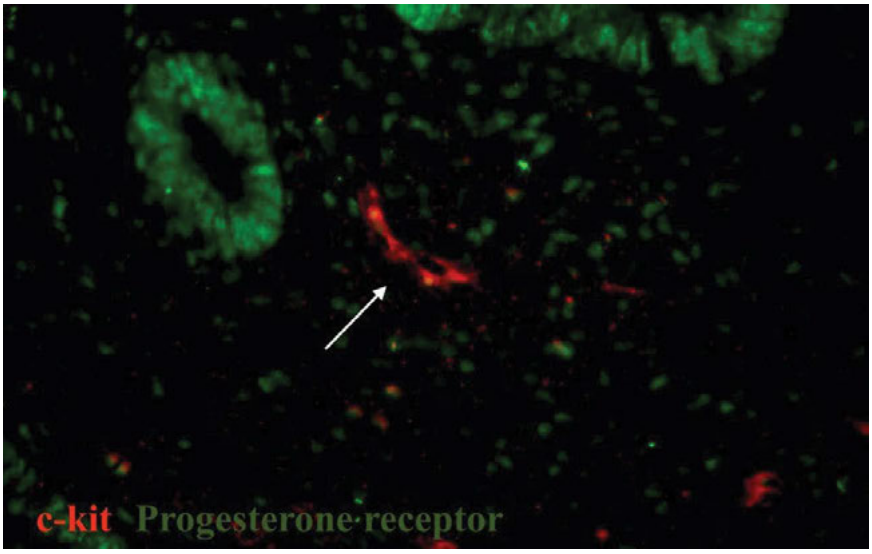

Fig. 3. Myometrial tissue sample stained for progesterone receptors (green, Alexa Fluor 488) and c-kit (red, Alexa Fluor 594). Uterine telocytes, which were double-positive for both markers, had oval-shaped bodies and long cellular lengths and were located close to blood vessels (indicated by arrow). Total magnification: 400×.

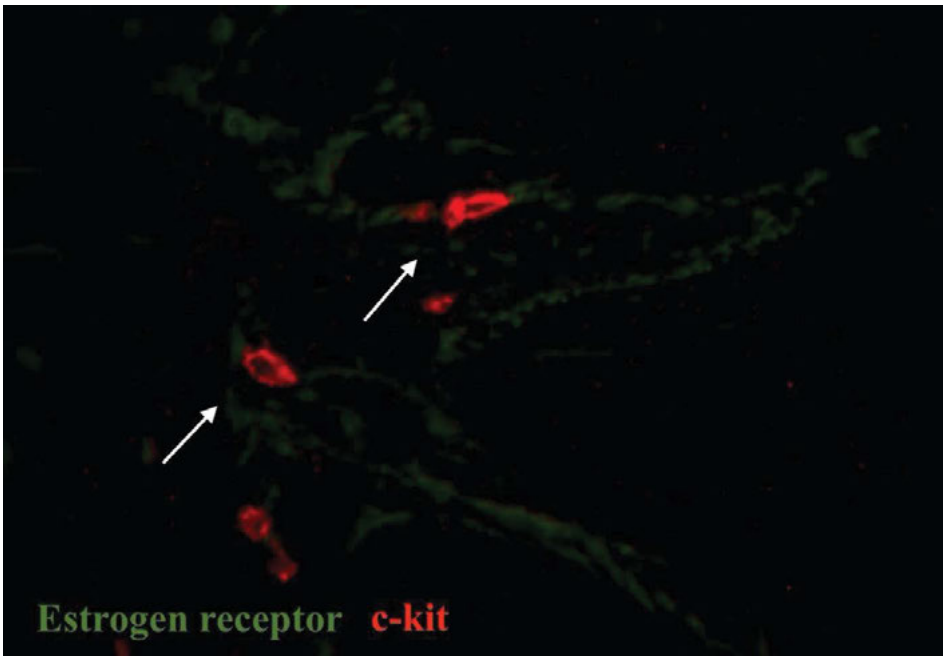

Fig. 4. Myometrial tissue sample stained for estrogen receptors (green, Alexa Fluor 488) and c-kit (red, Alexa Fluor 594). Two double-positive cells with long lengths, which were located close to each other, were detected near the capsule of the leiomyoma (are depicted by arrows). Total magnification: 400×.

Discussion

The first description of the coexpression of estrogen and progesterone receptors and telocytes in the female reproductive system was made by Cretoiu SM, who first focused her attention on this question more than ten years ago [25–27]. Since that time, several scientific groups have observed telocytes in the reproductive systems (male and female) of humans and animals (camel, cow, rat, rabbit, mouse, vole and turtle) [28–34]. Even such conditions as pregnancy have not been omitted from that point of view [21].

To date, how sex steroids impact cell proliferation and why their receptors are distributed on other cells, including telocytes, remain unclear. According to published data, oviduct TCs express estrogen and progesterone-A receptors [3, 21, 27]. PR-A expression was recognized by most anti-PR antibodies, whereas PR-B expression was not detected [3]. Telocytes are not always positive for sex steroid hormone receptors. Their immunopositivity is correlated with localization and probably the hormonal regulation of muscular contractions. For instance, telocytes of the gallbladder are negative for estrogen and progesterone receptors, while they are always strongly

positive in the uterus and oviduct [20]. The role of telocytes in myometrial contractions was previously excellently described. Therefore, gallbladder muscular contractions were shown to not be regulated by sex steroids, which play their own role in carcinogenesis in the gallbladder [35, 36]. Gupta *et al.* showed the absence of ER expression in benign gallbladder lesions, while PR shows wide variation in expression. The opposite situation has been observed in malignant cases, which show high expression of ER and PR in benign metaplastic lesions [37].

Our results demonstrated a decline in the expression of nuclear ER receptors in leiomyoma, which proves the benign nature of the pathology. The increase in PR receptor expression might stimulate growth factors with mitogenic activity, such as transforming growth factor-β3, basic fibroblast growth factor, epidermal growth factor, and insulin-like growth factor-I, which are also common in leiomyoma [3].

Levin hypothesized that the role of membrane estrogen receptors might be connected with the prevention of vascular injury or cardiac hypertrophy, sexual behavior, mechanisms of pain perception, osteoblast activity, neuroprogesterone synthesis, and fluid resorption in the colon [10]. Telocytes are widely present in the gastrointestinal tract and heart and are also involved in the activity of the autonomic nervous system [10, 37]. Their immunopositivity for estrogen receptors and localization might stress their possible role as conductors.

Horn *et al.* compared the number of uterine telocytes in the myometrium of nonpregnant premenopausal women without uterine endometriosis with that in postmenopausal women, the uterus with endometriosis and pregnant uteri. All four gynecological statuses are different in terms of the estrogen/progesterone balance. The highest level of progesterone in pregnant uteri was accompanied by the lowest number of telocytes. The highest number of telocytes (which was significant) was detected in the myometrium of nonpregnant premenopausal women, whose estrogen level is usually lower than that in patients with endometriosis based on the pathogenesis [38]. The number of telocytes is decreased in pregnancy [25]. We are not sure how the population of telocytes responds to pregnancy development. On the one hand, this rapid decline might be connected with the physiological enlargement of the uterus. However, this trend is reversible, and after delivery, the population of telocytes is restored in quantity. From another point of view, we observed a negative correlation between the number of telocytes and the level of progesterone or the expression of its receptors. This tendency is not typical for estrogen receptor expression.

Ben-Nagi *et al.* reported a reduction in the level of glycodelin in patients with submucosal leiomyomas. The secretion of this protein, which promotes angiogenesis and suppresses natural killer (NK) cells, is regulated by progesterone [7]. Immunopositivity of TCs for progesterone receptors and their reduction in number in leiomyoma might contribute to the local microenvironmental imbalance in glycodelin. TCs have a common dynamic in pathological conditions; fibrosis development is

accompanied by a decrease in TCs in psoriasis, leiomyomas, gallbladder disease, systemic sclerosis, and obstructive uropathy [39–43]. The common feature is that fibrosis and changes in sex steroid hormone/receptor expression are always correlated with the telocyte number.

Our study confirmed that because of their immunopositivity for various receptors, uterine telocytes might be considered conductor cells. In terms of in gynecological pathology, leiomyoma often coexists with an infertility problem. We are sure that further detailed analysis of the correlation between the estrogen/progesterone balance in the myometrium and the density of telocytes will provide essential knowledge regarding the pathogenesis of leiomyomas.

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Conflict of interest

None declared.

Author contributions

Veronika Aleksandrovych: 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, study supervision, and final approval of the manuscript. Anna Gil: analysis and interpretation of data, drafting of the manuscript, critical revision of the manuscript for important intellectual content, and final approval of the manuscript. Anna Wrona: editing and revising of the manuscript, analysis and interpretation of the data, and final acceptance of the manuscript.

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