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Development of a reporter cell line for visualizing PI3K signaling during MSCs adipogenic differentiation

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Abstract

Several hormones and paracrine factors like insulin, adiponectin, leptin, IGF-1, -2 activate phosphoinositide 3-kinase (PI3K) signaling pathway affecting adipogenic differentiation of multipotent mesenchymal stromal cells (MSCs). This study was aimed to develop an instrument for tracking a link between hormonal signaling and long-term cell response like cell differentiation using genetically encoded PH-GFP biosensor. Human Telomerase-Immortalized (hTERT) MSC was transduced by a lentiviral vector with the sequence based on pleckstrin homology domain of Bruton's tyrosine kinase and GFP (PH-GFP). Then, the cell line was separated using flow cytometry. MSC^{GFP-PH} were examined for the PDGF- and insulin-induced fluorescent responses, differentiation potential using standard differentiation protocols and proliferation rate applying neural networks for cell quantity counting. The obtained hTERT MSC^{GFP-PH} cell line retains intact adipogenic and osteogenic potential. It allows for the real-time detection of insulin and other PI3K-agonist signaling in living cells, both in the undifferentiated MSC state and during adipogenic differentiation. To simplify the detection of signaling events, we applied a computational approach to improve signal visualization and used a special metric to assess signaling activation. MSC^{GFP-PH} may be a useful instrument for investigating PI3K signaling in real time during long-term cellular processes such as adipogenic differentiation. This computational approach may represent a convenient option for detecting signaling by the PH-GFP biosensor at the single-cell level.

Keywords: Insulin, PI3K signaling, PIP3, Biosensors, MSC

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Разработка репортерной клеточной линии для визуализации PI3K-сигналинга в процессе адипогенной дифференцировки МСК

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Аннотация

Цель: ряд гормонов и паракринных факторов: инсулин, адипонектин, лептин, инсулиноподобный фактор роста-1, -2 — активируют сигнальный путь фосфоинозитид-3-киназы (PI3K) и оказывают значительное влияние на адипогенную дифференцировку мультипотентных мезенхимальных стромальных клеток (МСК). Настоящая работа была направлена на создание инструмента на основе генетически кодируемого биосенсора PH-GFP для определения взаимосвязи между гормональной сигнализацией и долгосрочным клеточным ответом, таким как дифференцировка клеток. **Методы.** Клеточная линия МСК, иммортализованная теломеразой человека (hTERT), была трансдуцирована лентивирусным вектором, несущим генетическую последовательность на основе домена плекстриновой гомологии тирозинкиназы Брутона, соединенного с зеленым флуоресцентным белком (PH-GFP). Отбор трансдуцированных клеток проводили методом проточной цитометрии. Полученная линия МСК^{GFP-PH} была охарактеризована по флуоресцентному ответу на PDGF и инсулин, дифференцировочному потенциалу с применением стандартных протоколов индукции дифференцировки, а также по скорости пролиферации с использованием нейронных сетей для подсчета клеток. **Результаты.** Было установлено, что клеточная линия hTERT МСК^{GFP-PH} обладает неизменным адипогенным и остеогенным потенциалом по сравнению с немодифицированной линией. hTERT МСК^{GFP-PH} позволяет регистрировать сигналинг инсулина и других агонистов PI3K в живых клетках в режиме реального времени как в МСК, находящихся в недифференцированном состоянии, так и в МСК в процессе адипогенной дифференцировки. Для упрощения анализа сигнальных событий был

применен вычислительный подход, обеспечивающий улучшенную визуализацию сигнала, а также введена специальная метрика для оценки активации PI3K сигнального пути. **Заключение.** Таким образом, МСК^{GFP-PH} представляет собой перспективную клеточную линию для изучения PI3K-сигналинга в реальном времени в условиях длительных клеточных процессов, таких как адипогенная дифференцировка. Предложенный вычислительный подход может оказаться удобным решением для детекции сигнальных событий с помощью биосенсора PH-GFP на уровне единичных клеток.

Ключевые слова: инсулин, PI3K-сигналинг, PIP3, биосенсоры, МСК

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Introduction

Adipose tissue is one of the key endocrine regulators in the human body. Throughout life, its functional integrity is maintained through continuous renewal by multipotent mesenchymal stromal cells (MSCs). They are self-renewing multipotent cells that can be isolated from the stroma of different organs, bearing certain immunohistochemical markers, capable of attaching to plastic, and of differentiating into adipocytes, osteoblasts, and chondroblasts [1]. These cells play an essential role in the maintenance of tissue homeostasis by creating a supportive microenvironment for the resident tissue progenitor cells, as well as coordinating their tissue renewal activity [2]. In adipose tissue, MSCs perform all these functions and serve as a source of adipocytes, thereby functioning as key regulators of adipose tissue homeostasis. In the process of adipogenic differentiation MSCs undergo two successive steps: 1) commitment of MSCs to preadipocytes, 2) subsequent terminal differentiation of preadipocytes into adipocytes [3].

The crucial drivers of such transitions are hormones and paracrine factors, such as insulin and insulin-like

growth factors 1/2, which upregulate the phosphoinositide 3-kinase (PI3K) signaling pathway. This signaling pathway controls the proliferation, differentiation, secretion of cytokines and growth factors, as well as the migration and angiogenic properties of MSCs [4]. The PI3K enzyme converts phosphatidylinositol 4,5-bisphosphate (PIP₂) into phosphatidylinositol 3,4,5-trisphosphate (PIP₃). PIP₃ then anchors its effector proteins near the plasma membrane in order to activate, stabilize, or co-localize them [5]. In PI3K-Akt signaling pathway, binding of 3-phosphoinositide-dependent protein kinase 1 (PDK1) to PIP3 leads to the subsequent phosphorylation of Akt (protein kinase B). Activated Akt participates in a large number of intracellular processes and phosphorylates various target proteins. Many of these targets are involved in the regulation of adipogenic differentiation. Forkhead box protein O1 (FOXO1) and GATA Binding Protein 2 (GATA2) are transcription factors, which inhibit transcription of PPARγ [6, 7]. Moreover, GATA2 also inhibits adipogenic differentiation by capturing proadipogenic transcription factors CCAAT-enhancer-binding

protein α (C/EBP α) and C/EBP β [8]. Akt-mediated phosphorylation of FOXO1 and GATA2 induces their translocation from the nucleus to the cytoplasm and subsequent degradation [9–11]. Also, Akt activates mammalian target of rapamycin complex 1 (mTORC1) through both direct phosphorylation of PRAS40, an endogenous mTORC1 inhibitor, and phosphorylation of Tuberous sclerosis proteins 1 and 2 (TSC1/2), which relieves inhibition of Rheb-GTP and allows mTORC1 activation, thereby promoting the expression of pro-adipogenic and lipogenic genes [12, 13]. mTORC1 promotes sterol regulatory element-binding protein 1 (SREBP1c) activation through two complementary mechanisms. First, mTORC1 facilitates the translocation of the SREBP1c–SCAP (SREBP cleavage-activating protein) complex from the endoplasmic reticulum to the Golgi apparatus, where SREBP1c undergoes proteolytic cleavage, releasing the transcriptionally active N-terminal domain that translocates to the nucleus [14]. Second, mTORC1-mediated phosphorylation of lipin-1 retains it in the cytoplasm, preventing its nuclear translocation and thus relieving lipin-1-dependent repression of SREBP target gene expression [15]. Nuclear SREBP1c then drives the expression of key lipogenic enzymes, including fatty acid synthase and acetyl-CoA carboxylase [16–18].

A key challenge in molecular endocrinology is elucidating the link between hormonal signaling and long-term cellular responses, such as adipogenic differentiation. Western blotting is a widely used approach for this purpose, as it enables detection of activated signaling intermediates (e.g., phosphorylated Akt) at discrete time points during adipogenic differentiation. However, since this method requires cell lysis, it precludes real-time observation of dynamic signaling events in living cells and cannot capture the full spatiotemporal complexity of processes lasting over days or weeks.

The aforementioned challenge can be overcome by using genetically encoded fluorescent biosensors (GEFBs). GEFBs comprise a binding domain, that binds to specific signaling molecules, and a reporter domain, that transduces these biochemical signaling events into a measurable fluorescent readout enabling visualization by fluorescent microscopy [19]. GEFBs are one of the most useful tools for visualizing hormonal signaling, owing to their interaction selectivity, high sensitivity, minimal phototoxicity and their capacity to enable the real-time visualization of signaling dynamics in living cells [20, 21].

In the present work, we developed a reporter MSC line for the real-time visualization of PI3K signaling in living cells during long-lasting cellular processes. This cell line is based on human Telomerase-Immortalized MSCs and genetically encoded fluorescent biosensor, which was first proposed by Varnai P. et al [22] and consists of the pleckstrin homology domain of Bruton's tyrosine kinase (BTK) as a binding domain and a green fluorescent protein (GFP) as a reporter domain. We assessed differentiation potential, fluorescent signal redistribution upon hormonal stimulation in both an undifferentiated state and during adipogenic differentiation, and proposed a computational approach for detecting these changes.

Materials and methods

Cell cultures

Two cell lines were used in this study. HEK293T (ATCC, #CRL-3216™) for lentiviral particle assembly. hTERT-immortalized adipose tissue-derived multipotent mesenchymal stromal cells (MSC^{hTERT}) served as the target cells for biosensor transduction and as a source of BTK cDNA. The Immortalized MSC^{hTERT} cultures were obtained and thoroughly characterized in our previous study [23].

HEK293T were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing high glucose (Gibco; Thermo Fisher Scientific, Waltham, MA, United States), supplemented with 10% fetal bovine serum (FBS) (HyClone, Washington D.C., U.S.), 1% antibiotic/antimycotic mixture (Gibco; Thermo Fisher Scientific, Waltham, MA, United States) and 1% L-glutamine (Gibco; Thermo Fisher Scientific, Waltham, MA, United States). For lentiviral particle production, the same DMEM high glucose medium was used but supplemented with 2% heat-inactivated FBS instead of 10%.

MSCs were cultured in the DMEM F12 medium (Paneco, Moscow, Russian Federation) containing 10% of FBS (HyClone, Washington D.C., U.S.) and 100 U/ml of penicillin/streptomycin (Gibco; Thermo Fisher Scientific, Waltham, MA, United States). The medium was changed every 2–3 days. Cells were passaged at ~80% confluency. All experiments were accomplished with cells from 15 to 35 passages.

PH-GFP sensor design and transduction

Total RNA was isolated from primary human mesenchymal stem cell (hMSC) cultures using the Direct-zol RNA Miniprep Kit (Zymo Research, #R2052) following the manufacturer's instructions. One mi-

crogram of total RNA was reverse-transcribed into complementary DNA (cDNA) using the MMLV Reverse Transcriptase Kit (Evrogen, #SK021) according to the manufacturer's protocol, using Oligo(dT)₁₅ primer (Evrogen, #SB001). The final reverse transcription reaction volume was 20 μ L. All RNA and cDNA manipulations were performed on ice.

The resulting cDNA was amplified for *BTK* using the forward primer 5'-GGACCGCTAGCCACCATGGCCGAGTGATTCTGGAGAG and the reverse primer 5'-GGACCGAATTACACACACACACACACACACTCGAGAGCGACCGGTGGATCCCCGTTCTC-CAAAATTTGGCAG with Phusion High-Fidelity PCR Master Mix with GC Buffer (Thermo Fisher Scientific, #F532L) according to the manufacturer's instructions. PCR was performed in a 50 μ L reaction volume in 200 μ L polypropylene tubes, including 2.5 μ L (5%) of freshly synthesized cDNA. Amplification conditions were as follows:

- Initial denaturation: 98 °C for 30 sec (1 cycle)
- Cycling: 35 cycles of 98 °C for 10 sec, 70 °C for 20 sec, and 72 °C for 40 sec
- Final extension: 72 °C for 10 min (1 cycle)
- Hold: 4 °C

Annealing temperatures for primer binding were predicted using SnapGene software (v. 5.3.1). The amplified PCR product was purified via 1% agarose gel electrophoresis in TAE buffer stained with ethidium bromide (1 μ g/mL) at 80 V/cm for 40 min. The DNA fragment containing the amplicon was excised under UV transillumination and purified using the GeneJET Gel Extraction Kit (Thermo Fisher Scientific, #K0692).

The purified *BTK* amplicon was digested with 10 U each of *NheI* and *EcoRI* in a 50 μ L reaction following the manufacturer's protocol for 6 hours. The restriction-digested fragment was cloned into the previously described LeGO-PKA-GFP vector [24] using T4 DNA ligase (Evrogen, #LK101L) according to the manufacturer's instructions, followed by transformation of chemically competent *XL1-Blue* cells (Evrogen, #CC001) using standard chemical transformation procedures. Colonies were selected on LB agar containing 100 μ g/mL ampicillin, and plasmid DNA was extracted from overnight cultures using the Plasmid Miniprep 2.0 Kit (Evrogen, #BC221L). The identity of the resulting LeGO-PH construct was confirmed by Sanger sequencing (Evrogen).

A mammalian codon-optimized *GFP* cDNA (*Aequorea victoria*) was subsequently inserted into the LeGO-PH vector. The *GFP* fragment was amplified using the primers 5'-GGACC CCCTCGAGAGCAAGGGCGAGGAGGAGCTGTTACCG and 5'-GGACCCGAATTCTCTCACTTGTACAGCTCGTC-CATGCCGAGAGAGTGATCC with Phusion High-Fidelity PCR Master Mix with GC Buffer (Thermo Fisher Scientific, #F532L) according to the manufacturer's instructions. The 50 μ L reaction was prepared in 200 μ L polypropylene tubes, using the previously described LeGO-PKA-GFP vector [24] as the template. PCR conditions were as follows:

- Initial denaturation: 98 °C for 30 sec (1 cycle)
- Cycling: 35 cycles of 98 °C for 10 sec and 72 °C for 50 sec
- Final extension: 72 °C for 10 min (1 cycle)
- Hold: 4 °C

The amplified *GFP* cDNA fragment was cloned into LeGO-PH at *XhoI* and *EcoRI* restriction sites, following the same procedures described for the *BTK* insertion. The sequence of the final LeGO-PH-GFP vector

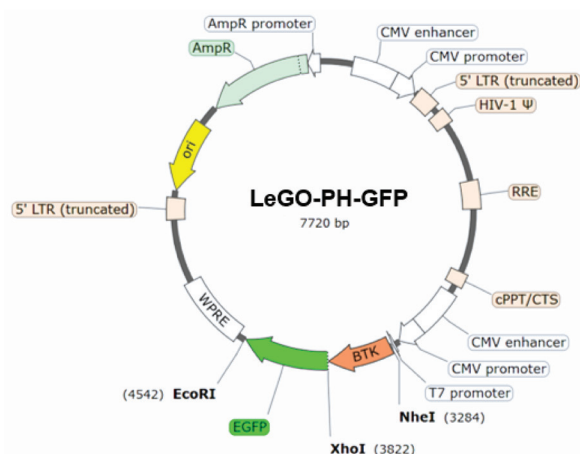


Fig. 1. The map of the LeGO-PH-GFP vector. AmpR – ampicillin resistance gene, CMV – cytomegalovirus, LTR – long terminal repeat, HIV1 ψ – ψ -RNA packaging signal of the human immunodeficiency virus type-1, RRE – Rev Response Element, cPPT/CTS – central polypurine tract / central termination sequence, WPRE – Woodchuck Hepatitis virus Posttranscriptional Regulatory Element, ori – origin of replication, BTK – PH-domain of Bruton's Tyrosine Kinase, eGFP – enhanced green fluorescent protein

Рис. 1. Схема вектора LeGO-PH-GFP. AmpR – ген устойчивости к ампициллину; CMV – цитомегаловирус; LTR – длинный концевой повтор; HIV1 ψ – сигнал упаковки РНК вируса иммунодефицита человека 1-го типа; RRE – элемент ответа на Rev; cPPT/CTS – центральный полипуринный тракт / центральная терминирующая последовательность; WPRE – посттранскрипционный регуляторный элемент вируса гепатита сурка; ori – точка начала репликации, BTK – домен плекстриновой гомологии тирозинкиназы Брутона, eGFP – улучшенный зеленый флуоресцентный белок

encoding the PIP₃ sensor was verified by Sanger sequencing (Evrogen). The map of the resulting LeGO-PH-GFP construct is shown in Figure 1.

Lentiviral particles (LVPs) encoding the PH-GFP PIP₃ sensor were produced in HEK293T cells and used to transduce MSC^{hTERT} cells (multiplicity of infection [MOI] ~ 20) as previously described [24, 25]. Control cells were generated by transducing MSC^{hTERT} with a GFP-encoding plasmid, followed by fluorescence-activated cell sorting to isolate GFP-positive populations.

Flow cytometry

Cells were rinsed with the Versene solution (Paneco, Moscow, Russian Federation) and then detached from culture dishes using 0.25% Trypsin solution (Paneco, Moscow, Russian Federation). Cells were solubilized in the Hanks Balanced Buffered Saline (HBSS, Paneco, Moscow, Russian Federation) supplemented with 2% BSA (Paneco, Moscow, Russian Federation). Then cells were analyzed using FACS ARIA III cell sorter (BD). PH-GFP biosensor fluorescence was excited using a 488 nm solid-state laser (>20 mW), and emission was detected through a 530/30 band-pass filter.

Proliferation rate assessment

MSC^{hTERT} and the obtained biosensor-expressing cell line were cultured in 12-well plastic plate for 72 hours in the medium DMEM F12 (Paneco, Russian Federation) containing 10% of FBS (HyClone, Washington D.C., U.S.) and 100 U/ml of penicillin/streptomycin (Gibco; Thermo Fisher Scientific, Waltham, MA, United States). The medium was changed every 2 days. Initial densities were 25 cells/mm², 50 cells/mm², 100 cells/mm². Images of the cells were taken every 24 hours throughout the observation period. Simultaneous imaging of 6×6 fields of view in Large Image mode was performed using an inverted widefield microscope Nikon Eclipse Ti2 equipped with an objective CFI Plan Fluor DLL 10X/0.3 (Nikon, Tokyo, Japan) and with digital monochrome camera Kinetix (Teledyne Photometrics, USA) or color camera Ri2 (Nikon, Tokyo, Japan).

The number of cells in each image was counted by Segment objects.ai model from NIS.ai module in NIS Elements AR software (version 5.42.02) fine-tuned to segment individual cells on phase contrast images. Fine-tuning was performed with default Train Segment objects.ai function on a set of 20 images 1024×1024 pixels annotated manually. Segmentation results were visually inspected and corrected. Then, the population doubling time of all cell lines was calculated using the following formula:

$$PDT = t \times \ln(2) / \ln(N_f / N_s),$$

where t is time between two time points of interest, N_s is the number of cells at the initial time point, and N_f is the number of cells after 72 hours of incubation

Cell differentiation

Cells were differentiated into adipogenic and osteogenic directions using standard protocols. Cells were cultivated in 12-well plastic culture plates until reaching confluency.

Adipogenic differentiation was induced by incubation in the growth medium based on DMEM with low glucose (HyClone, Washington D.C., U.S.) containing 10% of FBS (HyClone, Washington D.C., U.S.), 1% antibiotic/antimycotic mixture (Gibco; Thermo Fisher Scientific, Waltham, MA, United States), 1% L-glutamine (Gibco; Thermo Fisher Scientific, Waltham, MA, United States), 1 μM dexamethasone, 200 μM insulin, and 0.5 mM 3-isobutyl-1-methylxanthine (MilliporeSigma, Burlington, MA, USA), 100 μM indomethacin — agonist of PPARγ (MilliporeSigma, Burlington, MA, USA) for 14 days [26]. The growth medium was changed every 2–3 days. PI3K signaling was evaluated using the PH-GFP biosensor on day 14 of adipogenesis.

Osteogenic differentiation was induced by incubation in the growth medium based on DMEM with low glucose (HyClone, Washington D.C., U.S.) containing 10% of FBS (HyClone, Washington D.C., U.S.), 0.1 μM dexamethasone (MilliporeSigma, Burlington, MA, USA), 0.2 mM ascorbic acid (MilliporeSigma, Burlington, MA, USA), 10 mM of glycerol-2-phosphate (MilliporeSigma, Burlington, MA, USA), 100 mg/L calcium chloride (MilliporeSigma, Burlington, MA, USA), and 2 mM L-glutamine (MilliporeSigma, Burlington, MA, USA) for 14 days. Fresh medium was prepared immediately prior to each use, and the culture medium was replaced every 2–3 days.

Histological assessment of the differentiation effectiveness

Differentiation efficiency was evaluated on day 14 using Nile Red (MilliporeSigma, Burlington, MA, USA) staining for adipogenic differentiation and Alizarin Red S (MilliporeSigma, Burlington, MA, USA) staining for osteogenic differentiation.

For the adipogenic differentiation assessment, cells were washed with HBSS (Paneco, Moscow, Russian Federation) with 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, HyClone,

Washington D.C., U.S.) and then stained for 1h in a cell culture incubator in HBSS with 20 mM HEPES containing 1 μ M of Nile Red (MilliporeSigma, Burlington, MA, USA).

For the osteogenic differentiation assessment, cells were fixed with 70% ethanol for 1h, then washed 3 times with Milli-Q water, and stained with Alizarin Red S (MilliporeSigma, Burlington, MA, USA) for 30 minutes.

Differentiation efficiency was estimated via microscopy, using Nikon Eclipse Ti2 microscope (Tokyo, Japan). We used the simultaneous measuring of 6 X 6 fields of view in Large Image mode to increase the number of analyzed cells. Images were analyzed using NIS-Elements AR 5.42.02 (Nikon, Tokyo, Japan).

Since MSC^{GFP-PH} are fluorescent in the GFP channel in the undifferentiated state, we used an additional approach to visualization of adipogenic differentiation results with neural networks from NIS.ai module in NIS Elements AR 5.42.02. Phase-contrast images of differentiated cells were obtained with Nikon Eclipse Ti2 Microscope. Then NileRed images were predicted for these phase-contrast images by the Convert.ai model, which was trained on pairs of images phase-contrast — NileRed staining of differentiated MSC^{hTERT}, which are not fluorescent in the GFP channel in the undifferentiated state. Model training was performed according to NIS Elements software guidelines.

Registration of PI3K signaling using PH-GFP biosensor

PI3K signaling detection was performed on the MSC^{GFP-PH} lines both prior to and during adipogenic differentiation. In preparation for the experiment, the cells were cultivated in DMEM with low glucose (HyClone, Washington D.C., U.S.) without FBS for 24 hours. Beforehand the experiment cells were washed three times using HBSS (Paneco, Moscow, Russian Federation) containing 20 mM HEPES (HyClone, Washington D.C., U.S.). MSC^{GFP-PH} were stimulated with 100 nM insulin (Paneco, Moscow, Russian Federation). After 40 minutes of continuous signaling recording, cells were subsequently stimulated with 100 ng/mL platelet-derived growth factor (PDGF, Cell Signaling Technology Inc., Danvers, MA, United States) in order to register PI3K signaling. To assess the specificity of the PH-GFP biosensor, MSC^{GFP-PH-High} cells were pre-treated with the PI3K inhibitor LY294002 50 μ M for 1 h, after which

the biosensor response to 100 ng/ml PDGF stimulation (Cell Signaling Technology Inc., Danvers, MA, United States) was recorded. To test for nonspecific responses to mechanical stimuli, cells were rinsed with HBSS and the biosensor signal was recorded. Simultaneous imaging of 6 × 6 fields of view in Large Image mode was performed using a Nikon Eclipse Ti2 microscope (Tokyo, Japan) to increase the number of analyzed cells. The fluorescence of the biosensor was recorded using a CoolLED pE800-fura diode light source with an emission maximum of 470 nm and a set of filters (excitation 474/27 nm, dichroic mirror 506 nm, emission 536/40 nm). Movies were analyzed using NIS-Elements AR 5.42.02 (Nikon, Tokyo, Japan) software.

To visualize the spatial redistribution of signal intensity following hormone treatment, we performed a pixel-wise subtraction of the pre-treatment images from the post-treatment images. All further analysis was performed using NIS Elements AR 5.42.02. Images were aligned using the Align Current ND Document function and equalized using Equalize Intensity in Time function. Then the “Image Operations/Image × Image/Subtract” function from the GA3 module was used to create the subtraction map. For better visualization, “Red Below Green Above” LUT was used, where red represents decrease in signal intensity (negative values in subtraction map), green represents increase (positive values in subtraction map), while black color represents no change in signal intensity (values around 0 in subtraction map).

To quantify the temporal dynamics of intensity redistribution, we adapted an approach described previously [27], but instead of calculating periphery/center intensities ratio on a single intensity profile, we calculated periphery/center mean intensities ratio on the entire cell using binary mask. After the alignment and equalization of the time series images described above, a binary mask was manually generated for each cell using the Manual Segmentation module. Then this mask was partitioned into two regions: inner and outer. The inner region was created by applying Binary Processing/Circular erode function in GA3 module; values from 5 μ m to 10 μ m depending on cell shape were used as radius. The outer region was created using the Binary Operation/Subtract function between the initial mask and inner part. Then for each image in the time series a mean intensity value was calculated using Measurement/Object intensity/Mean function for outer and inner parts separately. Ratio of outer

to inner mean intensities normalized to 1 at the first frame was used as a metric of intensity distribution and plotted using Microsoft Excel.

Statistical analysis

Statistical analysis was performed using Statistica software (Data Analysis Software System, Version 12). The normality of the data distribution was assessed using the Shapiro-Wilk test. Comparison of multiple independent groups was performed using the Kruskal-Wallis test (one-way ANOVA on ranks)

with significance level $p < 0,05$. Subsequent post hoc pairwise comparisons of mean ranks for all groups were performed, with significance level corrected by Holm — Bonferroni adjustment.

Results

Development of PH-GFP reporter cell lines

The transduced cells were divided into two cell populations based on GFP fluorescence intensity (designated as «MSC^{GFP-PH-High}» and «MSC^{GFP-PH-Low}») using flow cytometry (Fig. 2a). The MSC^{GFP-PH-High}

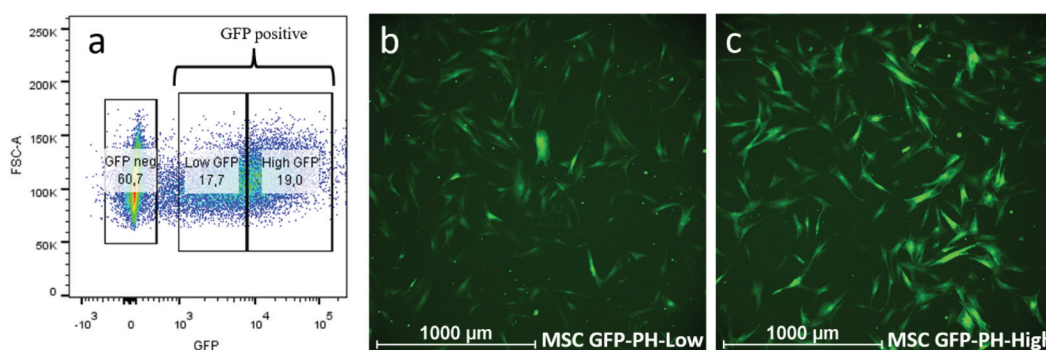


Fig. 2. Development of PH-GFP reporter cell lines. a — separation of transduced cells into MSC^{GFP-PH-High} (19% of total cells) and MSC^{GFP-PH-Low} (17,7% of total cells) populations; b, c — morphology of cultured MSC^{GFP-PH} (magnification: $\times 200$). The MSC^{GFP-PH-High} line exhibits a higher level of GFP fluorescence than the MSC^{GFP-PH-Low}

Рис. 2. Создание клеточных линий PH-GFP. а — разделение трансдуцированных клеток на МСК^{GFP-PH-High} (19% от общего количества клеток) и МСК^{GFP-PH-Low} (17,7% от общего количества клеток); б, с — морфология культивируемых клеток МСК^{GFP-PH} (увеличение: $\times 200$). Линия МСК^{GFP-PH-High} характеризуется более высоким уровнем флуоресценции GFP по сравнению с линией МСК^{GFP-PH-Low}

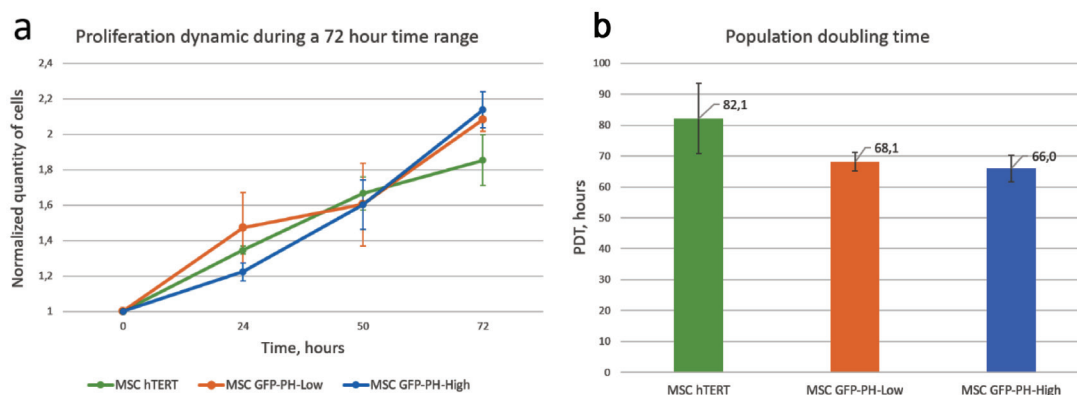


Fig. 3. Comparison of MSC^{hTERT} and MSC^{GFP-PH} proliferation over a 72-hour time period: a proliferation curves of MSC^{hTERT} and MSC^{GFP-PH}. Images of the cells were acquired every 24 hours during the observation period, b — population doubling time (PDT) of cell lines. PDTs of all cell lines were compared using Kruskal-Wallis test ($p = 0.041$) with subsequent *post hoc* multiple comparisons of mean ranks of all groups. No statistically significant differences were observed among groups following the *post hoc* analysis. Data are represented as mean $\pm$ standard deviation

Рис. 3. Сравнение пролиферации клеток МСК^{hTERT} и МСК^{GFP-PH} в течение 72 часов: а — кривые пролиферации клеток МСК^{hTERT} и МСК^{GFP-PH}. Съемку изображений клеток производили каждые 24 часа в течение периода наблюдения; б — время удвоения клеточных популяций. Сравнение между линиями проводили с использованием критерия Краскела — Уоллиса ($p = 0,041$) с последующими множественными апостериорными сравнениями. По результатам попарных апостериорных сравнений статистически значимых различий между группами не выявлено. Данные представлены как среднее значение $\pm$ стандартное отклонение

cell population exhibits a higher level of GFP fluorescence than the MSC^{GFP-PH-Low} cells (Fig. 2 b, c). This separation was performed to mitigate two possible problems. For many biosensors, low levels of expression can severely hinder the ability to detect cell response. At the same time, high levels of sensor expression may affect properties of cells

In order to evaluate the proliferation rate of the MSC^{GFP-PH}, population doubling time for all cell lines was calculated. No statistically significant differences were observed among MSC^{hTERT}, MSC^{GFP-PH-High}, and MSC^{GFP-PH-Low} (Fig. 3).

The reporter cell line reacts with PH-GFP sensor rearrangement on insulin stimulation

Stimulation with a PI3K signaling agonist leads to the conversion of PIP₂ to PIP₃ by PI3K, causing translocation of the PH-GFP biosensor from the cytoplasm to the plasma membrane thereby changing the fluorescent signal distribution [4, 22]. Downstream of PI3K activation, PIP₃ serves as a docking site for both Akt and PDK1 via their PH domains, colocalizing them at the plasma membrane and enabling PDK1-mediated phosphorylation of Akt. Full activation of Akt additionally requires phosphorylation by mTORC2, collectively leading to modulation of downstream effector activity. It has already been shown that stimulation with insulin, a PI3K signaling agonist, results in increased Akt phosphorylation in hTERT-immortalized MSCs expressing a biosensor based on the BTK PH domain [28].

To verify the specificity of fluorescent responses to hormones, we used three controls: 1) MSC^{hTERT} transduced with GFP, to confirm that the observed fluorescent signal rearrangement reflected PH-GFP redistribution rather than non-specific GFP reallocation; 2) MSC^{GFP-PH} treated by Hanks Balanced Buffered Saline to exclude mechanosignaling effects on sensor redistribution 3) MSC^{GFP-PH-High} incubated with the PI3K signaling inhibitor LY294002.

We successfully detected fluorescent responses to insulin and PDGF in MSC^{GFP-PH-High}, and these responses exceeded fluorescent signal rearrangements caused by mechanical stimulation and GFP redistribution upon the hormonal stimulation in control cell lines (Fig. 4 a, b, d, k, l, n and Fig. 5 a, b). Additionally, pre-treatment of MSC^{GFP-PH-High} with the PI3K inhibitor LY294002 completely abolished PH-GFP redistribution following PDGF stimulation, which further confirms the specificity of the PH-GFP sensor for PIP₃ in MSCs (Fig. 6). Visual

detection of MSC^{GFP-PH-Low} responses to hormone stimulation was challenging, thus, pixel-wise subtraction of the pre-stimulation image from the post-stimulation image was performed (Fig. 4 c, e, h, j). This revealed a redistribution of the fluorescent signal in each cell: fluorescence intensity in the central region decreased, while intensity in the peripheral region increased after insulin and PDGF stimulation in both MSC^{GFP-PH-High} and MSC^{GFP-PH-Low} (Fig. 4 c, e, h, j). This pattern of fluorescence redistribution is consistent with the established mechanism of PH-domain membrane translocation upon PIP₃ generation, as described previously [22]. Also, the images subtraction clearly demonstrated that neither GFP redistribution upon insulin and PDGF stimulation nor PH-GFP redistribution in response to mechanical stress occurred. These controls did not exhibit the redistribution pattern observed upon insulin and PDGF stimulation (Fig. 4 m, o and Fig. 5 c, f). Moreover, to get a quantitative representation of the fluorescent response, the periphery-to-center ratio of mean fluorescence intensity was calculated at each time point. As shown in Fig. 4 p, q, MSC^{GFP-PH-High} exhibited markedly more pronounced ratio dynamics compared to the control cell lines, whereas MSC^{GFP-PH-Low} responses to insulin only slightly exceeded the responses of the control cell lines. Therefore, unsorted cells may be suboptimal for hormone signaling research in the undifferentiated state of MSC^{GFP-PH}.

PH-GFP enables to detect hormonal signaling during adipogenic differentiation

To evaluate the suitability of using the MSC^{GFP-PH} lines as models for detecting hormonal signaling during MSC differentiation, we directed MSC^{GFP-PH} to adipogenic and osteogenic differentiation. Both MSC^{hTERT} and MSC^{GFP-PH} lines demonstrated equal adipogenic and osteogenic potential. No detectable differences were observed in lipid droplet accumulation (an adipogenic marker) or in the Alizarin Red S staining area, which reflects calcium deposits formation (Fig. 7).

We successfully detected fluorescent responses to insulin and PDGF after three weeks of adipogenic differentiation (Fig. 8 a, b, d, f, g, i). Pixel-wise subtraction maps confirmed the sensor redistribution from the cell center to the periphery (Fig. 8 c, e, h, j). Moreover, the periphery-to-center fluorescence intensity ratio dynamics also demonstrated an increase, reflecting the sensor redistribution to the plasma membrane and indicating a cellular response to hormonal stimulation (Fig. 8 k, l).

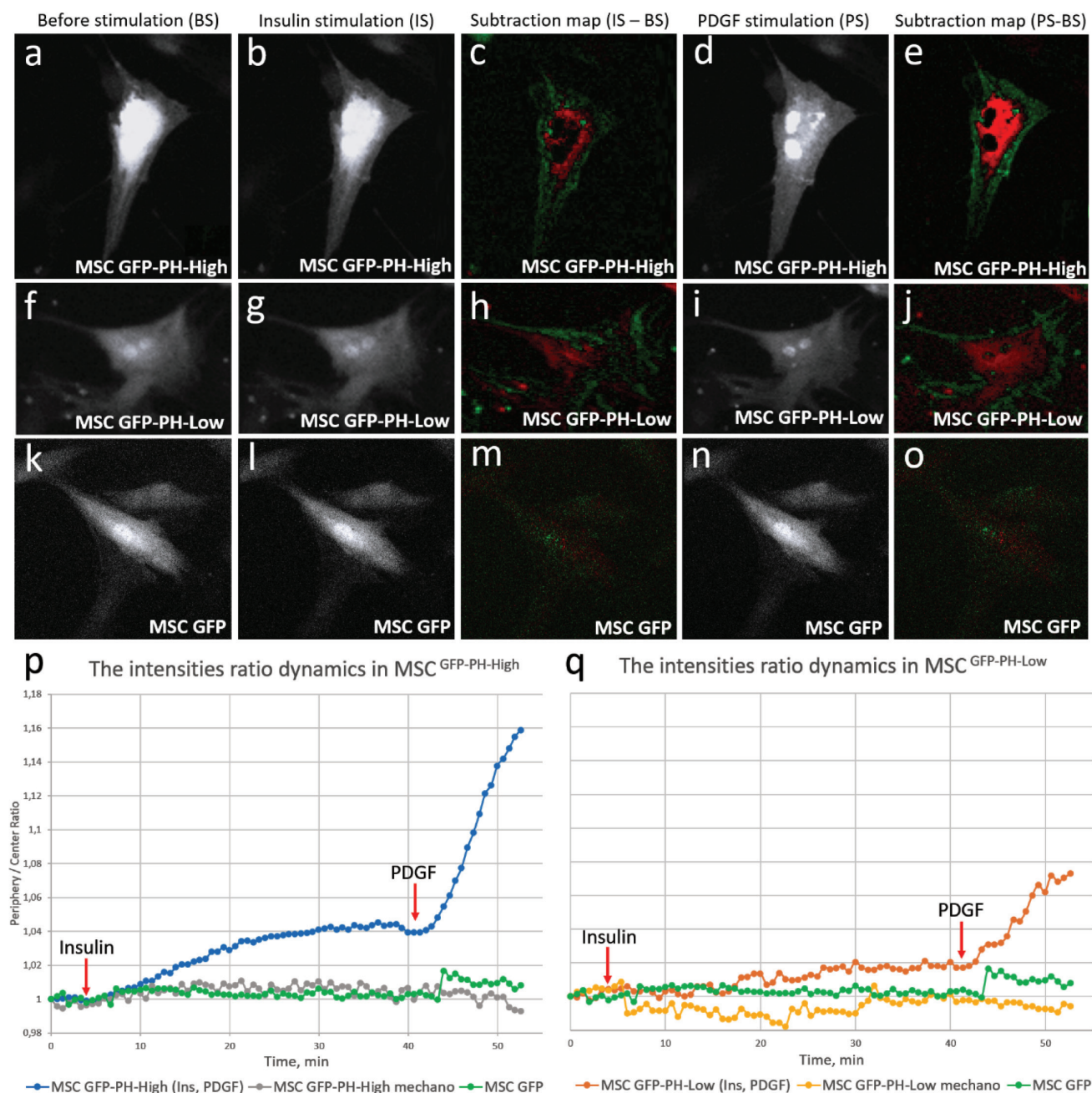


Fig. 4. Fluorescent signal redistribution following insulin and PDGF stimulation. Representative fluorescence images of MSC^{GFP-PH-High} (a, b, d), MSC^{GFP-PH-Low} (f, g, i), MSC^{GFP} (k, l, n). Representative images of the sensor redistribution, obtained via pixel-wise subtraction of the pre-stimulation images from the post-stimulation images for MSC^{GFP-PH-High} (c – insulin, e – PDGF), MSC^{GFP-PH-Low} (h – insulin, j – PDGF), and MSC^{GFP} (m – insulin, o – PDGF). Red and green colors indicate decreased and increased fluorescence intensity, respectively. Temporal dynamics of the periphery-to-center mean fluorescence intensity ratio during the hormonal response for MSC^{GFP-PH-High} (p) and MSC^{GFP-PH-Low} (q) cells

Рис. 4. Перераспределение флуоресцентного сигнала в МСК^{GFP-PH-High} (a, b, d), МСК^{GFP-PH-Low} (f, g, i) и МСК^{GFP} (k, l, n) в ответ на стимуляцию инсулином и PDGF. Репрезентативные изображения перераспределения сенсора, полученные путем попиксельного вычитания изображения клетки до гормональной стимуляции из изображения после гормональной стимуляции для МСК^{GFP-PH-High} (с – инсулин, е – PDGF), МСК^{GFP-PH-Low} (h – инсулин, j – PDGF) и МСК^{GFP} (m – инсулин, o – PDGF). Красным цветом отмечено уменьшение, зеленым – увеличение интенсивности флуоресценции при гормональной стимуляции. Временная динамика отношения средней интенсивности флуоресценции клетки на периферии к средней интенсивности в центре клетки в процессе ответа на гормоны представлена для МСК^{GFP-PH-High} на (p) и для МСК^{GFP-PH-Low} на (q)

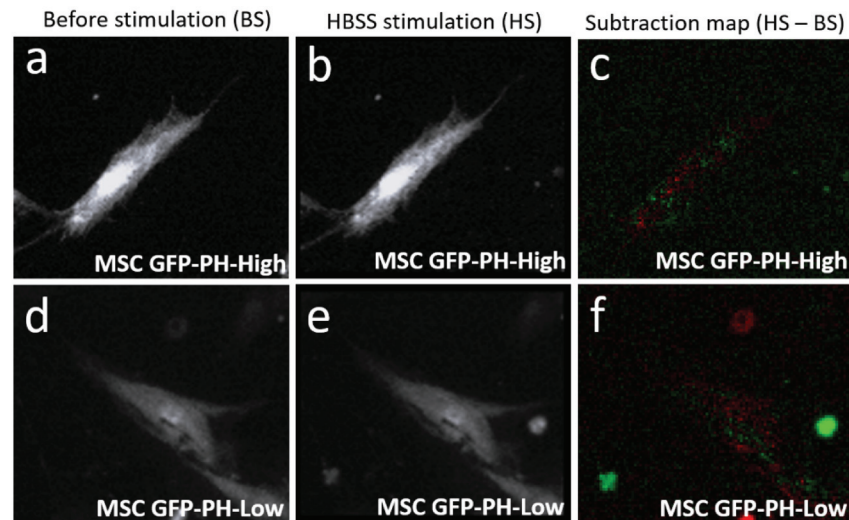


Fig. 5. Fluorescent signal redistribution following mechanical stimulation. Representative fluorescence images of (a, b) MSC^{GFP-PH-High} and (d, e) MSC^{GFP-PH-Low} cells following mechanical stimulation with Hank's Balanced Salt Solution. Representative images of the sensor redistribution induced by mechanical stimulation, obtained via pixel-wise subtraction of the pre-stimulation images from post-stimulation images for MSC^{GFP-PH-High} (c) and MSC^{GFP-PH-Low} (f). Red and green colors indicate decreased and increased fluorescence intensity, respectively

Рис. 5. Перераспределение флуоресцентного сигнала в МСК^{GFP-PH-High} (a, b) и МСК^{GFP-PH-Low} (d, e) в ответ на механическую стимуляцию раствором Хэнкса. Репрезентативные изображения перераспределения сенсора, вызванного механической стимуляцией, полученные путем попиксельного вычитания изображения клетки до механической стимуляции из изображения после механической стимуляции для клеток МСК^{GFP-PH-High} (c) и МСК^{GFP-PH-Low} (f). Красным цветом отмечено уменьшение, зеленым — увеличение интенсивности флуоресценции

Overall, both the MSC^{GFP-PH-High} and MSC^{GFP-PH-Low} cell populations enable the detection of hormonal signaling in living cells during adipogenic differentiation.

Discussion

The PI3K signaling pathway regulates crucial cell processes such as proliferation, survival, differentiation, secretion of cytokine and growth factors, and migration of MSCs [29]. Hormones and growth factors like insulin, adiponectin, IGF-1, -2, which are involved in adipogenic differentiation of MSCs, upregulate this signaling pathway [30].

Convenient tools enabling real-time monitoring of hormone-triggered intracellular signaling in living cells throughout long-lasting cellular processes remain limited. In this study, we developed an instrument that enables detection of PI3K pathway activation in response to specific hormonal stimuli in MSCs in real time.

We created an hTERT-immortalized MSCs line expressing a fluorescent biosensor to phosphatidylinositol 3,4,5-trisphosphate (a key mediator of the PI3K signaling), tested the cell line signaling properties in an undifferentiated state and during adipogenic

differentiation, and evaluated differentiation efficiency into adipogenic and osteogenic lineages. We also applied the computational approach to register fluorescent changes caused by hormone treatment.

Previous studies have demonstrated that genetically encoded fluorescent biosensors may affect stem cell survival and differentiation [31]. In case of PH-GFP usage in hTERT-immortalized MSCs, we did not observe any changes in proliferation rates or differentiation efficiency towards adipogenic and osteogenic directions between the obtained cell lines and the parental cell line. Although the immortalized MSC line Asc52Telo (ATCC, Virginia, USA) has been shown to exhibit decreased insulin sensitivity and consequently reduced adipogenic potential, the MSC^{hTERT} line retains intact insulin signaling and adipogenic potential [32]. Asc52Telo displayed elevated basal phosphorylated Akt levels compared to primary MSCs, with no increase upon insulin stimulation. In contrast, MSC^{hTERT} responded to insulin with insulin-induced Akt phosphorylation dynamics comparable to those of primary MSCs through passage 41 [23]. Beyond passage 41, MSC^{hTERT} also exhibited elevated basal Akt phosphorylation. However, insulin-stimulated increases in phosphorylated Akt were still detectable [23].

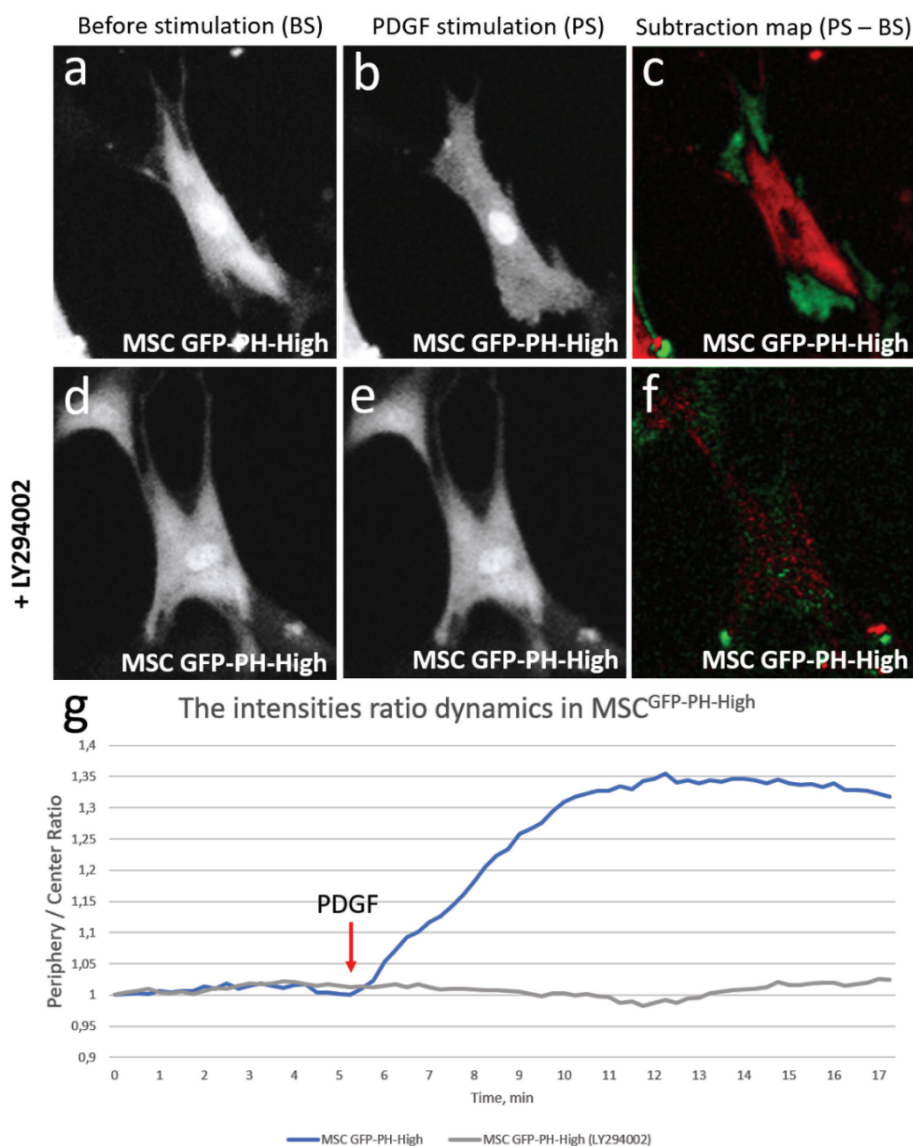


Fig. 6. PI3K inhibition abolishes PDGF-induced fluorescent signal redistribution. Following PDGF stimulation, MSC^{GFP-PH-High} cells pretreated with the specific PI3K inhibitor LY294002 (d, e) failed to exhibit the typical fluorescence redistribution pattern seen in untreated cells (a, b). (c, f) Representative images of sensor redistribution induced by PDGF stimulation, obtained via pixel-wise subtraction of the pre-stimulation image from the post-stimulation images of MSC^{GFP-PH-High}. Red and green colors indicate decreased and increased fluorescence intensity, respectively. Temporal dynamics of the periphery-to-center mean fluorescence intensity ratio during PDGF stimulation is depicted at (g)

Рис. 6. Ингибирование PI3K нивелирует PDGF-индуцированное перераспределение флуоресцентного сигнала. После стимуляции PDGF в клетках MSC^{GFP-PH-High}, обработанных специфическим ингибитором PI3K сигнального каскада LY294002 (d, e), не происходило характерного перераспределения флуоресценции, которое наблюдалось в необработанных клетках (a, b). (c, f) Репрезентативные изображения перераспределения сенсора под действием PDGF, полученные путем попиксельного вычитания снимков до и после стимуляции. Красный и зеленый цвета отражают снижение и увеличение интенсивности флуоресценции соответственно. Временная динамика отношения средней интенсивности флуоресценции на периферии и в центре клетки при воздействии PDGF изображена на панели (g)

Therefore, hTERT-immortalized MSC lines can be used for PI3K signaling research within an appropriate passage range, provided that insulin (or other PI3K agonist) signaling is carefully evaluated prior to transduction with GEFs.

We successfully detected insulin- and PDGF-induced PI3K signaling in undifferentiated state of MSC^{GFP-PH-High}. The PH-GFP binding to PIP₃ causes PH-GFP redistribution from the perinuclear area to the periphery of the cells owing to the plasma membrane

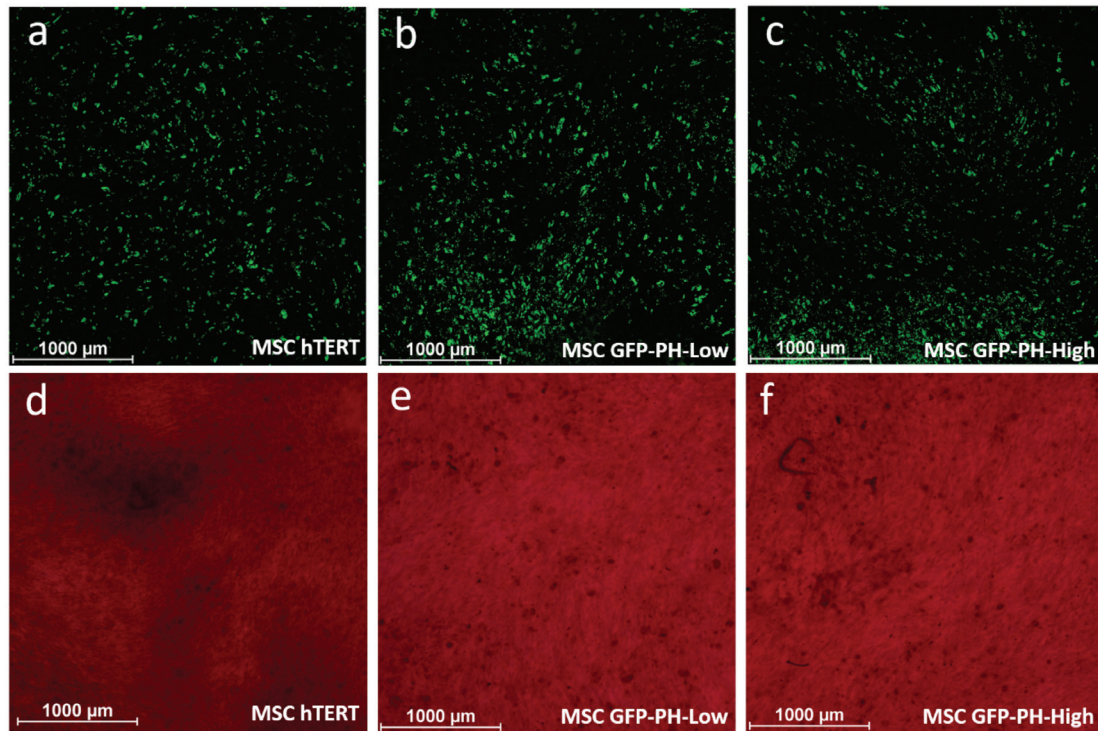


Fig. 7. Cells modified with PH-GFP retain intact adipogenic and osteogenic potential: a–c — analysis of adipogenic differentiation efficiency at day 14 using neural networks from NIS.ai module in NIS Elements for MSC^{hTERT} (a), MSC^{GFP-PH-Low} (b) and MSC^{GFP-PH-High} (c). Green color indicates lipid droplets; d–f — alizarin Red S staining at day 14 of osteogenic differentiation for MSC^{hTERT} (d), MSC^{GFP-PH-Low} (e) and MSC^{GFP-PH-High} (f)

Рис. 7. Клетки, модифицированные биосенсором PH-GFP, демонстрируют intactный адипогенный и остеогенный потенциал: a–c — анализ эффективности адипогенной дифференцировки на 14-й день с использованием нейронных сетей модуля NIS.ai в программе NIS Elements для MCK^{hTERT} (a), MCK^{GFP-PH-Low} (b) и MCK^{GFP-PH-High} (c). Зеленым цветом обозначены липидные капли; d–f — окрашивание ализариновым красным S после 14 дней остеогенной дифференцировки для клеток MCK^{hTERT} (d), MCK^{GFP-PH-Low} (e) и MCK^{GFP-PH-High} (f)

localization of PIP₃. Cells responded to insulin and PDGF with apparent changes in fluorescent signal distribution that significantly exceeded those caused by mechanical stimulation of MSC^{GFP-PH} and non-specific GFP reallocation in GFP transduced MSC^{hTERT}. Additionally, pre-treatment of MSC^{GFP-PH-High} with the PI3K inhibitor LY294002 completely abolished PH-GFP redistribution following PDGF stimulation, which further confirms the specificity of the PH-GFP sensor for PIP₃ in MSCs. The responses to PDGF were more distinct. We assume that these differences are attributable to the relatively low expression of insulin receptors on the MSC plasma membrane and relatively low insulin affinity to the insulin-like growth factor receptor, whereas PDGF receptor is abundantly represented on the MSC plasma membrane [32–36]. Furthermore, we detected distinct fluorescent responses to insulin and PDGF during adipogenic differentiation. Therefore, MSC^{GFP-PH} cell lines enable the detection of hormonal signaling events during adipogenic differentiation.

A versatile instrument for hormone signaling research should include a computational method for detection and quantitative representation of hormone-induced response. Firstly, we performed pixel-wise subtraction of pre-stimulation images from the post-stimulation images to obtain subtraction maps representing changes in fluorescence intensity in each pixel. This approach confirmed our findings of the sensor redistribution upon the hormonal stimulation in both the undifferentiated state of MSC^{GFP-PH} and during the adipogenic differentiation of MSC^{GFP-PH}. This proposed approach allows for a more straightforward visualization of fluorescence intensity redistribution, especially in cases of a weak cell response to a hormone. Secondly, we calculated the ratio between the average fluorescence intensity of the cell periphery and the average fluorescence intensity of the cell center in each time point during the registration of a response — a widely used metric for signal evaluation from the translocation-based biosensors [27]. Using

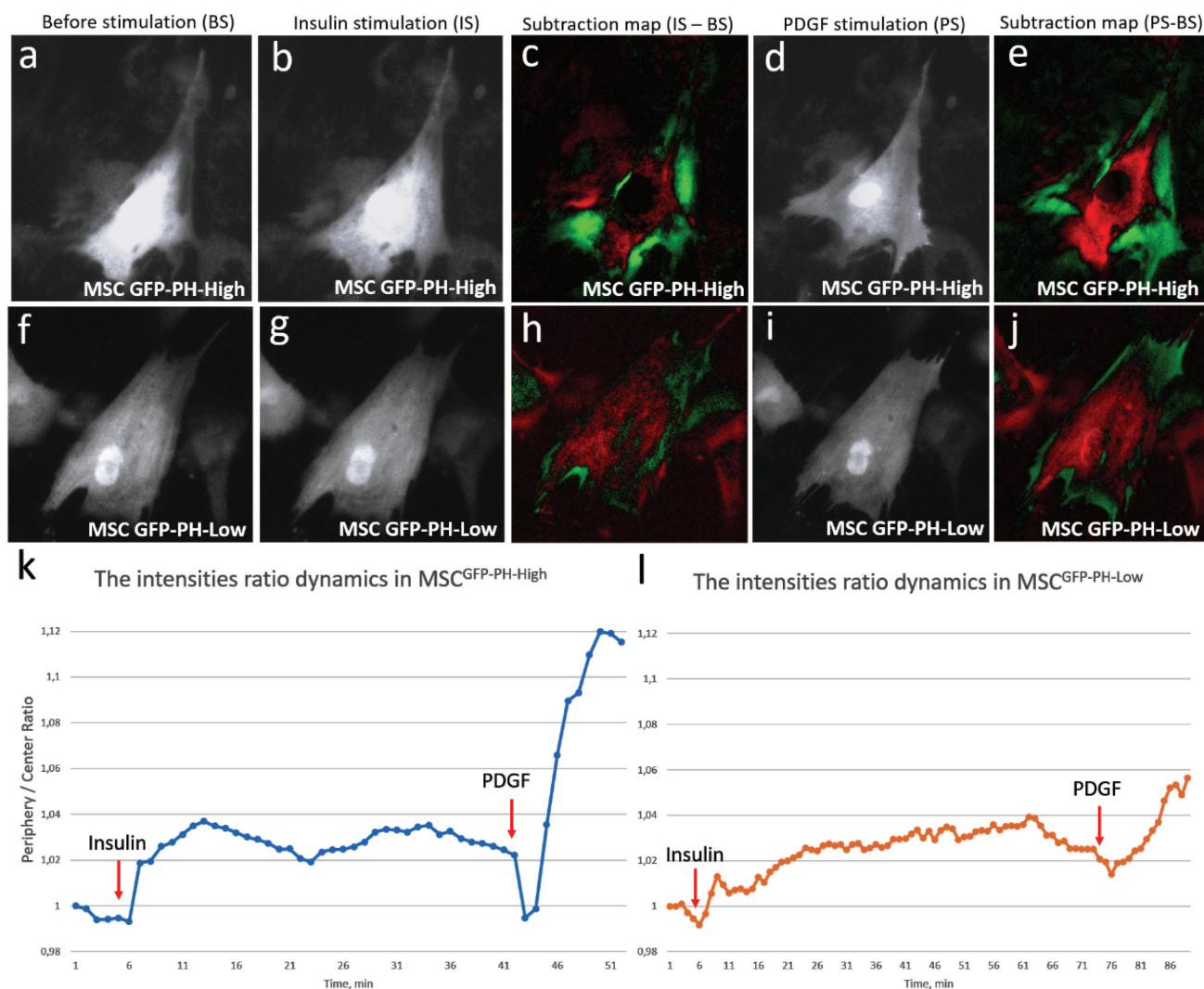


Fig. 8. Fluorescent signal redistribution following insulin and PDGF stimulation at day 14 of adipogenic differentiation. Representative fluorescence images of MSC^{GFP-PH-High} (a, b, d) and MSC^{GFP-PH-Low} (f, g, i). Representative images of sensor redistribution, obtained via the pixel-wise subtraction of pre-stimulation images from post-stimulation images for MSC^{GFP-PH-High} (c – insulin, e – PDGF) and MSC^{GFP-PH-Low} (h – insulin, j – PDGF). Red and green colors indicate decreased and increased fluorescence intensity, respectively. Temporal dynamics of the periphery-to-center mean fluorescence intensity ratio during the hormonal response for MSC^{GFP-PH-High} at (k) and for MSC^{GFP-PH-Low} at (l)

Рис. 8. Перераспределение флуоресцентного сигнала в ответ на стимуляцию инсулином и PDGF на 14-й день адипогенной дифференцировки. Репрезентативные флуоресцентные изображения МСК^{GFP-PH-High} (a, b, d) и МСК^{GFP-PH-Low} (f, g, i). Репрезентативные изображения перераспределения сенсора, полученные путем попиксельного вычитания изображения клетки до гормональной стимуляции из изображения после гормональной стимуляции для клеток МСК^{GFP-PH-High} (с – инсулин, е – PDGF) и МСК^{GFP-PH-Low} (h – инсулин, j – PDGF). Красным цветом отмечено уменьшение, зеленым – увеличение интенсивности флуоресценции при гормональной стимуляции. Временная динамика отношения средней интенсивности флуоресценции клетки на периферии к средней интенсивности в центре клетки в процессе ответа на гормон представлена для МСК^{GFP-PH-High} на (k) и для МСК^{GFP-PH-Low} на (l)

this approach, we demonstrated, that the hormonal stimulation caused an increase in the aforementioned ratio. PDGF stimulation induced more prominent and rapid changes in this ratio compared to stimulation in an undifferentiated state and during the adipogenic differentiation of MSCs. Thus,

this metric allows for the quantitative comparison of cellular responses to different hormones.

Overall, our findings suggest that the hTERT-immortalized MSCs retain intact differentiation potential after PH-GFP transduction. Cell sorting

of the total transduced cell population is recommended to isolate MSC^{GFP-PH-High} subpopulation for all subsequent experiments. The MSC^{GFP-PH-High} line can be used to test cellular hormonal sensitivity, monitor its dynamic changes, and elucidate hormone

effects during MSCs differentiation in living cells in real time. The applied computational approach serves as a helpful method to simplify the detection of cellular responses to hormones and to increase their detectability in living cells.

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