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Genetic Cell Reprogramming: A New Technology for Basic Research and Applied Usage[1]

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Abstract—Gene function disclosure and the development of modern technologies of genetic manipulations offered the possibility of genetic reprogramming application to alter cell specialization. With the involvement of a gene set that encodes the transcription factors responsible for the pluripotent state, any cell of an adult body could be reprogrammed into the embryonal state and pluripotency could be induced in this cell. Such reprogrammed cells were called induced pluripotent stem cells (iPSCs), and they are capable of again passing through all developmental stages. This provides new possibilities for studies of the basic mechanisms of developmental biology, the formation of specific cell types, and the whole body. In culture, iPSCs could be maintained permanently in a nontransformed state and permit genetic manipulations while maintaining their pluripotent properties. Such a unique combination of their properties makes them an attractive tool for studies of various pathologies and for the delineation of treatment approaches. This review discusses the basic and applied aspects of iPSCs biology.

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GENETIC REPROGRAMMING

Cells comprising various tissues of multicellular body undergo numerous divisions, starting from the moment of their conception (ovarian cell fertilization) until their biologic death. In the body cells gradually acquire more traits of terminal differentiation and, starting from the very early developmental stages, gradually lose the ability to transform into various specialized cell types. Generally, this is a unidirectional process: any differentiated cell does not go back and does not become a progenitor or a stem cell. Unidirectional movement is encoded by genetic programming, which is accomplished in natural conditions. Conrad Waddington, who was the first to offer the term "epigenetics" [1], suggested the idea of epigenetic regulation of a unidirectional developmental process in the 1950s. Recent advances in biology suggest that we can efficiently manage the cell status, altering its function and specialization.

In 2006, Japanese researchers reported on the production of pluripotent cell lines from terminally differentiated cells (murine fibroblasts) [2]. This became one of the most significant accomplishments in developmental biology in recent decades and represented the onset of a new era in research, as this technology made it possible to redirect the developmental program in an opposite way. A prerequisite for this discovery was the development of technologies of pluripotent stem cells' (PSCs) (murine and human embryonal stem cells (ESCs) [3, 4]) production and culture, reprogramming of the somatic cell nucleus by somatic cell nuclear transfer (SCNT), and cell merging, as well as the unraveling of molecular mechanisms of pluripotency.

Takahashi and Yamanaka examined various combinations of 24 transcriptional factors involved in the acquisition and maintenance of pluripotent state and determined a combination of factors Oct4, Sox2, Klf4, and c-Myc (now known as the "Yamanaka cocktail"), the expression of which in a somatic cell resulted in their transition to the pluripotent state. This process was called direct genetic reprogramming, i.e. reprogramming by a direct influence on the epigenetic state of an adult cell (in contrast to SCNT and cell fusion); cells obtained through such process were called "induced pluripotent stem cells" (iPSCs).

Reprogramming technology appeared to be universal, as it is possilbe to obtain pluripotent cells from different cell types, including skin fibroblasts and blood cells [5], nerve cells [6], and endothelial cells [7], as well as from the cells of different animals: rats [8], pigs [9], etc. In 2012, J. Gurdon (SCNT method) and Sh. Yamanaka (direct genetic reprogramming)

[1] All of the authors equally contributed to the writing of this paper.

received a Nobel Prize in Physiology and Medicine for the development of somatic cell reprogramming technology.

Unique components of the "Yamanaka cocktail" are involved in the maintenance of pluripotent state, and they play a significant role in regulation of the cell cycle, proliferation, and epigenetic traits of these cells.

Oct4 (Oct3, OTF3/4 or POU5F1) is a key factor of pluripotency. During the mammalian development, Oct4 is expressed in cells of the internal cellular mass of a blastocyst, while its expression is abolished in differentiated somatic cells. Oct4 is involved in normal differentiation of mammals: mouse embryos knocked-out at the Oct4 gene failed to form pluripotent cells of the internal cellular mass, resulting in embryos death after the blastocyst stage. Pluripotent stem cells are required to a maintain Oct4 protein concentration at a strictly specified level, since a decrease in this gene expression results in spontaneous differentiation into trophoblast cells and an increase leads to differentiation into a primitive ectoderm.

Sox2 is a transcriptional factor containing HMG-box. The level of its expression is extremely high in the embryonal pluripotent cell lines at the early stages of development; it is expressed in the embryonic and postembryonic tissues of the embryo and neural cell precursors. Sox2 regulates the expression of Oct4, which points to the important role of Sox2 in pluripotency maintenance. It forms heterodimers with Oct4, which controls the gene expression specific to ESC, such as *UTF1*, *Fgf4*, and *Fbx15*. Downregulation of Sox2 gene expression in ESC results in the loss of pluripotency and cell differentiation.

Klf4 is involved in many cellular processes, including development, proliferation, differentiation, and apoptosis. In developing murine embryos, Klf4 is expressed in the extraembryonic tissues and, later, in the intestinal tissues and skin cells. In the tissues of an adult body, Klf4 is expressed primarily in terminally differentiated cells of the intestinal epithelium and skin. Interestingly, Klf4 expression in nondividing cells is relatively high, while almost no expression of Klf4 was observed in actively proliferating cells. This transcription factor is important in the regulation of proliferation: activation of Klf4 expression in cultured cells results in the inhibition of DNA synthesis and cessation of the cell cycle.

C-Myc is a transcription factor that is important for the regulation of cellular processes such as proliferation, differentiation, and cell growth. It is also one of the most common activating proto-oncogens. It has been shown that *c-Myc* regulates the transcription of many genes using several mechanisms, including those that involve histone acetylases, DNA-methyltransferases, and chromatin remodeling enzymes.

Since its first application, reprogramming technology has been subjected to numerous alterations and additions, which were mainly related to the low efficacy of processes and protocol improvement for the potential clinical application of iPSCs. The first modifications were related to the presence of *c-Myc* oncogene in the Yamanaka cocktail; however, the reprogramming efficacy significantly decreased when only Oct4, Sox2, and Klf4 transcription factors were used [10]. The initial studies on reprogramming technologies have already shown a negative effect of aging on the efficacy of the pluripotent state induction [11]. Thus, an important step is the choice of cells for reprogramming, which should be readily available and free of accumulated DNA disturbances, such as damage by UV or other environmental factors [12].

The delivery of genetic factors via virus vectors, which are incorporated into the host genome, is the most efficient, fastest, and cheapest reprogramming method perfected in labs [13].

However, problems of genomic integration had to be overcome in order to establish the safety of these cells for application in medical practice. Over the last several years, numerous methods were developed to decrease the chance of transgenic effects on the host genome. They were based on nonintegration with the genome adenoviruses, transitory transfection by plasmids, a piggyback transposon system, and others. However, no methods exclude the risk of genome modification. At the same time, studies aimed at the formation of cells applicable for clinical studies and therapy required a method with a null chance of genomic integration. One possible way of excluding introduction of genetic modifications into the cell was the direct delivery of reprogramming proteins into the cell. This method was based on the technology of penetrating peptides, which were linked with proteins of the "Yamanaka cocktail" [14, 15]. The major limitations of this method were the very slow speed of iPSC formation and its extreme inefficacy, as well as the requirement of big quantities of highly purified recombinant proteins in repeated use.

Another method that excluded unexpected modifications of the genome was based on the use of a vector isolated from RNA-carrying Sendai virus, which is active solely in the cytoplasm, does not have a DNA stage, and cannot alter host chromosomes [16]. In addition, Sendai virus–based vectors were already used as a safe method in gene therapy in cystic fibrosis and for the delivery of vaccines [17, 18]. Even hematopoietic cells, which are stable to other nontransgenic methods of reprogramming and which represent an accessible source of patient cells, were sensitive to reprogramming by Sendai virus.

An alternative nonviral source of reprogramming factor delivery into the cell is a transfection of cells by the mRNA of transcription factor genes. mRNA is perfectly suitable for iPSC generation, as it is completely devoid of virus programs and genomic integration, and it is thus suitable for further application of these cells in personalized medicine. The initial

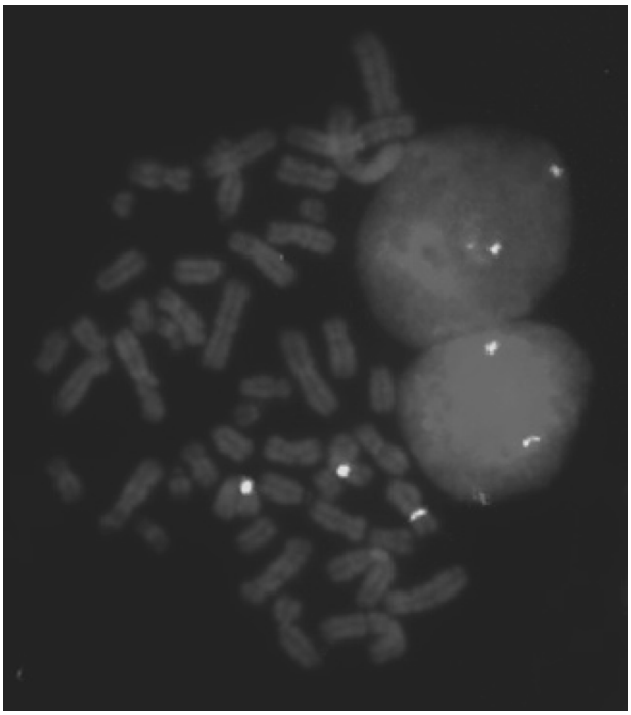

Fig. 1. Trisomy on chromosome X in the iPSC line CHE-fibro-iPS51 (passage 6) revealed by hybridization in situ with a DNA probe to the centromere region of chromosome X. Original fibroblasts had normal karyotype 46,XX.

attempts of pluripotent state induction by mRNA, including those at our lab, faced a problem of cellular immune response to exogenous RNA [19]. Synthetic mRNA was recognized by the cell as a viral genetic material, which finally resulted in interferon secretion, cell cycle arrest, and cell death. Despite this, a protocol for the successful generation of iPSCs using a modified mRNA was published in 2010 [20]. Researchers used two strategies to combat interferon response: introduction into the molecule of synthesized RNA of modified nucleotides, which mimic natural modifications of eukaryotic RNA, and inhibition of residual immune response by interferon-binding protein B18R. This protocol provided high efficacy of iPSC generation but was complicated technically, which limits its wide usage [21]. Independently of this protocol, highly efficient vectors for cell transfection by mRNA were produced in our lab, and the studies are presently being conducted for their application in the generation of nonintegrational iPSCs (Patent RU2399667). The major problem of the mRNA method application is the very short time of pluripotent factor induction, which is insufficient for reprogramming. This problem could be overcome by an increase in the expression level of synthesized mRNA and by repeated transfections. In 2013, Dowdy et al. suggested another way to solve this problem: reprogramming factors were introduced into the cell in the form of self-replicating mRNA, which was based on mRNA-containing virus of Venezuelan horse encephalitis [22]. Efficient expression and stability of mRNA in this case also depends on the presence of B18R protein, removal of which from the medium serves as a "natural" mechanism for complete elimination of the virus from the obtained iPSCs. The application of this method in other labs thus far was not documented but potentially could appear as the most simple and efficient technique of nontransgenic iPSC generation.

At present, the technology of genetic reprogramming by integrational or nonintegrational methods is available in many labs. Moreover, recent studies show that many cells inside or outside the body could be reprogrammed into other functional calls via genetic reprogramming technology [23, 24]. Thus, such definitions as "terminally differentiated cells" or "cell type" gradually lose their meaning and could be in the future be substituted for the names reflecting transit state of the cell inside or outside the body.

GENETIC STABILITY OF HUMAN PLURIOTENT STEM CELLS IN CULTURE

ESCs and iPSCs generally are characterized by rather stable karyotypes upon prolonged culturing, and a common cytogenetic analysis using GTG-bending does not reveal any changes in the majority of human PSC cell lines over a long time. The most commonly observed changes of a karyotype, which nevertheless are detected in human PSCs by methods of classic cytogenetics, are trisomy of chromosomes 12 and X, as well as trisomy of chromosome 17, whih are more specific to ESCs. These changes are related to the adaptive mechanism of human PSCs for culture conditions in vitro [25]. In monitoring the human iPSC line state cultured in our lab, we sometimes observed trisomy of chromosome 12 and X (Fig. 1), while in some cases several aneuploidy cells were present among cells with normal karyotype already at early passages after reprogramming (before passage 10).

In addition to trisomy, we sometimes observed chromosomal rearrangements, which were obtained in the course of culturing of PSCs. These rearrangements might give a selective advantage for carrying cells, as a complete displacement of cells with normal karyotype occurred after several passages. An example of such a rearrangement in ESCs could serve a complex rearrangement involving chromosome 4 and 9: 46,XX,del(4)(q25q31.1),dup(9)(q12q33), which was described by us [26]. An interesting example from the same paper is rearrangement with the formation of a circle chromosome: 46,XX,r(18)(::p11.31→q21.2::q21.2→p11.31::). Because of the instability inherent to chromosomal circles in mitoses, a circle r(18) in some cells can be lost, fragmented, amplified, or subjected to further rearrangements, and therefore up to 15% cells of a sub-line hESM01r18 in each passage had a karyotype different from the modal. Nevertheless, cells with karyotype 46,XX,r(18) are maintained

in the subline hESM01r18 as a modal class during many passages, which indicates that such a rearrangement provides a high selective advantage.

Studies on large numbers of ESCs by an international consortium [27] using more sophisticated methods of genetic analysis, namely genome wide SNP genotyping, has shown that during culture in vitro, PSCs obtain duplication of small region of chromosome 20 in the q11.21 bend. The length of the duplicated region in various PSC lines somewhat differs; however, it obligatorily includes the region where the *BCL2L1* gene is located. This gene is expressed in PSCs; the product of this gene is involved in apoptosis inhibition, and therefore amplification of this gene theoretically could provide a clonal advantage. During genome wide SNP genotyping of three lines of PSCs cultured in our lab, we also observed duplication of chromosome 20 in the q11.21 bend in one PSC line and in one iPSC line.

In addition to karyotype changes, which could be acquired during culture, iPSCs carry mutations that were present in the original cells. In addition, the process of cell reprogramming could result in DNA damage. It is known that increased expression of the reprogramming transgenes *MYC* and *KLF4*, which represent proto-oncogenes, could induce oxidative stress. Reprogramming also induces replicative stress, rearrangements of cellular response on DNA disturbances, and an extensive exchange in the transcriptional pattern of the cell. All this in total facilitates the induction of chromosomal rearrangements in the reprogrammed cells [28]. Interestingly, in immature iPSC clones, which did not develop complete silencing of reprogramming transgenes, we observed an increased frequency of chromatid type aberrations. Similar results were obtained in the study of Ramos-Mejia et al. [29]. This supports the notion that the introduction of reprogramming transgenes into somatic cells could result in significant genetic destabilization. In the study by Hussein et al. [30], the authors observed a higher frequency of small deletions and duplications in iPSCs at early passages as compared with the original fibroblasts and iPSCs at later passages. This also indicates genetic instability, to which cells are subjected at early stages of reprogramming. This effect decreases to a significant extent because of the negative selection of aberrant cells during culturing.

Genetic instability at early stages of reprogramming, the clonal nature of iPSCs, and their ability for fast iPSCs expansion with growth advantage in culture—all of these factors could facilitate a correction of an aberrant karyotype at the cellular level by reprogramming in the case above. Bershteyn et al. [31] have shown that the majority of obtained clones of iPSCs lost circular chromosome r(17) and obtained a second chromosome 17 with "normal morphology" during the generation of iPSCs from fibroblasts from patient cells with circle chromosome r(17) because of a disomic single parent. Although here it is worth noting that such a correction might occur only in circle chromosomes, which later are eliminated in all of the dividing cells, resulting in the constant formation of aneuploidy cells.

REPROGRAMMING ACCURACYAND THE GENETIC AND EPIGENETIC CRITERIA OF iPSC QUALITY

The technology of reprogramming has broad prospects for application in biomedicine. The main property of the obtained cells is their immunologic compatibility with a patient, which makes it possible to use their derivatives in therapy for various types of diseases. However, before a discussion of the clinical applications of iPSCs, it is necessary to prove their safety and the absence of an effect from the artificial reprogramming process on iPSC properties.

Tetraploid complementation provides a direct indication related to the pluripotency trait of the newly obtained murine iPSCs; however, it was not used for humans and other species. The quality of human iPSC lines could be assessed by genome-wide comparison of iPSCs with a "golden standard" of pluripotency, ESCs. In general, the properties of iPSCs correspond to those of ESCs. However, more sophisticated differences in the expression and promoter methylation patterns in various genes between ESCs and iPSCs were observed during analysis [32]. These differences partly accounted for the incomplete reprogramming of the obtained iPSCs, which lead in some clones to the preservation of gene expression of the somatic cells from which the iPSCs were obtained, the so-called "somatic memory." It is also partially explained by the incomplete removal of imprinting region methylation during reprogramming or, in contrast, by insufficient methylation of promoter of genes, the expression of which is typical for the corresponding type of differentiated cells. However, some differences were impossible to explain; therefore, they were explained by genetic differences between the ESC and iPSC lines or by the contribution of the reprogramming process itself. Indeed, comparative analysis on studies related to the search for differences between ESCs and iPSCs show that specific markers of reprogrammed cells were found only when a comparison of small number of ESC and iPSC lines was conducted. In the case of a comparison of DNA and transcriptome methylation level for a large number of ESC and iPSC lines obtained in different labs from different types of cells, they represent two noncrossing multitudes [33, 34]. This means that the present capacities of genome-wide analysis for a comparison of ESCs and iPSCs do not permit us to follow the traces of the reprogramming process or to distinguish them from the initial genetic differences of these cell lines. However, in the case of the application of individual iPSC lines for

therapy, it is necessary to know how similar they are to the pluripotent stem cells of the patient from which the iPSCs were obtained. To conduct such a comparison, a direct comparison of isogenic ESC and iPSC lines is required.

Studies comparing isogenic ESC and iPSC murine lines and almost isogenic ESC and iPSC human lines revealed differences in the regulation of imprinted genes (particularly, a cluster Dlk1-Dio3), as well as of genes located on the X chromosome. However, other studies have also noted a large variability among obtained iPSC lines, which prevents a conclusion on whether the observed differences in methylation between individual ESC and iPSC cell lines are a direct consequence of reprogramming and an evaluation of the contribution of the somatic cells. Moreover, genetic and epigenetic differences exist between different ESC lines, which though they are related to variations between individuals, complicate comparison with iPSCs.

A system developed in our lab for the comparison of isogenic lines of ESC and iPSCs obtained from various differentiated derivatives of this ESC line made it possible to conduct a genome-wide comparison of the methylation and expression of pluripotent cell lines. We have shown that there are no genes for which their expression differs in human iPSCs and isogenic ESCs. We found several differences characteristic to our isogenic system and significantly differentiating iPSCs and isogenic ESCs; however, the significance for nonisogenic PSC lines could be lower (Shutova et al., in press). In total, genome-wide studies of the expression and methylation of genes during reprogramming indicate that the existing differences are more occasional and do not result from reprograming technology application. Thus, we conclude that iPSCs exhibit all of the properties of the ESCs, including heterogeneity inside and between various lines.

INACTIVATION OF CHROMOSOME X DURING REPROGRAMMING

The induction of pluripotency in cells changes both the DNA methylation level and the pattern of histone modification as compared with the original somatic cells. One of the most interesting models for studies of epigenetic events occur during reprogramming is the X-chromosome status. It is well established that a process of dose compensation occurs in mammalian female somatic cells. It involves a set of sequential epigenetic events in the course of embryogenesis, which result in the formation of facultative heterochromatin, inactivation of one of the X chromosomes, and its transcriptional silencing in the differentiated cells. Thus, all of the somatic cells of each female mammalian tissue carry one active and one inactive X chromosome. Murine iPSCs obtained from female fibroblasts with one inactive X chromosome acquired two active X chromosomes; therefore, in the course of reprogramming, an inactive X chromosome was subjected to epigenetic alterations, resulting in its activation. This could be expected, as murine ESCs carry two active X chromosomes. It has been shown recently that reprogramming from somatic into the pluripotent state in murine cells is always accompanied by reactivation of the X chromosome in the female lines [35]. In the case of reprogramming of human somatic cells, the results were inconsistent. In 2010, it was shown in the lab of K. Plath that no reactivation of the X chromosome occurs during reprogramming [36]. In our studies on iPSC generation [37], we have shown the acquisition of active chromatin markers on the X chromosome, which resulted from reprogramming. It should be noted that variability of the X-chromosome status was observed also in human ESCs. Other researchers later showed that, indeed, during reprogramming of human cells, at least partial reactivation of the X chromosome could happen [38], but it is likely that the reactivation event occurs less often than the absence of reactivation during reprogramming.

Inactivation of the X chromosome is accompanied by a sequence of the following events: activation of protein-noncoding *XIST* gene expression, the RNA molecules of which cover the inactivated X chromosome (in exactly the same form from which it is expressed); theacquisition of heterochromatin-specific histone modifications (for example, H3K27me3) and the loss of active chromatin modifications (H3K4me2), and transcriptional silencing of the inactivated X chromosome. Its replication is transmitted to a later S-phase of the cell cycle. This is followed by the inclusion of histone macro-H2A into several nucleosomes, the formation of macro-chromatin bodies and, respectively, packaging of the X-chromosome territory, and finally, methylation of the promoters of genes located on the inactive X chromosome [39]. As a rule, some of these epigenetic events are used as markers of X-chromosome activity, but they do not reflect its real state every time. For example, in our study on human iPSC characteristics, it was shown that an inactive late-replicating X chromosome does not always have a compact chromosome territory [40]. With the characteristics of X-chromosome status in PSCs, scientists usually rely on such markers as the presence/absence of the XIST cloud or the focus of inactive chromatin H3K27me3. However, the absence of these markers may not mean the presence of a transcriptionally active X chromosome. In our study [41], we have shown that a better approach for the detection of X-chromosome reactivation could be early replication of the X chromosome and the acquisition of a cytosine modification in the form of 5-hydroxy methyl cytosine by X-chromosome DNA.

Thus, the inactivation status of the X chromosome reflects epigenetic events during reprogramming. Moreover, the definition of X-chromosome status becomes important during studies of X-linked diseases using iPSC lines for modeling of these diseases.

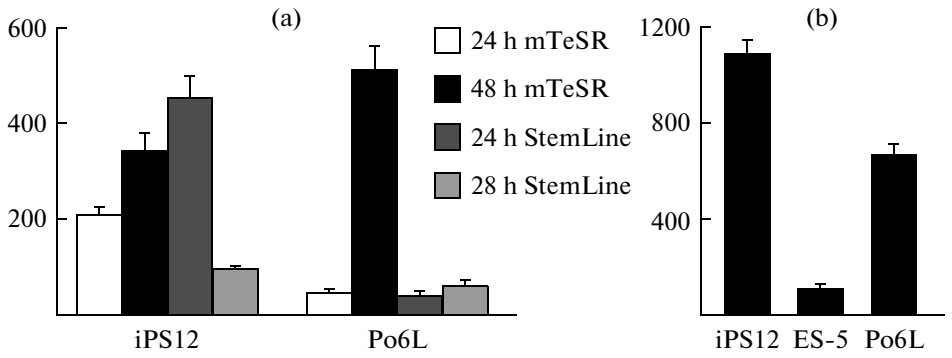

Fig. 2. Comparative analysis of the potential of iPSC differentiation into the blood cells. iPS12, iPSCs obtained from endothelium of umbilical cord; ES-5, ESC; Po6L, iPSCs obtained from skin fibroblasts. Differentiation was conducted through the stage of embryoid body (EB). (a) comparison of the number of hematopoietic colonies during differentiation in various media; (b) comparison of the number of hematopoietic colonies obtained from 500 thousand EB cells from various lines in optimal conditions.

DIFFERENTIATION POTENTIAL OF iPSCs

One of the most attractive properties of pluripotent stem cells is their capacity for transition into a differentiated state. During development in the body, this occurs in a niche that determines the execution of a genetic program of specialization. Formation of similar conditions outside the body will make it possible to obtain various specialized types of cells from pluripotent stem cell simultaneously. A significant number of protocols of directed iPSC degeneration have been developed, but we cannot discuss here even a small number of them. In the next chapter, we will discuss neuronal differentiation of iPSCs, which is important for studies of neurodegenerative diseases. However, we will discuss here the possibility of human iPSC differentiation into blood cells and epithelium of the eye retina.

It is well established that the requirements for donor blood is very high and is only partly satisfied. Moreover, there is a risk of recipient infection, while the difficulties with the selection of a blood stem cell donor reduce the chance of survival for oncohematologic patients. Studies of the process of hematopoiesis and its reproduction outside the body are of great importance for the development of biotechnological blood production techniques. In some studies, it was shown that iPSCs retain some traits similar to the original cell type. This phenomenon is called "somatic memory." The "somatic memory" of iPSCs can affect their properties, in particular, their capacity to differentiate [42]. In some studies the effect of basic culture conditions, the source of somatic cells, and the means of iPSC acquisition on their properties were explored, particularly with respect to their basic property: the iPSC capacity for differentiation. The obtained results are inconsistent: it has been shown, for example, that, in contrast to ESCs, the terminal stage is not achieved during erythroid differentiation of iPSCs although iPSCs efficiently differentiated into endothelial cells and erythroblasts, showing a higher level of apoptosis and limited proliferative activity [43, 44]. Comparison of the hematopoietic potential of 14 lines of human ESCs and iPSCs has shown that some cell lines possessed a high capacity for differentiation into blood cells, while others exhibited very low potential [45]. In contrast, other studies demonstrated that iPSCs did not differ from ESCs in their capacity to reach the terminal stage of erythroid differentiation and expressed a set of erythroid markers identical to differentiated derivatives of ESCs [46]. Like ESCs, all iPSCs produced CD34-positive hematopoietic precursor cells and CD31-positive endothelial cells upon coculturing with an OP9-line of stromal cells. When cultured in semi-solid media in the presence of hematopoietic growth factors, differentiated derivatives of iPSCs form all types of hematopoietic colonies. iPSCs obtained from various somatic cells (skin fibroblasts, embryonal and fetal mesenchyme stem cells) are subjected to erythroid differentiation with the same efficacy as ESCs, in spite of donor age and the type of somatic cells, while the end-products are mature erythroid cells, which express a level of embryonal and fetal globins corresponding to ECSs [47]. We have examined several protocols of iPSC differentiation into cells of the erythroid type and conducted a comparative analysis of iPSC lines obtained from the endothelium of the umbilical cord [37], skin fibroblasts, and ESCs [48] (Fig. 2). It appeared that iPSCs from the endothelium more efficiently differentiated into erythroid cells than iPSCs from fibroblasts or an ESC line. Endothelium and blood cells represent closer developmental cell types; the observed effect could be associated with "somatic memory." However, it is more likely in this case that other factors are involved, since previous genome-wide analysis of the methylation status and gene expression indicated a complete silencing of tissue-specific gene expression in the examined cell lines and revealed no significant differences between iPSC and ESC lines. Thus, there is no agreement at present on whether iPSCs and

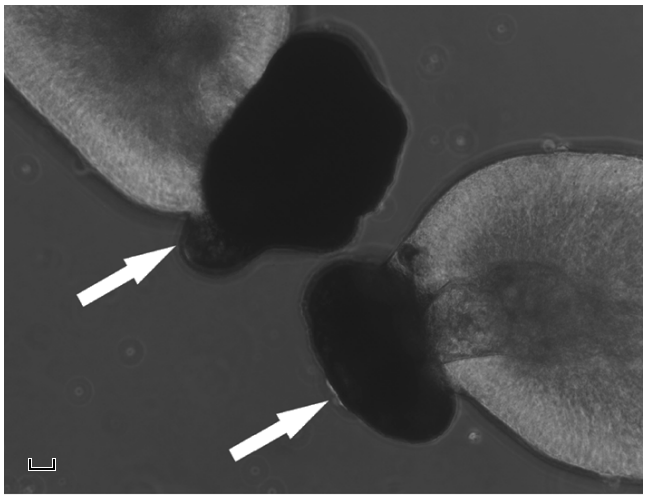

Fig. 3. Organoids containing tissues of the developing eye retina differentiated from human iPSCs. Arrows show the regions of the developing retinal pigment epithelium. Phase contrast. Bar = 100 μm.

ESCs differ in their capacity to differentiate and what the contribution of somatic iPSC precursors is.

The processes of eye retina degradation, which occur during age-related macular degeneration, as well as hereditary diseases of retina, are the major reasons of vision loss and blindness. A search for reliable sources for transplantation of eye retina components is an important medical task. PSCs could represent such a source, making it possible to study simultaneously the processes of eye development and to define the mechanisms of pathogenesis. Pigment epithelium of the retina could be easily obtained during directed differentiation of human ESCs and iPSCs and designated based on morphological criteria, the accumulation of brown pigment granules, and *RPE65* expression [49]. For example, retinal cells obtained from human ESCs were introduced into the retina of four- to six-week old mice with a knocked-out *crx* gene. These mice represent a model of inherited Leber amaurosis and do not have a detectable retina electrogram. Transplantation of retinal cells obtained from human ESCs resulted in the induction of b-waves on the retinogram; this indicated the appearance of functional photoreceptors, which formed correct contacts with bipolar cells [50]. It was shown that cells of retinal pigment epithelium differentiated from human iPSCs produce chromophore 11-*cis*-retinal and therefore are functionally active. After transplantation of these cells into the subretinal space of blind LRAT(–/–) and RPE65 (–/–) mice, their vision improved. These researchers have also shown that cells of pigment epithelium form functional contacts with recipient cells [51].

It is also worth noting the studies that demonstrated the possibility of organoid production, which imitates development of the eye bladder or even the optic cup from pluripotent stem cells [52, 53]. This supports the capacity of pluripotent stem cells, including iPSCs, for organogenesis in vitro. In our studies, we have shown that high-density culturing of ESCs and iPSCs in conditions that provide an advantage for the development of the neuro-epithelium over 90 days in culture resulted in the formation of complex, three-dimensional structures of neuro-epithelial origin consisting of developing eye tissues containing structured pigment epithelium and a multilayer retina (Fig. 3).

Phillips et al. [54] recently suggested an interesting cellular model of microphthalmia based on the production of organoids from iPSCs. In this study, the iPSCs were obtained from a biopsy of a patient with microphthalmia, which was induced by R200Q mutation in the homeodomain of the *VSX2* gene. The iPSC properties did not differ from iPSCs of healthy donor-siblings. During differentiation of mutant and normal iPSCs into organoids that model the optic cup, it was found that mutant cells have a slower growth rate and increased production of pigment epithelium cells as compared with the normal and slower differentiation of photoreceptors. In addition, mutant cells could not differentiate in bipolar cells, which was also observed previously in *VSX2* mutant mice.

In recent years, several groups of researchers obtained iPSCs for modeling various hereditary eye diseases. Thus, in a recent study [55] iPSCs were obtained from a patient with pigment retinitis with a mutation in the rhodopsin gene (E181K). The mutation was corrected in the patient iPSCs by homologous recombination and was introduced into control iPSCs. The cells were then differentiated into photoreceptors. Using the approach, the authors found decreased viability of photoreceptor cells carrying a E181K mutation, which correlated with an increased expression of markers of endoplasmic reticulum stress (ER-stress) and apoptosis. Screening for therapeutic agents has shown that rapamycin, PP242, AICAR, NQDI-1, and salubrinal facilitated survival of mutant photoreceptors obtained from iPSCs with corresponding downregulation of ER-stress and apoptosis expression markers.

An approach associated with the transplantation of retinal pigment epithelium obtained from human ESCs was explored at the level of clinical studies [56]. In the course of these studies, the safety of retinal pigment epithelium cell transplantation was demonstrated, and a positive effect of transplantation was noted. In September of 2014, clinical studies of pigment epithelial cells obtained from iPSCs from patients with age-related macular degeneration started in Japan. Experimental approaches associated with differentiation and transplantation of pigment epithelium were previously investigated in monkeys [57].

Further development of reprogrammed cell differentiation techniques and elucidation of the functional similarities of cells obtained in vitro and their natural analogs will permit an active launching of clinical

studies related to the transplantation of characterized cells in culture.

POSSIBILITIES FOR THE PRACTICAL USAGE OF GENETIC REPROGRAMMING TECHNOLOGY IN STUDIES OF NEURODEGENERATIVE DISEASES

The development of the majority of pathologies occurs gradually, while manifestation of the disease happens often at its terminal stage. Moreover, many tissues subjected to pathology remain inaccessible for studies during the whole process of the disease development. It is particularly important with respect to neurodegenerative diseases when pathological tissues are available for studies only *post mortem*. The technology of reprogramming of somatic cells to a pluripotent state and their consecutive directed differentiation into the required type of somatic cells, particularly into neuroglia cells, open essentially new possibilities in studies of pathologies. The study by Park et al. could be considered the beginning of the application of technology for the production and differentiation of iPSCs for the modeling of human diseases [58]. It reported for the first time on a successful production of iPSCs from patients with various hereditary diseases. Despite the short history of these studies, definite success has been achieved in modeling the use of iPSCs for such neural system diseases as schizophrenia, Alzheimer disease, Parkinson disease, and others. For example, Brennand et al., studied schizophrenia using differentiated from iPSCs neurons. For this purpose they reprogrammed fibroblasts obtained from patients with schizophrenia. The obtained iPSCs were differentiated into neurons. Mutant neurons exhibited fewer connections, fewer axons, and a decreased level of PSD95 protein expression and glutamate receptors as compared with wild-type neurons. Treatment of neurons with neuroleptic loxapine, which usually is used in schizophrenia therapy, resulted in amelioration of the observed phenotypic manifestations [59]. Alzheimer disease is actively studied. Highly purified cultures of neurons were obtained from iPSCs. Neurons from patients with Alzheimer disease demonstrated significantly higher levels of β-amyloid, phospho-Tau (Thr231), and active glycogen synthase kinase 3β (aGSK-3β) compared with normal neurons. An accumulation of Rab5-positive early endosomes, which were increased in size, was also noted. Treatment of neurons with inhibitors of β-secretase but not γ-secretase resulted in a significant decrease in the level of phospho-Tau (Thr 231) and aGSK-3β [60]. Kondo et al. [61] obtained iPSCs from patients with hereditary and sporadic forms of Alzheimer disease. After that, iPSCs were differentiated into neurons. Oligomers of β-amyloid, which were accumulated in neurons with (APP)-E693Δ mutation and in neurons obtained from iPSCs from patients with the sporadic form of Alzheimer disease, were found. The accumulation resulted in endoplasmic reticulum stress and oxidative stress. The discovered oligomers of β-amyloid were not resistant to proteolysis, and treatment by docosahexaenoic acid resulted in a decrease in the reaction to stress. The authors suggested that docosahexaenoic acid could be an effective drug for some subgroups of patients with Alzheimer disease. This was also supported by the results of clinical studies [61].

Certain accomplishments were achieved during the modeling of Parkinson disease with mutation in G2019S in the *LRRK2* gene. Neurons differentiated from iPSCs demonstrated an increased expression of key genes of response to oxidative stress and α-synuclein protein. The test for activation of caspase-3 demonstrated a higher sensitivity of mutant neurons to such chemical agents as hydrogen peroxide, proteasome inhibitor MG132, and 6-hydroxydophamine as compared to normal neurons [62]. Liu et al. found abnormalities in the architecture of the cell nucleus using the neurons obtained from iPSCs with G2019S mutation in the *LRRK2* gene. They later conducted an analysis of postmortem brain tissue sections from patients diagnosed with "Parkinson disease" and found a presence of abnormalities in cell nucleus [63]. iPSCs from patients with Parkinson disease were used to support the therapeutic action of previously discovered drugs. In this way the recovery of pathological phenotype to a normal state through the use of a chemical molecule, which was originally found during screening for a yeast model, as a potential remedy against Parkinson disease was confirmed [64]. A consortium for studies on Huntington disease reported on the production of 14 iPSC lines from patients with Huntington disease and from healthy subjects. Neurons differentiated from iPSCs demonstrated pathologic changes associated with the disease in electrophysiology, metabolism, and cell adhesion. Mutant neurons with an average and high number of repeats have also demonstrated an increased level of cell death as compared to the norm [65]. In our lab iPSCs, both from healthy donors and from patients with Parkinson, Huntington disease, and SOD1-associated side amyotrophic sclerosis, were successfully produced and characterized [66–68]. We have demonstrated the possibility of iPSC production from patients with SOD1-associated side amyotrophic sclerosis without the integration of genes of reprogramming factors into the genome, as well as the possibility of their differentiation into motor neurons, which is important for the development of methods of cell therapy for side amyotrophic sclerosis [68]. The obtained iPSC cell lines permit the study of neurodegenerative human diseases. Thus, dopaminergic neurons obtained from the iPSCs of patients with Parkinson disease (mutations in *LRRK2* and *PRKN* genes) expressed tyrosine hydroxylase and demonstrated spontaneous bioelectric network activity during culturing on a multielectrode template. In rats with a toxic 6-OHDA-model of Parkinson disease, transplantation of the obtained

dopaminergic neurons into the striate body resulted in strong improvement of the motor functions and a reduction in Parkinson disease symptoms [69].

CONCLUSIONS

The technology of genetic reprogramming, for which a Nobel Prize was awarded in 2012, offers great possibilities for fundamental studies in the area of gene regulation and in the exploration of interrelationships between genetics and epigenetics. In addition to basic studies, this technology permits the implementation of a wide range of applied studies, from the development of therapeutical drugs to the maintenance of planet biodiversity.

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