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MATURE RAT HEPATOCYTE DEDIFFERENTIATION INTO LONG-LIVED PROLIFERATING HEPATIC PROGENITOR CELLS

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Objective: to obtain long-lived proliferating cells with progenitor features by dedifferentiation of mature rat hepatocytes using combinations of small molecules. **Materials and Methods.** Hepatocytes isolated from rat liver by perfusion were cultured in the presence of a cocktail of three small molecules – Wnt signaling pathway activator (CHIR99021), TGF-β inhibitors (A83-01) and ROCK kinase (Y27632). The morphological characteristics and growth features of the culture were assessed using fluorescence and phase-contrast microscopy during cell culture. Cell proliferative activity was analyzed using real-time time-lapse imaging. The expression of surface and intracellular markers was analyzed using flow cytometry and high-resolution fluorescence microscopy. **Results.** Using a cocktail of small molecules, Y-27632, A-83-01, and CHIR99021, long-lived proliferating cells that express progenitor cell markers, such as α-fetoprotein and HNF4α, were obtained from mature rat hepatocytes. The cells had hepatocyte-like morphology and formed discrete clusters of proliferating cells, forming a single cell layer during culturing. Removal of the small molecules from the medium led to expansion of fibroblast-like cells and elimination of potentially progenitor hepatocyte-like cells. **Conclusion.** Proliferating progenitor cells can be obtained by dedifferentiation of mature hepatocytes.

Keywords: hepatocytes, dedifferentiation, hepatic progenitor cells, small molecules, signaling pathways, Y-27632, A-83-01, CHIR99021.

INTRODUCTION

Despite the fact that primary rat hepatocytes were first isolated from the liver more than 50 years ago [1], and the first report on isolation of hepatocytes from the human liver appeared 20 years later [2], issues on long-term culture of these cells and their preservation in functionally active state in vitro still remain unsolved. Meanwhile, it has been reliably proved that in conditions of chronic liver injury, hepatocytes are able to de-differentiate into proliferating bipotent progenitor/stem cells [3]. In this regard, coming up with ways to obtain progenitor cells from mature hepatocytes in vitro is of great importance. This may not only help to better understand the origin of hepatic progenitor cells and reprogramming mechanisms, but also offer an unlimited source of cells for generation of functional hepatocytes, which are widely used in pharmacology, clinical medicine, tissue engineering and disease modeling.

Over the past few years, scientists have managed to achieve certain successes in this direction. For example, protocols for transdifferentiation of mesenchymal stem cells into functionally active hepatocyte-like cells have been developed [4]. Many works have appeared in which hepatocytes were obtained from induced pluripotent stem cells [5], and a number of studies have described approaches to obtaining the so-called induced hepatocytes from various somatic cells [6]. All these methods, of course, have certain scientific interest, but at the same time have a number of limitations in terms of prospects of use in clinical practice. In particular, the use of genetic modifications for cell reprogramming poses a number of problems for the safety of their clinical use. One of the promising current approaches to address safety issues is cell reprogramming using the so-called small molecules. Small molecules are low molecular weight compounds that can include lipids, monosaccharides, secondary messengers, other natural compounds, as well as drugs and other xenobiotics. Small molecules have a number of undeniable advantages over any other way of regulating/altering cell fate: as a rule, they are permeable to cells; they are easier to synthesize and standardize; their cost is low, which allows using this method for mass production of cells with given properties. More importantly, the effects of small molecules can be regulated by varying their concentrations and combinations, thus providing a higher degree of temporal and spatial control over the

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function of the target protein or signaling pathway [7]. In 2017, Katsuda et al. [8] published a paper showing that it is possible to obtain proliferating hepatic bipotent progenitor cells from mature rat hepatocytes using a cocktail of small molecules such as Y-27632, A-83-01 (ROCK kinase and TGF-β inhibitors, respectively) and CHIR99021 (Wnt signaling pathway inhibitor) without any genetic modifications. The obtained cells, which the authors called chemically induced liver progenitors (CLiPs), had stem cell properties, i.e., they proliferated in culture and differentiated into hepatocytes and cholangiocytes. Around the same time, another work was published, which described a method for obtaining hepatic progenitor cells from mature murine hepatocytes by creating in vitro conditions simulating chronic liver injury conditions in vivo, when not only proliferation of mature hepatocytes occurs but also their reversible transformation into progenitor cells of the ducts occurs [9]. In addition to the aforementioned small molecules CHIR99021, A83-01 and Y27632, this medium also contained epidermal growth factor (EGF) and hepatocyte growth factor (HGF), Yap signaling activators – lysophosphatidic acid and sphingosine-1-phosphate. Proliferating duct-like cells (hepPDCs) obtained from hepatocytes in this manner were able to undergo more than 30 passages without obvious morphological changes or karyotype disruption. Gene expression profiles showed that these cells express markers of hepatocytes, cholangiocytes and hepatic progenitor cells, and are also capable of differentiating into mature functionally active hepatocytes [9]. Using a similar culture medium with slight modifications, the authors obtained progenitor cells from mature human hepatocytes after 2 years [10]. Also in 2019, the work of Kim et al. [11], who used a cocktail of two small molecules A83-01 and CHIR99021 in combination with EGF and HGF to obtain human hepatic progenitor cells from mature hepatocytes, which they named human chemically derived hepatic progenitors (hCdHs). The resulting hCdHs cells expressed hepatic progenitor cell markers and underwent about 10 passages in culture, maintaining a normal karyotype and the ability to differentiate into hepatocytes and bile duct epithelial cells in vitro. Gene profiling confirmed reprogramming at the transcriptional level, showing expression of the genes responsible for the progenitor state and suppression of mature hepatocyte genes. In intrasplenic transplantation in several animal models, hCdHs cells effectively repopulated the liver parenchyma [11]. More recently, work was published in which in a similar manner, using a cocktail of two small molecules A83-01, CHIR99021 and HGF, bipotent progenitor cells were derived from mature hepatocytes of nonhuman primates [12].

Taking into account the importance of the problem of obtaining proliferating liver cells in culture for solving the problems of cell biology, the lack of work on this topic in national science, as well as the importance of developing technology for cell mass reproduction and use in regenerative medicine, we aimed our work at obtaining proliferating cells with progenitor features by dedifferentiation of mature rat hepatocytes using combinations of small molecules.

MATERIALS AND METHODS

Rat liver cells were isolated by two-stage perfusion using a collagenase buffer, followed by washing and seeding on culture plates modified with bovine collagen type I. The first stage of perfusion was performed with 250 ml of Liver Perfusion Medium (LPM) (Gibco™, USA), the first two minutes at 5 ml/min, followed by the remaining volume at 25 ml/min. The second stage of perfusion was performed with Hanks' collagenase buffer (HBSS without Ca^{2+} and Mg^{2+}, without phenol red, (Gibco™, USA)) with addition of 0.03% type IV collagenase (PanEco, Russia) and 0.5% bovine serum albumin BSA (Diaem, Russia) in 250 ml/min at 25 ml/min. At the end of perfusion, the liver was placed in L-15 medium cooled to +2…+4 °C (Gibco™, USA) and transported from the operating room to a laminar box. Next, the Gilson capsule was opened with a scalpel, and the contents were crushed in a sterile Petri dish on ice. Then they were passed through a cell sieve with a pore size of 100 μm, washed with Hepatocyte Wash Medium (Gibco™, USA), cooled to +2…+4 °C. The cell suspension was centrifuged at +2 °C at 50 g for 5 minutes. The cell precipitate was resuspended in a fresh portion of medium and the procedure was repeated three times. Next, the precipitate was resuspended in William's E Medium (Gibco™, USA) with the addition of 5% FBS, (HyClone, UK), 1% antimycotic antibiotic (Gibco™, USA) and 200 mM L-glutamine (PanEco, Russia), after which live cell counts were made on a BioRad TS20 cell counter.

In order to determine the working concentrations of small molecules, we performed colorimetric analysis of cell proliferation in an MTT assay. As a cell model of hepatocytes, we used a human line HepG2, which was cultured under standard conditions (37 °C, 5%: CO_2) in complete growth medium based on DMEM/F12 (Gibco™, USA) with addition of 10% FBS, penicillin/streptomycin (100X, PanEco, Russia) and 200 mM L-glutamine (PanEco, Russia). For the test, serial dilutions of each inhibitor were added to the wells of a 96-well flat-bottom plate: for A-83-01, the concentration range was 2.5 μM to 0.03 μM, for CHIR99021 from 15 μM to 0.2 μM, for Y-27632 from 50 μM to 0.75 μM. Then, cell suspension (10,000 cells/well) was added to the wells. Each point in one experiment in four replicates. Intact cells cultured under the same conditions but without adding inhibitors were used as the control. The cells were incubated with small molecules for 4 days. After that,

culture medium was drained from the wells, 30 μl/well of MTT solution (5 μg/ml (Sigma, USA)) was added, and incubated in a CO_2 incubator for 2–4 hours until formazan crystals precipitated, which were then dissolved in 100 μl/well of DMSO (dimethyl sulfoxide; PanEco, Russia). Optical density (OD) was measured on a Tecan infinite M200 Pro plate reader (Tecan, USA) at 565 nm wavelength. Cell proliferation level was measured as described previously [13], using the formula:

(OD of induced cells minus OD blank) / (OD of control cells minus OD blank) × 100%, where OD blank is the optical density in the wells containing MTT and DMSO solution but no cells. OD of control cells is the optical density in the wells containing intact cells.

For surface modification, a solution of type I bovine collagen in cold (+2…+4 °C) DPBS at the rate of 12.5 μg of collagen per 1 cm^2 surface was added to culture vials and Petri dishes, then placed for 1 hour in an incubator at 37 °C. Then, the liquid was drained and the cell suspension was inoculated at a given concentration: 2×10^5 cells/cm^2. After two hours of incubation under standard conditions (37 °C, 5%: CO_2), William's E Medium and unattached cells were removed and Hepatozyme-SFM medium (Gibco™, USA) was added with 1% antibiotic antimycotic (Gibco™, USA), 200 mM L-glutamine (PanEco, Russia), a combination of small molecules in the following concentrations: 1.25 μM for A-83-01, 5 μM for CHIR99021, and 12.5 μM Y-27632. Control cells were cultured in the same growth medium without adding the small-molecule cocktail. After 48 hours, the growth medium was replaced with William's E Medium with the same additives. Cells were passaged when 50–70% confluence was achieved according to the standard technique using Versen solution (PanEco, Russia) and TrypLe reagent (Gibco™, USA).

At certain time intervals, cells on Petri dishes were stained with the Live/Dead Assay complex of vital dyes (Invitrogen, USA), which allowed us to evaluate the morphology of adherent cells and distinguish between live and dead cells. The study was performed using a Leica DMi8 Thunder super-resolution microscopic system equipped with a Leica DFC9000 GTC camera (Leica, Germany).

Cell proliferation was assessed using a microscopy system with automatic zeitrapher imaging (IncuCyte ZOOM, USA). The system allows us to evaluate the confluence of cell monolayer in real time with high accuracy and calculate the growth curves of cell culture.

For the analysis, we used passage 1 cells, which were seeded into 25 cm^2 culture vials at the rate of 4×10^3 cells/cm^2. Cell growth was studied on 4 types of media:

1. William's E Medium with addition of a small-molecule cocktail;
2. William's E Medium;
3. DMEM/F-12 Medium with addition of a small-molecule cocktail;
4. DMEM/F-12 Medium.

The analysis was performed in real time for 26 days. Growth curves were plotted automatically by the device's software.

For cytofluorometric analysis of expression of surface marker CD29, passage 1 cells cultured with addition of a small-molecule cocktail were removed from the plate, incubated with antibodies to CD29, labeled with FITC (BD Biosciences, USA) for 1 hour. After incubation, the cells were washed twice in DPBS, fixed in CytoFix (BD Biosciences, USA) for 15 minutes, and measured on a BD FACSAria III flow cytometry sorter (BD Biosciences, USA). At least 10,000 events were analyzed. Data was processed using FlowJo_V10 software.

The expression of intracellular markers in cells cultured in the presence of a small-molecule cocktail was analyzed by flow cytometry and fluorescence microscopy. For this purpose, cells were permeabilized in 0.1% Triton X-100 solution (Sigma, USA) for 10 minutes at room temperature. Then they were incubated with primary antibodies against Ki-67, α-fetoprotein (AFP), HNF4α, and cytokeratin 18 (all by SantaCruz Biotechnology, USA) for 1 hour, and then with FITC-labeled secondary anti-species antibodies (all by Sigma, USA) for 40 minutes. Cells stained only with secondary anti-species antibodies were used as controls for nonspecific binding. For fluorescence microscopy, cell nuclei were doped with DAPI (1 μg/mL) (4',6-diamidino-2-phenylindole; Invitrogen, USA) and the preparation was encased in fluorescence protector (Anti-Fade Fluorescence Mounting Medium, Abcam, USA). Fluorescence analysis and flow cytofluorimetry were performed as described above.

RESULTS AND DISCUSSION

At the first stage of work, we analyzed the effect of small molecules on the proliferation level of HepG2 cells and determined the optimal concentrations of small molecules for further work.

Fig. 1 shows that all the three small molecules in certain concentration ranges induced an increase in the proliferative level of the HepG2 cell line. Thus, the ROCK kinase inhibitor (Y27632) increased the level of cell proliferation in the concentration range from 0.75 to 12.5 μM (Fig. 1, a), the Wnt signaling pathway inhibitor (CHIR99021) stimulated proliferative activity in the range from 0.2 to 7.5 μM (Fig. 1, b), whereas the TGF-β inhibitor (A83-01) increased cell proliferation throughout the entire concentration range of 0.03 to 2.5 μM used (Fig. 1, b). Based on the data obtained and guided by previously published works in which the same small molecules were used to reprogram hepatocytes, the following working concentrations of small molecules were

determined: 12.5 μM for Y-27632, 5 μM for CHIR99021 and 1.25 μM for A-83-01.

Fluorescence and phase-contrast microscopy were used to analyze the effect of the small-molecule cocktail on formation of proliferating cell culture from rat hepatocytes. After isolation, rat hepatocytes were divided into two cultures. In the control culture, cells were cultured without addition of small-molecule cocktail, in the experimental culture, cells were cultured with addition of small-molecule cocktail in the working concentrations determined in preliminary experiments. Fluorescence microscopy of cells stained with the Live/Dead™ complex 24 hours after cell isolation showed that a significant number of viable cells were present in both the control and the experimental variant (Fig. 2).

It is noteworthy that already one day after isolation, the difference in the level of viability of control and experimental cultures was clearly visible. A much higher level of cell death can be observed in the control cells (Fig. 2, a) compared to the experimental cells that were cultured in the presence of the small-molecule cocktail (Fig. 2, b). Morphologically, two types of cells can be distinguished in the experimental culture: hepatocyte-like and oval-shaped cells.

On the fifth day of the experiment, even more significant differences in the experimental and control cell cultures were noted (Fig. 3, a, b). By this time, in the control culture, mainly single strongly expanded cells with signs of plasma and nuclear membrane disruption and increased vacuolization were preserved (Fig. 3, a). Whereas in the experimental culture along with the same mature cells, transitioning into the state of cell death, groups of newly formed cells were visualized (Fig. 3, b).

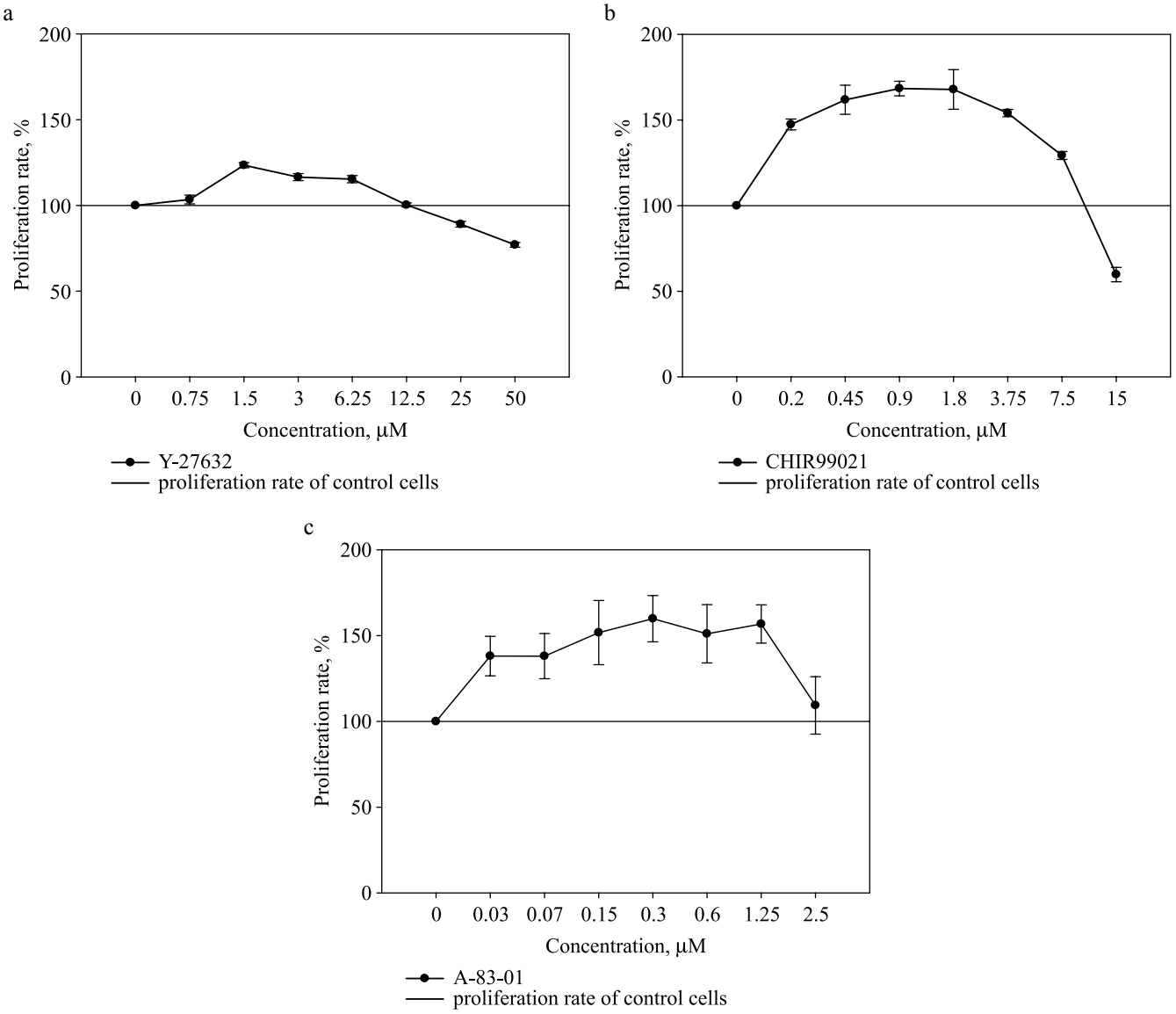

Fig. 1. Dependence of HepG2 cell proliferation level on serial dilutions of small molecules in the culture medium: a – in the presence of Y-27632; b – in the presence of CHIR99021; c – in the presence of A-83-01

By day 7 of the experiment, the process of cell death continued in the control culture (Fig. 3, c). In the experimental culture, proliferation enhancement with the formation of small cell clusters was clearly visualized (Fig. 3, d). On day 10 of the experiment, due to complete cell death in the control culture, only the experimental variant was monitored. During this time in the experimental culture, there was increased count of cells in the formed clusters, compaction of their structure and formation of the extracellular matrix (Fig. 4).

After 15 days of the experiment, there was cell expansion in clusters, which led to their outgrowth, fusion with neighboring clusters, and formation of a cell layer. The level of cell death in the culture was insignificant (Fig. 5).

For all subsequent experiments, cells after the first passage were used, which from the moment of isolation were continuously cultured in the presence of the small-molecule cocktail. To evaluate the effect of different growth media and the small-molecule cocktail on cell growth in culture, proliferation analysis was performed, and growth curves were plotted using a microscopy system with automatic zeitraffer imaging. Analysis of the growth of cell population on different media with and without addition of the small-molecule cocktail showed that the cells that were cultured on media (DMEM/F12 and William's E) without addition of small molecules demonstrated the most rapid growth and uniform expansion (Fig. 6, a and b).

However, visual analysis of images revealed that in both variants of the growth media in the absence of the small-molecule cocktail, growth of the cell population occurred due to expansion of fibroblast-like cells. Characteristic clusters of hepatocyte-like cells in these variants were absent throughout the experiment (26 days).

On the other hand, when cells were cultured in both types of growth media (DMEM/F12 and William's E) in the presence of small-molecule cocktail after the first passage, formation of hepatocyte-like cell clusters took place, the same as we observed in the initial experimental variant after isolation of hepatocytes before passage. Thus, it is shown that for maintenance of growth and proliferation of hepatocyte-like, but not fibroblast-like, cells, not only the initial exposure to the small-molecule cocktail is necessary, but also their constant presence in the growth medium throughout the entire period of cultivation. It is noteworthy that when cultured on a DMEM/F12 medium, cell cluster formation began significantly earlier (48 hours after cell planting), whereas in William's E medium, it was noted only on day 7 of culturing. The number of cell clusters in the field of view also differed. For each cell culture sample, 80 fields of view per 25 cm^2 culture vial were analyzed. When cultured on DMEM/F12 medium in the presence of small molecules, cell clusters occurred in 90% of the analyzed fields of view. When growing on William's E medium with addition of small molecules, this figure reached only 50%.

Phase-contrast microscopy of cells at different stages of cell culture growth allowed us to visualize formation of individual clusters. The center of such cluster formation is large, mostly irregularly shaped binuclear cells (Fig. 7).

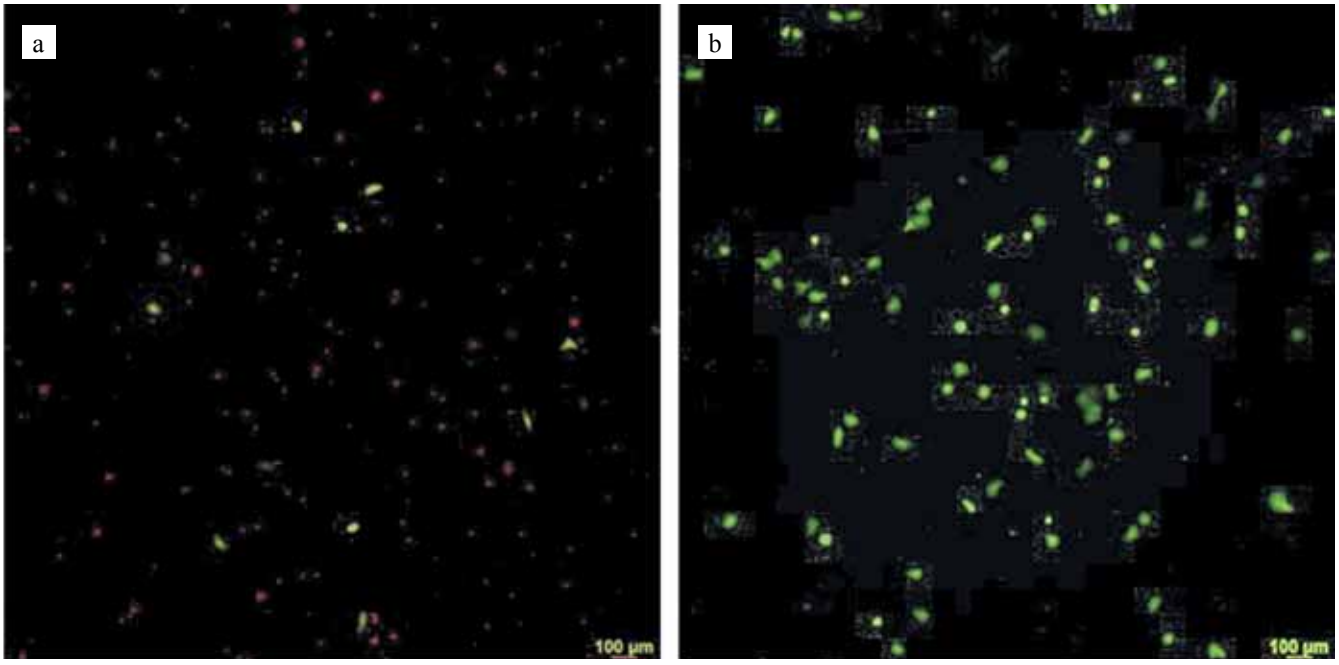

Fig. 2. Fluorescence microscopy of liver cells 24 hours after isolation. Live/Dead™ staining. a – control cells (without addition of the small-molecule cocktail), b – experimental cells (with addition of the small-molecule cocktail). Green color – live cells, red – cells in a state of death. 50× magnification

A significant number of cells containing two nuclei are found in the emerging cell cluster. It is known that one of the characteristic features of the liver is hepatocyte polyploidy, i.e. increased number of the set of chromosomes per cell. Polyploid hepatocytes can be presented in several variants: they can be tetraploid (bicore with a 2n set of chromosomes or unicore with a 4n set of chromosomes) or octaploid (bicore with a 4n set of chromosomes or unicore with an 8n set of chromosomes) [14]. The functional role of hepatocyte polyploidy is still not entirely clear. However, it has been shown that polyploid hepatocytes have increased proliferative capacity, expressing a number of genes associated with the passage of the cell cycle [15].

Further, in the process of culturing, there is active cell division and peripheral growth of the cluster with formation of significant clusters of densely packed cells (Fig. 8, a) at a later stage in close proximity with fibroblast-like cells (Fig. 8, б).

Expression of surface and intracellular markers was analyzed on cells after the first passages cultured with addition of the small-molecule cocktail.

CD29 or integrin β1 belongs to the family of integrin proteins that are involved in cell interactions with extracellular matrix proteins such as collagen, laminin, and fibronectin, as well as in heterodimers, participates in intercellular interactions, and plays a functional role in cell migration, adhesion, and survival. CD29 is widely

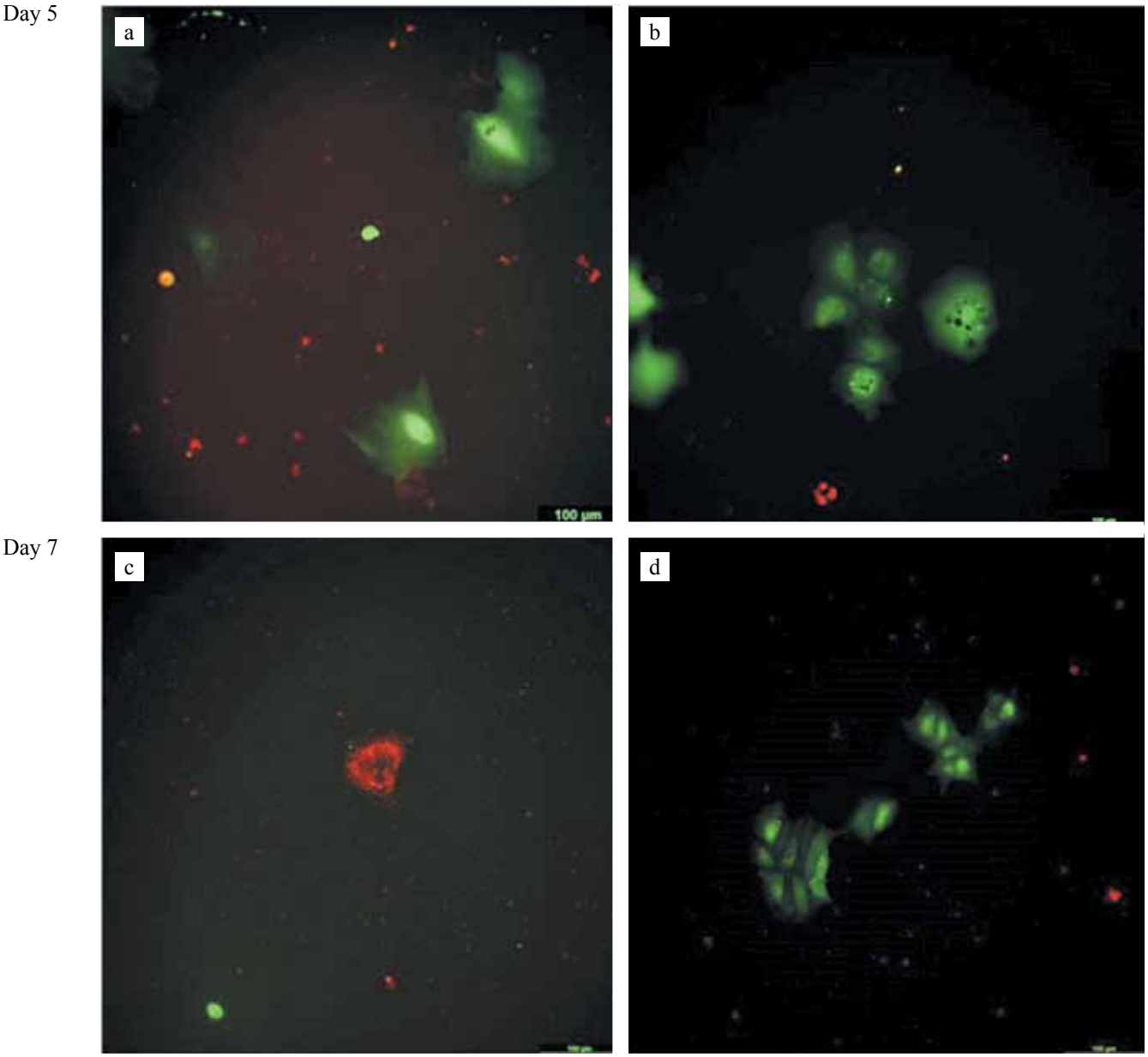

Fig. 3. Fluorescence microscopy of liver cells at day 5 (A and B) and day 7 (C and D) after isolation. Live/Dead™ staining. a, c – control cells (without addition of small-molecule cocktail), b, d – experimental cells (with addition of the small-molecule cocktail); b – group of newly formed cells; d – formation of cell clusters. Green color – live cells, red color – cells in a state of cell death. 200× magnification

expressed on various cell types, including mesenchymal and epithelial cells, as well as stem/progenitor cells of different origin [16]. So, CD29, along with CD44 and CD90, is often considered a marker of mesenchymal stem/stromal cells (MSCs) isolated from different tissue sources, both in humans [17] and in rodents [18]. In the adult liver, integrin β1 is expressed by mature hepatocytes [19], as well as by hepatic stem cells and hepatoblasts [20].

Using flow cytofluorimetry, we analyzed the expression of Integrin beta 1 (CD29) on the cell surface (Fig. 9, a).

As seen in Fig. 9, a, CD29 expression was observed on more than 60% of the cells. Morphologically, hepatocyte-like cells predominated in this culture (Fig. 9, b).

Deletion of integrin beta 1 in the embryonic liver leads to disruption of the normal development of hepatocyte polarity, lack of specification of intercellular contacts, and failure to form tubules. Directed deletion of integrin beta 1 in adult hepatocytes prevents restoration of normal hepatocyte architecture after liver injury followed by development of fibrosis [21]. So, CD29 expression on cells derived from rat hepatocytes after culturing them in the presence of small molecules may also ensure maintenance of the characteristic architecture of the forming clusters and promote proliferation.

The expression of intracellular markers in cells was analyzed using two methods. Using ultrahigh resolution fluorescence microscopy, the expression of such liver-associated proteins as cytokeratin 18, alpha-fetoprotein, HNF4α, and the proliferation cell marker Ki-67 was assessed.

Proliferation marker Ki-67 is expressed in the nucleus during the G1, S and G2-M phases of cell cycle in proliferating cells [22]. We showed that almost 100% of the cells express this protein (Fig. 10, a).

Cytokeratin 18 is a cytoskeleton protein and a major intermediate filament expressed in the liver [23]. Cytokeratin 18, along with cytokeratin 19, has been shown to be expressed in human hepatic stem cells similar to rodent oval cells [24]. We have shown that cytokeratin 18 expression is characteristic of all cells in culture that have hepatocyte-like morphology (Fig. 10, b).

Hepatocyte nuclear factor 4α (HNF4α) is an orphan nuclear receptor that is known to be a master regulator of hepatic differentiation processes because it regulates a large number of hepatocyte-specific genes [25]. HNF4α regulates important liver functions such as glycolysis, gluconeogenesis, fatty acid metabolism, bile acid synthesis, drug metabolism, apolipoprotein synthesis, ureogenesis and blood coagulation by regulating the transcription of multiple genes involved in each of these functions [26–28]. In addition to mature hepatocytes, HNF4α is expressed by human and rodent bipotent hepatoblasts during embryogenesis [29], as well as by hepatic stem cells in the adult organ [30].

We also detected HNF4α expression predominantly in cells forming characteristic clusters (Fig. 11, a).

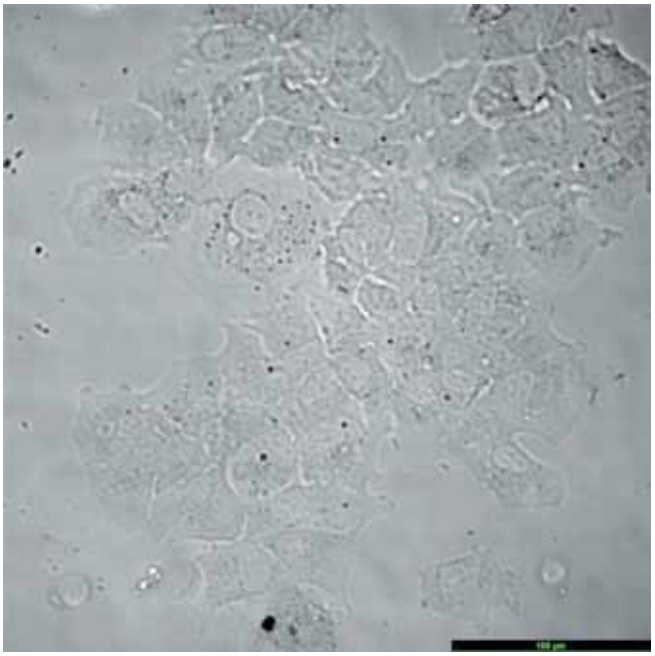

Fig. 4. Phase-contrast microscopy of a dense hepatocyte-like cell cluster at day 10 in the experimental culture. 200× magnification

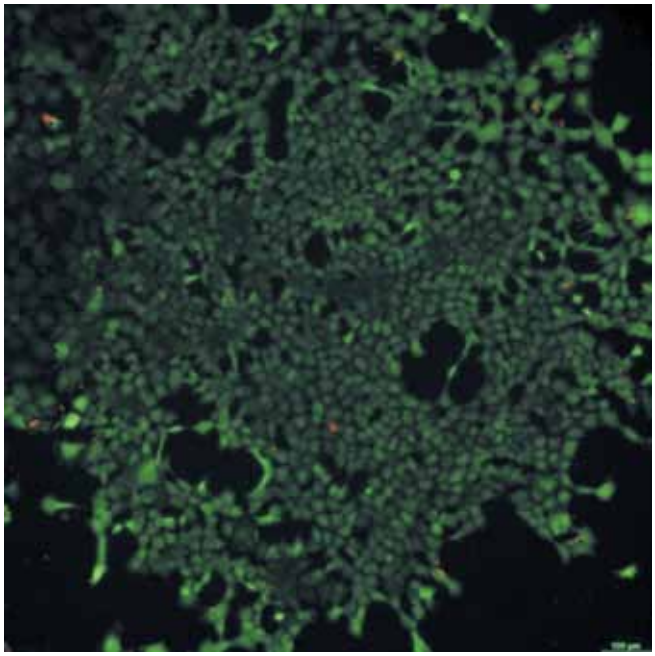

Fig. 5. Fluorescence microscopy of cells on day 15 after isolation. Live/Dead™ staining. Expansion of cells in clusters leading to formation of large cell clusters. Green color – live cells, red color – cells in a state of cell death. 100× magnification

The obtained images clearly show the nuclear localization of HNF4α, which is characteristic of normal hepatic cells. HNF4α expression in the cells cultured in the presence of the small-molecule cocktail indicates their hepatocytic origin and progenitor potential.

Alpha-fetoprotein (AFP) is one of the most studied markers of cell differentiation and tumor development. This protein is expressed by fetal and malignant hepatocytes and is not expressed by normal mature hepatocytes [31]. AFP is a major marker of oval cells [32] and immature hepatocytes [33]. Due to the fact that AFP expression strongly correlates with expression of fetal genes during ontogenesis, this protein serves as an ideal candidate

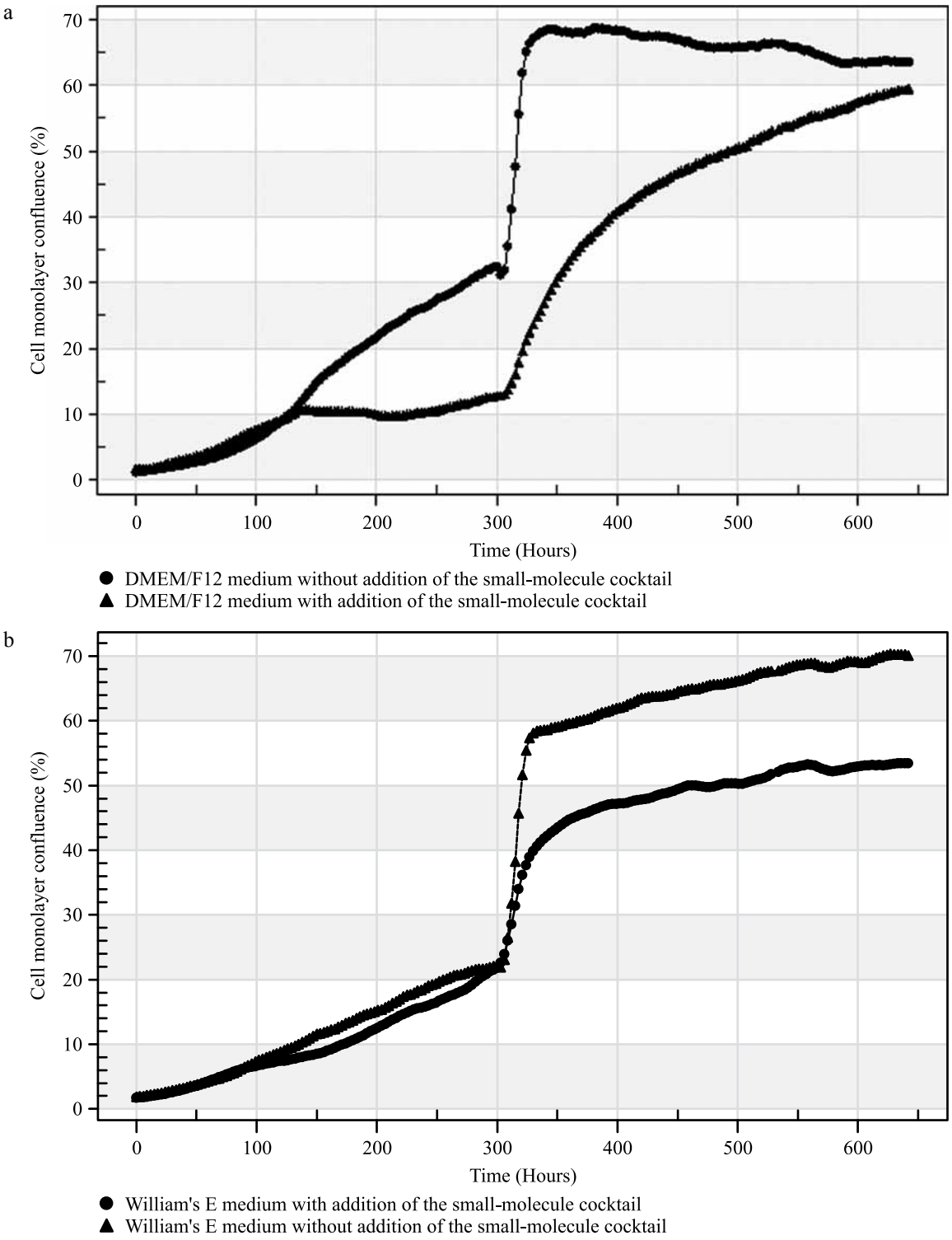

Fig. 6. Growth curves of cells at passage 1 cultured on different media for 26 days. The growth curves were constructed based on estimation percentage of the cell monolayer confluence due to photofixation of visual fields every 4 hours. a – cells were cultured on a DMEM/F12 medium; b – cells were cultured on William's E medium.

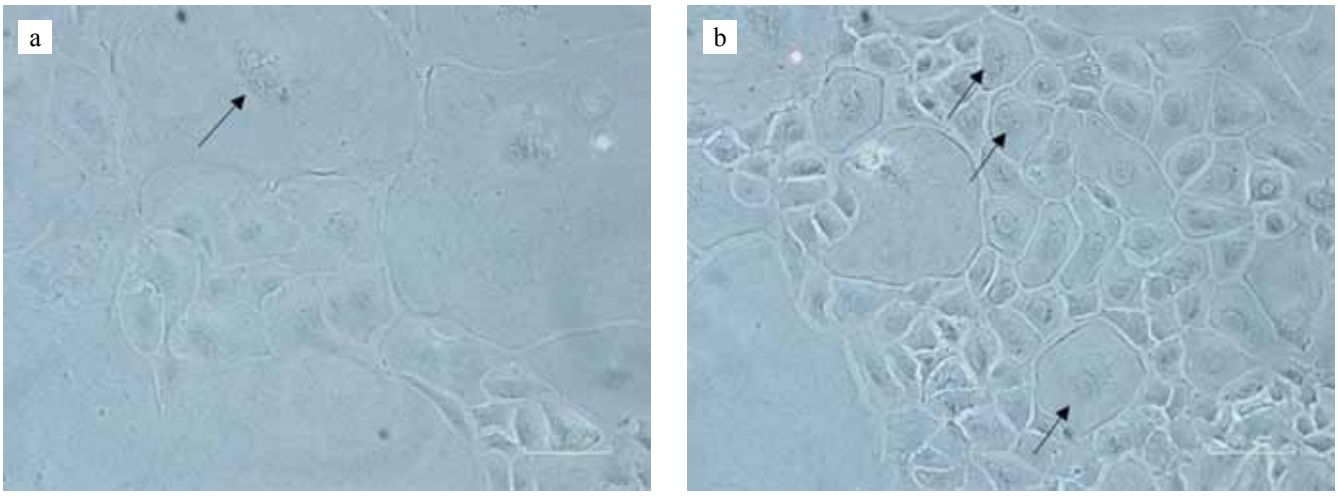

Fig. 7. Phase-contrast microscopy of hepatocyte-like cell clusters forming in the presence of a small-molecule cocktail. a – central binucleated cell, presumably giving rise to cluster growth. b – cell proliferation in a cluster; arrows indicate binucleated cells. 200× magnification

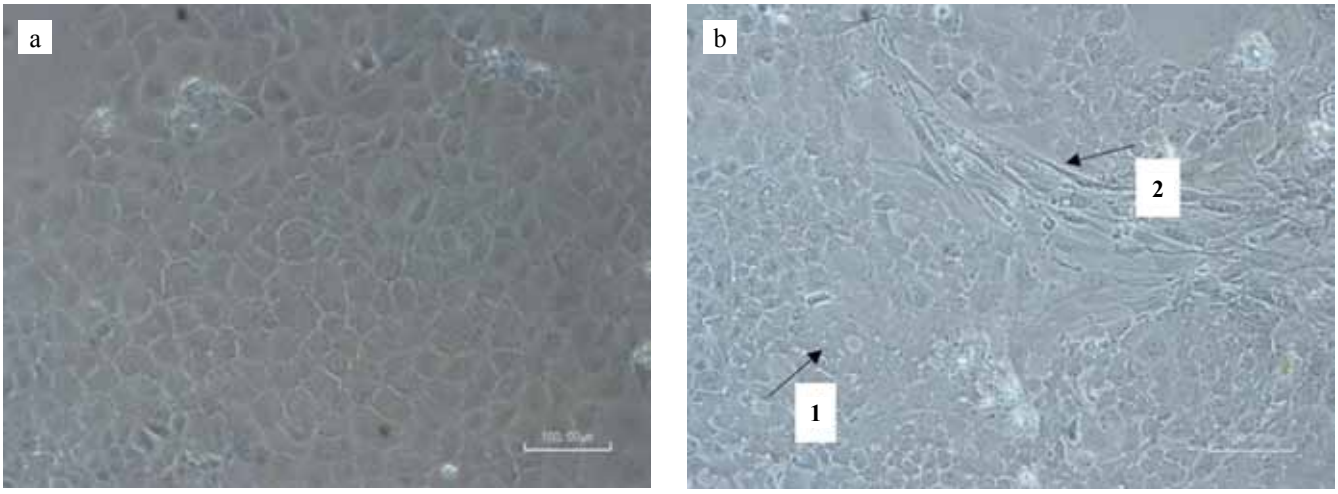

Fig. 8. Phase-contrast microscopy of the cell layer at late stages of culturing (47 days) in the presence of a small-molecule cocktail. a – dense hepatocyte-like cell cluster; 200× magnification. b – contact zone of different cell types within a single cell layer; 1 – hepatocyte-like cells, 2 – fibroblast-like cells. 100× magnification

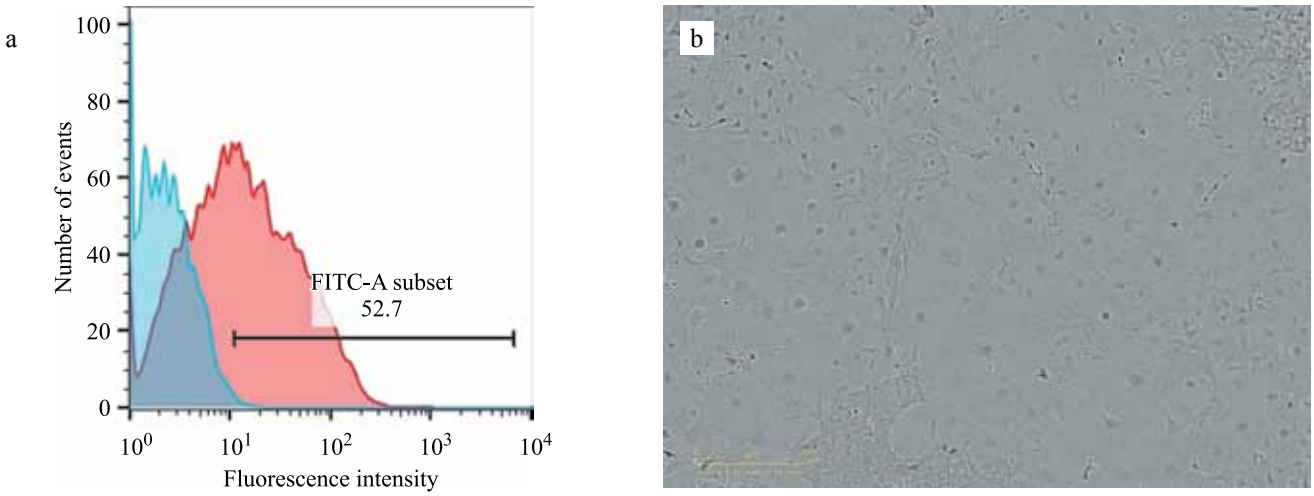

Fig. 9. a – cytofluorimetric analysis of CD29 expression. Blue peak – autofluorescence of control cells not stained with antibodies. Red peak – fluorescence of anti-CD29-FITC bound to the cells. b – phase-contrast microscopy of the culture. 100× magnification

marker for tracking and studying stem cell development, differentiation and redifferentiation pathways.

We analyzed α-fetoprotein expression using two methods, fluorescence microscopy and flow cytometry. For this purpose, we performed intracellular staining of cells with antibodies specific to AFP. The staining results are shown in Figs. 11, b and 12. When performing fluorescence microscopy, we observed a not very distinct pattern of AFP expression in cells, namely, very few cells were stained with antibodies to this protein, and its localization in most cells was predominantly perinuclear, and only in some cells AFP was distributed in the cytoplasm, which is typical for this protein (Fig. 11, b).

To confirm the presence of AFP expression, cytofluorimetric analysis of intracellular staining of cells with appropriate antibodies was performed (Fig. 12).

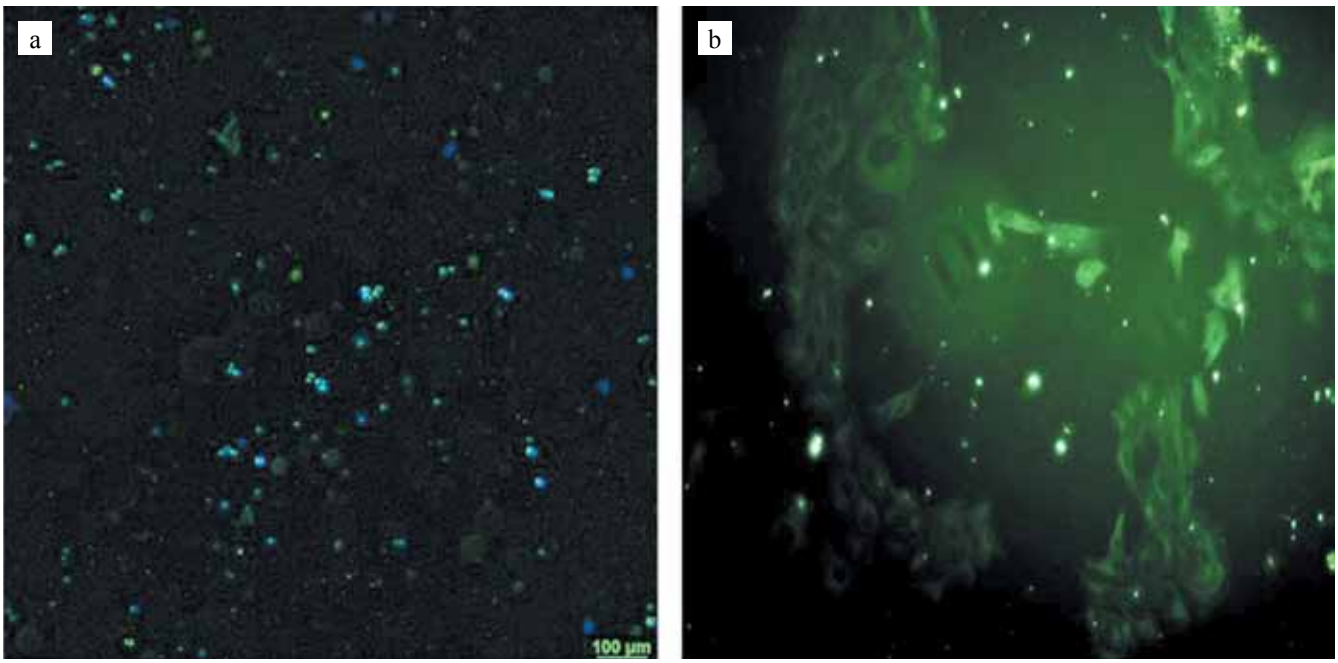

Fig. 10. Fluorescence microscopy of cells after culturing in the presence of small molecules. a – cells were stained with Ki-67 antibodies. Nuclei are stained with DAPI. 100× magnification. b – cells were stained with cytokeratin 18 antibodies. 200× magnification

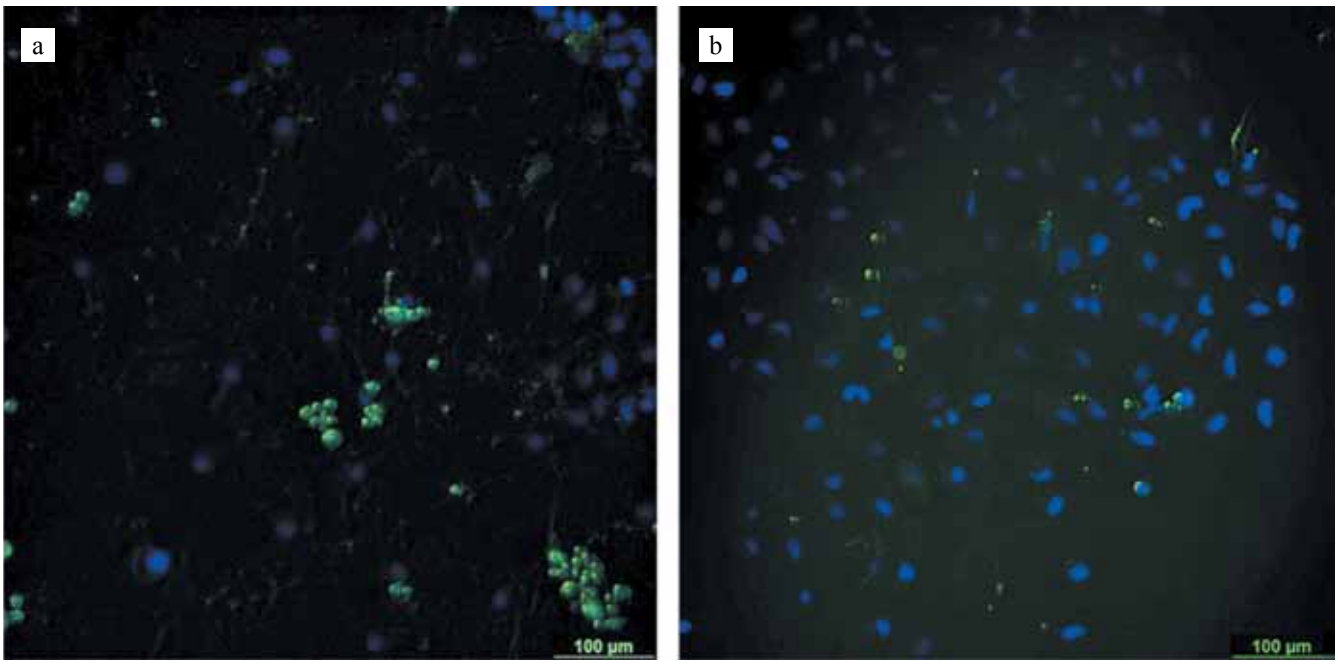

Fig. 11. Fluorescence microscopy of cells after culturing in the presence of small molecules. a – cells stained with anti-HNF4α. b – cells stained with AFP antibodies. Cell nuclei were stained with DAPI. 200× magnification

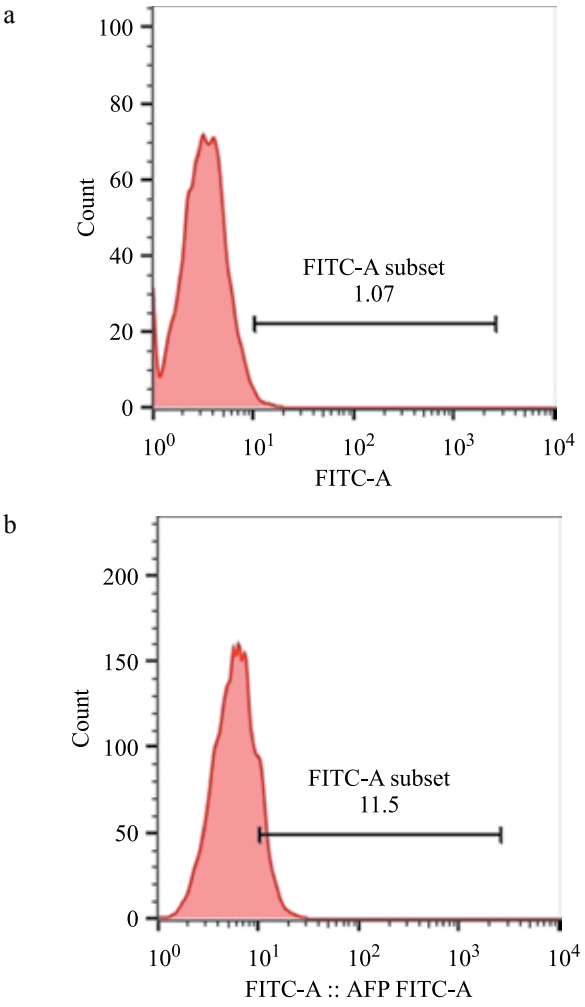

Fig. 12. Cytofluorimetric analysis of AFP expression in cells. a – control cells stained with anti-species secondary antibodies. b – cells stained with AFP antibodies

As follows from Fig. 12, about 10% of cells express α-fetoprotein, which confirms the result obtained by fluorescence microscopy. Thus, the presence of AFP expression in cells derived from mature rat hepatocytes after culturing in the presence of the small-molecule cocktail indicates their dedifferentiation/reprogramming into an immature progenitor state.

CONCLUSION

Induction of mature hepatocytes by a small-molecule cocktail may serve as one approach to reprogramming terminally differentiated cells into a progenitor state. It has been demonstrated that the presence of three small molecules Y-27632, A-83-01 and CHIR99021, in the medium allows to culture rat hepatocytes for a long time (over 2 months). At the same time, significant changes in their morphology occur and liver progenitor cell markers, such as AFP and HNF4α, start to be expressed. This result opens up new opportunities for studying the mechanism of dedifferentiation/reprogramming of mature hepatocytes. In our opinion, it is a starting point for further studies aimed at studying the properties of human hepatic progenitor cells, developing their production methods with subsequent application in the treatment of end-stage liver diseases, such as cirrhosis of various etiology and malignant tumors.

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REFERENCES

1. *Berry MN, Friend DS.* High-yield preparation of isolated rat liver parenchymal cells: a biochemical and fine structural study. *J Cell Biol*. 1969; 43: 506–520. doi: 10.1083/jcb.43.3.506. PMID: 4900611.
2. *Guguen-Guillouzo C, Campion JP, Brissot P, Glaise D, Launois B, Bourel M et al.* High yield preparation of isolated human adult hepatocytes by enzymatic perfusion of the liver. *Cell Biol Int Rep*. 1982; 6: 625–628. doi: 10.1016/0309-1651(82)90187-4. PMID: 6286153.
3. *Tarlow BD, Pelz C, Naugler WE, Wakefield L, Wilson EM, Finegold MJ et al.* Bipotential adult liver progenitors are derived from chronically injured mature hepatocytes. *Cell Stem Cell*. 2014; 15: 605–618. doi: 10.1016/j.stem.2014.09.008. PMID: 25312494.
4. *Afshari A, Shamdani S, Uzan G, Azarpira N.* Different approaches for transformation of mesenchymal stem cells into hepatocyte-like cells. *Stem Cell Res Ther*. 2020; 11: 54. doi: 10.1186/s13287-020-1555-8. PMID: 32033595.
5. *Kholodenko IV, Kholodenko RV, Manukyan GV, Lupatov AY, Yarygin KN.* Isolation of Induced Pluripotent Cells from Stromal Liver Cells of Patients with Alcoholic Cirrhosis. *Bull Exp Biol Med*. 2017; 163: 535–541. doi: 10.1007/s10517-017-3845-4. PMID: 28853085.
6. *Lim KT, Lee SC, Gao Y, Kim KP, Song G, An SY et al.* Small Molecules Facilitate Single Factor-Mediated Hepatic Reprogramming. *Cell Rep*. 2016; 15: 814–829. doi: 10.1016/j.celrep.2016.03.071. PMID: 27149847.
7. *Kim Y, Jeong J, Choi D.* Small-molecule-mediated reprogramming: a silver lining for regenerative medicine. *Exp Mol Med*. 2020; 52: 213–226. doi: 10.1038/s12276-020-0383-3. PMID: 32080339.
8. *Katsuda T, Kawamata M, Hagiwara K, Takahashi RU, Yamamoto Y, Camargo FD et al.* Conversion of Terminally Committed Hepatocytes to Culturable Bipotent Progenitor Cells with Regenerative Capacity. *Cell Stem Cell*. 2017; 20: 41–55. doi: 10.1016/j.stem.2016.10.007. PMID: 27840021.
9. *Wu H, Zhou X, Fu GB, He ZY, Wu HP, You P et al.* Reversible transition between hepatocytes and liver progenitors for *in vitro* hepatocyte expansion. *Cell Res*. 2017; 27: 709–712. doi: 10.1038/cr.2017.47. PMID: 28374751.

10. *Fu GB, Huang WJ, Zeng M, Zhou X, Wu HP, Liu CC et al.* Expansion and differentiation of human hepatocyte-derived liver progenitor-like cells and their use for the study of hepatotropic pathogens. *Cell Res*. 2019; 29: 8–22. doi: 10.1038/s41422-018-0103-x. PMID: 30361550.
11. *Kim Y, Kang K, Lee SB, Seo D, Yoon S, Kim SJ et al*. Small molecule-mediated reprogramming of human hepatocytes into bipotent progenitor cells. *J Hepatol*. 2019; 70: 97–107. doi: 10.1016/j.jhep.2018.09.007. PMID: 30240598.
12. *Hee Hong D, Lee C, Kim Y, Lee SB, Han SC, Kim SJ et al.* Generation of Hepatic Progenitor Cells from the Primary Hepatocytes of Nonhuman Primates Using Small Molecules. *Tissue Eng Regen Med*. 2021; 18: 305–313. doi: 10.1007/s13770-020-00327-8. PMID: 33591557.
13. *Kholodenko IV, Kalinovsky DV, Svirshchevskaya EV, Doronin II, Konovalova MV, Kibardin AV et al.* Multimerization through Pegylation Improves Pharmacokinetic Properties of scFv Fragments of GD2-Specific Antibodies. *Molecules*. 2019; 24: 3835. doi: 10.3390/molecules24213835. PMID: 31653037
14. *Zhang S, Lin YH, Tarlow B, Zhu H.* The origins and functions of hepatic polyploidy. *Cell Cycle*. 2019; 18: 1302–1315. doi: 10.1080/15384101.2019.1618123. PMID: 31096847.
15. *Katsuda T, Hosaka K, Matsuzaki J, Usuba W, Prieto-Vila M, Yamaguchi T et al.* Transcriptomic Dissection of Hepatocyte Heterogeneity: Linking Ploidy, Zonation, and Stem/Progenitor Cell Characteristics. *Cell Mol Gastroenterol Hepatol*. 2020; 9: 161–183. doi: 10.1016/j.jcmgh.2019.08.011. PMID: 31493546.
16. *Togarrati PP, Dinglasan N, Desai S, Ryan WR, Muench MO.* CD29 is highly expressed on epithelial, myoepithelial, and mesenchymal stromal cells of human salivary glands. *Oral Dis*. 2018; 24: 561–572. doi: 10.1111/odi.12812. PMID: 29197149.
17. *Kholodenko IV, Kurbatov LK, Kholodenko RV, Manukyan GV, Yarygin KN.* Mesenchymal Stem Cells in the Adult Human Liver: Hype or Hope? *Cells*. 2019; 8: 1127. doi: 10.3390/cells8101127. PMID: 31546729.
18. *Zou W, Zhao J, Li Y, Wang Z, Yan H, Liu Y et al.* Rat Bone Marrow-Derived Mesenchymal Stem Cells Promote the Migration and Invasion of Colorectal Cancer Stem Cells. *Onco Targets Ther*. 2020; 13: 6617–6628. doi: 10.2147/OTT.S249353.
19. *Terry C, Hughes RD, Mitry RR, Lehec SC, Dhawan A.* Cryopreservation-induced nonattachment of human hepatocytes: role of adhesion molecules. *Cell Transplant*. 2007; 16: 639–647. doi: 10.3727/000000007783465000. PMID: 17912955.
20. *Turner RA, Mendel G, Wauthier E, Barbier C, Reid LM.* Hyaluronan-supplemented buffers preserve adhesion mechanisms facilitating cryopreservation of human hepatic stem/progenitor cells. *Cell Transplant*. 2012; 21: 2257–2266. doi: 10.3727/096368912X637000. PMID: 22472355.
21. *Masuzaki R, Ray KC, Roland J, Zent R, Lee YA, Karp SJ.* Integrin β1 Establishes Liver Microstructure and Modulates Transforming Growth Factor β during Liver Development and Regeneration. *Am J Pathol*. 2021; 191: 309–319. doi: 10.1016/j.ajpath.2020.10.011. PMID: 33159885.
22. *Sasaki K, Murakami T, Kawasaki M, Takahashi M.* The cell cycle associated change of the Ki-67 reactive nuclear antigen expression. *J Cell Physiol*. 1987; 133: 579–584. doi: 10.1002/jcp.1041330321. PMID: 3121642.
23. *Chougule P.* Sumitran-Holgersson S. Cytokeratins of the liver and intestine epithelial cells during development and disease. in: Hamilton G. Cytokeratins – Tools in Oncology. InTech, 2012; 118–158.
24. *Masson NM, Currie IS, Terrace JD, Garden OJ, Parks RW, Ross JA.* Hepatic progenitor cells in human fetal liver express the oval cell marker Thy-1. *Am J Physiol Gastrointest Liver Physiol*. 2006; 291: G45–54. doi: 10.1152/ajpgi.00465.2005. PMID: 16769813.
25. *Walesky C, Apte U.* Role of hepatocyte nuclear factor 4α (HNF4α) in cell proliferation and cancer. *Gene Expr*. 2015; 16: 101–108. doi: 10.3727/105221615X14181438356292. PMID: 25700366.
26. *Hayhurst GP, Lee YH, Lambert G, Ward JM, Gonzalez FJ.* Hepatocyte nuclear factor 4alpha (nuclear receptor 2A1) is essential for maintenance of hepatic gene expression and lipid homeostasis. *Mol Cell Biol*. 2001; 21: 1393–1403. doi: 10.1128/MCB.21.4.1393-1403.2001. PMID: 11158324.
27. *Inoue Y, Yu AM, Inoue J, Gonzalez FJ.* Hepatocyte nuclear factor 4 alpha is a central regulator of bile acid conjugation. *Journal of Biological Chemistry*. 2004; 279: 2480–2489. doi: 10.1074/jbc.M311015200. PMID: 14583614.
28. *Inoue Y, Peters LL, Yim SH, Inoue J, Gonzalez FJ.* Role of hepatocyte nuclear factor 4 alpha in control of blood coagulation factor gene expression. *Journal of Molecular Medicine – Jmm*. 2006; 84: 334–344. doi: 10.1007/s00109-005-0013-5. PMID: 16389552.
29. *Gordillo M, Evans T, Gouon-Evans V*. Orchestrating liver development. *Development*. 2015; 142: 2094–2108. doi: 10.1242/dev.114215. PMID: 26081571.
30. *Li K, Zhang H, Wang Y, Wang Y, Feng M.* Differential expression of HNF4alpha isoforms in liver stem cells and hepatocytes. *J Cell Biochem*. 2006; 99: 558–564. doi: 10.1002/jcb.20939. PMID: 16639723.
31. *Khamzina L, Borgeat P.* Correlation of alpha-fetoprotein expression in normal hepatocytes during development with tyrosine phosphorylation and insulin receptor expression. *Mol Biol Cell*. 1998; 9: 1093–1105. doi: 10.1091/mbc.9.5.1093. PMID: 9571242.
32. *Fausto N.* Liver regeneration and repair: hepatocytes, progenitor cells, and stem cells. *Hepatology*. 2004; 39: 1477–1487. doi: 10.1002/hep.20214. PMID: 15185286.
33. *Giri S, Acikgöz A, Bader A.* Isolation and Expansion of Hepatic Stem-like Cells from a Healthy Rat Liver and their Efficient Hepatic Differentiation of under Well-defined Vivo Hepatic like Microenvironment in a Multiwell Bioreactor. *J Clin Exp Hepatol*. 2015; 5: 107–122. doi: 10.1016/j.jceh.2015.03.003. PMID: 26155038.

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