ISSN 0026-8933, Molecular Biology, 2021, Vol. 55, No. 5, pp. 645–669. © Pleiades Publishing, Inc., 2021.
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REVIEWS

Transcription Factors of Direct Neuronal Reprogramming in Ontogenesis and Ex Vivo

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Received October 11, 2020; revised December 14, 2020; accepted December 15, 2020

Abstract—Direct reprogramming technology allows several specific types of cells, including specialized neurons, to be obtained from readily available autologous somatic cells. It presents unique opportunities for the development of personalized medicine, from in vitro models of hereditary and degenerative neurological diseases to novel neuroregenerative technologies. Over the past decade, a plethora of protocols for primary reprogramming has been published, yet reproducible generation of homogeneous populations of neuronally reprogrammed cells still remains a challenge. All existing protocols, however, use transcription factors that are involved in embryonic neurogenesis. This is presumably be the key issue for obtaining highly efficient and reproducible protocols for ex vivo neurogenesis. Analysis of the functional features of transcription factors in embryonic and adult neurogenesis may not only lead to the improvement of reprogramming protocols, but also, via cell marker analysis, can exactly determine the stage of neurogenesis that a particular protocol will reach. The purpose of this review is to characterize the general factors that play key roles in neurogenesis for the embryonic and adult periods, as well as in cellular reprogramming, and to assess correspondence of cell forms obtained as a result of cellular reprogramming to the ontogenetic series of the nervous system, from pluripotent stem cells to specialized neurons.

Keywords: embryonic neurogenesis, adult neurogenesis, morphogenic factors, proneuronal factors, direct reprogramming, neural stem cells, neural progenitor cells, induced neurons, PAX6, MSI1, GSX2, DLX, ASCL1, NGN2, BRN2, MYT1l, NEUROD1

DOI: 10.1134/S0026893321040087

INTRODUCTION

Until recently, it has been difficult to conduct research in the field of regenerative therapy for incurable diseases of the central nervous system (CNS) due to the impossibility of obtaining the necessary types of nervous tissue cells, including all transitional forms: from pluripotent stem cells to highly specialized neurons. The development of induced pluripotent stem cell (iPSCs) technology and direct cell reprogramming technology (direct production of progenitor cells, i.e., without the induced pluripotency stage) has led to the onset of a new era in the field of neuroregeneration [1–5]. It is known that most neurodegenerative disorders result in preferential damage to particular subtypes of neurons. For example, dopaminergic neurons of the mesencephalon degenerate in Parkinson's disease, striatal GABAergic neurons (γ-aminobutyric acid (GABA) is a neurotransmitter) are damaged in Huntington's disease, and motor neurons degenerate spinal muscular atrophy and amyotrophic lateral sclerosis [6]. Cell reprogramming opens up prospects for reconstruction of lost specialized neurons and the development of patient-specific cell models, including those of genetic and sporadic diseases, to study the intimate mechanisms of pathogenesis and develop new methods of therapy for currently incurable neuropsychiatric and neurodegenerative disorders [7, 8].

Some researchers note that cell development is controlled by the spatial-temporal context of the environment as well as biochemically [9–11]. In this context, the iPSC production technique and direct cell reprogramming are different in principle, because iPSC production is accompanied by the "zeroing" of the epigenetic age of transformed cells [10]. Direct reprogramming of somatic cells of an adult patient makes it possible to bypass the unstable and potentially

Abbreviations: GABA, γ-aminobutyric acid; NSC, neural stem cells; RG, radial glia; BMP, bone morphogenetic protein; drNPCs, directly reprogrammed neural precursor cell; DV, dorsal-ventral (axis); FGF, fibroblast growth factors; iPSCs, induced pluripotent stem cells; LGE, lateral ganglionic eminence; NCCs, neural crest cells; RA, retinoic acid; RC, rostral-caudal (axis).

unsafe iPSC stage but maintain the basic epigenetic "picture" of original cell [11]. Retention of age-related peculiarities determines the preferential application of directly reprogrammed neural precursor cells (drNPCs) [2], e.g., in the study of age-related neurodegenerative diseases such as Alzheimer's and Huntington's diseases [11].

The discovery of many transcription factors and quite a number of small molecules currently used for direct reprogramming is owed to the study of the development of the nervous system. Moreover, at present it is becoming obvious that iPSC or drNPC technologies for nerve regeneration aimed at recovering the pyramidal tract in case of acute spinal cord injury or stroke cannot be developed without deep understanding and modeling of the processes of embryonic neurogenesis. Mere implantation of syngeneic embryonic neural stem cells (NSC) does not lead to complete regeneration of the adult CNS as a result of epigenetic signal mismatch, the absence of gradients of morphogenetic factors and other important components of the cellular microenvironment, without which neuroregeneration is impossible [9–11].

The review is aimed at characterizing the general factors that play the key roles in embryonic and adult neurogenesis, as well as in cell reprogramming, and assessing the similarity of the cells obtained by reprogramming to the forms of the ontogenetic series of the nervous system: from pluripotent stem cells to specialized neurons.

EMBRYONIC NEUROGENESIS

In embryogenesis, the development of the nervous system, or neural induction, begins at the gastrula stage, when the neural plate folds to form a neural groove, which later will develop into the brain and the spinal cord. The sources of different types of spinal cord cells in vertebrates are neuromesodermal progenitors localized in the caudal lateral epiblast [12–14]. The process of neural induction is determined by the gradients of several morphogenetic factors along the rostral-caudal (RC) and dorsal-ventral (DV) axes, which provides the radial and longitudinal/tangential migration of progenitor cells. The major morphogens responsible for the pattern of formation of the RC-axis include the factors of the Wnt signaling pathway, retinoic acid (RA) and the Fibroblast Growth Factors (FGF), while the pattern of formation of the DV-axis involves the factors of the Wnt and SHH[1] signaling pathways, as well as the bone morphogenetic proteins (BMP) (Fig. 1a).

[1] The works considered in the review were performed in different species of animals, with species-specific designations for orthologous genes and encoded proteins. To make the text clutter free, we give the gene and protein names according to the GeneCards database of human genes.

The main caudalizing morphogens include proteins of the WNT, FGF and RA families. WNT1 and FGF-8 are produced by cells at the mesencephalon–metencephalon boundary and are necessary to impart identity to the mesencephalon (midbrain) and the rhombcephalon (hindbrain) by regulating the genes *OTX1* (the encoded protein, homeobox protein OTX1, determines the forebrain and midbrain boundaries) and *GBX2* (the encoded protein, gastrulation brain homeobox 2, determines the hindbrain boundaries) [15]. Forced activation of the Wnt pathway by the small molecule CHIR99021 contributes to the formation of regional neural precursors; at the same time, the higher the dose of CHIR99021 (and the activity of WNT, respectively), the greater the caudal identity of the cells [16]. This dose-dependent effect made it possible to obtain neural progenitors identical to the cells of the forebrain, midbrain and hindbrain, as well as the anterior surface of the spinal cord [16]. The accuracy of the dose-dependent effect of CHIR99021 on the morphogenic properties of WNT proteins is so high that progenitors specific for very close areas, e.g., the ventral mesencephalon and the subthalamic nuclei, can be obtained by adjusting the concentration of this molecule [17]. RA functions in the reverse, rostral direction and weakens the effects of the Wnt and FGF signaling pathways [18, 19]. At the molecular level, RA inhibits *FGF8* gene expression, thereby preventing further elongation of the caudal part of the spinal cord [20, 21].

The SHH transcription factors are produced by the chord and mesodermal tissue under the ventral pole of the nerve tube and, due to activation of the SHH signaling pathway, form the basic ventral pattern, while the BMP transcription factors, in particular, BMP2, BMP4, BMP5, BMP7, etc., are secreted in the dorsal part of the chord (roof plate), and those of the WNT family, on the contrary, determine the dorsal pattern [13, 22, 23]. These morphogens form antiparallel gradients of signals controlling the work of transcription factors, and their expression, in turn, divides the spinal cord into 14 separate domains in the DV-direction with different types of cells [24, 25]. For example, the SHH gradient, due to the activation of transcription factors of the following families: PAX (paired box), NKX (NK2 homeobox 1), DBX (developing brain homeobox protein) and IRX (iroquois homeobox protein) in the ventral part of the spinal cord, determines the formation of progenitors of motor neurons and interneurons (V0–V3), as well as floor plate cells. On the contrary, neural crest cells and dI1–dI6 interneuron progenitors are generated in the dorsal part of the nerve tube under the influence of the BMP gradient [26]. The embryonic hindbrain includes two main regions: dorsal (pallium) and ventral (subpallium). The anterior and lateral parts of the dorsal hindbrain form the neocortex and the paleocortex; the posterior and medial areas later on develop into the hippocampus (archicortex), the cortical border zone (one of the

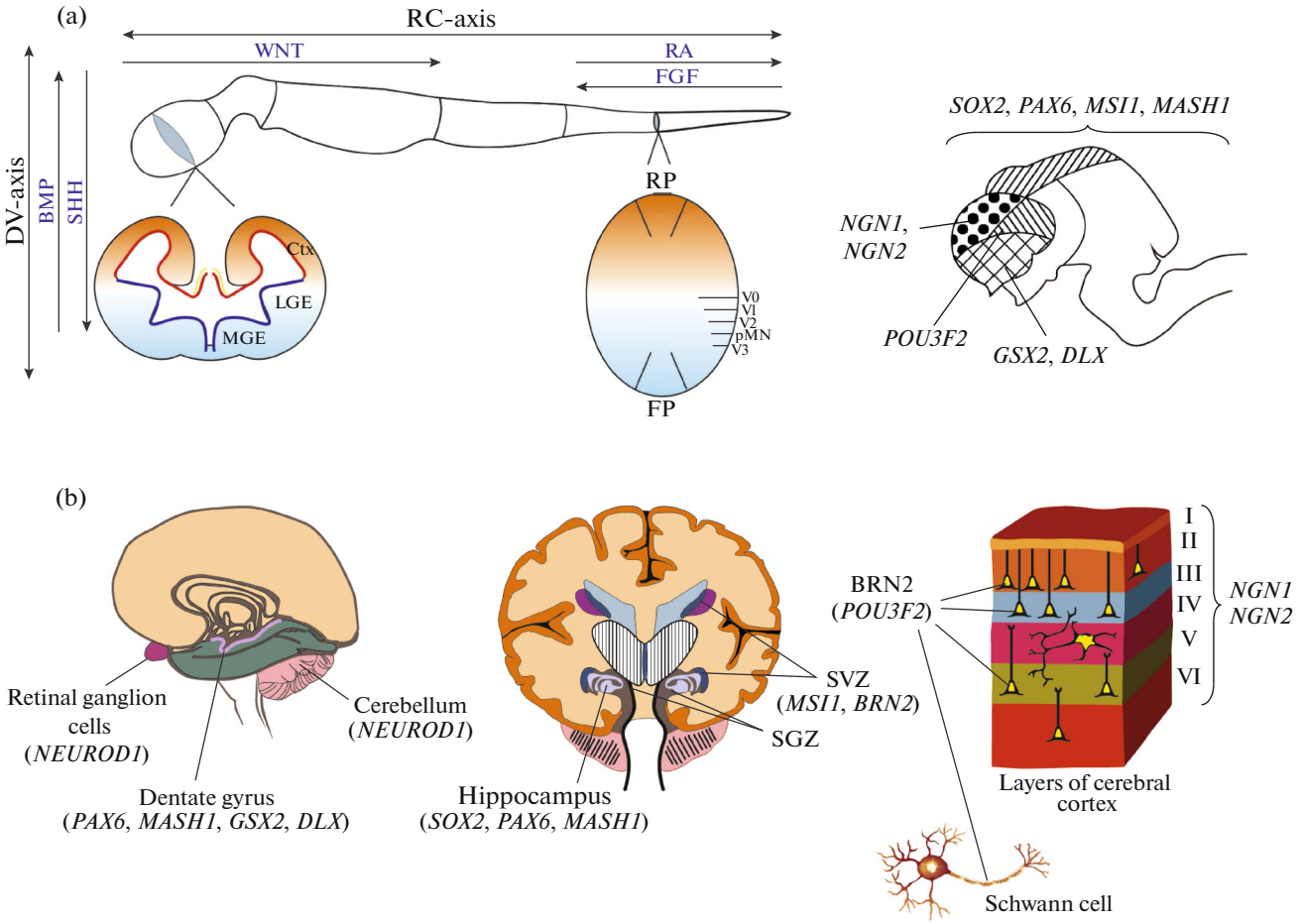

Fig. 1. The factors of neurogenesis in the human embryonic and adult brain. (a) The main morphogenic gradients of the embryonic brain at the early stage of development and the zones of functioning of proneural transcription factors in the embryonic brain at the stage of brain vesicles (on the right). (b) The expression domains and the targets of proneural transcription factors in the adult brain. Longitudinal section: the dentate gyrus, the retina and the cerebellum. Cross section: the hippocampus, the subgranular (SGZ) and subventricular/subependymal (SVZ) zones. The diagram of the cortical layer shows that the neurogenin-1 and -2 genes (*NGN1* and *NGN2*) are expressed in pyramidal neurons of all cortical layers of the adult brain and in astrocytes of cortical layer IV of the brain, while the gene of protein BRN2 (*POU3F2*) is expressed in pyramidal neurons of cortical layers II, III and V of the adult brain, as well as in Schwann cells. Designations: Ctx, cerebral cortex; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; RP, roof plate (the dorsal region of the spinal cord); FP, floor plate (the ventral plate); V0–V3, ventral interneurons; pMN, motoneuron progenitors.

major sources of the Cajal–Retzius cells) and the choroid plexus [27]. The lateral, medial and caudal ganglionic eminences formed in the ventral part of the hindbrain are involved in the formation of basal ganglia, GABAergic interneurons of the cortex, and the rostral migratory stream [28–31]. The formation of these domains in the embryonic brain is controlled by the WNT, BMP, SHH, FGF and PAX6 morphogens. PAX6, WNT and BMP protein expression primarily determines the formation of the dorsal hindbrain [29, 32], while the increase in the SHH level determines the formation of its ventral parts [33]. PAX6 is a transcription factor, the expression of which forms the border between the dorsal and ventral parts of the brain along with expression of the *GSX2* gene, with its product GSH2 localized in the ventral part of the hindbrain [34]. The dorsal border of *GSX2* expression is adjacent to the ventral border of the pallial PAX6 domain with a narrow overlap, while cross-repressive interactions between *GSX2* and *PAX6* establish the pallial–subpallial boundary [35–37].

SPECIFICATION OF TERMINAL NEURONAL SUBTYPES

In addition to the morphogens determining the RC- and DV-orientation, there are quite a number of transcription factors ensuring the specialization of the forming neurons. This process has been best studied in the hindbrain, because the cortex is formed from its dorsal part. Primary cortical neurons originate from actively dividing radial glia in the deeper layer (layer

VI) and only then in the upper layer (layer I), followed by sequential generation of neurons in layers V, IV, III and II. First there is activation of the FOXP2 (forkhead box protein P2) and CTIP2 factors responsible for the formation of neurons in layers V–VI; this is followed by the emergence of cells expressing POU3F3 (POU class 3 homeobox 3), CUX1/2 (Cut-like homeobox 1/2) and LHX2 (LIM homeobox 2) in layer IV, then cells expressing LMO3 (LIM domain only protein 3) and TLE3 (transducin-like enhancer protein 3) in layer III and, finally, cells expressing PLXND1 (Plexin D1) in layer II [38]. It has been shown that PAX6, NGN1 (Neurogenin 1) and NGN2 determine the formation of glutamatergic neurons and inhibit astroglial differentiation [39–42]. PAX6 can activate neurogenesis both by the NGN-dependent mechanism and then via the activation of the neuronal differentiation factors NeuroD, TBR1, and TBR2 (T-box brain protein 1/2) [39], and by the NGN-independent mechanism [32]. The NGN1 and NGN2 activities are induced by WNT [42, 43]. Ganglionic eminences give rise to somatostatin-expressing GABAergic and cholinergic interneurons and neuropeptide Y-immunopositive (NPY) interneurons [31, 44]. One of the main inducers of GABAergic neurons is transcription factor ASCL1 (Achaete-scute homolog 1; the *MASH1* gene) [40, 45–47]. GABAergic and cholinergic neurons are characterized by expression of the factors LHX6 [48, 49] and LHX8 or ISL1 [48, 50], respectively. The genes responsible for neuronal specialization in the ventral region of the hindbrain encode the DLX (*Distal-less* homeobox) family of transcription factors [51, 52] and the SHH signaling pathway: GLI1, GLI2 and GLI3 [53, 54]. The formation of dopaminergic neurons of substantia nigra of the midbrain involves the FGF2, FGF8 and WNT factors. They influence the formation of tyrosine hydroxylase-positive neurons expressing the *PITX3* (pituitary homeobox 3) and *NR4A2* (encoding the nuclear receptor related 1 (NURR1) protein) genes [55].

Thus, as a result of the spatiotemporal organization of the above transcription factors (master regulators) and the secondary morphogenetic and specializing factors triggered by the latter, neural induction leads to formation of the whole diversity of neurons and glial cells in the terminal structures of the brain and the spinal cord. In this review, we will not dwell in detail on the factors of specialization of terminal neural subtypes in the context of direct cell reprogramming, because the protocols of terminal differentiation are universal and do not depend on the method of reprogramming.

PECULIARITIES OF NEUROGENESIS IN THE ADULT BRAIN

Neurogenesis in the adult brain implies the emergence of functionally active neurons *de novo* from progenitor cells (radial glia, NSC), which occurs throughout the life span in the subventricular (SVZ) and subgranular (SGZ) regions of the brain [56, 57]. Neurogenesis, which was discovered for the first time in singing birds in the 1970s, was then found in the SVZ and SGZ of the brain of small mammals and a little later in the same zones of the primate and human brain (see reviews [58, 59]). Recently it has been reported that neurogenesis occurs also in other areas of the mammalian brain such as the hypothalamus, nigrostriatal system, amygdaloid body and different cortical areas, though it is still too early to state that it is neurogenesis and not cell migration from the SVZ and SGZ [57]. The main functions of neurogenesis in the adult brain are the regeneration of olfactory cells and the formation of new neurons and glyocytes in the structures performing the function of memory and other cognitive processes, and maintaining neural plasticity in general [57–59]. Under pathological conditions, all processes in the niches of stem cells of the brain undergo substantial changes. For example, neurogenesis in the adult brain is activated in response to the mass death of neurons in case of strokes, traumatic brain injuries and neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, epilepsy, etc. [57–59].

Neural stem/progenitor cells of the subependymal and subgranular zones originate from embryonic NSCs formed in the early neurogenesis and then resting right up to the postnatal period [60]. These cells undergo several postnatal divisions before giving rise to type I radial glial cells (RGCs) in the SGZ or to their analogs, B1 cells, in the SVZ, which form the niche of stem cells of the adult brain. It is commonly supposed that NSCs in the SVZ and SGZ of the adult brain perform obligate expression of GFAP (glial fibrillary acidic protein) and nestin and optional expression of SOX2 (sex determining region Y (SRY)-box 2) or SHH, which demonstrates the heterogeneity of their population [61].

In the SVZ, type I RG cells undergo asymmetric division, which results in the formation of type II progenitor cells with the astroglial phenotype (2a), as well as intermediate neural progenitors (2b cells) giving rise to young neurons [62]. Proglial 2a cells are characterized by the expression of SHH, WNT, insulin-like growth factor 1 (IGF-1), FGF-2, SOX2 and nuclear protein TLX (T Cell Leukemia Homeobox) [61]. The activity of the SHH, WNT, IGF-1 and FGF-2 pathways is necessary for the differentiation and maturation of type II cells, while the local changes in IGF-1 concentration determine the direction of migration [63]. These cells also express GABA receptors and respond to the presence of GABA in the ambient medium [62]. In addition to GABA, the proliferation and maturation of type II cells are also regulated by dopamine and serotonin, as is demonstrated by expression of the genes of dopamine (D2/D3) and serotonin (5-HT) receptors in these cells [64, 65]. Proneural 2b cells, in addition to the factor common

for all type II cells, express the *DCX* (Doublecortin), *NT3* (neurotrophin-3), *TBR2*, *MASH1*, *NEUROD1* and *PROX1* (prospero homeobox protein 1) genes [61]. The 2b cells are later on differentiated into type III cells (neuroblasts and predifferentiated neurons). The early stage of formation of these cells is referred to as the post-mitotic maturation phase [62]. In this process, type III cells change their spatial orientation to more vertical within the SGZ and acquire a polar form, releasing dendrites into the dentate molecular layer and axons moving to the layer of pyramidal cells in the CA3 area [66]. Then these cells mature, forming dendritic spines and elongating axons to the CA2 zone [61]. Young type III cells continue the expression of proneural genes active in type I and II cells (*DCX*, *WNT*, *NOTCH*, *GABA*, *NEUROD1* and *PROX1*) [67], which is then replaced by the expression of mature neuronal genes such as *BDNF* (encoding the brain-derived neurotrophic factor), *RELIN* (reelin), *CREB* (cAMP response element-binding protein), *RBFOX3* (RNA binding FOX-1 homolog 3), *CALB2* (calretinin), and *AP1* (activating protein 1) [67]. Reelin is an extracellular matrix glycoprotein that regulates the processes of neuronal migration and positioning and the formation of dendritic spines [68]. CREB phosphorylation activates transcription of the genes of other key molecules such as c-FOS, JUN-B, BCL-2, GDNF (glial cell-derived neurotrophic factor) and different neurotrophins for the regulation of neuronal survival and regeneration [69]. The Wnt signaling cascade activates AP-1, which is a heterodimer consisting of c-FOS and c-JUN subunits, and influences neuronal proliferation, differentiation and apoptosis [70].

The SVZ of lateral ventricles, or the subependymal zone, contains an ample quantity of NSCs, so-called type B1 RG cells, which are morphologically similar to adult radial astrocytes, express the *GFAP* genes and the genes of glutamate/aspartate transporters and the brain lipid-binding protein, and contact capillaries through their processes [57, 71, 72]. Activated B1 cells express nestin and divide asymmetrically with the formation of B2 cells, which also have the astroglial phenotype and interact with capillaries but do not contact the apical surface of the ependyma, as well as with the formation of transit-amplifying intermediate type C cells expressing the ASCL1 and DLX markers. Type C cells divide asymmetrically several times and form a pool of type A cells (neuroblasts), which then migrate via the rostral migratory stream to the olfactory bulbs [57, 71, 73]. Type A cells are characterized by the expression of DCX, CRMP-4 (collapsin-response mediator protein 4), PSA-NCAM (prostate-specific antigen–neural cell adhesion molecule), ganglioside 9-O acetyl GD3, and other cell adhesion proteins and integrins providing effective cell migration to the olfactory bulbs, which is regulated by factors such as TN-C (tenascin-C) and PK2 (prokineticin-2) [57].

Neurogenesis in the adult brain is usually considered as a limited and simplified continuation of embryonic neurogenesis [13]. The similarity between embryonic and adult neurogenesis, in particular, is that neurons originate from RG cells in both the embryonic and adult brain [74]. However, there are also fundamental differences between adult and embryonic neurogenesis, including both peculiarities of the environment and in the key molecular mechanisms. For example, transcriptome analysis has shown that embryonic RG cells are most similar to neuroblasts and transit-amplifying progenitor cells (TAP cells), while the pattern of expression of RG cells in the adult brain is more consistent with differentiated astrocytes and ependymal cells [9]. The latter play an important role in the formation of the niche of stem cells in the subependymal space and the SGZ of the adult brain [75] and are absent in the environment of NSCs of the early embryonic brain [76]. This peculiarity of the microenvironment probably results in the fact that embryonic NSCs are differentiated mostly in the neuronal direction, while adult NSCs preferentially perform gliogenesis [9].

Transplantation experiments have shown that the cultivated embryonic and adult NSCs, which are easily differentiated into neurons in vitro, change the direction of differentiation towards gliogenesis after having been implanted into the brain parenchyma of an adult animal [13]. It is interesting that transplanted cells can produce neurons only in the dentate gyrus of the hippocampus, though even there adult NSCs are surrounded by the glial cells absent at embryonic stages such as mature oligodendrocytes, NG2-glia and astrocytes [13]. This phenomenon is associated with the specialized mechanisms of suppression of gliogenesis due to activation of the BMP signaling pathway [13, 77]. In addition, it has been shown that the transplantation of neurogenically active cells into the CNS zones, where natural neurogenesis does not occur in adults, leads to impaired differentiation of transplanted cells, so that they either remain stem cells or are differentiated into glia [78]. The researchers who have demonstrated a dramatic decrease in expression of proneurogenic transcription factors such as PAX6, ASCL1 and NGN2 after transplantation in vivo from high activity in vitro have arrived at the same conclusion [79, 80].

It is interesting that the embryonic radial glia originally has an enhanced level of expression of neurogenic transcription factors compared to the glia in the adult brain [9]. NSCs in the adult brain are capable of neurogenesis only via intermediate forms and without additional transcriptional support at the subsequent stages can change direction to gliogenesis [81]. It has been shown that the factors regulating neurogenesis at the early stages of development, such as PAX6, GSX2, DLX, ASCL1, NGN2 and NEUROD1, are also involved in the regulation of adult neurogenesis [9, 82]. A significant difference is that the embryonic radial glia is originally disposed to direct neuronal differentiation, while adult NSCs require a series of inter-

mediate divisions to produce the necessary level of proneural transcription factors [81, 83–85].

One more substantial difference between adult and embryonic neurogenesis is related to the peculiarities of cell cycle regulation. For example, the NSC cycle in the embryonic period lasts from 10 to 18 h, as it is necessary to produce a rather large volume of nerve tissue within a short period of time, while the cell cycle of radial glial cells in the dentate gyrus, SVZ and SGZ of the adult brain takes from several days to two weeks [86–88].

The data presented above lead to the conclusion that adult NSCs are a peculiar cell pool distinguished at the early stages of development, which performs it own functions and has its own proliferation and differentiation mechanisms. The key feature of NSCs in the niche of stem cells of the adult brain is the preferential disposition to gliogenesis but not to neuronal differentiation [13, 89]. This disposition and "gliogenicity" of the microenvironment of the adult brain should be taken into account in the development of all neuroregeneration technologies including neuronal differentiation.

PRONEURAL TRANSCRIPTION FACTORS IN REPROGRAMMING

In contrast to the iPSC technology for obtaining pluripotent cells analogous to embryonic stem cells (ESC), direct reprogramming can produce both progenitor cells and neuroblasts or neurons at different stages of maturation [2, 90, 91]. Based on the modern concepts of embryonic and adult neurogenesis, we have obtained an ontogenetic series of CNS cells (from pluripotent ESCs to specialized neurons) and attempted to systematize the published protocols of reprogramming in the context of correspondence of resultant cells to some particular positions in the ontogenetic series (Fig. 2, Table 1).

Among transcription factors playing key roles in embryonic and adult neurogenesis, master regulators such as SOX2 [142] and PAX6 [43] are used for neural reprogramming. The latter, in turn, regulates the expression of the *NEUROG2* gene, its protein product NGN2 being actively involved in the regulation of NSC proliferation [143]. One more general condition for successful neural reprogramming, in addition to the expression of SOX2, PAX6 and NGN2, is suppression of the function of the transcription repressor REST (RE1-silencing transcription factor), also known as neuron-restrictive silencer factor (NRSF). The *REST* gene product suppresses the expression of neurospecific genes by attracting other corepressors: mSin3A/B [144], N-CoR, CtBP [145] or CoREST, blocking the promoters of coding and noncoding target genes [43]. As a negative master regulator of neurogenesis, REST plays the fundamental role both in maintaining the resting NSC pool and in post-mitotic neurons, where it regulates the expression of the genes important for synaptic plasticity. REST is active in all non-neural cells and tissues, where it also suppresses the expression of neurospecific genes. This is precisely why the proneural reprogramming of somatic cells is impossible without the inactivation of this suppressor [144].

In addition to the above-mentioned master factors such as SOX2 and PAX6, there are many genes or cofactors under their control, which are involved in neurogenesis, that are potential reprogramming factors. Analysis of the published data has shown that the factors such as MSI1 (Musashi RNA binding protein 1), ASCL1, BRN2, neurogenins, NEUROD1, MYT1L (myelin transcription factor 1 like), GSX2, DLX, as well as microRNA and small molecules, are also used in different techniques of direct reprogramming. Let us describe these factors more thoroughly.

Transcription factor SOX2

SOX2 (SRY-box 2) is a multifunctional transcription master factor involved in the maintenance of stem cell proliferation and pluripotency, the development of the nervous system, and many other processes in the body [146, 147]. Some studies have shown that the level of *SOX2* gene expression in ESCs is strictly regulated and minor changes lead to significant variations in the direction of differentiation, because SOX2, as opposed to OCT4, is an active participant of mesenchymal–epithelial transition [148, 149]. SOX2 also prevents cell cycle exit and differentiation of NSCs [150, 151]. It has been shown that the *SOX2* knockout considerably reduces the activity of type I cell proliferation in the SGZ. Interestingly, some neurons and glial cells maintain enhanced expression of *SOX2*, which is necessary for their functioning [152]. SOX2 directly regulates the expression of the SHH pathway and simultaneously suppresses the expression of *NEUROD1* and *WNT* in order to maintain the self-renewal ability of NSCs [153]. Moreover, SOX2 controls the expression of the nuclear receptor TLX, which in turn maintains the proliferation and self-renewal of adult NSCs [43].

Bergsland et al. [154] have shown that SOX2 proteins trigger the genetic program of the neural profile in cells by activating neuronal enhancers and proneural genes. In particular, SOX2 expression was related to activation of the following genes: *SOX1*, *SOX3*, *OLIG2* (oligodendrocyte transcription factor 2), *NCAM* (neural cell adhesion molecule), *PAX6*, etc. Thus, the sequential coordination of neuronal differentiation from stem cells can be initiated by the expression of *SOX2*, which confirms its role as a master gene.

In embryonic neurogenesis, SOX2 is expressed in developing cells of the nerve tube and in proliferating progenitors of nerve cells. In addition to the fact that SOX2 is a component of the well-known "Yamanaka cocktail", which is necessary for iPSC generation [1],

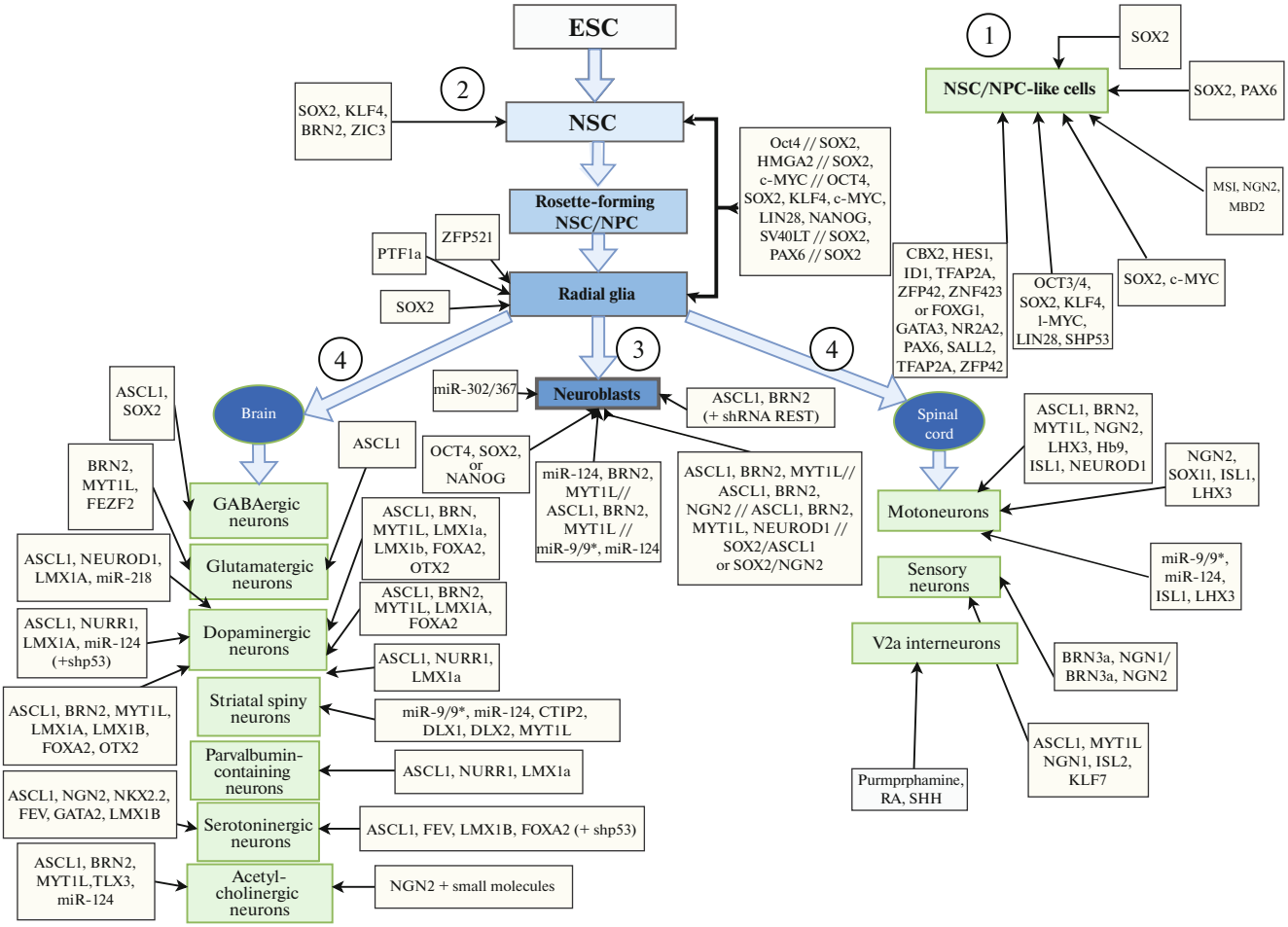

Fig. 2. The ontogenetic series of cells from pluripotent ESC to specialized neurons and the direct reprogramming protocols. The arrows show the correspondence of terminal cell types obtained by reprogramming to particular positions in the ontogenetic series. The specified protocols make it possible to obtain a mixed/heterogenous population of NSCs, NPCs, TAP cells, neuroblasts (1); characterized NPCs (2); predifferentiated neurons (3); and terminally differentiated neurons of the brain and the spinal cord (4). Designations: ESC, embryonic stem cell; TAP, transit-amplifying progenitors; NPC, neural progenitor cell; NSC, neural stem cell.

it is actively used in drNPCs. For example, under certain cultivation conditions, *SOX2* expression induced the transformation of mouse and human fibroblasts into NSCs [104, 155]. It has been shown that SOX2 is one of the few transcription factors, the induction of which is necessary and sufficient for the direct reprogramming of mouse astrocytes [156, 157]. In fact, the same results were obtained by another research team [104] using the transformation of cortical NG2-glia into neurons with the involvement of a retroviral vector with the *SOX2* gene. Ring et al. [104] have shown that the ectopic expression of *SOX2* is sufficient for reprogramming not only mouse but also human embryonic fibroblasts into NSCs. It seems that SOX2, being the primary transcription factor, is able to interact with locally untwisted DNA regions [104, 158]. SOX2 turned out to stimulate proneural induction through interaction with another primary factor: ASCL1, as well as PAX6 and NR2E1 (nuclear receptor subfamily 2 group E member 1) [159, 160]. Karow et al. [161] succeeded in reprogramming the cells of the primary culture of human pericytes into induced neurons using the SOX2 and ASCL1 factors. Lujan et al. [162] have shown that lentiviral transduction of the SOX2, BRN2 and FOXG1 (Forkhead box protein G1) factors is sufficient for conversion of mouse embryo fibroblasts into induced neural progenitor cells, with the potential of further differentiation into neurons, astrocytes and oligodendrocytes. Interestingly, the SOX2 and FOXG1 combination resulted in the formation of induced neural progenitor cells that gave rise to astrocytes and functional neurons. Separately, transcription factors FOXG1 and BRN2 also generated neural progenitor cells, but the formed neurons proved to be less mature compared to the effect of SOX2.

Another research team obtained NPCs from adult mouse fibroblasts using a combination of five transcription factors: SOX2, BRN2, TLX, c-MYC and

Table 1. The list of direct reprogramming protocols for Figure 2

Target cell type	Reprogramming factors	Literature source
NSC	SOX2, KLF4, BRN2, ZIC3	Thier M.C. et al. [92]
	OCT4	Zhu S. et al. (2014) [93]
	SOX2, HMGA2	Yu K.R. et al. (2015) [94]
	SOX2, c-MYC	Sheng C. et al. (2018) [95]
	OCT4, SOX2, KLF4, c-MYC, LIN28, NANOG, SV40LT	Cheng L. et al. (2017) [96]
	SOX2, PAX6	Connor B. et al. (2018) [97]
	SOX2	Kim B.E. et al. (2018) [98]
NSC/NPC-like cells	CBX2, HES1, ID1, TFAP2A, ZFP42, ZNF423 or FOXG1, GATA3, NR2A2, PAX6, SALL2, TFAP2A, ZFP42	Hou P.S. et al. (2017) [99]
	OCT3/4, SOX2, KLF4, l-MYC, LIN28, shp53	Capetian P. et al. (2016) [100]
	SOX2, c-MYC	Giorgetti A. et al. (2012) [101]
	SOX2, c-MYC	Castaño J. et al. (2016) [102]
	MSI, NGN2, MBD2	Ahlfors J.-E. et al. (2019) [2]
	SOX2, PAX6	Maucksch C. et al. (2012) [103]
	SOX2	Ring K.L. et al. (2012) [104]
Radial glia	ZFP521	Shahbazi E. et al. (2016) [105]
	PTF1a	Xiao D. et al. (2018) [106]
	SOX2	Mirakhori F. et al. (2015) [107]
Neuroblasts	miR302/367	Ghasemi-Kasman M. et al. (2015) [108]
	OCT4, SOX2 or NANOG	Corti S. et al. (2012) [109]
	miR124, BRN2, MYT1L	Yoo A.S. et al. (2011) [110]
	miR124, BRN2, MYT1L	Ambasudhan, R. et al. (2011) [111]
	miR-124, ASCL1, BRN2, MYT1L	Lau S. et al. (2014) [112]
	miR9/9*, miR124	Huh C.J. et al. (2016) [113]
	ASCL1, BRN2, MYT1L	Torper O. et al. (2013) [79]
	ASCL1, BRN2, MYT1L	Pereira M. et al. (2014) [114]
	ASCL1, BRN2, MYT1L	Vierbuchen T. et al. (2010) [115]
	ASCL1, BRN2, NGN2	Meng F. et al. (2012) [116]
	ASCL1, BRN2, NGN2	Mertens J. et al. (2015) [117]
	ASCL1, BRN2, MYT1L, NEUROD1	Pang Z.P. et al. (2011) [118]
	ASCL1, BRN2, MYT1L, NEUROD1	Matsuda T. et al. (2019) [119]
	SOX2/ASCL1 or SOX2/NGN2	Araújo J. et al. (2018) [120]
	ASCL1, BRN2 (+ shRNA REST)	Drouin-Ouellet J. et al. (2017) [121]

Table 1. (Contd.)

Target cell type	Reprogramming factors	Literature source
GABAergic neurons	ASCL1, SOX2	Karow M. et al. (2012) [122]
Glutamatergic neurons	BRN2, MYT1L, FEZF2	Miskinyte G. et al. (2017) [123]
	ASCL1	Chanda S. et al. (2014) [124]
Dopaminergic neurons	ASCL1, NEUROD1, LMX1A, miR218	Rivetti di Val Cervo et al. (2017) [125]
	ASCL1, NURR1, LMX1A, miR124 (+shp53)	Jiang H. et al. (2015) [126]
	ASCL1, BRN2, MYT1L, LMX1A, LMX1B, FOXA2, OTX2	Pereira M. et al. (2014) [114]
	ASCL1, BRN2, MYT1L, LMX1A, LMX1B, FOXA2, OTX2	Torper O. et al. (2013) [79]
	ASCL1, BRN2, MYT1L, LMX1A, FOXA2	Pfisterer U. et al. (2011) [127]
	ASCL1, NURR1, LMX1a	Caiazzo M. et al. (2011) [128]
Striatal medium spiny neurons	miR-9/9*, miR-124, CTIP2, DLX1, DLX2, MYT1L	Victor M.B. et al. (2014) [129]
Parvalbumin-containing interneurons	ASCL1, NURR1, LMX1a	Pereira M. et al. (2017) [130]
Serotoninergic neurons	ASCL1, NGN2, NKX2.2, FEV, GATA2, LMX1B	Vadodaria K.C. et al. (2016) [131]
	ASCL1, FEV, LMX1B, FOXA2 (+ shp53)	Xu Z. et al. (2016) [132]
Acetylcholinergic neurons	ASCL1, BRN2, MYT1L, TLX3, miR-124	Liang X.G. et al. (2018) [133]
	NGN2 + small molecules	Liu M.L. et al. (2013) [134]
Motor neurons	ASCL1, BRN2, MYT1L, NGN2, LHX3, Hb9, ISL1, NEUROD1	Son E.Y. et al. (2011) [135]
	NGN2, SOX11, ISL1, LHX3	Liu M.L. et al. (2016) [136]
	miR-9/9*, miR-124, ISL1, LHX3	Abernathy D.G. et al. (2017) [137]
	NGN2, SOX11, ISL1, LHX3	Tang Y. et al. (2017) [138]
Sensory neurons	BRN3a, NGN1 or BRN3a, NGN2	Blanchard J.W. et al. (2015) [139]
	ASCL1, MYT1L, NGN1, ISL2, KLF7	Wainger B.J. et al. (2015) [140]
V2a-interneurons	Purmprphamine, RA, SHH	Brown C.R. et al. (2014) [141]

BMI1 (B lymphoma Mo-MLV insertion region 1 homolog) [163]. The properties of resultant cells were analogous to those of primary NPCs, including the level of proliferation and self-renewal and the efficiency of differentiation. Han et al. [164] investigated the possibility of direct reprogramming of mouse fibroblasts into neural cells and showed that the combination of the major factors (SOX2, c-MYC and KLF4 (Kruppel like factor 4)) and two supplementary factors (BRN4 and E47) provided successful reprogramming. However, differentiation of the resultant NPCs into oligodendrocytes was impaired. Finally, Maucksch et al. [103] have shown that the combined ectopic expression of *SOX2* and *PAX6* with nonviral delivery induces the transformation of adult human fibroblasts into neural progenitor cells. The resultant NPCs expressed the markers of neural precursor cells and differentiated into functional neurons and astrocytes but not oligodendrocytes.

Transcription Factor PAX6

PAX6 (aniridia type II protein) is a tissue-specific transcription factor, it is one of the coordinating genes for eye and nervous system development in the embryonic period. The *PAX6* gene is expressed in NSCs and at the initial stages of embryonic neurogenesis, forming the pluripotent potential of cells. During differentiation and maturation, *PAX6* expression is inhibited by some microRNAs, in particular, miR-7a [165, 166], because the continuous expression of *PAX6* blocks differentiation [167]. However, in adult neurogenesis, this regulatory mechanism functions differently, providing enhanced expression in NPCs and

limited expression in NSCs with the involvement of the same miR-7a [166].

PAX6 performs numerous functions in the developing brain: it regulates the cell cycle, neurogenesis and gliogenesis, forms spatiotemporal patterns, and even triggers the generation of specialized subtypes of neurons during interaction with NGN2 [167, 168]. PAX6 triggers expression of the genes necessary for proliferation and proneural differentiation: *HMGA2* (high mobility group AT-hook 2), *CDK4* (cyclin-dependent kinase 4), *GADD45G* (growth arrest and DNA damage inducible gamma), *NEUROD1, SSTR2* (somatostatin receptor *2*) and *HES6* (HES family BHLH transcription factor 6) [152]. In addition, PAX6 regulates the expression of the *NEUROG2* gene, while its protein product NGN2 actively participates in the regulation of NSC proliferation and differentiation [153].

It is interesting that quite a number of analogous mechanisms are triggered also in the adult brain of mammals in response to traumatic death of neurons in vivo [80, 169]. It has been shown that PAX6 functions in the adult mammalian brain only together with the BRG1 (Brahma-related gene-1)-containing BAF complex of the SWI/SNF family of chromatin remodeling factors [81]. This is apparently determined by the ability of PAX6 to bind only with the free DNA prevalent in the embryonic period but absent in the adult brain. PAX6 and BRG1-containing BAF complex form a heterodimer and activate the regulatory network of three neurogenic factors: BRN2, SOX4/11 and NFIA/B (nuclear factor 1 A/B-type) [81]. It should be noted that these mechanisms do not work in the embryonic brain, because in the latter, as already mentioned, free chromatin is prevalent and neurogenesis occurs in the neurogenic medium that does not require additional proneural support [169, 170]. Thus, it seems that only in the adult brain the activation of PAX6 is associated with massive chromatin remodeling for activation of the underlying regulatory network.

There are not as many examples of direct reprogramming with the involvement of PAX6 as would be desirable, but several research teams have succeeded in obtaining functional neurons from glial cells (astrocytes and oligodendrocyte progenitors) using the forced expression of this factor [81, 171, 172] (Fig. 2).

Transcription Factor MSI1

MSI1 (RNA-binding protein Musashi homolog 1) binds RNA via two conservative tandem motifs. This protein is permanently expressed in NSC/progenitor cells: from the embryonic [173] to the adult stage [174]. It is necessary to maintain the stem properties of NSCs, probably through intensification of NOTCH signal transduction via the repression of mRNA m-NUMB translation [175]. Mutation in the *MSI1* gene results in the development of autosomal recessive primary microcephaly. In the adult mammalian brain, MSI1 is present in ependymal cells, subependymal cells and astrocytes, in the niches of stem cells, but not in the microglia, oligodendrocytes, or mature neurons [173, 174]. It has been shown that cluster-forming proliferating cells in the SVZ after acute or chronic ischemia, or focal forebrain ischemia, express the *MSI1* gene but do not express *GFAP* [176].

In view of the fact that *MSI1* gene expression is typical only of NSCs, its application as a direct reprogramming factor is rather limited, though in some works *MSI1* was included in reprogramming cocktails [2]. The primary transcription factors MSI1 and NGN2 and secondary BRN2 were used by the authors to convert pluripotent mesenchymal human stem cells into viable functional NSCs positive to the major markers (SOX2, NESTIN, β-III-TUBULIN and GFAP) and differentiate them into neurons, astrocytes and oligodendrocytes [2].

Transcription Factor ASCL1

ASCL1 (the *MASH1* gene product) belongs to the bHLH ("helix-loop-helix") family. The successful binding of ASCL1 to DNA requires dimerization with other bHLH proteins. ASCL1 plays a key role in neuronal differentiation and in the induction of olfactory and vegetative neurons [157, 177]. The differences in ASCL1 functions between embryonic neurogenesis and neurogenesis in the adult brain are very interesting. For example, the reduced activity of ASCL1 in the embryonic period does not lead to any serious consequences, but the inhibition of ASCL1 in the cells of the dentate gyrus of the adult brain results in almost complete cessation of neurogenesis [178]. The ectopic expression of *MASH1* is sufficient for the stimulation of neuronal differentiation of embryonic stem cells, NSCs and early postnatal astroglial cells [124]. Similar to SOX2, ASCL1 is the primary transcription factor, so it initially does not need any cofactors [179, 180]; however, the reprogramming only by ASCL1 proved to be efficient for mouse but not human somatic cells [161]. The direct reprogramming of human somatic cells requires at least two transcription factors, e.g., SOX2 and ASCL1 [161]. However, the findings of the research team headed by M. Werning [124] cast doubt on this fact: they have succeeded in obtaining TUJ1- and MAP2-positive induced neurons from embryonic and postnatal fibroblasts using only the ASCL1 factor, though with much lower efficiency compared to mouse cells. Based on the above, the authors have come to the conclusion that ASCL1 is one of the necessary and sufficient factors for direct proneural reprogramming. Their further experiments with single-cell RNA sequencing have shown [181] that the overexpression of proneural primary factor ASCL1 results in well-defined initialization, forcing the cells to exit from the cell cycle and to refocus gene expression in the proneural direction through activation of

the genes of the underlying signaling cascade. At first, the primary gene response is quite homogeneous; however, later on the competing myogenic program decreases reprogramming efficiency. It is interesting that the FOXO3 (forkhead box O3) factor, which plays an important role in the negative control of neurogenesis in the embryonic and postnatal development of the brain, also has common target genes with ASCL1 and inhibits the ASCL1-dependent neurogenesis [182, 183]. Successful direct reprogramming of umbilical cord multipotent mesenchymal stem cells into functional neurons was performed using a cocktail of three transcription factors: ASCL1, SOX2 and NGN2 [120]. In combination with Brn2/BRN2 and Myt1L/MYT1L or with Lmx1a/LMX1A and Nurr1/NURR1 (nuclear receptor related 1 protein), Ascl1/ASCL1 induced the neural conversion of mouse and human fibroblasts in culture [115, 128, 179]. The resultant cells were denominated as induced neural cells. These two combinations of three transcription factors also directly converted astrocytes and NG2-glial cells into neurons in the striate body of an adult mouse, through these neurons were neither DARPP32 (dopamine- and cAMP-regulated neuronal phosphoprotein)-positive striatal projection neurons nor dopaminergic neurons [184]. A very similar cocktail of factors was used by M. Werning's team [3] to obtain functional induced neurons from human blood cells. This is a combination of the BRN2, ASCL1, MYT1L and NGN2 (BAMN) factors. They also demonstrated the possibility of converting mouse functional hepatocytes into induced neurons using the Brn2 (BRN2), Ascl1 (ASCL1) and Myt1L (MYT1L) factors [185]. Though Ascl1 (ASCL1) only was sufficient for the generation of induced neurons, exogenous Myt1L (MYT1L) considerably increased reprogramming efficiency and functional maturation of the cells obtained [186]. Some research groups reported that the evident neural induction by ASCL1 was observed only when transduction was performed with an adenoviral vector [80, 187]; when using an analogous construct with a lentiviral or retroviral vector, ASCL1 stimulated gliogenesis [187].

Transcription Factor BRN2

BRN2 (the *POU3F2* gene product) is a POU-III neural transcription factors and is expressed in postmitotic pyramidal neurons of cortical layers II, III and V as well as in progenitor cells of the SVZ, playing a key role in adult neurogenesis [188, 189]. BRN2 binds to a recognition sequence consisting of two separate subdomains: GCAT and TAAT with a nonconservative spacer region of 0, 2 or 3 nucleotides in between [190]. BRN2, together with other transcription factors, regulates a number of genes responsible for neurogenesis, e.g., *Delta1* encoding the NOTCH ligand [191]. It is involved in the development of the neocortex in mice and is associated with a single nucleotide polymorphism rs1906252, which determines the cognitive phenotype, i.e., the rate of information processing [192]. It has also been shown that BRN2, together with BRN1, regulates the radial migration of postmitotic neurons, and their loss results in the laminar inversion of cerebral cortex [193]. Moreover, BRN1/2 is necessary for the migration of layer V cells and for the factual production of layer II–IV cells [194], though the mechanisms of this process are still nuclear. In addition to proneural differentiation, BRN2 plays an important role in the development of Schwann cells [195]. The role of BRN2 in neurogenesis is confirmed by the fact that it can be used for reprogramming fibroblasts into neurons in vitro in combination with the expression of *MASH1* and *MYT1L* [115, 179, 184]. In spite of the crucial role of BRN2 in neurogenesis, its target genes and method of action have as yet been little studied. In view of the fact that BRN2 is not a primary transcription factor, it is incapable of independent reprogramming of somatic cells: it needs a "guide" to interact with DNA [179].

Neurogenins

Neurogenins, similar to ASCL1, are transcription factors containing the bHLH structural motif. This family includes 3 factors: neurogenin-1 (NGN1), neurogenin-2 (NGN2) and neurogenin-3 (NGN3). Among them, only the former two are involved in the development of the nervous system. NGN1 is present in the cerebral cortex and stimulates differentiation due to binding with its cofactor complex CBP/p300/SMAD1 [196]. Just as ASCL1, neurogenins bind to DNA by forming a dimer with another bHLH-type factor. The CBP/p300/SMAD1 coactivator complex is related to the activity of the BMP signaling pathway; therefore, in the presence of NGN1, BMP stimulates neuronal differentiation. At the same time, NGN1 indirectly blocks the differentiation of astrocytes due to isolation of the CBP/p300/SMAD1 complex from DNA of the genes involved in gliogenesis, such as STAT transcription factors. In the embryonic forebrain, NGN1 in combination with NGN2 and PAX6 is associated with dorsal pattern formation and neuronal specification.

NGN2 activates proneural gene expression and controls neuronal specification by inhibiting the expression of glial genes in NSCs [196]. Interestingly, transcription factor OLIG2 stimulates the expression of NGN2 in NSCs, though both NKX2.2 (homeodomain transcription factors 2.2) and OLIG2 per se stimulate gliogenesis [197]. It is believed that NGN2 is involved in the specification of motor neurons and ventral interneurons [198]. NGN2 is one more primary transcription factor and, in combination with other factors or small molecules, can reprogram human fibroblasts into cholinergic neurons in vitro [2, 120] and human blood cells into induced neurons [184]. The retroviral expression of NGN2 in proliferating cells induced the formation of neurons in the spi-

nal cord, the striatum and the cortex [58, 163]. Such neural induction in vivo can be additionally enhanced by growth factors or via joint expression with BCL2 [199]. This procedure resulted in the formation of mainly glutamatergic pyramidal neurons in the cerebral cortex [163] or GABAergic but DARPP32-negative neurons in the striated body [80].

Transcription Factor NEUROD1

NEUROD1 (the transcription neurogenic differentiation factor 1) also belongs to the bHLH structural family. Just as NGN1, NEUROD1 binds to the CBP/p300 coactivator complex, promoting the regulation of several cell differentiation pathways in the nervous system, including those involved in the formation of early retinal ganglion cells, inner ear sensory neurons and granular cells forming either the layer of the cerebellum or the layer of the dentate gyrus of the hippocampus [200]. NEUROD1 is also necessary for morphogenesis and dendrite maintenance in the cerebellar cortex [200]. Ectopic expression by a retroviral vector with the *NEUROD1* gene made it possible to reprogram the cortical astrocytes into glutamatergic neurons and the NG2-glial cells into glutamatergic and GABAergic neurons [125, 201]. These converted neurons gave spontaneous and evoked synaptic responses, which indicated their integration into local neural circuits. M. Werning's team [115] used the NEUROD1, ASCL1 and LMX1A transcription factors, as well as microRNA miR-128, for reprogramming human astrocytes in vitro and mouse astrocytes *in vivo* into dopaminergic neurons. It should be noted that reprogramming efficiency increased under the influence of low-molecular weight compounds, which promoted chromatin remodeling and activation of the TGF-β, SHH and WNT signaling pathways. They also demonstrated the possibility of direct reprogramming of fetal and postnatal human fibroblasts into induced neurons using a cocktail of the BRN2, ASCL1, MYT1L and NEUROD1 factors [118].

Transcription Factor GSX2

GSX2 (GS homeobox 2, the *GSX2* gene product) is one of the transcription factors binding the DNA 5'-CNAATTAG-3' sequence and responsible for ventralization of the telencephalon during embryonic development, where it forms the early specification of progenitors of the lateral ganglionic eminence (LGE), the medial ganglionic eminence (MGE) and the ventricular septum [34] and, depending on the stage of development, determines neuronal specification. *GSH2* is necessary for the development of striatal projection neurons and olfactory bulb interneurons, which are the two major derivatives of the LGE [202]. Similar to *PAX6* and *EMX2* (empty spiracles homeobox 2) in the dorsal hindbrain, the *GSX2* gene is necessary not only for the formation of patterns of LGE precursors, but also for the control of their proliferation [203]. *GSX2* can be expressed in several regions. The area with the highest *GSX2* expression determines the dorsal region of LGE, which is the main source of interneurons, while the more ventral region of LGE is responsible for the production of neurons of the striated body [82]. An analogous situation is observed in distribution of the GSX2 expression in the dentate gyrus of the adult brain: the dorsolateral region is more often enriched in GSX2-positive NSCs [82]. It is interesting that a small area of PAX6-positive cells is localized more dorsally than GSX2-positive NSCs in the dentate gyrus and, in the central area of reduced GSX2 expression, its closest homolog GSX1 and NKX2.1 are expressed. Thus, 4 different subdomains are formed in the dentate gyrus of the adult brain: PAX6-positive, PAX6/GSX2-positive, GSX1-positive lateral and medial subdomains, and GSX1/NKX2.1-positive ventral subdomain [37, 82]. It has been shown that the inactivation of PAX6 and GSX2 results in the loss of particular subsets of neurons, specifically tyrosine hydroxylase-positive and calretinin-glomerular neurons, demonstrating the significance of the regional identity of NSCs in both the embryonic and adult brain [9, 82, 204]. However, GSX2 inactivation in the embryonic brain results in compensatory expansion of the zones of the PAX6-positive dorsal domain and the GSX1-positive ventral domain, which is not observed in the adult brain [82]. In addition, in the case of GSX2 inactivation, the proportion of lost populations of neurons in the adult brain is much less than in the embryonic brain, suggesting a limited role of GSX2 in the adult brain [82]. In regenerative medicine, it is very important that GSX2 participates in specific regulation of neurogenesis in response to brain injury in adult mammals. It has been shown that the expression of *GSX2* in the dentate gyrus during postischemic neurogenesis affects the subdomains where it is normally absent, while the targeted inhibition of the *GSX2* expression in NSCs of the dentate gyrus stops posttraumatic neurogenesis [82]. One more difference between *GSX2* expression in the embryonic and adult brain is the limited distribution of *GSX2* among NSC subpopulations of the dentate gyrus: in the embryonic brain, *GSX2* expression lasts until the final stages, while in the adult brain it is restricted by transition from NSCs to neuroblasts via the stage of transit-amplifying progenitor cells [82]. Interestingly, at this stage GSX2 almost simultaneously both activates and blocks further neurogenesis, i.e., for NSCs to be transferred into transit-amplifying progenitor cells and neuroblasts, it is necessary first to activate and then to inhibit the expression of this transcription factor. It is supposed that the basic role of GSX2 in the adult brain is the involvement of NSCs in the cell cycle.

Transcription Factor DLX

Transcription factors of the DLX family (DLX1, DLX2, DLX5 and DLX6) regulate differentiation of NSCs in the preoptic area into GABAergic neurons [205]. DLX1 and DLX2 are functionally excessive, because their separate inhibition does not lead to serious impairments in differentiation, but simultaneous knockout of their genes considerably reduces the efficiency of formation of GABAergic neurons [206]. In relation to GSX2, the DLX1 and DLX2 factors are involved in the underlying signaling cascade, and their expression depends on the level of GSX2. Thus, during the transition from transit-amplifying progenitor cells to neuroblasts, the expression of *GSX2* is replaced exactly by the expression of *DLX*. In addition, the ectopic expression of *GSX1* or *GSX2* can induce the steady-state expression of *MASH1* and *DLX* in many areas of the hindbrain [207]. A similar relationship between *GSX1* and *GSX2*, *MASH1* and *DLX* in the ascending and descending directions has been found in the adult human brain. The expression of *MASH1* and *DLX* decreases in the dorsolateral area of the dentate gyrus during the inhibition of GSX2 [82]; conditional inactivation of ASCL1 and DLX leads to a serious loss of neurogenesis in adult NSCs [178]. In general, these results show that the *GSX2/MASH1/DLX* genetic cascade is involved in the coordination of neurogenesis in the embryonic and adult brain. The studies on direct reprogramming and the role of ASCL1 in the latter have shown that activation of the complex of underlying genes *ZFP238*, *SOX8* and *DLX3* is the critical point of function of the ASCL1 gene network [181].

Transcription Factor MYT1L

The primary transcription factor MYT1L (myelin transcription factor 1 like) is an ontogenetic repressor of REST (RE1-silencing transcription factor) and Groucho (transducin-like enhancer; TLE) blocking the activation of proneural gene networks. The repressor function of MYT1L is mediated by recruiting the SIN3B-containing complex through binding to the previously uncharacterized N-terminal domain [186]. In accordance with its repressor function, the MYT1L binding sites are similar in neurons and fibroblasts, being mostly in an open chromatin configuration. MYT1L inhibits the Notch signal transduction pathway by suppressing the expression of several of its members including HES1 [186]. The *MYT1L* knockdown in the developing mouse brain simulates the phenotype mediated by activation of the Notch pathway, confirming that MYT1L allows embryonic neurons to avoid activation of the Notch pathway during normal development. The depletion of MYT1L in the primary postmitotic neurons activates non-proneural programs and impairs the expression and function of neuronal genes [186]. It has been described above how MYT1L can be used in direct reprogramming, where it acts as a primary transcription factor modeling chromatin configuration and suppressing the myogenic programs of the cell [180, 185–187].

MicroRNA

In addition to transcription factors, microRNA is also used for direct proneural reprogramming. This is a class of noncoding RNAs, 20–25 nucleotides in length, which regulate the stability and translation of their mRNA target through binding to its 3'-untranslated region (UTR) or to the coding sequence [208]. MicroRNAs regulate various biological processes at all stages of development, and many different microRNAs are involved in neurogenesis. Among them, there is miR-19 that stimulates NSC proliferation and the expansion of radial glial cells in embryonic neurogensis [208]. The miR-17-92 cluster, on the contrary, inhibits the expression of *TBR2,* preventing the conversion of radial glial cells into neuronal intermediate progenitor cells [209]. The molecules miR-184, miR-let-7b, miR-137, miR-9 and miR-124 that are discussed below have a regulatory effect on neurogenesis in adults by targeting different neuronally expressed genes [210–213].

miR-184 is expressed under the influence of MBD1 (methyl-CpG-binding domain protein 1), which can modify chromatin structure by interacting with SETDB1, histone-lysine-N-methyltransferase. Enhanced expression of miR-184 decreases the level of MBD1 mRNA with formation of a negative feedback loop, which promotes proliferation and inhibits differentiation of NSCs [213]. The known target of miR-184 is the *NUMB* gene playing a key role in the function of embryonic NSCs and the development of the cortex [214]. On the contrary, miR-let-7b contributes to the suppression of proliferation and stimulates neuronal differentiation via interaction with the *TLX* and *CCND1* genes, the latter coding for cyclin D1 [212].

miR-137 is expressed in the brain and promotes NSC differentiation via the regulatory loop with transcription corepressor TLX by decreasing the level of mRNA of lysine-specific histone-demetylase-1 (LSD1), which in turn suppresses the transcription of miR-137 [211]. It is known that MSI1 and miR-137 have the opposite effects on cells [215]. It has also been shown that miR-137 dysfunction promotes the development of some types of human cancer such as neuroblastoma [216] and glioblastoma multiforme [217]. It has been shown that miR-137 can directly target histone-demetylase JARID1B (the *KDM5B* gene), which results in differentiation of mouse ESCs [218]. miR-137 is expressed in the mitotic phase of the cell cycle and is strongly activated during ESC differentiation into neural cells [219]. This activation leads to the repression of two ESC transcription factors: KLF4 and TBX3, which are exposed to the direct effect of miR-137. The opposite effect of miR-137 on the modulation of proliferation and differentiation has been shown in NSCs of the adult brain. For example, miR-137 intensifies proliferation and inhibits differentiation via posttran-

scriptional suppression of *EZH2* [220]. Based on these results, it would be logical to assume that the function performed by miR-137 is determined by context. Interestingly, the presence of at least one functional allele of miR-137 is important for normal embryonic development [221]. In addition, miR-137 is involved in the development of many mental disorders such as schizophrenia, major depressive disorder, Huntington's disease [221], etc.

miR-9 is one of the most highly expressed ancient microRNAs in the developing and mature brain of vertebrates [222, 223]. It is a universal multi-type microRNA regulating quite different processes. Its expression levels are dynamically regulated during brain development and during induced in vitro neurogenesis [224]. For the overwhelming majority of microRNAs, only one strand (5' or 3') is retained after association of their duplexes with the RISC complex; in the case of miR-9, the guide strand can be generated from either the 5'- (miR-9-5p/miR-9) or 3'-end (miR-9-3p/miR-9*) depending on the gene under consideration [225]. The expression of miR-9 occurs for the first time in the middle of embryogenesis, after specialization of the major subregions of the brain, first in the hindbrain and then spreading to more caudal regions of the brain and the spinal cord. In the entire CNS, the expression of miR-9 is associated mainly with the areas of ventricular NSCs [226], though some neurons also express miR-9, especially in the dorsal hindbrain and the spinal cord [224]. miR-9 expression determines active neurogenic areas and is regulated by the Notch signaling cascade [226]. Experiments in vitro have shown that miR-9/9* promotes differentiation of adult NSCs, but only together with small forskolin or RA molecules [227]. In addition, there is a possibility of direct reprogramming of human fetal fibroblasts into postmitotic neurons by lentiviral vectors containing miR-9/9* and miR-124 via the activation of NEUROD2. However, this conversion depends on expression of all three microRNAs [110]. Surprisingly, the inhibition of miR-9 induces enhanced proliferation of embryonic NSCs [226] or NSCs of adult mice [227] though followed by the resumption of differentiation [226], suggesting the facultative control of miR-9 over NPCs and NSCs. It has been reported that miR-9 can perform the opposite functions depending on the cellular context, which is probably related to the differential expression of mRNA targets and the synergism between miR-9 and other mRNA-regulating factors [224]. miR-9 has a lot of mRNA targets, including the *HES* genes, which are the main effectors of Notch signal transduction and the inhibitors of differentiation via the repression of proneural genes such as *MASH1* [228]. Some other targets of miR-9 also control NPC proliferation. They include transcription factors FOXG1 [224], GSX2 [224], TLX/NR2E1 [229] and ZIC5 [229]. miR-9 and TLX form a negative feedback loop in order to promote premature differentiation of neurons [229, 230]. It is interesting that miR-9 is also involved in the remodeling of the microRNA landscape in nerve cells, for example by inhibiting the pluripotent factors LIN28A and LIN28B, which are RNA-binding proteins that block the processing of some microRNAs including miR-let-7 [230]. miR-9 stimulates neuronal differentiation by inhibiting the expression of proliferation factors and progenitor-specific epigenetic factors [226]. The expression of miR-9 can be also detected at the later stages of cell development. For example, miR-9 is temporally expressed during the differentiation of spinal cord motor neurons localized in the lateral motor column and innervating the muscles of the limbs. This effect of miR-9 is due to its interaction with its targets: *FOXP1* and *ISL1/2*, which are expressed in motor neurons [231]. In addition, it has been shown that miR-9 is involved in the maturation of cortical neurons: its expression has been detected in the axons and dendrites of different neurons [224].

miR-124 is another microRNA widespread in neural cells. miR-124 is believed to promote NSC differentiation by inhibiting the expression of *SOX9*, as is confirmed by the maintenance of NSC pluripotency under the conditions of miR-124 knockdown in the SVZ [232]. The expression of miR-124 has been detected in the residential microglia of the CNS, probably due to horizontal transfer of microRNA from neurons to glia [233]. As a result, macrophages suppress the markers of activation of class II major histocompatibility complex (MHC) and CD45 in microglial cells [234]. In addition to the classical experiment that has proven the efficiency of direct reprogramming of human fibroblasts using miR-9/9* and miR-124 [110], there is a study that has demonstrated the possibility of direct reprogramming of primary dermal fibroblasts of an adult person into functional neurons using a "cocktail" of miR-124 and the transcription factors MYT1L and BRN2 [111].

Small Molecules

The epigenetic method of direct reprogramming is implemented through small molecules: low-molecular weight compounds affecting the major cell signaling pathways, chromatin accessibility and state, metabolism, cytoskeletal activity, etc. [235, 236]. In spite of the fact that some studies have demonstrated the self-sufficiency of small molecules as direct proneural reprogramming factors [237, 238], the method is characterized by low reproducibility, insufficient specificity and, as a consequence, high variability of results [239]. Hence, most researchers use small molecules in combination with other reprogramming factors. The highly important auxiliary trend in direct reprogramming is believed to be the change in the epigenetic background of a primary cell, most often of mesodermal origin. First and foremost, it can be achieved by using molecules that increase the accessi-

bility of DNA of transformed cells: valproic acid, N-phthalyl-*L*-triptophan (RG108)) [239], and inhibiting mesodermal differentiation. For example, the Noggin protein and small molecule A-83-01 inhibit signal transduction by TGF-β, SMAD and ALK (anaplastic lymphoma kinase) [239]. Transformation efficiency is considerably increased by the molecules supporting vital activity and proliferation, such as forskolin mediating the activation of adenylate cyclase [134, 240] and Y-27632 inhibiting RHO-associated protein kinase (ROCK) [241]. In addition, there are small molecules exerting more obvious effects on the mesoderm-to-ectoderm transition. For example, CHIR99021 inhibits GSK3β (glycogen synthase kinase 3 beta), thereby activating the Wnt signaling pathway which is involved, as mentioned above, in the control of neurogenesis, while isoxazole-9 (ISX9), via indirect activation of transcription factors of the MEF2 (myocyte enhancer factor-2) family, regulates the activity of neural transcription factors such as NEUROD, ASCL1 and BRN2 [242–245]. Auxiliary factors considerably facilitate transformation and targeted differentiation and minimize the number of transcription factors. For example, NGN2, ASCL1, Noggin protein and a cocktail of small molecules (CHIR-99021, SB-431542, LDN-193189, A-83–01, forskolin and dibutiryl-cAMP) have been successfully used for reprogramming human fibroblasts to functional neurons [246].

CONCLUSIONS

The analysis of numerous reprogramming protocols has shown that almost all of them are based on the primary and secondary transcription factors mediating neurogenesis in the embryonic and adult brain. SOX2, PAX6, MSI1 ASCL1, BRN2, neurogenins, NEUROD1, MYT1L, GSX2 and DLX most often ensure the success of reprogramming. The cell types obtained as a result of direct reprogramming are highly diverse: from stem-like neural progenitors/neural progenitor cells and cells with characteristics of the radial glia to neuroblasts and "young" neurons expressing the basic neuronal markers. In much fewer studies, direct reprogramming yielded terminally differentiated types of neurons: intermediate, motor, sensory neurons and those performing specialized neurotransmission. In contrast to the iPSC technology, the epigenetic context of initial cells is of great significance in drNPC production. The important aspects include inactivation of the suppression of neurospecific genes in the initial somatic cells, as well as overcoming of the initial "gliogenicity" of microenvironment of the adult brain. The microenvironment can be modeled by different approaches, e.g., by using hydrogels containing proneural growth factors, microRNA and small molecules, extracellular matrix proteins, biologically active self-assembling peptides, etc. [247–251].

ACKNOWLEDGMENTS

The authors are grateful to Maria Andreevna Lagar'kova for her consultative assistance in writing the review.

FUNDING

The work was supported by the Russian Science Foundation, project no. 16-15-10432.

COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflict of interest. This article does not contain any studies involving animals or human participants performed by any of the authors.

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Translated by E. Makeeva