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Induced pluripotent stem cell generation using mRNAs: the effect of valproic acid, 5-azacytidine and ascorbic acid

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ABSTRACT

In the bourgeoning fields of tissue engineering and regenerative medicine, induced pluripotent stem cells (iPSCs) technology with gene therapy are promising candidates for alternative stem cell source and cell transplantation. In this study, small molecules as anti-oxidant; ascorbic acid (ASA), histone deacetylase inhibitors (HDACi); Valproic acid (VPA), and DNA methyltransferase inhibitors (DNMTi); 5-Azacytidine (5-AzaC) were examined during the generation of murine iPSCs using mRNA of Yamanaka factors from mouse embryonic fibroblasts (MEFs). These modulators were selected based on their well-known effect on the epigenetic status and chromatin modification during early reprogramming. iPSC generation was performed by using synthesized mRNAs of Yamanaka factors Oct4, Sox2, c-Myc, and Klf4 (OSCK) as a standard reprogramming strategy. Both morphological changes and the expression level of the pluripotency markers were examined. 5-AzaC with 1 μM concentration has a slightly toxic effect on the cells, affecting its proliferation and growing efficiency. In contrast, the use of VPA or ASA led to a two-fold increase in the number of iPSC colonies. The iPSCs cultured with the addition of VPA or ASA showed a high expression of the tested pluripotency markers, with a significant increase, more than that of the cells cultured with the addition of 5-AzaC. These findings shed light on the role of ASA, VPA, and 5-AzaC during murine iPSCs generation using a mRNA reprogramming strategy.

Key words: iPSCs; 5-azacytidine; valproic acid; ascorbic acid; epigenetics

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Introduction

Stem cell therapy is recognized as a potential approach that paves new avenues for regenerative therapy in veterinary medicine at both experimental and clinical levels (FORTIER and TRAVIS, 2011).

Generation of induced pluripotent stem cells (iPSCs) from somatic cells is still inefficient and leads to long stepwise complex events. However, the remarkable work of Yamanaka's discovery of iPSC in stem cell therapy (TAKAHASHI and YAMANAKA, 2006), achieved through viral integration, may create alterations in chromosomes, which increase the risk of tumorigenesis, raising

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questions about iPSC safety for regenerative medicine (VOGA et al., 2020). Several safer strategies have been developed to overcome the potentially harmful effect of using viral integrating methods in reprogramming. These non-integrating safe strategies, such as episomal plasmid (OKITA et al., 2011), protein (ZHOU et al., 2009; KIM et al., 2009), and mRNA (WARREN et al., 2010; EL-SAYED et al., 2014) transfection, still have significant genetic instability. Besides, several reports have demonstrated that the reprogramming may be inadequate at both transcriptional and epigenetic levels (XU et al., 2012; LEE et al., 2012; JI et al., 2016). These findings shed light on reprogramming quality. Consequently, more research must be conducted to make cellular reprogramming more efficient.

Recently, treatments with small molecules were performed to enhance the reprogramming process (KOCHEGAROV, 2009; LI and DING, 2010). They are unique in driving the reprogramming process to be faster, more efficient, and directed through its ability to control protein function (SPRING, 2005). For these reasons, small molecules are used to replace other transcription factors, and they in turn improve the reprogramming process. They play a key role in the change of the DNA or histone modifications by influencing reprogramming through regulation of the epigenetic enzymes or mechanisms (MORGAN et al., 2005; ZHU et al., 2020). Many compounds are well- known to facilitate iPSC generation, and the compounds commonly used are histone deacetylase inhibitors (HDACi) and DNA methyltransferase inhibitors (DNMTi) (HUANGFU et al., 2008a), such as Valproic acid (VPA) and 5-Azacytidine (5-AzaC), respectively. These molecules alone or in combination with other key factors could improve reprogramming effectiveness by decreasing the methylation level of H3K9di-Me, H3K9mono-Me, or the agonist of the L-calcium channel (Bayk8644) (MIKKELSEN et al., 2008; HUANGFU et al., 2008a; JI et al., 2016).

VPA directly inhibits histone deacetylases and relieves HDAC-dependent transcriptional repression by inducing hyper-acetylated histones against class I and class IIb HDAC families (KAZANTSEV and THOMPSON, 2008). VPA treatment improved the expression of four reprogramming factors strategy by more than 100-fold (HUANGFU et al., 2008a). Furthermore, VPA successfully improved the generation of iPSCs with only recombinant proteins (ZHOU et al., 2009). This demonstrates that chromatin modification is a basic step during pluripotency induction in somatic cells. The distinguished role of VPA is thought to be from its other effects, alongside its HDACi activity (PANDA et al., 2020).

A nucleotide analogue, 5-AzaC, has promoted overall reprogramming efficiency through hindering the methylation of DNA nucleotides (HUANGFU et al., 2008a). DNA methylation has been shown to impede full reprogramming, and produced only partially reprogrammed cells (DE CARVALHO et al., 2010; GOMES et al., 2017). Moreover, its biological activity is associated with its incorporation into cellular DNA and/or RNA.

Vitamin-C-dependent H3K36 demethylases have been found to control and improve the reprogramming process (WANG et al., 2011; CIMMINO et al., 2018). Ascorbic acid (ASA) has several other actions, which could improve reprogramming efficiency, such as reducing the DNA damage through its suppressive effect on p53, which is a known factor in cell senescence induction (ESTEBAN et al., 2010). Moreover, ASA is a co-factor that implicates a variety of diverse enzymes, such as the epigenetic regulators of histone, collagen prolyl hydroxylases, and DNA methylation.

It is also interesting to mention that epigenetic modifiers (VPA, 5-AzaC and ASA) not only enhance reprogramming efficiency but also increase the reprogramming quality (POLO et al., 2010; D'ANIELLO et al., 2017). On the basis of previous research data, these three small molecules (VPA, 5-AzaC and ASA) were selected to study their reprogramming efficiency during the generation of iPSCs using mRNAs of Yamanaka factors (OSCK) as a safe reprogramming strategy. To our

knowledge, this is the first study that has tested the effect of these three molecules on the regeneration of iPSCs by mRNAs strategy.

Materials and methods

Reagents. All reagents were obtained from Gibco; Thermo Fisher Scientific, Inc., (Waltham, MA, USA) unless stated elsewhere in the text.

Isolation of MEF and cell culture condition. Animals were brought from the Laboratory Animal Centre, Jiangsu University (Zhenjiang, China). Animal care was provided according to the U.S. National Institute of Health guidelines (NIH Pub. No. 85-23, revised 1996). All the experiments including animal use were reviewed and then approved by the Institutional Animal Care and Use Committee, College of Animal Science and Technology, Yangzhou University. Briefly, embryos from pregnant (C57/BL) mice at 13 days post-coitum were harvested. After processing, they were incubated at 37°C in 0.25% Trypsin/ EDTA for 15 min. with gentle shaking. Afterward, trypsin action was neutralized by adding an equal amount of MEF culture medium, and cells were dissociated by pipetting several times. After filtration, the cell suspension was centrifuged at 1000 rpm for 7 min to collect the cell pellet. The cell pellet was suspended in the culture medium, and cells were seeded on gelatin-coated dishes at 37 °C with 5% CO_2. MEF cells at P3 were cultured in a medium composed of high glucose DMEM medium, 10% FBS (Hyclone, USA), 4 mM L-glutamine, and 1:100 penicillin-streptomycin. MEF cells after transfection were maintained on a feeder layer of MEF cells inactivated with mitomycin-C. After transfection, cells were cultured in an iPS culture medium containing DMEM, supplemented with 15% FBS, 1000 U/mL LIF (Sigma, USA), 2 mM L-Glutamine, 1×10^{-4} M 2-mercaptoethanol (Bio Basic Inc., USA), 1% penicillin-streptomycin, and 1×10^{-4} M non-essential amino acids (M7145, Sigma, USA).

Generation of murine iPSCs using mRNAs of Yamanaka Factors. The technique of murine iPSC generation using mRNAs of the four Yamanaka factors (OSCK) was performed according to our lab protocol (EL-SAYED et al., 2014). The four genes were ligated to the expression vector (pCDNA3) using (T4 DNA) ligase enzyme (Takara, China). To synthesize the mRNA of OKSM, the previously designed plasmids were linearized, followed by RNA transcription using AM1345 (Ambion®, USA). After the linearization step, the transcribed mRNA was purified using AM1908 (Ambion®, USA). Afterward, RNA transfection was performed by cationic lipid delivery vehicles using MIR2225 (Mirus Bio, USA). Briefly, about 0.25 µg of the mRNA of each gene was diluted in 100 µL of Opti-MEM, and then 2µL BOOST reagent, followed by the addition of 2 µL of TransIT®–mRNA. After gentle mixing, this complex was incubated for 5 min. at 25°C and then added to the culture medium. The RNA transfection step was performed in the normal culture medium. Later, the culture medium was changed after 12 hrs to a new one without the transfection factors.

Characterization of the generated iPSCs. Apoptosis and dead cell detection. An Annexin V–FITC Apoptosis Detection Kit (BD Biosciences, USA) was used for the detection of apoptotic cells, according to the manufacturer's instructions. The analysis was performed by flow cytometry within one hour, where the fluorescence was detected by a FACS Aria flow cytometer using the FACSDiva software (Becton-Dickinson Immunocytometry System, CA, USA). The controls were used to set up compensation and quadrants for flow cytometry: (1) Cells were unstained; (2) Stained with FITC Annexin V without PI; (3) Stained with PI and without FITC Annexin V); and (4) Stained with both FITC Annexin V and PI.

Quantitative Real-Time PCR (qRT-PCR). The extraction of total RNA from the cell samples was done using the TRIZOL® reagent (Invitrogen). One µg of RNA was reverse-transcribed into cDNA using Fast Quant RT Kits (Tiangen, China). The qRT-PCR analysis of the cDNA samples was carried out in a 7500 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) using Super Real Premix plus (FP205, Tiangen, China). Standard PCR conditions were: 95 °C for 15 min.; followed by 40 amplification cycles (95 °C for 10 sec.; 60 °C for 32 sec.). The primers were designed and manufactured in Takara, China. The sequences

of the qRT-PCR primers are listed in Table 1. Calculation of the relative quantification was done with $2^{-\Delta\Delta Ct}$ (LIVAK and SCHMITTGEN, 2001) and normalized to the internal control Gapdh mRNA level in each sample. Data are presented as levels relative to the expression level in the control of non-transfected MEF cells.

Alkaline phosphatase (ALP) staining. According to the manufacturer's protocol, iPSCs were stained using the ALP staining kit (SiDanSai, Beijing, China).

Bisulfite genomic sequencing. Treatment with bisulfite was performed using D5001 (ZYMO RESEARCH, USA). The PCR primers used for this reaction are listed in Table 2. Cloning of the amplified products was done into TOP10 (Vazyme Biotech, Nanjing, China). Ten random clones were chosen and picked up for the sequencing step with the M13 (F) and M13 (R) primers for each gene (Invitrogen Co., Shanghai, China).

*Treatment with small molecule*s. MEFs were prepared and transfected as described previously. Cells cultured in an iPS culture medium without the addition of small molecules were considered as the positive control group (OSCK). Otherwise, the selected small molecules were added to the iPS medium one day after the last transfection according to the specified groups under examination. In the Valproic acid group (OSCK + VPA), 100 µM of Valproic acid (Sigma, USA) was added to the iPS medium for 5 days consecutively (PARK et al., 2015). In the 5-Aza-cytidine group (OSCK + 5-AzaC), 1 µM of 5-AzaC (Sigma, USA) was added to the iPS medium for 5 days as modified according to Park et al., (PARK et al., 2015). In the Ascorbic acid group (OSCK+ ASA), 10 µg/mL of ASA (Sigma, USA) was added until the end of the experiment (ESTEBAN et al., 2010). The negative controls were non-transfected, non-treated MEF cells (negative control group), and non-transfected

Table 1. Primers used for quantitative real-time PCR (qRT-PCR)

Gene	Primer Sequence
Oct4	**F:** CAGACCACCATCTGTCGCTTC **R:** AGACTCCACCTCACACGGTTCTC
Sox2	**F:** GGTTACCTCTTCCTCCCACTCCAG **R:** TCACATGTGCGACAGGGGCAG
c-Myc	**F:** CCTAGTGCTGCATGAGGAGACAC **R:** TCCACAGACACCACATCAATTTCTT
Klf4	**F:** ACAGCCACCCACACTTGTGACTA **R:** GGCGAATTTCCACCCACAG
Nanog	**F:** GCAAGCGGTGGCAGAAA **R:** TCCAGATGCGTTCACCAGATAG
Rex1	**F:** GGGCGAGCTCATTACTTGCAG **R:** AGGACACTCCAGCATCGATAAGAC
Gapdh	**F:** TGTGTCCGTCGTGGATCTGA **R:** TTGCTGTTGAAGTCGCAGGAG

Table 2. DNA methylation primers used for (bisulfite sequencing)

Gene	Forward Primer	Reverse Primer
Rex-1	5'TATATTAATGTTGGAAAAAGTTTAGGTAAT3'	5'AACTCCTTAAACCCCTCCCTTTTTAAATAA3'
N-Myc	5'TTAGAGAGTTAGTATTTTGAGGGAGAATTT3'	5'AAACCCTTTTTCAACACACATTCTCAATAA3'

MEFs that were treated with small molecules (MEFs + VPA; MEFs+ 5-AzaC; and MEFs+ASA, respectively).

Statistical analysis. Statistical analyses between groups were performed using SPSS® software V.21 (IBM Corp., Armonk, NY, USA). All quantitative data were expressed as the means ± standard deviation (n=3). Statistical significance was determined using one-way analysis of variance followed by Tukey's post hoc test. P-value was calculated at three levels ($P < 0.05$), ($P < 0.01$) and ($P < 0.001$). Statistical illustrations were performed using GraphPad Prism software V.6 (GraphPad Software, La Jolla, CA, USA).

Results

Generation and characterization of iPSC colonies. According to our lab protocol (EL-SAYED et al. 2014), five consecutive transfections were done to obtain pluripotent clones. The morphological appearance of the MEF cells showed remarkable changes from spindle shape to compact round epithelial shape, which increased by time. On the 8th day post-transfection, small colony-like structures began to appear. By the 15th day, these colonies became larger with clearly defined borders. The nuclear/cytoplasm ratio also increased over time. Approximately 100 to 130 colonies per reprogramming line were obtained. These colonies

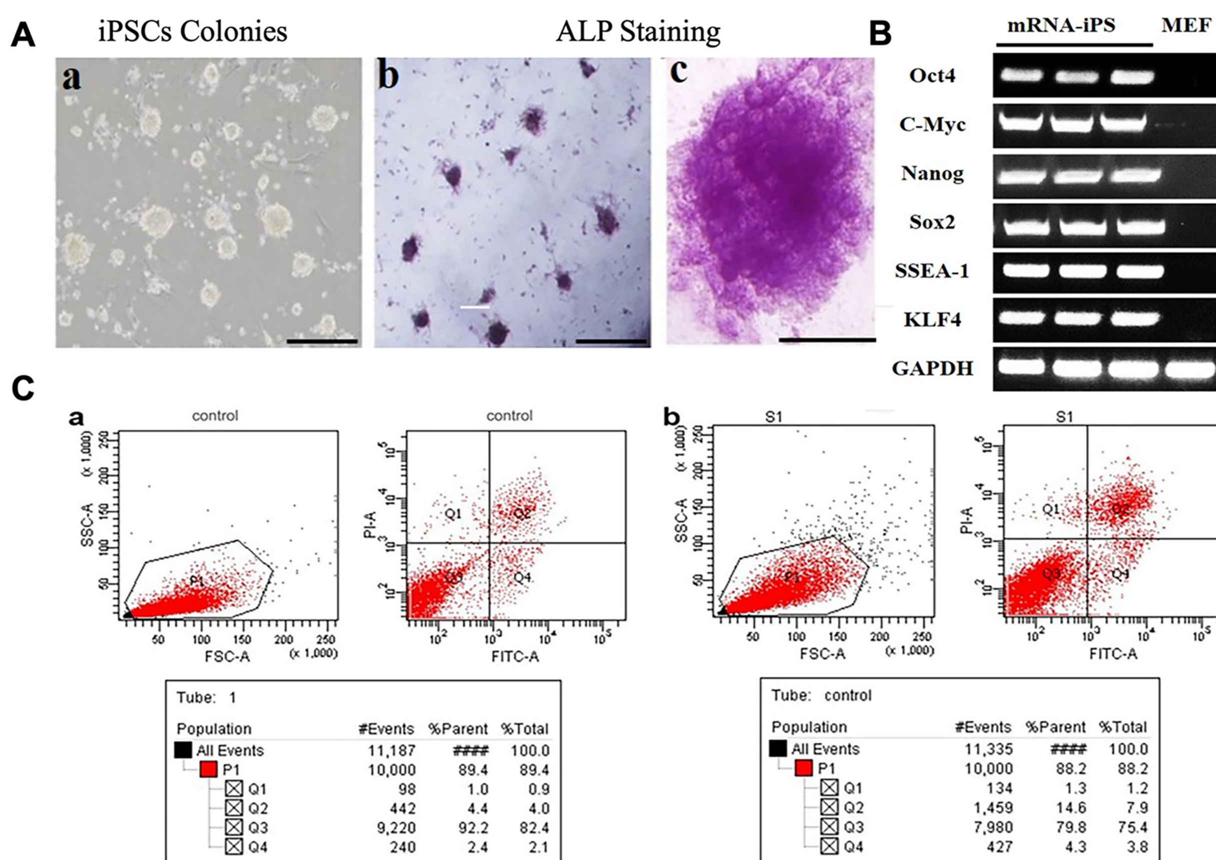

Fig. 1. Characterization of the mRNA iPSCs generated. (A) Cellular morphological changes from the characteristic spindle shape of MEF cells to ES-like colonies (a), which were positively stained with ALP staining (b) magnification (200X) (c) (Scale bar: 100 μm). (B) RT-PCR analysis of pluripotency marker gene expression (Oct4, Nanog, Sox2, Klf4, Rex1, and cMyc) in the iPSCs generated from 3 mRNA iPS clones and control non-transfected MEF cells. The results showed that iPS colonies expressed all the pluripotency markers, in contrast to MEF cells. (C) Annexin V-FITC analysis of the MEF cells after the 5th transfection showed the Annexin V-FITC analysis of the control (a) and transfected cells (b). The results revealed that the percentage of live cells in the transfected cells was similar to that of the control cells, and there was a non-significant increase in the number of dead cells.

were positive for ALP staining (Fig. 1A). The iPS colonies were immune stained for pluripotency markers (Sox2, Nanog, Oct4, Klf4, SSEA-1, and c-Myc), and the obtained colonies were positive for all of them, while the control cells were negatively stained for these markers (EL-SAYED et al., 2014). For further confirmation, the expressions of the pluripotency markers in the newly generated cells were tested using the reverse transcription PCR (RT-PCR) reaction. The RT-PCR results confirmed the previous immune staining results with positive expression of the pluripotency markers (Oct4, Rex-1, Klf4, c-Myc, and Nanog) in comparison with the negative expression of these markers in the started MEF as illustrated in Fig. 1B.

For examination of the health of the transfected cells in response to the transfection, the cells were collected after the 5th transfection and the percentages of live healthy cells and of apoptosis were detected using Annexin V-FITC using the FACS technique (Fig. 1C). The percentage of live cells in the transfected cells was similar to that of the control cells without a marked increase in dead cells.

Epigenetic status changes of MEF cells toward iPSC generation. Previously epigenetic status changes in the transfected cells using the mRNA of Yamanaka factors was tested by examining the methylation patterns of the promoter regions of both Oct4 and Nanog (EL-SAYED et al. 2014). Here, an additional two different pluripotency markers (Rex1 and N-Myc) were examined for the changes in their methylation patterns in the promoter regions during reprogramming. The promoter regions were analyzed at different time points following transfection (on the 1st, 6th, 9th, 12th and 15th days). Rex1 and N-Myc loci showed a gradual increase in the percentage of de-methylation from the first day until the maturation of the iPSC colonies on the 15th day. The percentage of un-methylation at CpG dinucleotides in both the Rex1 and N-Myc gene upstream regions increased respectively, indicating a gradual decrease in the percentage of methylation

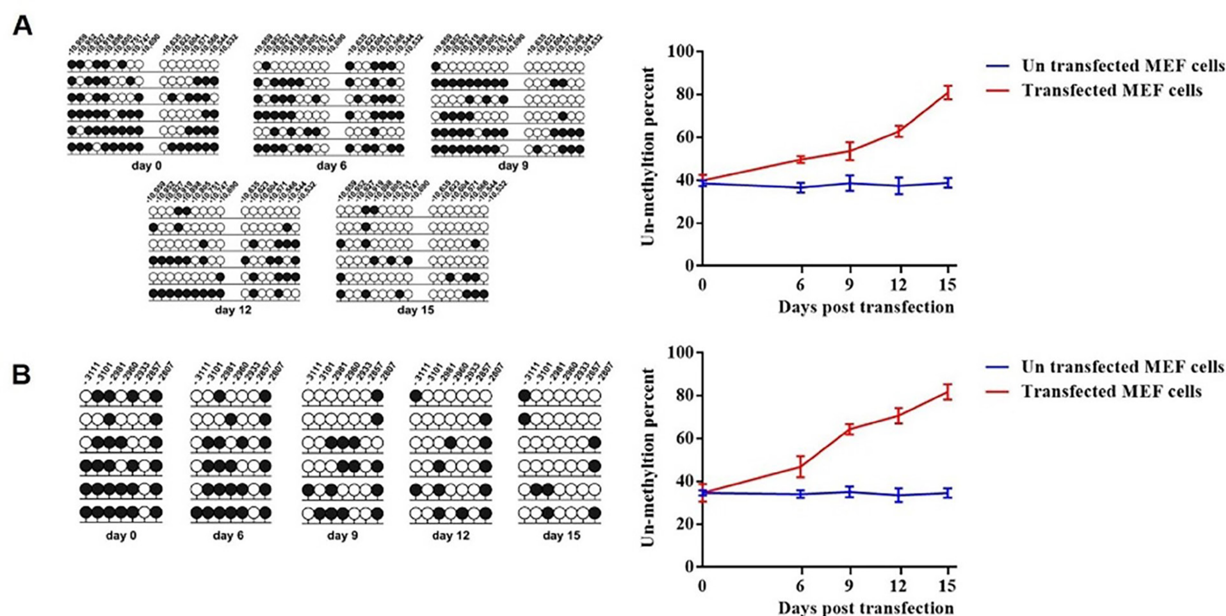

Fig. 2. Global analysis and the overall pattern of the tested loci of Rex1 and N-Myc promoters. (A) The CpG methylation of Rex1 and a diagram showing the demethylation percentage at different time points during reprogramming. (B) The CpG methylation of N-Myc and a diagram showing the demethylation percentage at different time points during reprogramming. The percentage of un-methylation in both genes' upstream region increased, indicating the decrease in the percentage of methylation from the transfection day until day 15.

from the beginning of transfection until the 15th day (Fig. 2).

Effect of small molecules on the reprogramming efficiency. The cellular morphological changes were recorded in all groups; where the cell morphology of MEF (the mesenchymal spindle-shaped appearance) changed to a compact round epithelial cell morphology. It was clearly shown that the transfected cells treated with ASA showed better growing and proliferation. In addition, it was observed that the 5-AzaC had a slightly toxic effect on the cells, affecting its proliferation and growing efficiency during the reprogramming period (Fig. 3). Both VPA and ASA led to the earlier appearance of the iPSC colonies with more efficiency than in the control group. Their use led to a two-fold increase in the number of colonies, that reached 200 to 230 colonies per 10^5 primary transfected cells.

The effect of each treatment on the expression level of the pluripotency genes Oct4, Sox2, Nanog and Rex-1 was evaluated through qRT-PCR. The expression level of each transcription factor was detected on days 8, 12, 15, and 18 respectively from the last transfection (Fig. 4). In comparison with the non-transfected, non-treated MEF or the non-transfected treated MEF cells, it was clear that there was high upregulation of the expression level of all pluripotency markers in the transfected cells, whether treated or non-treated by the small molecules at all the examined times. The transfected cells cultured with the addition of VPA or ASA showed a high expression of the pluripotency factors tested, with a significant increase level, more than that of the cultured transfected cells in the condition of 5-AzaC. The 5-AzaC group also showed a higher level than the transfected cells without any treatment, but the

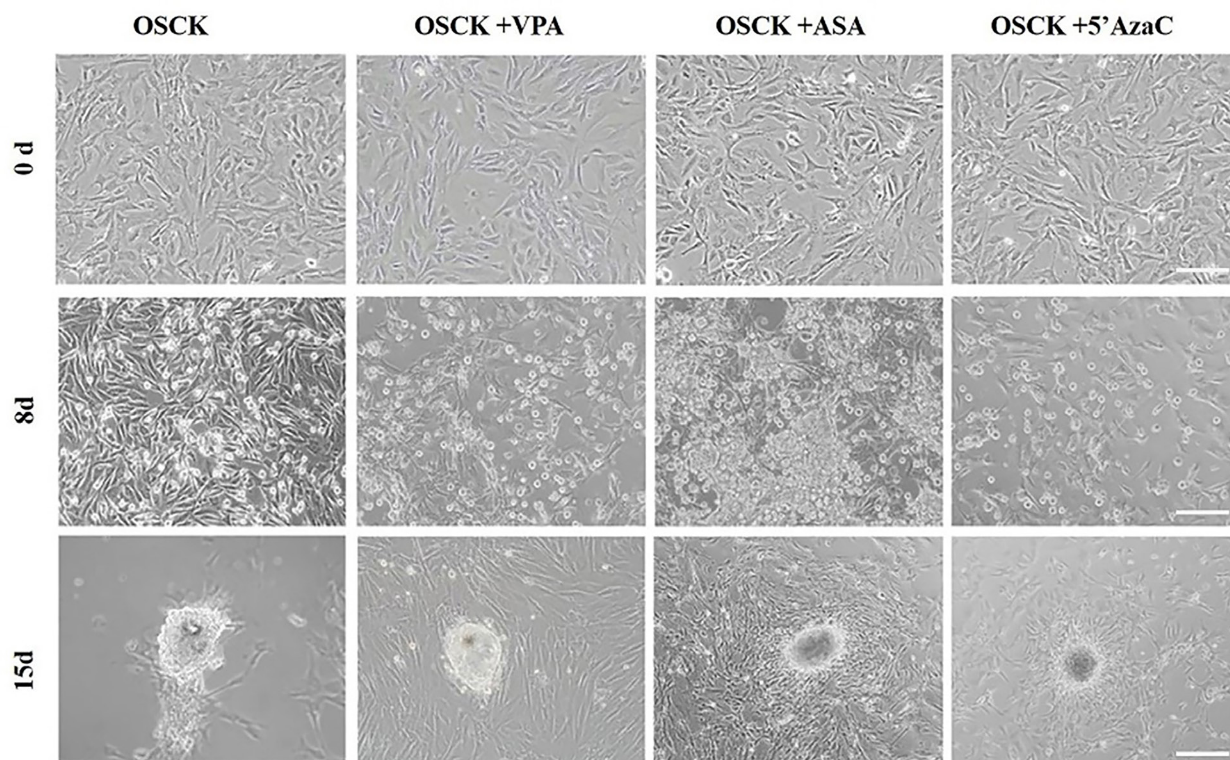

Fig. 3. Cellular morphological changes during reprogramming under the effect of the small molecules. The effect of the addition of different small molecules, Valproic acid (VPA), Ascorbic acid (ASA), and 5-Azacytidine (5'AzaC), on the cellular morphology, showed in the different groups at different time points from the transfection day (0 d), during reprogramming (day 8), and after the formation of iPSC colonies (day 15). (Scale bar: 100 μm).

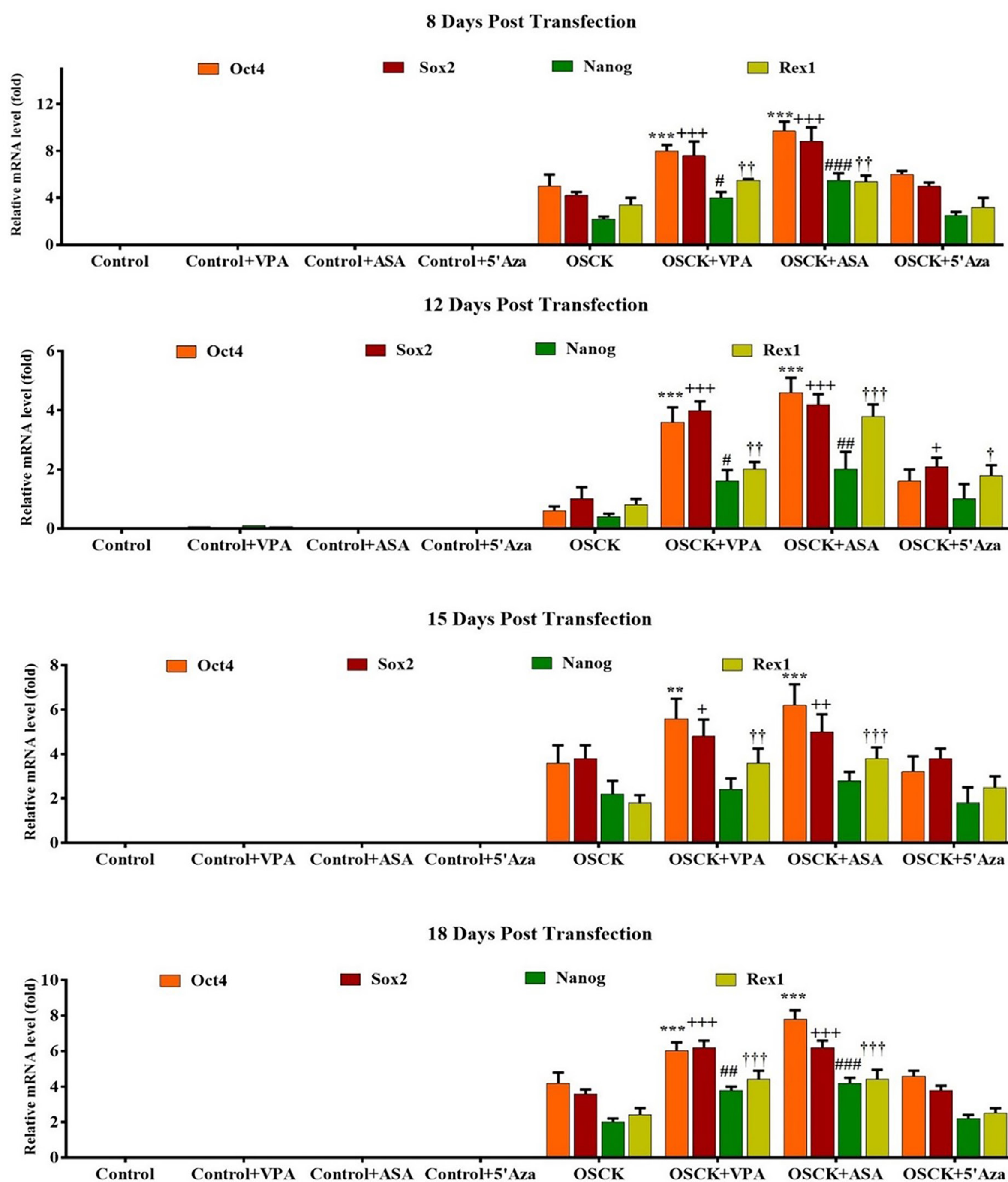

Fig. 4. The effect of the small molecules on the expression level of the pluripotency markers (Oct4, Sox2, Nanog, and Rex1) in the transfected cells with mRNA of Yamanaka factors at different intervals, under the effect of Valproic acid (VPA), Ascorbic acid (ASA), and 5-Azacytidine (5' AzaC). Oct4 in different groups vs. Oct4 in the control group: $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$); Sox2 in different groups vs. Sox2 in the control group: $^{+}P < 0.05$, $^{++}P < 0.01$ and $^{+++}P < 0.001$); Nanog in different groups vs. Nanog in the control group: $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$); Rex1 in different groups vs. Rex1 in the control group: $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ and $^{\dagger\dagger\dagger}P < 0.001$).

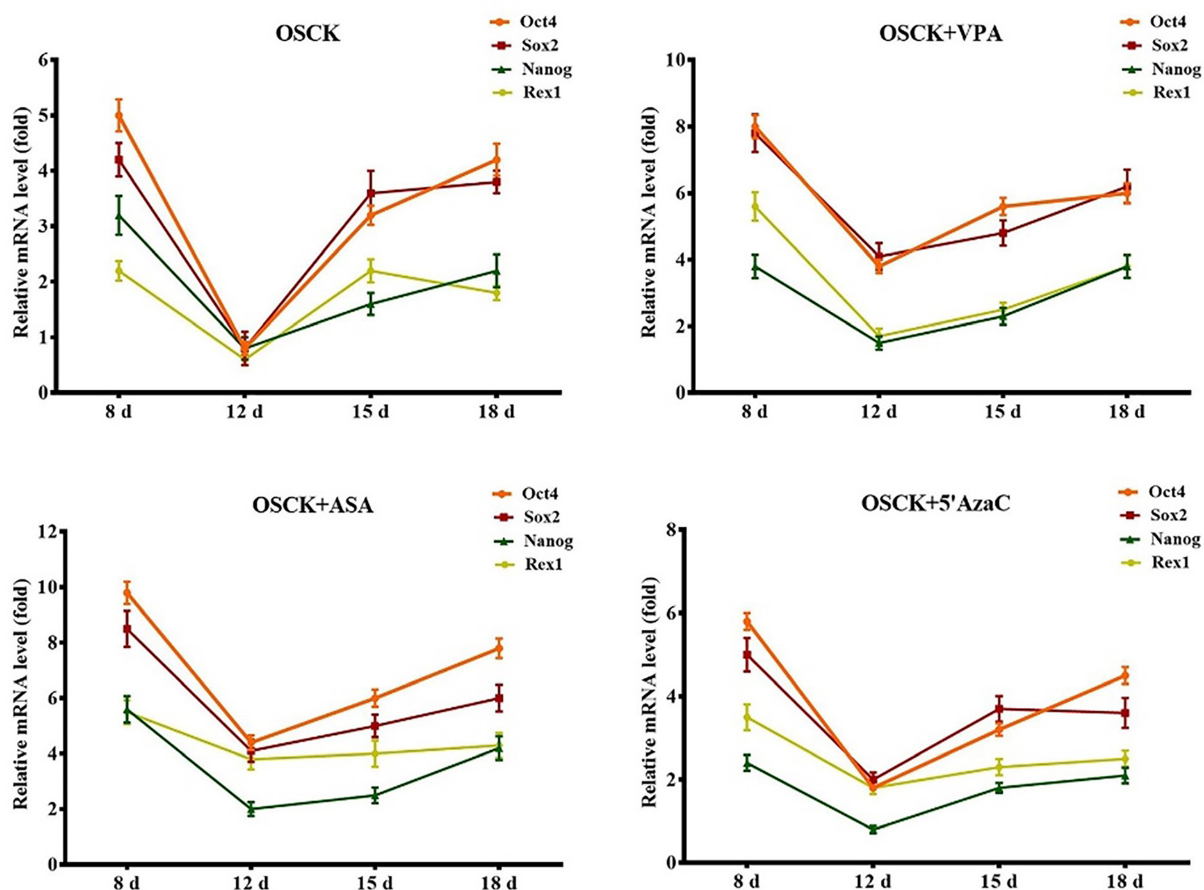

Fig. 5. The expression level curve of the pluripotency factors (Oct4, Sox2, Nanog, and Rex1) throughout the examined timeline, either with or without the effect of the small molecules Valproic acid (VPA), Ascorbic acid (ASA), and 5-Azacytidine (5'AzaC).

difference was without significance (Fig. 4). These results revealed that all treated groups have the same expression level curve for each transcription factor throughout the timeline examined, which began with an increased level on day 8 which decreased on day 12, and this was followed by a second wave of increase on day 15 due to intrinsic cell expression (Fig. 5).

Discussion

In this study, small molecules (VPA, ASA and 5-AzaC) promoted iPSC generation through the mRNA approach. These findings could solve the inefficiency problem related to non-viral reprogramming strategies. The results were in line with previous work, which showed that small molecules could improve the proliferation, pluripotency, and differentiation ability of iPSCs and made them resemble ESCs. Moreover, they play an essential role during reprogramming and long-term culture by inhibiting the genomic instability (GORE et al., 2011; HUSSEIN et al., 2011; PETERSON and LORING, 2014).

Many inhibitors of various cell signaling pathways and modulators of the epigenomic state have been used to improve the reprogramming effectiveness and the dynamics of pluripotency initiation (HUANGFU et al., 2008a; AASEN et al., 2008; TROKOVIC et al., 2013; CHEN, 2020). For this reason, in this study some small molecules

were examined that are well known for their action on the epigenomic landscape, either on histone modification or on DNA methylation. The effect of these compounds on the genetic expression of the iPSCs markers during the reprogramming process was compared to non-transfected MEF cells (either treated or non-treated by these small molecules). All the transfected cells showed a high expression level of the pluripotency factors examined. This increase in the expression resulted from both the effects of the externally transfected factors and of the endogenously induced factors. This increase was followed by a decrease after a certain period due to the removal of the residual effect of the externally introduced factors. After that, the endogenously produced factors increased, which resulted from the reprogrammed pluripotent cells. Concerning the small molecules' effectiveness, the finding of each group was compared with the group which contained only transfected cells without any treatment. Throughout the specified times tested, it was obvious that both the VPA group and the ASA group had improved MEF reprogramming to iPSCs. This was manifested by a significant increase in the expression levels of the markers used, which was even observed during the decreasing periods. These molecules kept the expression of these factors at a higher level than in the non-treated group, in contrast to 5-AzaC, which led to a non-significant increase. In the case of 5-AzaC, it had some toxic effect on the cells in accordance with the findings of PARK et al. (2015), so it was used in this experiment at a low concentration, which emphasized the non-significant increase in the pluripotency factors' expression in the 5-Aza-C group.

HDACi compounds (VPA, Suberoylanilide hydroxamic acid, and Trichostatin A) were found to increase the induction of mouse and human iPSCs even with decreased reprogramming factors (Oct4 and Sox2) (HUANGFU et al., 2008a; HUANGFU et al., 2008b). The VPA treated group showed an approximately more than two-fold increase in the expression of pluripotency factors in comparison with the control group. However, the VPA findings were not as high as in a previous study that showed a 200-fold increase (LIN et al., 2009; HUANGFU et al., 2008a). This could be attributed to species differences, cell age, and different reprogramming strategies.

ASA caused a robust increase in the expression level, more than VPA. This was in agreement with the finding of ESTEBAN et al. (2010), who found that ASA had a more potent effect than VPA in reprogramming enhancement. In this experiment, ASA was added throughout the experiment time to achieve its full action and potential, as was found before (ESTEBAN et al., 2010). It has been shown that ASA is a co-factor in reactions controlled by dioxygenases, including histone demethylases (SHI, 2007). It was also considered that ASA might influence the reprogramming process by increasing those enzymes' activities. This process is essential for the development and regulation of the embryonic cell's master gene expression (CLOOS et al., 2008). Surprisingly, ASA also has an anti-aging effect, which promotes the reprogramming process (MASSIP et al., 2010). ASA, which is known for its antioxidant function, is one content of Knockout serum replacement (KSR) and is considered the key player in its effect during the reprogramming period. Although the ASA effect is mainly independent of reactive oxygen species modulation, it was also expected to act through decreasing p53 levels (ESTEBAN et al., 2010; CHEN et al., 2020), which is not desirable for reprogramming, especially for clinical use (MARIÓN et al., 2009). From all the above-mentioned data, we can conclude that using ASA during reprogramming is safe and could improve reprogramming efficiency, particularly during a cell-therapy strategy.

Recently it was reported that mRNA transfection in combination with 5-aza-29-deoxycytidine, BIX-01294, and VPA, could improve the pluripotency factors' activation (PLEWS et al., 2010), which is in agreement with the results of this study. They found that the previous combination induced a certain degree of reprogramming, releasing both DNA damage response and cell cycle arrest. These results may explain the non-extreme elevation in reprogramming induction. In our current study, we were aiming to check the individual effect of each molecule on reprogramming efficiency to avoid the toxic effect of several factors at the same time. But this point will be under further investigation, to

check the effect of combination of these molecules together, especially since they have different mechanisms of efficiency enhancement.

Conclusion

On the basis of these results, for the first time iPS cells generated by mRNAs of Yamanaka factors were promoted using small molecules (VPA, ASA and 5-AzaC). These molecules could promote the efficient expression of the pluripotency-associated genes during the reprogramming of MEFs into iPSCs using the mRNAs of Yamanaka factors, to overcome the decreased reprogramming efficiency. However, caution should be taken while using these molecules due to the toxic effect of some of them.

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SAŽETAK

Ubrzani razvoj u područjima tkivnog inženjerstva i regenerativne medicine, potaknuo je tehnologiju pluripotentnih matičnih stanica (iPSCs) koja zajedno s genskom terapijom predstavlja obećavajući izvor matičnih odnosno transplantacijskih stanica. U ovom su radu, za vrijeme stvaranja mišjih iPSC-a upotrebom mRNA Yamanaka faktora od mišjih embrionalnih fibroblasta (MEF), istraženi učinci različitih modulatora: malih molekula kao antioksidansa, askorbinske kiseline (ASA), inhibitora histonske deacetilaze (HDACi), valproične kiseline (VPA), inhibitora DNA metiltransferaze (DNMTi) i 5-azacitidina (5-AzaC). Ovi su modulatori odabrani zbog njihova dobro poznatog učinka na epigenetski status i modifikaciju kromatina za vrijeme ranog reprogramiranja. Stvaranje iPSC-a postignuto je upotrebom sintetiziranih mRNA Yamanaka faktora Oct4, Sox2, c-Myc i Klf4 (OSCK). Istražene su i morfološke promjene i razina ekspresije markera pluripotencije. 5-AzaC s koncentracijom od 1 μM imao je mali toksičan učinak na stanice, utječući na proliferaciju i njihov rast. Nasuprot tome, upotreba VPA-a ili ASA-e dovela je do dvostrukog povećanja broja iPSC kolonija. iPSC kultura s dodatkom VPA-a ili ASA-e pokazala je visoku ekspresiju testiranih markera pluripotencije, sa znakovitim višom razinom u odnosu na stanice kojima je dodan 5-AzaC. Ovi rezultati rasvjetljuju ulogu ASA-e, VPA-a i 5-AzaC-a za vrijeme stvaranja mišjih iPSC-a primjenom strategije reprogramiranja mRNA.

Ključne riječi: iPSCs; 5-azacitidin; valproična kiselina; askorbinska kiselina; epigenetika