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Communication

Single Ion Channel Recording in 3D Culture of Stem Cells Using Patch-Clamp Technique

Vladislav I. Chubinskiy-Nadezhdin[1*#], Anastasia V. Sudarikova[1#], Mariia A. Shorokhova[1], Valeria Y. Vasileva[1], Zuleikha M. Khairullina[1], Yuri A. Negulyaev[1]

[1] Institute of Cytology, Russian Academy of Sciences, 194064 Tikhoretsky Ave. 4, St. Petersburg, Russia
[#] Equal contribution
[*] Correspondence: vchubinskiyl@incras.ru

Abstract: Tri-dimensional (3D) cell aggregates or spheroids are considered to be closer to physiological conditions than traditional 2D cell culture. Mesenchymal stem cells (MSCs) assembling in spheroids have increased the survival of transplanted cells. The regulation of the biological processes that maintain crucial physiological reactions of MSCs is closely related to the functioning of ion channels. The pattern of expression, role and regulatory mechanisms of ion channels could be significantly different in 3D compared to 2D culture, and, thus, needed to be properly analyzed on the level of ionic currents. We developed a specific approach that allowed us to register, for the first time, endogenous ion channels in endometrial MSCs (eMSCs) assembled in spheroids. Particularly, using the single-channel patch-clamp technique, we have recorded the activity of ion channels and observed their functional interplay in mechanosensitive clusters. Our experimental protocol could be applied for identification and studying of ion channels in 3D cell cultures.

Keywords: 3D cell culture; patch-clamp; spheroids; single-channel recording; ion channels; mesenchymal stem cells

1. Introduction

Mesenchymal stem cells (MSCs) are multipotent stromal cells with high proliferative activity and the ability to migrate and differentiate into various cell types, including adipocytes, osteoblasts, chondrocytes, and myocytes [1,2]. Currently, MSCs as a tool for cell therapy of various diseases is the subject of a large number of studies. The source of stem cells can be such adult tissues as bone marrow, adipose tissue [3], umbilical cord blood [1], placenta [4], endometrium [5], and others [6]. MSCs isolated from menstrual blood (endometrial MSCs, eMSCs) have good prospects for future clinical applications because they can proliferate faster than MSCs from other sources, have low immunogenicity [7] and lack of tumorigenicity [8], as well as due to their non-invasive isolation process and the relatively few ethical issues [9,10]. Traditionally, two-dimensional (2D) adhesive culture conditions have been used as the standard method for MSCs culturing in vitro, but are very far from in vivo conditions and the cell properties differ significantly from the properties of cells in the body. Compared to 2D cell culture, 3D cell aggregates or spheroids are considered to be closer to physiological conditions. Recent publications provide evidence that MSCs (and eMSCs) assembling in 3D multicellular spheroids have increased survival of transplanted cells and therapeutic potential due to secretion of more trophic factors (antitumorigenic, angiogenic, anti-inflammatory) and other factors participating in tissue regeneration [11-13].

The regulation of biological processes that maintain crucial physiological reactions of MSCs such as cell proliferation, migration, differentiation, gene expression, cell volume regulation and other is closely related to the functioning of ion-transporting membrane proteins, in particular, to the activity of various types of ion channels. The expression pattern of ion channels has been reported to significantly vary in stem cells from different

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origins and sources [14]. Despite the large number of works devoted to the study of the functional role of ion channels in various types of stem cells, there is few data on the involvement of ion channels in the physiological functions of eMSCs from menstrual blood. In our previous works using single-channel patch-clamp analysis, the functional expression of several types of endogenous ion channels in eMSCs was detected [15-17]. In particular, we identified Ca^{2+}-permeable mechanosensitive stretch-activated channels (SACs) and their Ca^{2+}-mediated functional coupling with Ca^{2+}-activated potassium channels of big conductance (BK, KCa1.1). These channels may be involved in the mechanotransduction processes mediating their contribution to the regulation of migration, differentiation and proliferation of stem cells [15]. Currently, there are no publications concerning the role of ion channels and their activity in MSCs (and eMSCs) assembled in spheroids. It is known that the organization of stem cells in 3D culture affects cell microenvironment, their morphology, polarization, actin cytoskeleton and cell to cell interactions within the spheroid [18,19]. Thus, the functional expression, mechanisms of regulation and role of endogenous ion channels could be significantly different in 3D compared to 2D culture and needed to be thoroughly analyzed. Moreover, selective modulation of ion channel activity in spheroids could be a promising approach to modulate their reactions during spheroid-based cell therapy. Here, using single-channel patch-clamp technique we developed a specific approach that allowed us to register, for the first time, the activity of endogenous ion channels in eMSCs cultivated in 3D conditions.

2. Materials and Methods

Cells. Human endometrial mesenchymal stem cells (eMSCs, line №2804) were isolated from desquamated endometrium of menstrual blood and characterized [20] in the Department of Intracellular Signaling at the Institute of Cytology of the Russian Academy of Sciences (St. Petersburg, Russia). These cells meet minimal criteria of the International Society for Cell Therapy for multipotent MSCs [20,21], express mesenchymal surface markers (CD13, CD29, CDD44, CD73, CD90, CD105), are negative for hematopoietic markers (CD11b, CD34, CD45, CD117, CD130, HLA-DR class 2), and are capable of differentiation in the adipogenic and osteogenic directions [20]. eMSCs were cultured in DMEM/F12 medium (Gibco, USA) with 10% fetal bovine serum (HyClone, USA), 1% antibiotic-antimycotic mixture, and 1% GlutaMAX (Gibco, USA). Cell passaging was performed twice a week at a ratio of 1:3–1:4 using a 0.05% trypsin–EDTA solution (Invitrogen, United States).

Formation of spheroids. Spheroids (3D culture) were formed from a suspension of eMSCs (2D culture) from 4 to 11 passages using the hanging drop method. Drops of 35 μl volume containing 7000 cells were placed on the cover of 10 cm Petri dishes (Corning, NY, USA) and inverted. Cells under the action of surface tension and gravity aggregated in hanging drops for 48 hours. After that, the drops with formed spheroids were collected, and the resulting spheroids were subjected to further analysis. 40 min before patch-clamp experiments spheroids were plated on coverslips pre-coated with poly-DL-lysine (Sigma-Aldrich, USA). The mean size (± S.D.) of the spheroids was 230 ± 32 μm (n=16). eMSCs in spheroids retain all main properties of eMSCs in 2D culture including differentiation potential, and expression of CD markers, except for CD146 [13].

Electrophysiology. To record unitary ion currents from eMSC spheroids a cell-attached configuration of the patch-clamp technique was used. Gap-free single-channel currents were recorded with Axopatch 200B amplifier (Molecular Devices Corp., USA) interfaced via a Digidata 1550A low-noise digitizer to the computer running the pClamp 10.7 software (Molecular Devices Corp., USA). Signal sampling was performed at a frequency of 10 kHz and low-pass filtering at 0.3 kHz using an 8-pole Bessel filter. Micropipettes were made at P-97 Flaming/Brown puller from borosilicate glass capillaries (BF 150-110-10, Sutter Instrument, USA) and had resistance from 8 to 10 MΩ when filled with standard extracellular solution. The standard pipette solution contained (in mM): 145 NaCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES/TrisOH. To nullify the resting membrane potential the chamber was

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filled with typical potassium-containing bath solution (in mM): 145 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES/TrisOH. Channel activity was recorded at various membrane potentials from -70 to +100 mV. Single-channel current data were analyzed and processed using Clampfit 10.7 (Molecular Devices Corp., USA) and Qtiplot (Iondev SRL., Romania). The images of the spheroids during patch-clamp experiments were acquired using Touptek 5.1 Mpix CMOS camera (Hangzhou Touptek Photonics Co. LTD, China) controlled via ToupCam Software (Touptek, China). To register transmembrane ion currents, the specific method of forming a high resistance gigaseal contact (patch) between the recording micropipette and the plasma membrane of eMSCs grown as spheroids was developed (see Results and Discussion).

3. Results and Discussion

eMSCs spheroids are densely packed and could slightly differ in its size and shape, thus the precise focal plane in which the patch pipette contacts the surface of the spheroid is hardly to be reliably determined. Thus, stem cells in spheroids could not be successfully patch-clamped using a "canonical" approach on 2D cell cultures: the problem of correct determination of the focus and patch pipette position relative to the spheroid will result to pipette contamination and breakage. To overcome these major limitations, we have developed a specific approach that allows the formation of stable patches on eMSC spheroids with a significant rate of success. Particularly, the focal plane of the inverted microscope in patch-clamp setup is changed by re-focusing from the surface of the cover glass (Figure 1A, focal plane 1, which is routinely used for patch-clamp experiments on 2D cells) to the new focal plane that is slightly higher than the coverglass (focal plane 2, Figure 1B). After selecting the spheroid of interest (Figure 1B), it is translocated to the left plane of the field of view using the X-Y stage of the inverted microscope. Then, using the micromanipulator, the patch pipette is moved to the focal plane 2: the pipette tip is clearly seen under the microscope (Figure 1C). After that, spheroid is accurately moved using X-Y stage towards (from left to right) the pipette tip, and its position is continuously controlled (Figure 1D). In parallel, the pipette resistance (R) is monitored in pClamp 10.7 software, and indication of the initial contact between the pipette tip and the cell from the spheroid surface is the increase of R from 8-10 MΩ (typical R of pulled pipettes, see Materials and Methods) to 12-15 MΩ (Figure 1E). After that, a "negative pressure" is applied via patch pipette that results in a further increase of the R indicating the start of the process of tight contact formation between the pipette and the cell in spheroid. When R-value reaches 200-220 MΩ, the pressure is removed, and it results in the further increase of R, and a rapid establishment of the giga-seal (of electrical resistance about 3-5 GΩ) that allows to record single ion currents from the membrane fragment (cell-attached configuration) of the cells in the 3D spheroids (Figure 1F). Practically, our technique is based on the fixation of the patch pipette in the focal plane with further movement of the spheroid towards the pipette using the microscope stage rather than the "canonical" approach of the pipette to the cells using the manipulator. Until our work, patch-clamp recording was performed only on stem cells that spread from the spheroid's body after several days (3 to 8 days) from plating of spheroids on coverslips. Evidently, those cells could not be considered as an intact part of the whole 3D culture [22,23]. In contrast, our technique could be successfully utilized for patch-clamp recording from cells on the surface of 3D culture. Also, our assay is practically invariant to any differences in form of spheroids and could be applied to perform patch-clamping of spheroids of any size and shape. Importantly, a single spheroid could be used for patch-clamping several times, however, exactly same region for the contact of the pipette with the cell surface should be avoided.

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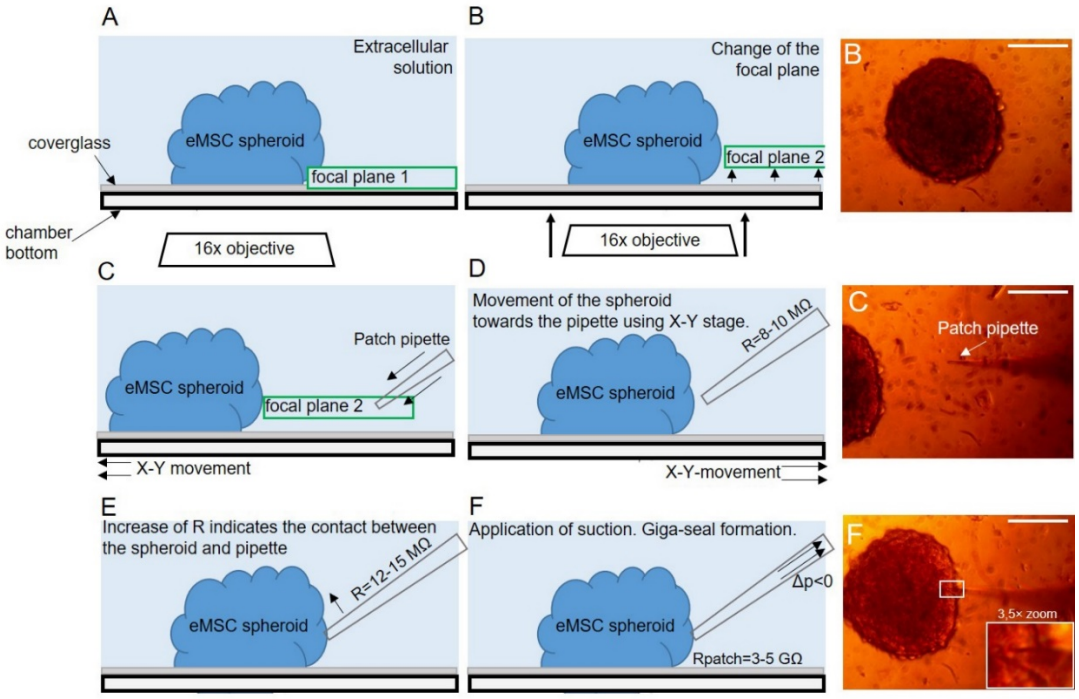

Figure 1. Step-by-step protocol of the cell-attached patch formation on eMSCs assembled in spheroids. On the right side of the scheme, the microscopic images of the spheroid and patch pipette are shown. The scale bar is 100 μm. (A). The focal plane of the inverted microscope is changed from the surface of the cover glass (focal plane 1) to the new one that is slightly higher (focal plane 2, (B)). (C). The spheroid is relocated leftwards using the X-Y stage, then the patch micropipette is moved to the focal plane 2; the tip of the pipette is in focus. (D). The X-Y stage of the microscope with the spheroid is slowly moved towards the pipette tip. (E). The increase of the resistance of the pipette (R) indicates the initial contact between the pipette tip and the spheroid. (F). The application of "negative pressure" via patch pipette results in the formation of a giga-seal between the pipette and the cell on the surface of the spheroid.

Using a developed approach, we were able to obtain stable patches (n=62) on eMSCs spheroids in about 60-70% of experiments. We probed, for the first time, to stimulate the activity of SACs in response to membrane stretch in eMSCs grown in 3D spheroids. Firstly, we registered the basal activity of ion channels in a wide range of membrane potentials from -70 mV to +100 mV in the absence of mechanical stimuli ("suction"). In about of 50% patches (29 from 62), the background channel activity was low or absent. In 13 experiments the increase of membrane potential higher than +50 mV resulted in stimulation of outward currents in voltage-dependent manner (increase in the number of channel openings, Figure 2A), that is characteristic feature of BK channels previously reported to be expressed in eMSCs [15,16].

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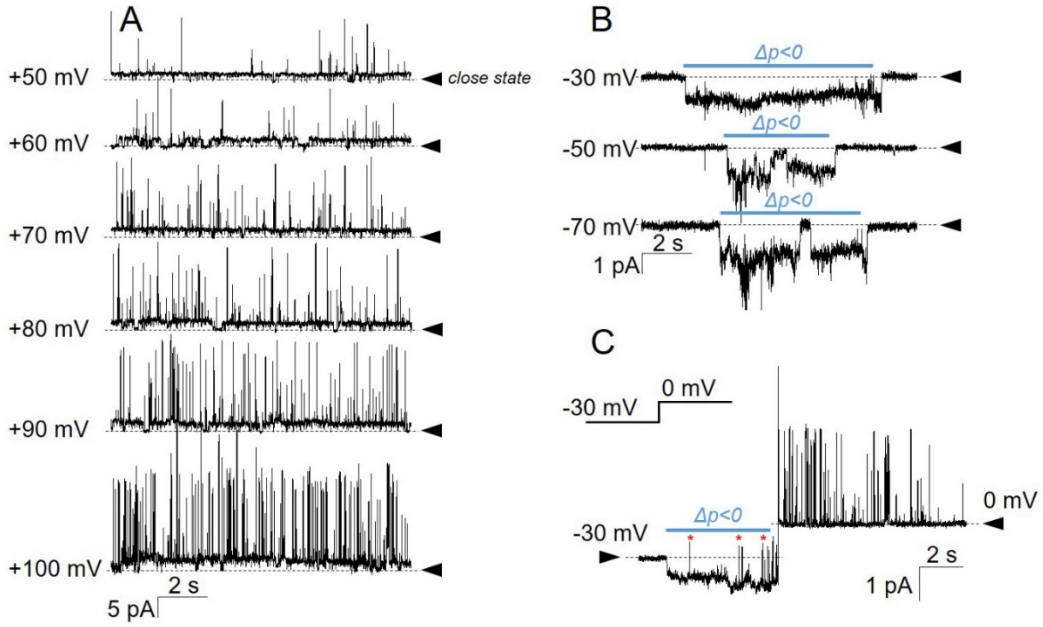

Figure 2. Single ion channel activity in the plasma membrane of eMSCs spheroids. (A). The typical voltage-dependent activity of BK channels at membrane potentials from +50 mV to +100 mV in the cell-attached configuration. The holding membrane potentials are indicated near the current traces. Close state (zero current) is indicated by black arrows. (B). Activation of SACs by membrane stretch in the plasma membrane. Application of a mechanical stimulus (suction, $\Delta p<0$) is shown by line under current traces. (C). Functional activity of two different types of ion channels in membrane fragment. The application of mechanical stimulus induces the activity of SACs (inward currents) followed by the activation of BK channels (outward currents). Asterisks (*) indicate outward BK channel openings together with inward SAC activity at -30 mV. Only BK channels are observed after rapid switching of the membrane potential from -30 mV to 0 mV as SACs have a reversal potential close to zero (for details, see [15]).

Then, we applied membrane stretch via patch pipette mostly in the negative range of membrane potentials to elicit inward SAC currents, as SACs in eMSCs have a reversal potential near zero [15]. In several patches (16 from 62), the application of suction resulted in activation of SACs (Figure 2B) with characteristics close to those reported previously in eMSCs. Interestingly, the percent of "SAC-positive" patches was lower (about 25% compared to 50-60% in 2D cell culture, [15]) in the spheroids, but this observation requires further analysis and more experimental data. More importantly, in number of patches (7 from 16 with SACs) inward SAC currents were followed by outward BK channel activity (Figure 2C). Previously, we have shown that Ca^{2+} entry via SACs stimulated the activity of BK channels in eMSCs under 2D culture conditions [15]. The observed phenomenon indicates that native SACs and BK channels are functionally active in the plasma membrane of eMSC spheroids and that these two types of channels form functional complexes in 3D culture. In sum, (1) we have established a specific approach for patch-clamp recording from the cells in 3D culture, (2) have recorded, for the first time, the activity of native ion channels (SACs and BK) from the spheroids and (3) evidenced the principal similarity between local mechanisms of Ca^{2+} signaling realized by Ca^{2+}-permeable SACs and Ca^{2+}-activated BK channels in the cells grown under 2D and 3D conditions. Our specific protocol could be successfully applied in further studies that could be aimed at comparing the ionic mechanisms of cell signaling and ion channel regulation between 2D cells and spheroids and in revealing the functional role of ion channels in the physiological reactions of 3D cultures in course of spheroid-based cell therapies.

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Data Availability Statement: The data are avaliable within the article and upon request.

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