

Increased Migration Ability of Adenomyosis-Derived Endometrial Mesenchymal Stem Cells

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Abstract—Adenomyosis is a form of endometriosis—a gynecological disease associated with abnormal functional activity of endometrial cells. Endometrial stem cells can play a key role in the pathogenesis of this disease. Despite the numerous studies that have been conducted of cultures of endometrial mesenchymal stem cells (eMSCs) obtained from patients with adenomyosis, information on their phenotypic and functional properties is very contradictory. In this work, a comparative study of morphological and migratory characteristics of human endometrial mesenchymal stem cells isolated from the menstrual blood of healthy donors (eMSCs) and a donor with adenomyosis (eMSCs-A) was performed. eMSC migration was evaluated by the method of “wound healing” using live-cell microscopy. It was established that the rate of wound healing of eMSCs-A is significantly higher compared to normal cells, which indicates an increased migration potential of eMSCs-A in adenomyosis. However, when transferring cells to a serum-free medium, eMSC-A migrated more slowly than normal cells. As a result of the assessment of morphological characteristics, it was found that eMSCs-A are smaller (in area and perimeter) than normal eMSCs, while the remaining morphometric parameters reflecting cell polarization did not differ. The data obtained allow the use of eMSCs in culture as a model for elucidating the membrane and intracellular mechanisms that underlie changes in cellular mechanics, motility, and invasive activity in various pathologies, including adenomyosis.

Keywords: endometrial mesenchymal stem cells, adenomyosis, endometriosis, cell motility, migration potential

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Adenomyosis is a common gynecological disease affecting 6–10% of women of reproductive age. In the medical literature, this form of endometriosis is characterized by pathological germination of the functional layer of the uterus (endometrium) into the muscle layer—the myometrium (Sasson, Taylor, 2008). In the myometrium, endometrial cells form foci of inflammation, which can contribute to the formation of tumors and lead to infertility. Despite much attention having been given to the study of the etiology of the disease, the cellular mechanisms of adenomyosis remain poorly understood. Endometrial tissue is renewed throughout the reproductive life of a woman. Human endometrium contains a stem cell population that is responsible for its ability to regenerate (Nikoo et al., 2014). Since adenomyosis is associated with abnormal functional activity of endometrial cells,

endometrial stem cells may play a key role in the pathogenesis of this disease (Sasson, Taylor, 2008; Dhesi, Morelli, 2015).

Judging by the literature, most experimental work has been carried out on endometrial mesenchymal stem cells (eMSCs) isolated from patients' endometrial tissues as a result of surgical intervention (for example, Banu et al., 2008; Chen et al., 2010). Stem cells isolated from menstrual blood are a relatively new source of mesenchymal stem cells and have prospects for use in cell therapy. Due to their availability and noninvasive isolation protocols, eMSCs obtained from the desquamated endometrium of patients with adenomyosis can be used to study the cellular and molecular aspects of this diseases (Shilina et al., 2015; Chen et al., 2019).

Despite the numerous studies of eMSCs that have taken place over the past 10 years, information on their morphological and functional characteristics in the norm and in a pathological state is very contradictory. Thus, in a number of works, it has been shown that the phenotypic profile, the capacity for targeted differen-

Abbreviations: eMSC—endometrial mesenchymal stem cell isolated from the menstrual blood of healthy donors, eMSC-A—eMSC isolated from menstrual blood of a donor with adenomyosis, FBS—fetal bovine serum.

tiation, and the proliferation rate of cells during adenomyosis do not differ from normal ones (Chen et al., 2010; Kao et al., 2011a, b). At the same time, morphological differences were found between eMSCs obtained from healthy donors and from patients with endometriosis (Nikoo et al., 2014). It was reported that, with endometriosis, eMSCs are less polarized than are normal fibroblast-like eMSCs. In addition, it has been shown that, in adenomyosis, eMSCs have higher proliferation and invasive activity (Banu et al., 2008; Nikoo et al., 2014).

Earlier, we isolated a cell line from the menstrual blood of a donor with adenomyosis, characterized it in detail, and investigated the karyotype of these cells (Shilina et al., 2015). The objective of this paper is to explore the morphological and migratory properties of cell cultures obtained from healthy donors and from a patient with adenomyosis under normal culture conditions and under conditions of serum starvation and, on the basis of the data obtained, evaluate the possibilities of using eMSCs as a model to elucidate the mechanisms underlying the changes in mechanical properties, mobility, and invasive activity in adenomyosis.

MATERIALS AND METHOD




Cell Culture

Studies were performed on eMSC lines obtained from desquamated endometrium in menstrual blood (Zemelko et al., 2011). Cells were cultured in DMEM/F12 medium (Gibco, United States) containing 10% bovine fetal serum (FBS) (HyClone, United States), as well as 1% antibiotic solution and 1% GlutaMAX solution (Gibco, United States). Cells were seeded twice a week in a ratio of 1 : 3–1 : 4 using 0.05% trypsin with EDTA (Invitrogen, United States). In the work, eMSCs isolated from the menstrual blood of healthy donors (eMSCs, lines 2304, 2804) and a donor with adenomyosis (eMSC-A, line 0404) (Shilina et al., 2015) were used.

Assessment of Migration of eMSCs by the Method of Wound Healing

eMSCs were sown in a sterile cultural four-well plate (Nunc, United States) with special silicone inserts for creating an experimental wound (Ibidi, Germany) in full medium. After reaching the confluent monolayer, the inserts were removed, thereby simulating the creation of a wound (approximately 400–500 μm wide), and the cells were washed with phosphate-buffered saline (PBS). PBS was then replaced with complete or serum-free medium, depending on the experimental issues. Using an Axio Observer Z1 microscope (Zeiss, Germany) and an incubation chamber to maintain cultivation conditions (37°C, 5% CO_2) took a photograph with a frequency of 1 frame in 30 min for 24 h using the AxioVision 4.8.2 software. A

Table 1. Parameters used to evaluate cell morphology

Shape	Parameter and its meaning	
	circularity	ratio of large to small diameter
	0.03	25.31
	0.17	5.656
	0.95	1.04

10 \times lens was used. The change in the wound area relative to the starting point in percent was then evaluated, the wound area was measured in the ImageJ program (NIH, United States), and the obtained numerical values were processed in Excel (Microsoft, United States). Images in transmitted light were obtained using a Carl Zeiss Primovert microscope (Carl Zeiss, Germany) equipped with a 20 \times lens and a digital CCD camera.

Cell Morphology Parameters

Using the ImageJ software, using the built-in “cell shape parameters” function, the area, perimeter, diameter ratio, and roundness were calculated. Cells were circled manually using the “freehand selection tool.” The “ratio of diameters” value calculates the ratio of the long and short diameters (D) of a virtual ellipse entered by the program into the selected cell. The “roundness” parameter shows the difference between the shape of the selection (the selected shape) and an ideal circle, for which the roundness value is 1. For clarity, Table 1 presents examples of morphological parameters of selected cells of various shapes.

The parameters obtained for at least 15 cells in each experiment were compared using the one-way ANOVA analysis of variance. The significance of data differences was evaluated at a significance level of 0.05. The values were subordinate to the normal distribution according to the Shapiro–Wilk test. Differences or their absence between specific groups were determined using the Bonferroni test. To confirm the equality of the variances of the estimated parameters, the Levene criterion was used. Data are presented as mean values and their standard errors.

RESULTS

In the present work, morphological differences and the migratory potential of eMSCs isolated from the menstrual blood of healthy donors (lines 2304, 2804) and a donor with adenomyosis (eMSCs-A) were investigated. Microscopy showed that eMSCs-A are smaller than healthy ones (Fig. 1a). Indeed, a computerized assessment of cell shape showed that eMSCs-A

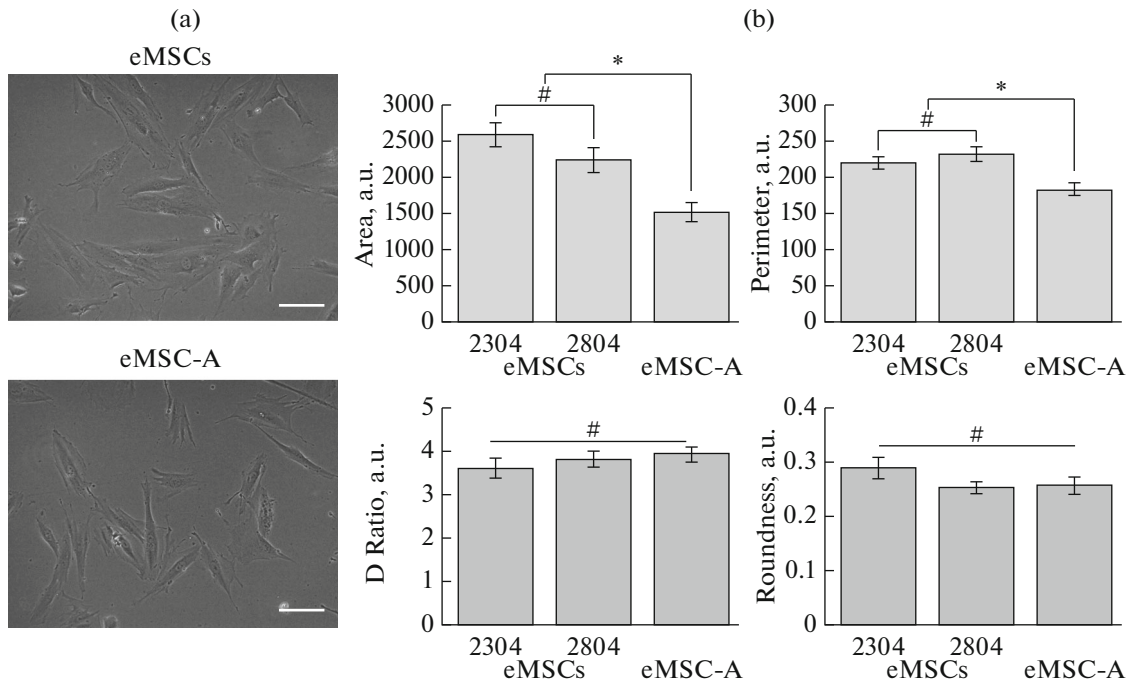


Fig. 1. (a) Morphological characteristics of eMSCs from healthy donors (lines 2804 and 2304) and from donor with adenomyosis (eMSC-A). (b) Values of cell parameters. eMSCs have a smaller area and perimeter than healthy eMSCs. Lens: 20 \times . D, ratio of long to short diameter. Mean values and their errors are given for at least 15 cells in each group. Differences are significant at $*p < 0.05$. #, differences are unreliable.

have a smaller area and perimeter than do normal eMSCs, while the remaining morphometric parameters reflecting fibroblast-like cells did not differ (Fig. 1b).

The migratory ability of healthy MSCs and eMSCs-A evaluated using the experimental wound healing method and live-cell time-lapse microscopy. Figure 2a presents the results of a typical (repeating) experiment for healthy eMSCs and eMSCs-A at the initial time (0 h) and 24 h after the start of filming. Averaged data showing a decrease in wound area for both cell lines in control conditions (in complete medium) are shown in Fig. 2b. 24 h after the start of the experiment, the area of the eMSC-A experimental wound was $31.3 \pm 8.1\%$ ($n = 5$) relative to the starting point, while in healthy cells the area was $47.2 \pm 5.4\%$ ($n = 5$). Thus, data analysis showed that the migration capacity of eMSCs-A is significantly higher than that of normal ones. Experimental area wounds in eMSCs-A decreased 1.3 times faster than in healthy ones. In addition, we did not reveal significant differences in the proliferation rate of the studied cell lines.

It is known that most techniques using mesenchymal stem cells in cell therapy require strict regulatory compliance and require the use of serum-free media. Serum in the culture medium may increase the risk of immune reactions and, thus, interfere with the use of cells in therapy (Yoshida et al., 2018). In addition, the transfer of cell cultures to serum-free medium for a

long time (24–36 h) is used when synchronizing them or modifying the lipid composition of cell membranes (Chubinskiy-Nadezhdin et al., 2013). Therefore, the next goal was to study the effect of FBS on cell migration. It turned out that the transfer of all lines of eMSC cells to serum-free medium affected their migratory ability, but in different ways (Fig. 3a). 24 h after the start of the experiment, the rate of healing of the experimental wound for eMSCs-A decreased 1.3 times in serum-free medium compared with the control (medium with 10% FBS): the wound area was $45 \pm 9.8\%$ ($n = 5$) relative to the initial value. The migratory capacity of healthy eMSCs in serum-free medium, on the contrary, increased compared to control conditions: the wound area after 24 h was $22.4 \pm 10.5\%$ ($n = 5$) relative to the starting point. Thus, it was found that, in medium without FBS, the migration rate of eMSCs-A is significantly lower (1.5 times) than healthy eMSCs.

The detected effect of serum on the dynamics of wound healing could potentially be associated not only with the influence of the serum-free medium on the eMSC migration mechanism itself, but also be a consequence of changes in the shape, degree of spreading and other characteristics of cells after prolonged cultivation in serum-free medium. To test the effect of serum-free medium on cell morphology, we analyzed changes in the form of eMSC-A and healthy eMSCs after 24 h of cultivation in medium without

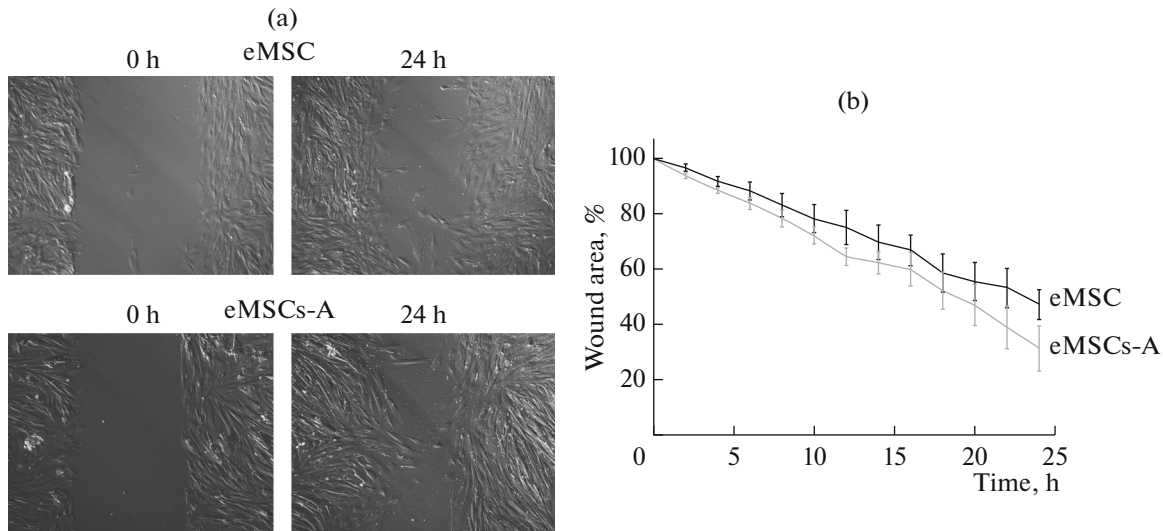


Fig. 2. Migration of normal eMSCs and eMSCs-A in a wound healing model. (a) Images showing the area of the experimental wound at the starting point (0 h) and 24 h after the start of the experiment. Lens: 10×. (b) Curve of wound healing rate averaged over five experiments for each cell line. The values of the wound area at each moment of time are normalized to the initial area at the beginning of the experiment. Vertical lines, errors of the mean.

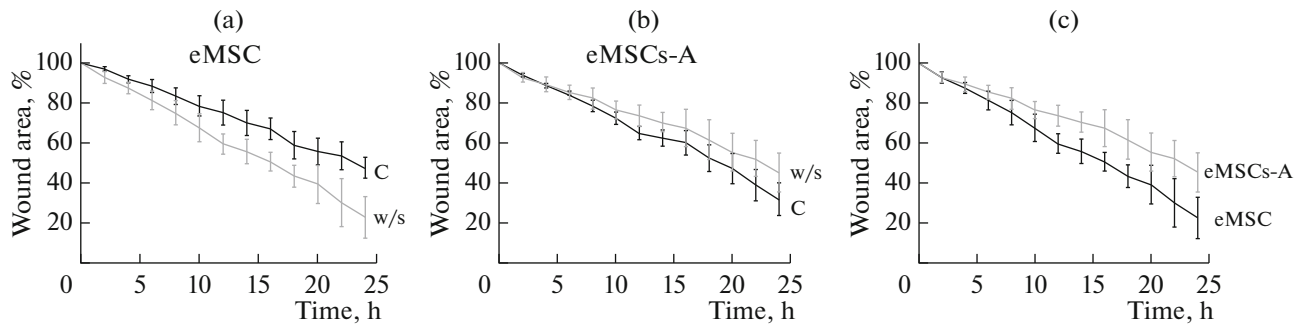


Fig. 3. Migration rate of normal eMSCs and eMSCs-A in control (C, medium containing 10% serum) and medium without serum (w/s, a, b). (c) Comparison of migration curves of normal eMSCs and eMSCs-A in serum-free medium.

FBS. The results of morphometric analysis did not reveal significant changes in the area and perimeter of cells in such conditions. The expected changes in cell proliferation when transferred to serum-free medium are unlikely to make a significant contribution to the dynamics of wound healing within 24 h of observation. It is most likely that the observed dynamics of wound healing under serum-free conditions is due to a change in cell motility.

DISCUSSION

In this work, we used previously characterized by markers, karyotype, and ability to directed differentiation of the eMSC cell line (Zemelko et al., 2011; Shilina et al., 2015). We have demonstrated that healthy eMSCs differ from adenomyosis in size, both under normal conditions and in serum-free medium. Thus, the area and perimeter of eMSC-A cells is smaller than

that of normal eMSCs, while the remaining morphological parameters remain unchanged. Changes in the morphology of stem cells obtained from the menstrual blood of donors with endometriosis have been found by Iranian authors (Nikoo et al., 2014). The functional significance of such changes is not yet clear. However, on the basis of theoretical and experimental assumptions, it can be suggested that the shape of the cell can modulate signal transmission due to changes in the curvature of the plasma membrane and cellular mechanics (Lloyd, 2013).

The results of our experiments show that, under standard conditions, in the presence of 10% FBS, eMSCs-A have a significantly greater migratory ability than healthy eMSCs. The revealed difference cannot be related to the proliferation rate of the studied cell lines, since it was shown that the population doubling time is practically the same and amounts to 23–25 h

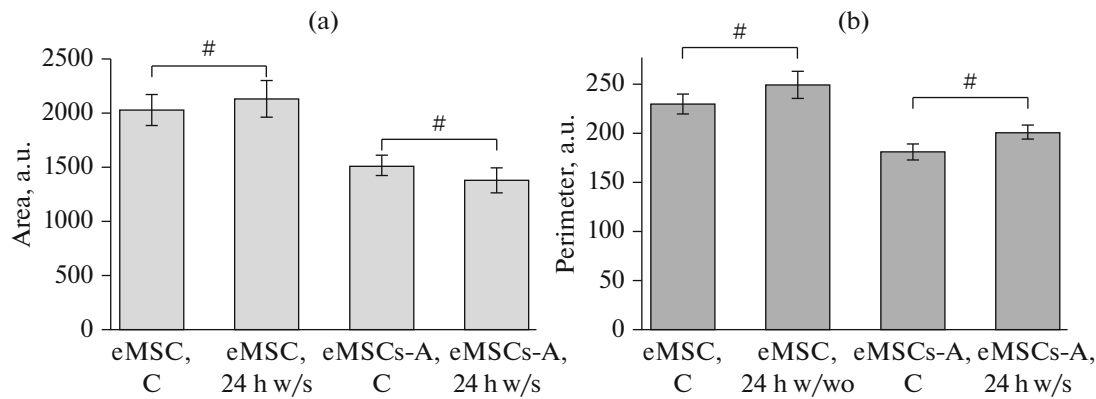


Fig. 4. Transfer of normal eMSCs and eMSCs-A to serum-free medium (w/s, 24 h) does not affect their morphometric parameters—(a) area and (b) perimeter. C, control (medium containing 10% serum). Differences are not significant when $^{\#}p < 0.05$.

(Zemelko et al., 2011; Shilina et al., 2015). At the same time, increased proliferative activity was observed in a number of studies on MSCs from donors with endometriosis (for example: Nikoo et al., 2014). Similar data regarding increased endometrial stem cell motility in adenomyosis have been reported for cell lines obtained using various protocols (Chen et al., 2010; Kao et al., 2011a; Nikoo et al., 2014). In all cases, MSCs from patients with endometriosis had a higher migration and invasive potential compared to MSCs from healthy donors. The data obtained allow to use the cell lines studied by as an adequate model for the analysis of mechanisms, underlying changes in cellular mechanics and motility in adenomyosis.

Data were obtained on stem cell cultures indicating likely changes in lipid metabolism associated with endometriosis. Cyclooxygenase-2 (COX-2) has been shown to be involved in an increase in the migratory and invasive ability of MSCs obtained from ectopic endometrioma (Kao et al., 2011b) and stem cells isolated from the menstrual blood of patients with endometriosis (Nikoo et al., 2014). Moreover, it has been suggested that the high invasive capacity of eMSCs-A may be partially connected with a higher level of indolamine 2.3-dioxygenase-1 (IDO1) (Nikoo et al., 2014). The increased migratory and invasive potentials are considered to be factors contributing to the malignant transformation of endometrial cells, which causes particular interest in identifying the molecular determinants of endometriosis—in particular, adenomyosis. Therefore, the fundamental changes in the migration of endometrial stem cells in a serum-free medium (FBS) that we discovered using live-cell microscopy are especially important. Transfer to a serum-free medium significantly affected the rate of wound healing of normal eMSCs and eMSCs-A; moreover, this happened in a multidirectional manner. According to our data (Fig. 3c), eMSCs-A migrated in the absence of serum much more slowly than did normal cells.

The use of FBS as an additive for the growth of human cells and tissues is widespread in both basic research and clinical approaches. However, it is known that FBS poses a risk of immune reactions and, thus, may interfere with the use of MSCs for the treatment of diseases. The effect of serum-free medium on the rate of migration and invasion is described in works on various cells and primary cultures. The presence of serum in the medium stimulates the migration and invasion of various cell lines of HeLa, SiHa, and murine fibroblasts (Brink et al., 2005; Hegera et al., 2018). It is reported that serum-free culture medium can enhance the immunosuppressive and antifibrotic capabilities of MSCs and is useful for the cultivation of clinically used MSCs (Solmesky et al., 2010; Gottipamula et al., 2014; Yoshida et al., 2018). However, the authors did not find the effect of serum-free medium on the migratory ability or the rate of engraftment of MSCs in rats (Yoshida et al., 2018). It is interesting to note that no work has described an increase in the migratory capacity of cells during serum starvation. In our work, it was first discovered that the migratory capability of eMSCs-A in serum-free medium (within 24 h) decreased, while, in normal eMSCs, on the contrary, it increased compared to control conditions. The serum withdrawal effect is an interesting result of the work, but requires further special analysis.

FUNDING

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COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflict of interest.

The authors did not conduct experiments involving animals or human beings. Cell lines obtained and characterized previously (Shilina et al., 2015) were used.

REFERENCES

- Banu, S.K., Lee, J.H., Starzinski-Powitz, A., and Arosch, J.A., Gene expression profiles and functional characterization of human immortalized endometriotic epithelial and stromal cells, *Fertil. Steril.*, 2008, vol. 90, pp. 972–987.
- Brink, H.E., Stalling, S.S., and Nicoll, S.B., Influence of serum on adult and fetal dermal fibroblast migration, adhesion, and collagen expression, *In Vitro Cell. Dev. Biol. Anim.*, 2005, vol. 41, pp. 252–257.
- Chen, Y.-J., Li, H.-Y., Chang, Y.-L., Yuan, C.-C., Tai, L.-K., Lu, K.H., Chang, C.-M., and Chiou, S.-H., Suppression of migratory/invasive ability and induction of apoptosis in adenomyosis-derived mesenchymal stem cells by cyclooxygenase-2 inhibitors, *Fertil. Steril.*, 2010, vol. 94, pp. 1972–1979.
- Chen, L., Qu, J., and Xiang, C., The multi-functional roles of menstrual blood-derived stem cells in regenerative medicine, *Stem Cell Res. Ther.*, 2019, vol. 10, no. 1. <https://doi.org/10.1186/s13287-018-1105-9>
- Chubinskiy-Nadezhdin, V.I., Efremova, T.N., Khaitlina, S.Y., and Morachevskaya, E.A., Functional impact of cholesterol sequestration on actin cytoskeleton in normal and transformed fibroblasts, *Cell Biol. Int.*, 2013, vol. 37, pp. 617–623.
- Dhesi, A.S. and Morelli, S.S., Endometriosis: a role for stem cells, *Womens Health*, 2015, vol. 11, pp. 35–49.
- Gottipamula, S., Ashwin, K.M., Muttigi, M.S., Kannan, S., Kolkundkar, U., and Seetharam, R.N., Isolation, expansion and characterization of bone marrow-derived mesenchymal stromal cells in serum-free conditions, *Cell Tissue Res.*, 2014, vol. 356, pp. 123–135.
- Hegera, J.I., Froehlich, K., Pastuscheka, J., Schmidta, A., Baera, C., Mrowkab, R., Backschc, C., Schlußnera, E., Markerta, U.R., and Schmidta, A., Human serum alters cell culture behavior and improves spheroid formation in comparison to fetal bovine serum, *Exp. Cell Res.*, 2018, vol. 365, pp. 57–65.
- Kao, A.P., Wang, K.H., Chang, C.C., Lee, J.N., Long, C.Y., Chen, H.S., Tsai, C.F., Hsieh, T.H., and Tsai, E.M., Comparative study of human eutopic and ectopic endometrial mesenchymal stem cells and the development of an in vivo endometriotic invasion model, *Fertil. Steril.*, 2011a, vol. 95, pp. 1308–1315.
- Kao, A.P., Wang, K.H., Long, C.Y., Chai, C.Y., Tsai, C.F., Hsieh, T.H., Hsu, C.Y., Chang, C.C., Lee, J.N., and Tsai, E.M., Interleukin-1beta induces cyclooxygenase-2 expression and promotes the invasive ability of human mesenchymal stem cells derived from ovarian endometrioma, *Fertil. Steril.*, 2011b, vol. 96, pp. 678–684.
- Lloyd, A.C., The regulation of cell size, *Cell*, 2013, vol. 154, pp. 1194–1205.
- Nikoo, S., Ebtekar, M., Jeddi-Tehrani, M., Shervin, A., Bozorgmehr, M., Vafaei, S., Kazemnejad, S., and Zarnani, A.-H., Menstrual blood-derived stromal stem cells from women with and without endometriosis reveal different phenotypic and functional characteristics, *Mol. Hum. Reprod.*, 2014, vol. 20, pp. 905–918.
- Sasson, I.E. and Taylor, H.S., Stem cells and the pathogenesis of endometriosis, *Ann. N.Y. Acad. Sci.*, 2008, vol. 1127, pp. 106–115.
- Shilina, M.A., Domnina, A.P., Kozhukharova, I.V., Zenin, V.V., Anisimov, S.V., Nikolsky, N.N., and Grinchuk, T.M., Establishment and characterization of a novel human endometrial mesenchymal stem cell line from a patient with adenomyosis, *Cell Tissue Biol.*, 2016, vol. 10, no. 1, pp. 10–17.
- Solmesky, L., Lefler, S., Jacob-Hirsch, J., Bulvik, S., Rechavi, G., and Weil, M., Serum free cultured bone marrow mesenchymal stem cells as a platform to characterize the effects of specific molecules, *PLoS One*, 2010, vol. 5, no. 9, e12689. <https://doi.org/10.1371/journal.pone.0012689>
- Yoshida, K., Nakashima, A., Doi, S., Ueno, T., Okubo, T., Kawano, K.-I., Kanawa, M., Kato, Y., Higashi, Y., and Masaki, T., Serum-free medium enhances the immunosuppressive and antifibrotic abilities of mesenchymal stem cells utilized in experimental renal fibrosis, *Stem Cells Transl. Med.*, 2018, vol. 7, pp. 893–905.
- Zemelko, V.I., Grinchuk, T.M., Domnina, A.P., Artsybasheva, I.V., Zenin, V.V., Kirsanov, A.A., Bichevaya, N.K., Korsak, V.S., and Nikolsky, N.N., Multipotent mesenchymal stem cells of desquamated endometrium: isolation, characterization, and application as a feeder layer for maintenance of human embryonic stem cells, *Cell Tissue Biol.*, 2012, vol. 6, no. 1, pp. 1–11.