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BIOCHEMISTRY, BIOPHYSICS AND MOLECULAR BIOLOGY

Investigation of the Effect of α-Melanocyte-Stimulating Hormone on Proliferation and Early Stages of Differentiation of Human Induced Pluripotent Stem Cells

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Abstract—We have studied the influence of α-melanocyte-stimulating hormone (α-MSH) on proliferation and early stages of differentiation of human induced pluripotent stem cells (iPSc). We have demonstrated that α-MSH receptor genes are expressed in undifferentiated iPSc. The expression levels of MCR1, MCR2, and MCR3 increased at the embryoid body (EB) formation stage. The formation of neural progenitors was accompanied by elevation of MCR2, MCR3, and MCR4 expression. α-MSH had no effect on EB generation and iPSc proliferation at concentrations ranging from 1 nM to 10 μM. At the same time, α-MSH increased the generation of neural rosettes in human iPSc cultures more than twice.

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α-Melanocyte-stimulating hormone (α-MSH) is a component of the melanocortin system of the body, which also includes the adrenocorticotropic hormone (ACTH), five melanocortin receptors (MCR1–MCR5), β-, and γ-MSHs, and endogenous melanocortin receptor antagonists—the agouti protein and AGRP (the agouti-related protein) [1]. In interacting with each other, they form the peripheral and central signaling systems, which control a wide range of the key physiological processes in the body. In particular, in binding to corresponding receptors, melanocortins and their fragments exert nootropic, neuroprotective, and neurotrophic effects [2, 3]. α-MSH exhibits a high affinity to four types of receptors: MCR1, MCR3, MCR4, and MCR5. It is known that all types of melanocortin receptors (MCR1–MCR5) are functionally linked to adenylate cyclase and realize their effect primarily through the regulation of the cAMP-dependent signaling pathway [4]. It should be noted that MCR1, MCR3, MCR4, and MCR5 receptors exhibit a high level of intrinsic, ligand-independent activity (i.e., stimulate cAMP production in the absence of agonist).

It was shown that some melanocortins increase neural survival, stimulate neurite growth, and enhance the expression of the neuron-specific protein B-50 in primary cultures of rat brain neurons [5]. It was also shown that α-MSH stimulated the growth of processes in Neuro 2A (mouse neuroblastoma) cells and that the addition of the type 4 receptor antagonist D-Arg8 $ACTH_{4–10}$ blocked the stimulatory effect of the peptide [6]. Experiments in the primary cultures isolated from the embryonic rat brain showed that α-MSH not only increased the proliferative activity of the cell population but also increased the number of secondary neurospheres, which indicates that α-MSH has a stimulatory effect on cell self-renewal [7]. α-MSH and peptide semax (Met–Glu–His–Phe–Pro–Gly–Pro), an analogue of the N-terminal fragment 4–10 of ACTH, decelerated the death of primary cultures of neurons and astrocytes of the rat brain, which was accompanied by an increase in the expression of the brain-derived neurotrophic factor BDNF [8, 9]. Administration of melanocortins in animals with induced lesions of peripheral nerve fibers accelerated axonal regeneration and enhanced the growth of neural processes in the central nervous system [5]. Using the mouse model of Alzheimer's disease (TgCRND8 mice), it was shown that α-MSH suppresses anxiety and improves the spatial memory of animals. This result was due to the neuroprotective effects of the peptide, aimed at improving the GABAergic system functioning [10].

Earlier, we studied the effect of α-MSH on the initial stages of differentiation of mouse embryonic stem cells (ESCs) [11]. The studies showed that α-MSH stimulates neural differentiation of mouse ES cells in vitro but has no significant effect on their proliferation and the formation of embryoid bodies (EBs) [11].

One of the most important discoveries in the biology made in the past decade was the obtaining of human induced pluripotent stem cells (iPSCs) [12]. Currently, iPSCs have occupied the key place in the

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studies of the molecular basis of the pathogenesis of hereditary and sporadic human diseases and in screening the potential drugs and have opened the prospects for their use in clinical practice in regenerative medicine [13]. Through directed differentiation of iPSCs, it is possible to track the changes in the levels of expression of tissue-specific genes and proteins, which is extremely important for understanding the molecular and genetic processes in cells both in health and disease.

The aim of this work was to study the effect of α-MSH on the initial stages of differentiation of human iPSCs and to analyze the expression of melanocortin receptor genes in these cells.

Cultivation of human iPSCs. iPScs, which were obtained as described earlier [14], were cultured in 35- and 60-mm Petri dishes (Greiner, Germany) on Matrigel substrate (Corning, United States) in mTeSR medium (STEM CELL Technologies, United States). The medium was replaced with a fresh one once a day. Passaging was performed using dispase at a concentration of 1 mg/mL (Gibco, United States). Cells were incubated with the enzyme at 37°C for 7–10 min in a CO_2 incubator. Then, the cells were washed from dispase 5 times with DMEM medium (PanEco, Russia). Cell colonies were mechanically removed with the wide end of a 200-μL plastic tip to 1 mL of mTeSR medium and plated onto a new Matrigel-coated Petri dish containing 1 mL of mTeSR medium. Cells were passaged at a ratio of 1 : 2 or 1 : 3 every 5–7 days.

Determination of proliferative activity of human iPSCs. Human iPScs were replated at a density of 40 thousand cells/well in a Matrigel-coated 24-well plate in mTeSR medium. Then, 24 h after seeding the cells, α-MSH (Sigma, United States) and antibiotic geneticin G418 (Gibco) were added to final concentrations of 10^{-5}, 10^{-7}, and 10^{-9} M and 50, 100, and 200 μg/mL, respectively. The cells were incubated for 3 days, the medium was once a day replaced with a fresh one containing α-MSH and geneticin. The proliferative activity of the cells was evaluated on day 3 after seeding by direct counting of cells under an Olympus SKH41 microscope (Olympus, Japan) in a standard hemocytometer chamber. In addition, cell viability was evaluated cytochemically with 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide (MTT) [10].

Obtaining embryoid bodies. iPSC colonies were removed from the plate surface with dispase (1 mg/mL) by incubating them with the enzyme solution at 37°C for 7–10 min. After incubation, the enzyme solution was removed, the dishes washed 5 times with 1 mL of DMEM and filled with 1 mL of a culture medium for obtaining EBs, which contained DMEM/F12 (Gibco), 20% fetal calf serum (HyClone, United States), 2 mM L-glutamine (ICN Biomedicals, United States) 0.1 mM β-mercaptoethanol (Sigma), 1% nonessential amino acid mixture (PanEco), and penicillin and streptomycin (50 U/mL and 50 μg/mL, respectively; PanEco). The colonies were scraped off with a 200-μL plastic tip, gently dissociated into fragments (approximately 400–600 cells each), and transferred to a 24-well plate with an extremely low adhesion (Costar, United States). Simultaneously with seeding, α-MSH was added to final concentrations of 10^{-5}, 10^{-7}, and 10^{-9} M. The control wells were filled with an equal volume of phosphate buffered saline (PBS). The formed EBs were counted 48 h after seeding.

Differentiation of iPSCs to neural rosettes. iPScs were replated into a Matrigel-coated 24-well plate in mTeSR medium at a density of 40 thousand cells per well in 1 mL of medium. After the cells formed a 70–80% monolayer, mTeSR medium was changed for a medium containing DMEM/F12, 2% serum substitute (Gibco), 1 mM nonessential amino acids (PanEco), 2 mM L-glutamine, 1% N2 Supplement (Life Technologies, United States), and penicillin and streptomycin at the above concentrations. Simultaneously with changing the medium, α-MSH was added to a final concentration of 10^{-9} M to the experimental wells, and an equal volume of PBS was added to the control wells. The medium was replaced with a fresh one every 48 h. On day 7 of culturing, the cells were fixed with 4% paraformaldehyde, and immunocytochemical analysis with the antibodies to beta-III-tubulin and Sox1 (Abcam, United States) was performed.

Differentiation of iPSCs in neural progenitors. iPScs were cultured in mTeSR medium to 80% confluence, after which the medium was replaced with a neural medium containing DMEM/F12, 2% serum substitute (Gibco), 1 mM nonessential amino acids (PanEco), 2 mM L-glutamine, penicillin and streptomycin (50 U/mL and 50 μg/mL, respectively), 1% N2 Supplement (Life Technologies), 10 μM SB431542 (Stemgent, United States), and 80 ng Noggin (Peprotech, United States). The medium was replaced with a fresh one daily for 7–10 days. The formed neural rosettes and ridges were separated mechanically and transferred into a 24-well plate with an extremely low adhesion (Costar), where neurospheres were then formed, which were cultured in the plate for another 5–7 days. Neurospheres were collected to centrifuge tubes, dissociated to a monocell suspension with trypsin (0.25%), and plated in the neural medium into Petri dishes coated with Matrigel.

Determination of expression of melanocortin receptor genes. Total RNA was isolated and reverse transcription and polymerase chain reaction were performed as described in [15]. The structure of the primers used in this study is shown in the table.

Statistical analysis was performed using the standard statistical software package Statistica 7.0 for WinXP.

At the initial stage of work, we used real-time PCR to study the expression of the genes encoding α-MSH receptors MCR1, MCR2, MCR3, MCR4, and

Table 1. The structure of the primers used in this work

Gene	Primers	Annealing temperature, °C
18 S	5'-CGGCTACCACATCCAAGGAA-3' 5'-GCTGGAATTACCGCGGCT-3'	60
MCR1	5'-GCAGCAGCTGGACAATGTCA-3' 5'-ATGAAGAGCGTGCTGAAGACG-3'	60
MCR2	5'-ATCACCTTCACGTCGCTGTT-3' 5'-CGTTATTCCCATGGATTCTA-3'	60
MCR3	5'-CGGTGGCCGACATGCTGGTAAGTG-3' 5'-TGAGGAGCATCATGGCGAAGAACA-3'	64
MCR4	5'-CAATAGCCAAGAACAAGAATC-3' 5'-GACAACAAAGACGCCAATCAG-3'	60
MCR5	5'-CATTGCTGTGGAGGTGTTTCT-3' 5'-GCCGTCATGATGTGGTGGTAG-3'	60

MCR5 in undifferentiated human iPSCs, EBs, and neural progenitors derived from these cells.

Data shown in Fig. 1 demonstrate an elevated expression of MCR1, MCR2, and MCR3 at the stage of EB formation. At the stage of formation of neural progenitors, an increased expression of MCR2, MCR3, and MCR4 receptor genes was observed. The level of expression of the MCR5 receptor gene virtually did not change at the stages of formation of EB and neural progenitors. Earlier, using the mouse ESC model, we showed that some genes encoding MCRs for α-MSH are expressed starting from the EB formation step and that their expression continues during the differentiation of ESCs [11].

The detection of expression of a number of melanocortin receptor genes led us to assume that α-MSH can influence the proliferation, EB formation, and the initial stages of differentiation of human iPSCs through these receptors.

When studying the effect of α-MSH on proliferation and its potential cytotoxic activity with respect to iPSCs, this peptide was used in a wide concentration range from 1 nM to 10 μM, which are used in experiments both in vitro and in vivo [6, 8]. The results showed that α-MSH at these concentrations had no effect on the proliferative activity of iPSCs, whereas the well-known cytotoxic agent G418 reduced the number of living cells in a dose-dependent manner (Fig. 2).

Figure 3 shows the results of experiments on studying the effect of α-MSH at different concentrations on the formation of EBs in human iPSC cultures. As can be seen from this figure, the peptide had no significant effect on the EB formation. Similar results were obtained earlier in the mouse ESC model: the effect of α-MSH on proliferation and EB formation was not detected [11].

Next, we studied the effect of α-MSH on the formation of neural rosettes in the iPSC cultures, in which neural progenitors were formed. The number of neural rosettes formed in wells was counted under a microscope. The presence of rosettes was also confirmed by staining the cells with the antibodies to tubulin beta-III and Sox1. It was found that α-MSH at a concentration of 1 nM caused a more that twofold increase in the formation of neural rosettes (16 ± 3) in human iPSC cultures compared to the control (PBS, 6 ± 1 neural rosettes). It should be noted that, in the cell cultures derived from the embryonic rat brain, α-MSH also caused a nearly twofold increase in the number of secondary neurospheres [7]. In the future, it is important and interesting to study the effect of α-MSH and other melanocortin peptides on the end-stage differentiation of iPSCs in the neural direction, especially taking into account the fact that the positive

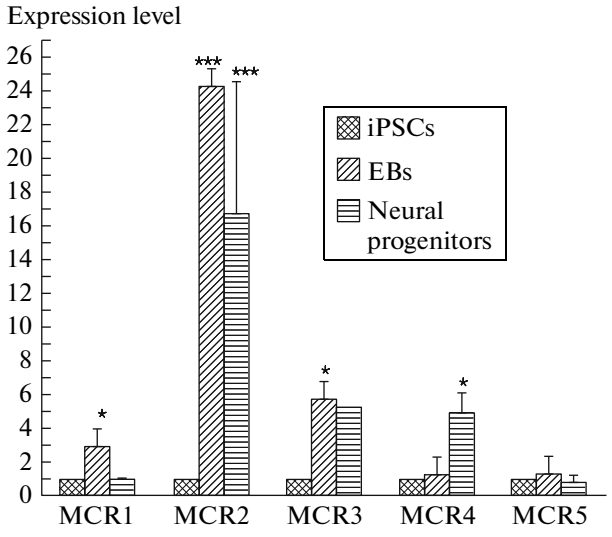

Fig. 1. Gene expression of melanocortin receptors at different stages of differentiation of human iPSCs. The expression level in iPSCs was taken as unity; the levels in EBs and neural progenitors were determined with respect to it. $*p < 0.05$, $***p < 0.001$. Data are represented as $M \pm m$, $n = 6$.

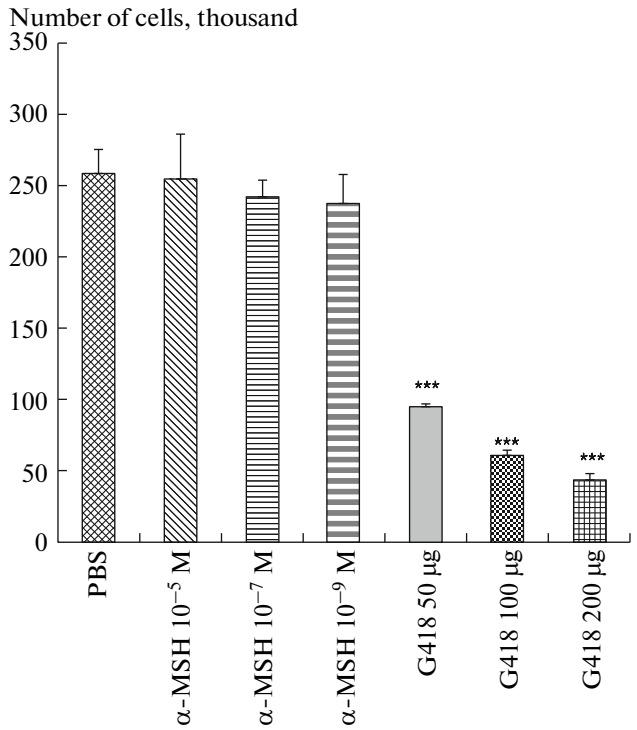

Fig. 2. Effect of α-MSH and G418 on the proliferative activity of iPSCs. ***$p < 0.001$. Data are represented as $M \pm m$, $n = 6$.

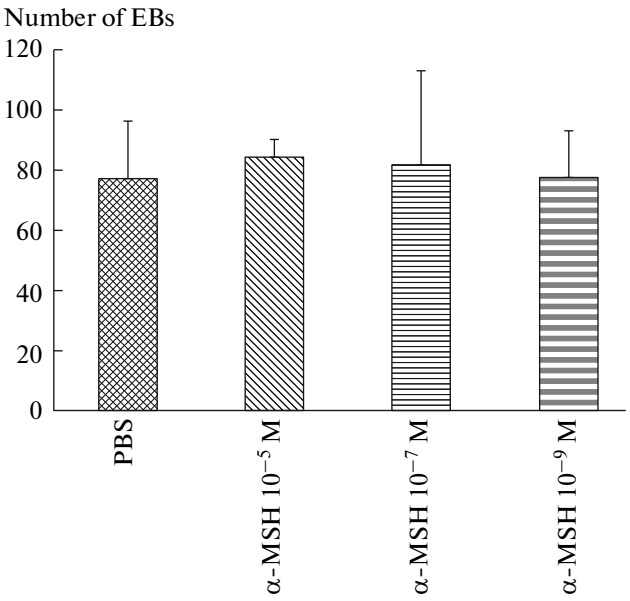

Fig. 3. Effect of α-MSH on the formation of embryoid bodies. The number of EBs formed from 100 thousand iPSCs 2 days after seeding. Data are represented as $M \pm m$, $n = 4$.

effects of melanocortin peptides in the mouse ESC model were described in our earlier study [11].

Thus, this is the first study to demonstrate the expression of α-MSH receptors both in undifferentiated iPSCs and at the early stages of their differentiation and to show that α-MSH stimulates the formation of neural rosettes in these cells in vitro.

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