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CELL REPROGRAMMING: THERAPEUTIC POTENTIAL AND THE PROMISE OF REJUVENATION FOR THE AGING BRAIN

Running title: Cell reprogramming for the aging brain

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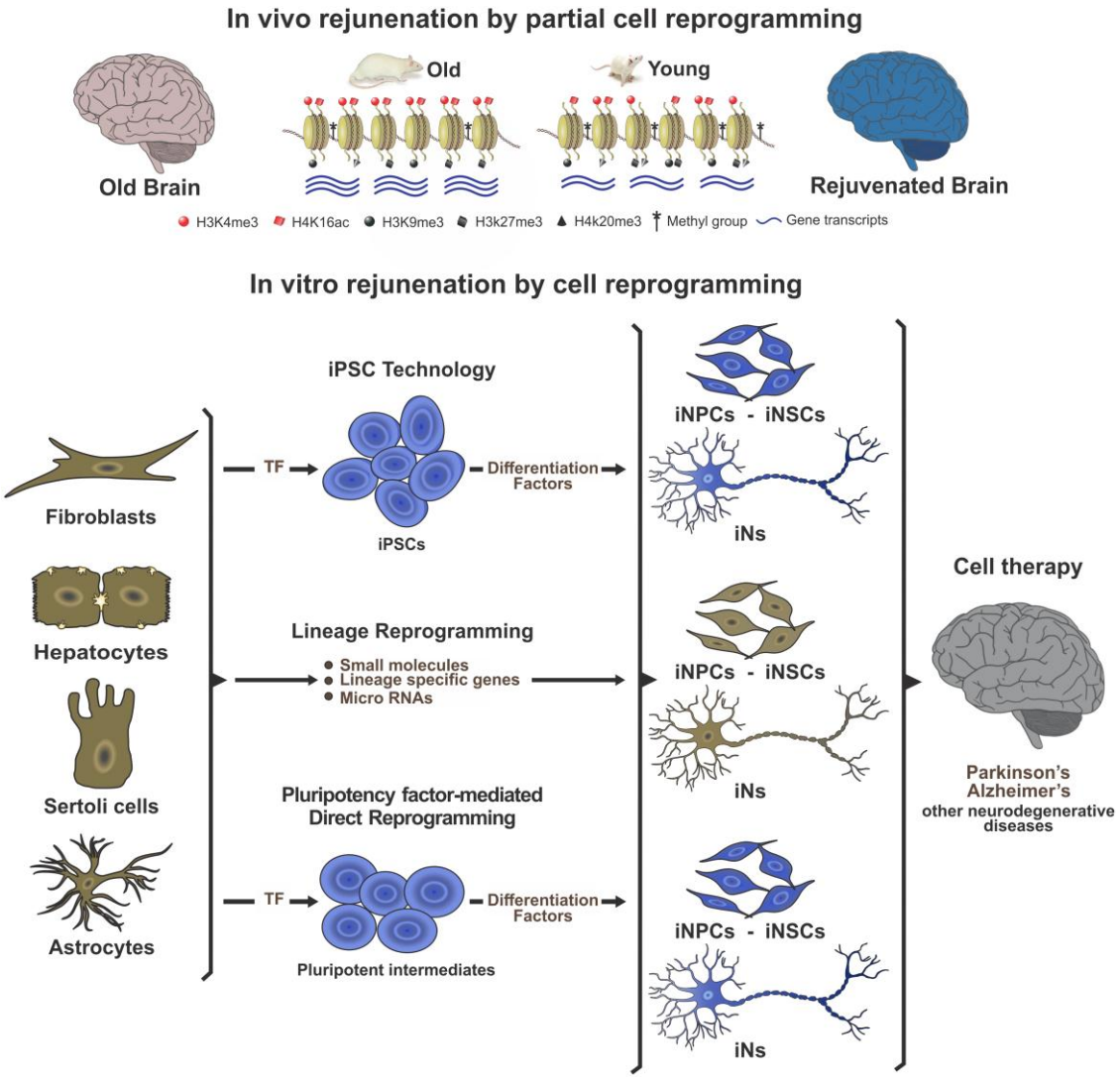
Graphical Abstract. Upper diagram- The red and black symbols on the histone tails represent the respective chromatin-activating and -repressor marks. Stemmed stars represent DNA methylation marks. Blue wavy lines depict gene transcripts (RNAs). The rejuvenated brain on the right is shown in blue color. **Lower diagram-** On the left, the cells of origin are in brown color, in the center, pluripotent intermediates are displayed in blue and on the right, rejuvenated cells are displayed in blue whereas nonrejuvenated cells are represented in brown.



HIGHLIGHTS

- Brain aging is associated with an increase in neurodegenerative pathologies.

- Regenerative medicine offers novel approaches for the treatment of these pathologies.
- Generation of neural cells for treating the aging brain is of clinical interest.
- Direct reprogramming paradigms to generate neural cells are particularly relevant.
- Rejuvenation by cell reprogramming promises revolutionary therapies for the brain.

ABSTRACT

Aging is associated with a progressive increase in the incidence of neurodegenerative diseases, with Alzheimer's (AD) and Parkinson's (PD) disease being the most conspicuous examples. Within this context, the absence of efficacious therapies for most age-related brain pathologies has increased the interest in regenerative medicine. In particular, cell reprogramming technologies have ushered in the era of personalized therapies that not only show a significant potential for the treatment of neurodegenerative diseases but also promise to make biological rejuvenation feasible. We will first review recent evidence supporting the emerging view that aging is a reversible epigenetic phenomenon. Next, we will describe novel reprogramming approaches that overcome some of the intrinsic limitations of conventional induced–pluripotent-stem-cell technology. One of the alternative approaches, lineage reprogramming, consists of the direct conversion of one adult cell type into another by transgenic expression of multiple lineage-specific transcription factors (TF). Another strategy, termed pluripotency factor-mediated direct reprogramming, uses universal TF to generate epigenetically unstable intermediates able to differentiate into somatic cell types in response to specific differentiation factors. In the third part we will review studies showing the potential relevance of the above approaches for the treatment of AD and PD.

(194 words)

Key words: brain aging - cell reprogramming - rejuvenation - direct reprogramming - transdifferentiation – Parkinson´s disease – Alzheimer´s disease

1. AGE-RELATED NEURODEGENERATIVE DISEASES

Aging is associated with cognitive decline and a progressive increase in the incidence of neurodegenerative diseases. In the central nervous system (CNS), dopaminergic (DA) and cholinergic neurons are among the most susceptible cells to the deleterious effects of aging **(Grothr *et al.*, 2011; Fischer *et al.*, 1992; de Rijk *et al.*, 1995)**. Thus, the basal forebrain cholinergic system undergoes mild neurodegenerative changes during normal aging as well as severe atrophy in Alzheimer´s Disease (AD), the most common form of neurodegeneration and dementia among older people. In fact, the cholinergic degeneration in AD appears to take place against a background of age-associated atrophy and the exacerbated degeneration occurring can be detected at very early stages of cognitive impairment **(Grothr *et al.*, 2011)**. In rats, aging is paralleled by degenerative and/or atrophic changes in the forebrain cholinergic system and these morphologic changes are associated with a decline in spatial learning ability **(Fischer *et al.*, 1992)**.

Parkinson's disease (PD) is a neurological disorder associated with degeneration and progressive loss of DA neurons in the midbrain substantia nigra, which leads to a profound reduction of dopamine levels in the striatum **(Gaillard and Jaber, 2011)**. When 50-60% of the nigral DA neurons have degenerated and 70-80% of DA terminals in the striatum have been depleted, motor symptoms -like resting tremor, muscular rigidity, bradykinesia and postural instability- become evident **(Bernheimer *et al.*, 1973)**. PD affects 0.3-1.0% of the human population and is the most conspicuous reflection of the vulnerability of DA neurons to aging, the only unequivocal risk factor for PD **(de Rijk *et al.*, 1995)**. In rats, aging is associated with a progressive

degeneration and loss of a different group of central DA neurons, namely, the hypothalamic tuberoinfundibular dopaminergic (TIDA) neurons, whose physiologic role is to exert a tonic inhibitory control of prolactin secretion and lactotropic cell proliferation in the anterior pituitary gland **(Sarkar *et al.*, 1982)**. Progressive dysfunction and loss of TIDA neurons during normal aging in the female rat is paralleled by chronic hyperprolactinemia **(Goya *et al.*, 1990)** and the development of pituitary prolactinomas **(Cónsole *et al.*, 1997)**. Although aging rats do not display parkinsonian symptoms even at advanced ages (32 months), they lose 35-40% of the nigral DA neurons and show a marked deficit in motor performance **(Sánchez *et al.*, 2008)**. In humans, normal aging is also associated with a progressive decline in motor performance and a gradual loss of nigral DA neurons **(Rudow *et al.*, 2008)**. Therefore, progressive deterioration of cognitive function and gradual decline in central DA activity seem to represent basic features of normal aging in both laboratory rodents and humans. Exacerbation of these processes would lead to AD and PD, respectively. Within this context, cell reprogramming emerges as a powerful technology that promises to make possible the implementation of personalized regenerative medicine aimed at preventing or delaying the progression of AD and PD. Furthermore, the recently emerging evidence that partial cell reprogramming may be capable of rejuvenating old animals **(Ocampo *et al.*, 2016b)** brings about the promise of achieving, perhaps in the not-too-distant future, the ultimate cure for age-related brain (and other) disorders, rejuvenation. We will begin by reviewing this fascinating new avenue of regenerative medicine.

2. REJUVENATION BY CELL REPROGRAMMING

2.1 The Road to Cell Reprogramming

The generation of induced pluripotent stem cells (iPSCs) from somatic cells has demonstrated that somatic mammalian cells from adult individuals can be reprogrammed to a pluripotent state by overexpression of a small number of embryonic transcription factors (TF) **(Takahashi and Yamanaka, 2006)**. The achievement of induced pluripotency constitutes the synthesis of scientific advances and technologies that were developed over the last six decades. Among the pioneering efforts that paved the path to cell reprogramming, the work of John Gurdon and collaborators in the sixties should be mentioned. Their seminal studies in frogs demonstrated that animal cloning is possible **(Gurdon 1962; Gurdon 2006)**. Mammalian cloning by somatic cell nuclear transfer (SCNT), was achieved in 1996 with the birth of Dolly, the sheep **(Wilmut *et al.*, 1997)**. In the 1980s, it was discovered that a single TF, MYOD, can convert fibroblasts into skeletal muscle cells demonstrating that cell fate can be changed through the overexpression of specific TF **(Davis *et al.*, 1987; Choi *et al.*, 1990; Weintraub *et al.*, 1989)**. These findings challenged the fundamental doctrine that terminally differentiated cells are irreversibly determined in their specialization. The discovery of animal cloning strongly suggested that the somatic cell genome is remarkably plastic. Another transcendent implication of the cloning experiments was that in the cytoplasm of a mature oocyte there are molecules capable of reprogramming a somatic nucleus, setting in motion the developmental program for a new individual. The identity of the above molecules began to emerge in 2006, when Takahashi and Yamanaka demonstrated that the transfer of the four pluripotency genes *oct4, klf4, sox2 and c-myc* (OKSM genes), to mouse fibroblasts can reprogram them, taking the cells to a stage (the iPSC stage) in which they behave as embryonic stem cells (ESCs) **(Takahashi and Yamanaka, 2006)**. These discoveries have opened a panorama of unimagined possibilities for the development of personalized therapeutic approaches **(Bayart and Cohen-Haguenauer, 2013; Nedelec *et al.*, 2013)**.

2.2 Aging and Epigenetics: the Promise of Rejuvenation by Cell Reprogramming

In light of a growing body of reports demonstrating that reprogramming of somatic cells from aged individuals rejuvenates them to their embryonic stage (a topic to be reviewed briefly below), the emerging view is that aging is a reversible epigenetic phenomenon where age-related DNA damage seems to play no significant role **(Rando *et al.*, 2012; Ocampo *et al.*, 2016a; López León & Goya, 2017)**.

Studies in model organisms like yeasts, worms and flies have shown that aging is associated with progressive changes in chromatin regulation. In young cells the genome is in a relatively high level of repression that is in part achieved by DNA methylation and relatively high levels of histone H3 trimethylated at lysine 9 (H3K9me3) and at lysine 27 (H3K27me3) and histone H4 trimethylated at lysine 20 (H4K20me3), all of which are associated with transcriptionally repressed chromatin, as well as relatively low levels of histone H3 trimethylated at lysine 4 (H3K4me3) and histone H4 acetylated at lysine 16 (H4K16ac), both of which are associated with active chromatin. Aging seems to be associated with a progressive derepression of the transcriptional activity of chromatin, which is effected in part by reduction in DNA methylation (although paradoxically, DNA of specific gene groups becomes hypermethylated at CpGi), decrease of epigenetic repressor marks like H3K9me3, H3K27me3 and H4K20me3 as well as an increase in the levels of activation marks like H3K4me3 and H4K16ac **(Fig.1, upper scheme)**. Acetylation and methylation of this group of histones is specifically achieved by a number of protein groups associated with chromatin, like the histone methyl transferases and histone demethylases, histone deacetylases (including sirtuins), the polycomb complex, trithorax groups and others. A detailed description of the mechanisms involved in altering the epigenetic state of chromatin during aging is beyond the scope of this review and the reader is referred to a number of

comprehensive reviews **(Greer *et al.*, 2010; Han and Brunet, 2012; Benayoun *et al.*, 2015; Sen *et al.*, 2016)**. These epigenetic marks are influenced by both endogenous (e.g., hormones) and exogenous (e.g., nutrition) environmental factors **(Sen *et al.*, 2016)**.

A cluster of recent studies (see below) strongly suggests that when cells from old individuals are reprogrammed to the iPSC stage many, if not all epigenetic marks of aging are erased **(Fig. 1, lower scheme)**. Furthermore, if iPSCs derived from aged individuals are re-differentiated to the original cell type, the rejuvenated cells display the structural and functional features of young wild-type counterparts. Thus, it has been shown that reprogramming skin fibroblasts from aged humans to the iPSC stage and differentiating them to induced neurons (*i.e.*, iPSC-derived neurons) rejuvenates their transcriptome signature and their nucleocytoplasmic compartmentalization (NCC) making them comparable to that of wild-type fibroblasts from young donors **(Mertens *et al.*, 2015)**. Induced neurons (iNs) were also generated by transdifferentiation, a process that bypasses the pluripotency stage. Transdifferentiated iNs retain the transcriptome profile of old fibroblasts and showed a disrupted NCC as old fibroblasts do **(Mertens *et al.*, 2015)**. The authors conclude that dedifferentiation to the pluripotency stage is necessary to fully erase all marks of aging in the epigenome. In another study, skin fibroblasts from old and centenarian normal human donors were dedifferentiated to the iPSC stage and redifferentiated back into fibroblasts. In the rejuvenated fibroblasts it was found that the telomere length was restored and that gene expression profiles, oxidative stress levels and mitochondrial metabolism was found to be comparable to that of fibroblasts from young humans **(Lapasset *et al.*, 2011)**. In an interesting study with skin fibroblasts from healthy old humans, it was observed that the lower oxygen consumption typically observed in the mitochondria of cells from old individuals was restored to youthful levels after the old cells were dedifferentiated to

iPSCs and differentiated back to rejuvenated fibroblasts. The damage in mitochondrial DNA was comparable in fibroblasts from young donors and rejuvenated fibroblasts from old donors. It was also found that in the old fibroblasts there was no damage to the nuclear GCAT gene which encodes an enzyme involved in the synthesis of glycine; rather, the gene was epigenetically down regulated in those old cells. Rejuvenation reversed this repression and restored normal glycine levels in the mitochondria of rejuvenated fibroblasts **(Hashizume *et al.*, 2015)**. In another study, it was reported that overexpression of the pluripotency factor NANOG in senescent or progeroid myogenic progenitors reversed cellular aging and fully restored the ability to generate contractile force. This transformation was mediated by the reactivation of the Rho-associated–protein-kinase (ROCK) and the transforming-growth-factor-ß (TGF-β) pathways **(Mistriotis *et al.*, 2017)**.

Rejuvenation by cell reprogramming is one of the new horizons opened by iPSC technology. A particularly promising report appeared recently **(Ocampo *et al.*, 2016b)**, showing that cyclic partial reprogramming in transgenic progeric mice carrying a Tet-On regulatable cassette harboring the four Yamanaka pluripotency, genes whose expression is activated in cycles consisting of a two-day period of transgene overexpression (effected by administration of the antibiotic doxycycline (DOX) via the drinking water) followed by a 5-day period or repression (effected by DOX removal), can prolong their survival time. Analysis of internal organs revealed that many signs of senescence disappeared in adult (senile) mice after a few cycles of partial reprogramming **(Ocampo *et al.*, 2016b)**. At the time of this writing this is, to our knowledge, the first report showing that *in vivo* rejuvenation can be achieved by a partial reprogramming approach **(Fig. 1, lower scheme)**. Although the authors do not report an assessment of the brains of the rejuvenated animals, it seems likely that some aging markers may have been attenuated in their brain. It is reasonable to expect that

there will be numerous follow-up *in vivo* studies using cell reprogramming to achieve *in vivo* rejuvenation, an expectation that bears the implication that this approach will be tried in animal models of neurodegenerative diseases. Considering the ever-accelerating rate of cell reprogramming research, there is a clear promise that in the not-too-distant future reprogramming-based medical technologies will be developed not only to treat so far incurable age-related brain diseases like AD and PD but also to reverse the functional declines (like memory impairment) that occur in the human brain during normal aging.

3. TOWARDS iPSC-FREE CELL REPROGRAMMING STRATEGIES

The first cell reprogramming strategy mediated by iPSC was the one developed by the Yamanaka's group whose approach, as mentioned above, involves converting a somatic cell line into iPSCs, which subsequently can be re-differentiated to specific somatic cell types of interest. This method is still the most widely used strategy to convert a somatic cell type into another and is the only procedure so far used to rejuvenate cells and animals (*cf.*, section 2.2 above). Since its discovery in 2006, iPSC technology has been extensively characterized - for instance, it has been demonstrated that cellular origin influences the *in vitro* differentiation potentials of iPSCs into embryoid bodies and different cell types. However, it is also known that continuous passaging of iPSCs largely attenuates these differences **(Polo et *al.*, 2010)**. High fidelity protocols to differentiate iPSC into neural cells that closely resemble their endogenous counterparts have been developed. Thus, it was recently reported that iPSC-derived cortical neurons from myoclonus-dystonia (M-D) patients with mutations (W100G and R102X) in the maternally imprinted *ε-sarcoglycan (SGCE*) gene were generated and properties such as imprinting, mRNA and protein expression were analyzed. Comparison of the promoter during reprogramming and differentiation demonstrated a tissue-independent

differential methylation. DNA sequencing with methylation-specific primers and cDNA analysis in patient neurons indicated selective expression of the mutated paternal *SGCE* allele. A phenotypic characterization of the neurons showed the suitability of iPSC-derived cortical neurons with *SGCE* mutations for M-D research and, in more general terms, encouraged the use of iPSC-derived cellular models to study epigenetic mechanisms impacting on health and disease **(Grütz *et al*., 2017)**. In another instance of an improved protocol for generation of iPSC-derived neurons, a fully defined feeder- and xenogeneic-free protocol for the generation of ventral midbrain dopamine (vmDA) neurons from human pluripotent stem cells (hPSCs) has been recently reported **(Niclis *et al.*, 2017)** (*c.f.*, section 5.1 for further details).

Despite the advantages of iPSC technology, the procedure as a whole is arduous, lengthy and costly. Since the protocols to generate iPSCs comprise a number of steps, the efficiency with which the final cell type is generated may be low. Furthermore, a number of concerns about safety of iPSC-derived cells need to be addressed before these cells can be used clinically **(Okano *et al.*, 2013)**. It is important to note that a commonly used assay for demonstrating pluripotency is the capacity to forming teratomas **(Patel and Yang, 2010)**. Therefore, the tumorigenic potential of contaminating iPSCs that fail to differentiate increases the risk for any clinical application of somatic cells generated by this procedure **(Miura *et al.*, 2009)**. These hurdles have led to the emergence of other ways of reprogramming cells that involve direct conversion between cell types. These strategies are outlined below and illustrated in **Figure 2**.

3.1 Lineage Reprogramming

An alternative cell reprogramming approach, known as **lineage reprogramming (LR)** or transdifferentiation, emerged in 1987 **(Davis *et al.*)** for the generation of specific cell types. This method consists of the direct conversion of one somatic cell type into

another one by ectopic expression of multiple lineage-specific TF or microRNAs (miRNA) without the cell passing through the pluripotent stem cell stage **(Ieda *et al.*, 2013; Sancho-Martinez *et al.*, 2012)**. This strategy uses factors that show specific expression in target cells. Thus, the adenoviral-mediated gene transfer of a combination of three TF was able to reprogram efficiently pancreatic exocrine cells into functional β cells in mice, which constituted the first documented evidence of cell reprogramming *in vivo* by defined factors **(Zhou *et al.*, 2008)**. A number of studies have demonstrated that LR can yield a diverse range of medically relevant cell types, such as cardiomyocytes and neurons **(Ieda *et al.*, 2010; Kim *et al.*, 2011a; Vierbuchen *et al*., 2010)**. The transdifferentiated cells exhibit a functionality equivalent to that of the cells differentiated from iPSCs or their wild-type counterparts and show no tumorigenicity when transplanted *in vivo* **(Liu *et al.*, 2012a; Matsui *et al*., 2012)**. However, cells generated by this procedure may demonstrate residual memory of cells of origin and identity differences as well as restricted proliferative potential, limited cell type diversity and even senescence **(Margariti *et al.*, 2012)**, which may in turn substantially compromise their potential application in regenerative therapy. Furthermore, when LR is applied to somatic cells from old donors to generate neural cells, the induced neurons will retain the age-related features of the donors **(Mertens *et al*., 2015)**, which represents an additional limitation for their use in cell therapy. Despite these limitations, particularly the lack of rejuvenation in neural cells derived by LR, in the following sections we include *in vitro* and *in vivo* reports documenting the application of induced neural cells generated by LR to models of PD and AD as we consider that the results achieved should be known by the readers.

3.2 Pluripotency factor-mediated Direct Reprogramming

Another approach to regenerative medicine, termed direct reprogramming or **pluripotency-factor-mediated direct reprogramming** (PDR) has also emerged **(Kim**

***et al.,* 2012)**. This procedure uses fully differentiated somatic cells and converts them into other somatic cell types by transient expression of pluripotency genes, usually for 3 to 5 days, thus generating epigenetically unstable (EU) intermediates which are responsive to appropriate cocktails of specific differentiation factors **(Kim *et al*., 2011b; Efe *et al*., 2011; Ma *et al.,* 2013)**.

The PDR strategy originated from initial observations indicating that iPSCs are generated in either a sequential or stochastic manner **(Stadtfeld *et al.,* 2008; Brambrink *et al.,* 2008; Hanna *et al.,* 2009)**. This led Kim *et al*. **(2012)** to hypothesize that it could be possible to manipulate cells at an early, epigenetically highly unstable state, induced by the Yamanaka pluripotency factors. In the presence of appropriate differentiation factors, these EU intermediates can be subsequently induced to differentiate into various somatic cell lineages with more stable epigenetic profiles **(Artyomov *et al.,* 2010)**. This new method has several advantages such as the use of a universal pluripotency gene set and the ability to generate rejuvenated multipotent progenitor cell populations capable to differentiate into various tissue-specific destination cells under specific conditions **(Kim *et al.,* 2012; Kim *et al.,* 2011b; Efe *et al*., 2011)**.

Although the initial thinking was that in PDR the EU intermediates bypassed the pluripotency state **(Kim *et al.,* 2012)**, subsequent studies showed that when PDR is used to generate reprogrammed cardiomyocytes or neural stem cells (NSC), most of the induced cells derived from mouse fibroblasts by OSKM-induced conversion pass through a transient pluripotency state as evidenced by the reactivation of the X chromosome and expression of endogenous NANOG and OCT4 **(Maza *et al.,* 2015)**. The evidence that EU intermediates pass through a transient pluripotency state, has been further strengthened by a study showing that when PDR is used to generate induced neural stem cells (iNSCs) from mouse fibroblasts, iNSC colonies repress retroviral

transgenes and reactivate silenced X chromosomes, both of which are hallmarks of pluripotency **(Bar-Nur *et al.,* 2015)**. In view of this evidence we now consider the terms **UE intermediates** and **pluripotent intermediates** to be interchangeable.The fact that PDR involves a transient pluripotency stage makes it conceivable that unlike LR, PDR applied to somatic cells from old donors may erase at least part of the epigenetic marks of aging. This hypothesis is supported by a report cited above documenting that transient overexpression of the OSKM genes not only can rejuvenate somatic cells from progeric mice but is even able to rejuvenate the progeric mice themselves, increasing their survival time **(Ocampo *et al.,* 2016b)**.

Lineage reprogramming and PDR share in common a direct conversion from one cell type into another relying on specific signals to help the original cells reach the desired cell-type destination and both are patient-specific. In contrast to lengthy iPSC-mediated cell reprogramming, in the direct approaches the conversion usually occurs in a short period of time and the induced cells have no tumorigenic potential. A significant difference between PDR and LR is that the former generates multipotent precursor cells whereas the later drives the somatic cells of origin directly to terminal cells **(Prasad *et al.,* 2016).**

4. GENERATION OF NEURAL CELLS BY LR AND PDR

There is a keen interest in reprogramming somatic cells to mature neurons and neural precursors, which can later be used for implementing cell therapy for neurodegenerative pathologies like AD and PD. This section will review PDR and LR procedures used to generate them, leaving for later sections on specific brain pathologies the reviewing of procedures to obtain specific neuronal cell subtypes like DA neurons.

It has been demonstrated that nonneural somatic cells can be transdifferentiated into functional neurons by lineage-determining TF **(Kim *et al.*, 2011a)**. Initially, mouse **(Vierbuchen *et al.*, 2010)** and human **(Pang *et al*., 2011; Ambasudhan *et al.*, 2011)** fibroblasts were efficiently converted into functional neurons *in vitro* by transfer of the genes for some neuronal lineage-specific TF and/or miRNA, under precisely defined conditions. Embryonic and postnatal mouse fibroblasts could give rise to iNs by transferring only three neuronal-specific TF, namely BRN2 (encoded by POU3F2), achaete-scute homologue 1 (ASCL1) and myelin TF 1-like protein (MYT1L), a cocktail known as BAM **(Vierbuchen *et al.*, 2010)**. The resulting iNs display typical neural morphologies and express multiple neural markers. When human fibroblasts are combined with the TF neurodifferentiation D1 (NEUROD1), BAM can convert human fibroblasts into iNs, which are also able to generate action potentials and form functional synapses with murine cortical neurons in coculture **(Pang *et al.*, 2011)**. Shortly after this discovery, a number of groups reported successful generation of iNs from human fibroblasts **(Ambasudhan *et al.*, 2011: Pfisterer *et al.*, 2011)**. These findings clearly show that the overexpression of a limited number of 'master' factors is sufficient to drive relatively fast and direct specific lineage changes in cells derived from different embryonic layers. In effect, lineage conversion is not restricted to the same germ layer or lineage, since mesodermal cells like fibroblasts can give rise to neurons, which are cells derived from the ectoderm. Furthermore, terminally differentiated hepatocytes, derived from the endoderm, were converted into iNs by the same neuronal TF **(Marro *et al.*, 2011)**. The generation of iNs seems to follow a definite sequence of epigenetic events. In the case of the BAM-mediated reprogramming of mouse fibroblasts to iNs, ASCL1 may act as a triggering TF which later recruits BRN2 to their binding site, with MYT1L being required at later stages of maturation **(Wapinski *et al.*, 2013)**. So, ASCL1 emerged as a master TF that exerts a

central role for different neuronal fates depending on the timing of expression and the starting cell type. Additional studies have demonstrated that expression of subtype-specific regulatory factors in human and mouse fibroblasts results in the establishment of specific neuronal subtypes, like DA and glutamatergic neurons (See PD and AD sections).

The generation of iNs from nonneural lineages may have significant applications for studies of neural development and neurological disease modeling because they can be derived from patients. In principle, iNs can be also used for regenerative medicine. However, they are not adequate for transplantation treatments **(Rhee *et al.*, 2011)** because, owing to their limited ability to proliferate, only few cells usually survive and become functionally integrated to the brain, showing limited effectiveness. In addition, because differentiated cells are postmitotic and nondividing, the process of generating sufficient numbers of cells for further basic and clinical applications constitutes a significant challenge. Within this context, stem and progenitor cells emerge as more suitable cell types for obtaining adequate cell stocks and for proper *in vivo* integration. Consequently, a number of studies have focused on the generation of iNSCs, which maintain the properties of multipotentiality and self-renewal, and induced neural progenitor cells (iNPCs) from fibroblasts by both PDR and LR (**Kim *et al.*, 2011b; Wang *et al.*, 2012; Ring *et al*., 2012; Lujan *et al*., 2012; Han *et al.*, 2012; Thier *et al.*, 2012)**. The PDR strategy was used for the first time by Kim *et al*. **(2011b)** who demonstrated that transient induction of the four Yamanaka pluripotency factors for 3-5 days followed by appropriate signaling inputs, can efficiently convert fibroblasts into functional iNPCs. For example, when EU mouse fibroblasts induced by the OKSM reprogramming factors are grown in a medium that contains the neurogenic molecules, fibroblast growth factor 2 (FGF2), fibroblast growth factor 4 (FGF4) and epidermal growth factor (EGF), colonies of cells showing many of the features of NPCs are

generated after 8-9 days in culture. This process is highly efficient and specific, requiring only a single step that is completed within 13 days. Compared with iNs, iNPCs have the distinct advantage of being expandable *in vitro* and of retaining the ability to give rise to multiple neuronal subtypes and glial cells **(Reynolds and Weiss, 1992)**. However, the generated populations are usually heterogeneous and the iNPCs have shown poor multipotentiality **(Kim *et al.*, 2011b)**. Another group reported the direct conversion of human primary fibroblasts into stably expandable iNPCs by timely restricted expression of the four Yamanaka factors **(Meyer *et al.*, 2015)**. The generation of tripotent iNPCs from adult human fibroblasts by direct reprogramming using OCT4 alone, was also reported **(Mitchell *et al.*, 2014)**. Because NSCs endogenously express a subset of the Yamanaka reprogramming factors, SOX2, KLF4, and c-MYC, but not OCT4, Thier *et al*. **(2012)** investigated whether curtailed reprogramming with restricted OCT4 activity could convert mouse fibroblasts into iNSCs. Those authors demonstrated that when the expression of *oct4* is selectively repressed after day 5, keeping the other three genes overexpressed, colonies of iNSCs are generated. The generation of iNSCs from human and mouse fibroblasts by direct reprogramming with a single factor, SOX2, was also reported **(Ring *et al.*, 2012)**. The iNSCs express NSC markers and resemble endogenous NSC in their morphology, self-renewal ability and gene expression profiles. They can differentiate into several types of mature neurons which indicate multipotency. Implanted iNSCs survive and integrate in mouse brains and, unlike iPSC-derived NSCs, they are not tumorigenic. These iNSCs, morover, exhibited, an extensive self-renewal capacity as compared with the limited passaging ability of iNPCs **(Kim *et al.*, 2011b)**, which could be expanded for only a few passages thus lacking the capacity for permanent self-renewal, a critical requirement for clinical applicability. Morover, iNPCs apparently lacked the potential to differentiate into oligodendrocytes. Thus, self-

renewable and multipotent iNSCs devoid of tumorigenic potential may be directly generated from fibroblasts by restricted overexpression of pluripotency genes.

Alternative approaches combined Yamanaka's factor expression with early neural TF, which then help push the EU intermediates into NPCs. In this field, Lujan *et al.* **(2012)** demonstrated that defined sets of TF highly expressed in NPCs are sufficient to transdifferentiate mouse fibroblasts into proliferative iNPCs. Towards this goal, the authors started with a set of candidate genes for TF and using stepwise elimination, they found that SOX2 and forkhead box protein G1 (FOXG1) are capable of generating self-renewing bipotent iNPCs, that give rise to astrocytes and functional neurons. When the gene for BRN2 was added, tripotent iNPCs were generated which could also be differentiated into oligodendrocytes. Furthermore, FOXG1 and BRN2 alone had the ability to induce NPC-like cells, which generated less mature neurons, although those precursors did produce astrocytes and also oligodendrocytes capable to integrate into demyelinated Shiverer mouse brains. However, the resulting population was heterogeneous and the issues of contaminating pluripotent SCs persistence with teratoma formation after transplantation of iNPCs in animal models, were reported by this group **(Lujan *et al.*, 2012)**.

A similar reprogramming strategy for the direct conversion of fibroblasts into iNSCs started with three pluripotency TF together with eight neural specific TF **(Han *et al.*, 2012)** and by systematic elimination, the list of effective factors could be reduced to a smaller number. Considering that neurogenin 2 (NGN2) and LIM3 are specific for more differentiated cell types such as motor neurons **(Marro *et al.*, 2011)**, these factors were excluded from the reprogramming cocktail. By this procedure, stable cell lines that highly resemble NSCs in morphology, marker gene expression, differentiation potential and self-renewing capacity, were successfully generated with two final reprogramming

cocktails, a 4-factor or a 5-factor combination (4-factor plus E47). In sum, the authors demonstrated that SOX2, c-MYC, KLF4 and BRN4 are active factors sufficient to induce transdifferentiation of mouse fibroblasts into iNSCs, in what was a gradual process in which the fibroblast transcriptional program was silenced over time. In another transdifferentiation approach, Sheng *et al.* **(2012a)** demonstrated that Sertoli cells which are derived from mesoderm, can be directly converted into iNSC/iNPCs expressing multiple NSC-specific markers, exhibiting a global NSC gene-expression profile and being capable of self-renewal and differentiating into glia and electrophysiologically functional neurons. This was achieved by overexpressing nine NSC-specific reprogramming factors. Ectopic expression of the reprogramming genes *oct4, sox2* and *nanog* into astrocytes in specific cytokine-containing cultures activated the neural stem gene program and generated induced cells expressing neural stem/precursor markers **(Corti *et al.*, 2012)**. **Figure 3** displays a graphic summary of the studies outlined in this section.

The ectopic expression of brain-enriched miRNAs, miR-9/9* and miR-124 promote the direct conversion of human fibroblasts into immature neurons, a process facilitated by NEUROD2 **(Yoo *et al.*, 2011)**. The addition of the neurogenic TF ASCL1 and MYT1L enhanced the conversion rate and maturation of the converted neurons, whereas expression of these TF alone without miRNAs was ineffective. The combination of miR-124 with the TF BRN2 and MYT1L can convert adult human fibroblasts into functional iNs without the addition of any pioneer TF like ASCL1 **(Ambasudhan *et al.*, 2011)**. These human iNs exhibit typical marker gene expression, neuronal morphology, fire action potentials, and produce functional synapses between each other. The coexpression of miR-9/9*-124 with BCL11B, DLX1, DLX2 and MYT1L (TF highly expressed in the developing striatum), can guide the conversion of adult and postnatal human fibroblasts into an enriched population of neurons comparable to striatal medium

spiny neurons (MSNs) **(Richner *et al.,* 2015)**, a neuronal subpopulation that has a crucial role in motor control and displays a selective susceptibility to cell death in Huntington's disease. When transplanted in the mouse brain, the reprogrammed human cells persisted in situ for over 6 months, exhibiting membrane properties comparable to native MSNs and extended projections to the normal anatomical targets of MSNs **(Victor *et al.,* 2014)**.

Because this direct conversion bypasses pluripotent/multipotent stem cell stages, miRNA-mediated directly reprogrammed neurons would retain the age signature of the original donor. Using a miRNA-based cellular reprogramming approach, human fibroblasts obtained from donors ranging from neonates to near centenarians can be efficiently converted into neurons that retain multiple age-associated signatures **(Huh *et al.,* 2016)**. To assess cellular age, Huh *et al.* **(2016)** used the epigenetic clock method, considered to be a highly accurate biomarker of age based on DNA methylation **(Horvath , 2013)**. They evaluated age-associated signatures based on the level of gene expression, miRNAs, and cellular readouts considered to be hallmarks of aging **(López-Otín *et al.,* 2013)** to finally demonstrate the maintenance of the age of the original donor cells during neuronal conversion. These directly converted human neurons can be advantageous for studying age-related neuronal disorders, while iPSC-derived cells are unsuitable in recapitulating phenotypes specifically observed in aged cells **(Mattson and Magnus, 2006; Vera and Studer, 2015)**. As mentioned above, induction of pluripotency in adult fibroblasts reverts cellular age to an embryonic stage erasing the epigenetic marks of aging **(Lapasset *et al.,* 2011; Patterson *et al.,* 2012)**. This feature (youth) remains so even after neuronal differentiation **(Miller *et al.,* 2013)**.

4.1 Generation of neural cells by non-integrative direct cell reprogramming strategies

The clinical applications of iNs or their precursors are strongly compromised in most of the reprogramming strategies by the fact that they involve integrative gene transfer with the well-known risk of gene silencing or insertional mutagenesis. We hypothesize that adenoviral vectors constitute a safer reprogramming alternative and have thus, designed a regulatable bidirectional helper-dependent adenovector expressing the four OKSM pluripotency genes as well as the gene for humanized green fluorescent protein (hGFP) which should allow us to implement a direct reprogramming protocol in a non-integrative fashion in order to generate fibroblast-derived iNPCs and terminally differentiated neurons **(López-León *et al.*, 2014)**. In order to overcome the lower transduction efficiency of adenovectors as compared to retroviral vectors, we can use the magnetofection technique during cell reprogramming. Magnetofection is based on complexing adenoviral vectors to magnetic nanoparticles which under the influence of a magnetic field, efficiently enter the target cells. This procedure markedly increases the transduction efficiency of adenovectors in cell culture **(Schwerdt *et al.*, 2012)**.

Alternative integration-free strategies for direct reprogramming including plasmid vectors, targeted gene repression by miRNA or small molecules, have been explored. The generation of integration-free iNSCs from mouse fibroblasts by nonviral episomal vectors containing the genes for the TF BRN4, SOX2, KLF4 and c-MYC has recently been reported **(Kim *et al.*, 2016)**. The episomal vector-derived iNSCs closely resemble control NSCs and iNSCs generated by retrovirus in morphology, gene expression profile, epigenetic status, and self-renewal capacity. Furthermore, the episomal vector-derived iNSCs are functionally mature, as they could differentiate into all the neuronal cell types *in vivo* as well as *in vitro*. Another study established a vector-mediated reprogramming protocol based on the expression of OCT3/4, SOX2, KLF4, L-MYC, Lin28 and a small hairpin RNA directed against p53 (sh-p53). In order to obtain iNSCs from adult human fibroblasts a defined culture medium in combination with the

neurotrophins bFGF, EGF and FGF4 was used after transduction. The cultivation was performed without the addition of small molecules **(Capetian *et al.*, 2016)**.

Finally, the successful generation of iNs from human and mouse fibroblasts by chemical approaches was achieved by identifying optimal cocktails of small molecules without needing additional genetic factors. In effect, Li *et al.***(2015)** showed that 4 small molecules (Forskolin, N-cyclopropyl-5-(2-thienyl)-3-isoxazolecarboxamide (ISX9), the glycogen-synthetase inhibitor CHIR99021 and the bromodomain-protein inhibitor I-BET151) were enough for mouse fibroblast conversion to iNs. Hu *et al*. **(2015)** reported that human fibroblasts could be converted into neurons using a cocktail of small molecules (valproic acid (VPA), CHIR99021, the kinase inhibitor RepSox, Forskolin, the anthrapyrazolone kinase inhibitor (SP600125), the protein-kinase inhibitors GO6983 and Y-27631, and the AMP-kinase inhibitor dorsomorphin) as could fibroblasts from familial AD patients. These iNs can be used for *in vitro* disease modeling and drug screening. Interestingly, it was shown that the sequential addition of a cocktail of small molecules -the bone-morphogenic-protein–receptor inhibitor LDN193189, the activin-receptor-like kinase inhibitor SB431542, the retinoic-acid analogue TTNPB, the protein-kinase inhibitor thiazovivin, CHIR99021, VPA, the γ-secretase inhibitor DAPT, the smoothened-protein agonist SAG and the hedgehog-signalling–pathway inhibitor Purmorphamine- which inhibit glial- but activate neuronal signaling pathways through epigenetic and transcriptional modulation, can reprogram human astrocytes into functional neurons **(Zhang *et al.*, 2015)**. These human iNs could survive more than 5 months under cell culture conditions.

Small molecule-mediated reprogramming was also reported for generation of iNSCs. Thus, a novel chemical cocktail-comprised of the activin-receptor-like–kinase inhibitor A83-01, CHIR99021, sodium borate (NaB [*sic*]), lysophosphatidic acid (LPA), the

cyclic-AMP–phosphodiesterase inhibitor Rolipram and SP600125, enabled the generation of expandable human iNSCs from fibroblasts transduced with OCT4 alone **(Zhu *et al.*, 2014)**. In the same year, Cheng and colleagues used three small molecules, VPA -CHIR99021 and RepSox- to induce NPCs from somatic cells **(Cheng *et al.*, 2014)**. More recently, a cocktail of nine components -CHIR99021, LDN193189, A83-01, retinoic acid, another smoothened-protein agonist, HhAg1.5, the DNA-methyltransferase inhibitor RG108, the monoamine-oxidase inhibitor Parnate™ (tranylcypromine), the mammalian-autophagy inducer 6-bromo-N-2-propenyl-4-quinazolinamine (SMER28) and bFGF- was used to generate iNSCs from mouse fibroblasts in a more efficient approach **(Zhang *et al.*, 2016a)**.

4.2 Advances in *in vivo* reprogramming for neural regeneration

Recent advances suggest that direct *in vivo* reprogramming of endogenous cell populations may provide a promising prospect for cell-based clinical regenerative therapy through neuron replacement **(Chen *et al.*, 2015; Li and Chen, 2016)**. Glial cells constitute the most abundant cells in adult brains and several groups have reported the successful TF-based *in vivo* reprogramming of glial cells to neurons or iNPCs.

Astrocytes appear as the natural *in vivo* cell target to reprogram into neuronal cells not only because of the capacity of those glial cells to become reactive after damage or neurodegeneration and their abundance in the CNS, but also because a series of *in vitro* studies demonstrated their direct conversion into functional iNs. The overexpression of the TFs PAX6, NEUROG2, ASCL1 or DLX2 can convert astroglia from the early postnatal cortex to functional iNs by LR **(Berninger *et al.*, 2007; Heinrich *et al.*, 2010; Heins *et al.*, 2002**). As mentioned above the feasibility of directly reprogramming astrocytes into iNSCs and iNs by overexpression of OCT4, SOX2 and NANOG, has been also demonstrated **(Corti *et al.*, 2012)**. The feasibility of converting resident

astrocytes directly into neural cells in the adult brain *in vivo* was assessed. Endogenous astrocytes can be reprogrammed to proliferating neuroblasts by delivering SOX2 **(Niu *et al.,* 2013)** and thereafter can be further differentiated to functional neurons that integrate into neural networks in the brain. It was subsequently shown that astrocytes can be reprogrammed into neurons with SOX2, within the context of adult spinal cord injury **(Su *et al.,* 2014)**. Guo *et al.,* **(2014)** demonstrated that cortical glial cells activated by injury or disease could be reprogrammed *in vivo* by NEUROD1 into functional neurons. Torper *et al.* **(2013)** furthermore showed that astroglia can be reprogrammed *in vivo* into neurons in the adult brain by overexpression of the TFs, ASCL1, BRN2 and MYT1L.

Another study demonstrates that a single, nontoxic adeno-associated viral vector can reprogram striatal oligodendrocytes *in vivo*, such that they transdifferentiate into functional neurons **(Weinberg *et al.,* 2017)**. A key feature of this reprogramming approach involves the ability to preferentially targeting oligodendrocytes *in vivo* in the rat striatum and the cell-specific suppression of polypyrimidine tract-binding (PTB) protein expression. Xue *et al.* **(2013)** reported that suppression of PTB protein expression in cultured mouse embryonic fibroblasts caused their differentiation into functional, NeuN-positive neurons.

An electrochemical method of gene delivery *in vivo* to rapidly manipulate gene expression specifically in postmitotic neurons has been developed. With this technique, mouse spiny neurons were specifically reprogrammed during the first postnatal week by ectopic expression of the layer 5B output neuron-specific TF, Fezf2 **(De la Rossa *et al.,* 2013)**. These findings suggest that the identity of postmitotic neurons can be reprogrammed *in vivo* long after they are born. These postmitotic neurons exhibit a high level of functional plasticity in which transcriptional programs can be activated so that

they fundamentally reassign their morphology, physiological properties and circuit identity in a coordinated and functionally meaningful manner. In closing the section a brief comparison between *in vivo* and *in vitro* reprogramming is in order. A major advantage of *in vivo* cell reprogramming is that it uses resident brain cells, mainly astrocytes, to generate specific neuronal cell populations. This strategy avoids the risk of contamination of the induced neural cells with exogenous components like, for instance, those coming from MEF co-cultures and ensures the fidelity of the neuronal cells generated. In more general terms, neural cells generated by *in vivo* reprogramming are never exposed to *in vitro* environments and are therefore less likely to acquire non-physiologic features. Furthermore, the therapeutic effects of *in vivo* reprogramming can be readily assessed in the experimental animals. As a counterbalance to these advantages the *in vivo* delivery of reprogramming TF is much less efficient than delivery *in vitro*. Thus, the risk of attaining therapeutically insufficient numbers of induced neural cells is a concern. With *in vitro* reprogramming the opposite occurs. Furthermore, *in vitro* reprogramming is technically less challenging and the reprogrammed cells are easier to characterize. However, the therapeutic effectiveness of the new cells needs to be subsequently checked *in vivo*.

5. REGENERATIVE MEDICINE FOR THE AGING BRAIN

5.1 Therapeutic potential of cell reprogramming for PD

The advent of cell reprogramming techniques has enabled PD researchers to derive iPSCs as well as iNs and iNSC/iNPCs from easily accessible somatic cells from patients. Currently, DA neurons can be derived through differentiation from iPSCs **(Wernig *et al.*, 2008)** and by direct conversion from fibroblasts **(Kim *et al.*, 2011a; Pfisterer *et al.*, 2011; Rhee *et al.*, 2011; Caiazzo *et al.*, 2011; Liu *et al.*, 2012b)**.

DA neurons derived from ESCs have proved to be functional when grafted into parkinsonian rats **(Kriks *et al*., 2011; Kim *et al*., 2002)**. This enabled the differentiation of mouse iPSCs into DA neurons in order to evaluate their therapeutic potential in a rat model of PD **(Wernig *et al.* 2008)**. After treatment of iPSC-derived neural precursor cells with sonic hedgehog (SHH) and FGF8, most cells differentiated into a βIII-tubulin-positive phenotype with neuronal morphology, which showed markers typically expressed in midbrain DA neurons. They demonstrated functional recovery of parkinsonian rats after cell transplantation. Since teratoma formation after transplantation seems to be a frequent complication for cell therapy, fluorescence-activated cell sorting (FACS) was used to eliminate the undifferentiated cell fraction. No tumor formation was observed after 8 weeks of transplantation whereas the animals exhibited a similar recovery rate in a behavioral test compared with animals receiving unsorted cells. This study revealed that iPSC-derived DA neurons display synaptic integration and dopaminergic function after transplantation into the adult brain in a rat model of PD.

More recently, derivation of human DA neurons from iPSCs was achieved **(Kriks et al., 2011)**. Midbrain precursors were derived from human iPSCs and exposed to SHH and canonical WNT signaling. After 25 days, transdifferentiated induced DA (iDA) neurons were observed. These cells exhibited molecular, biochemical and electrophysiologic profiles which confirm their cell identity and could be maintained *in vitro* for several months. The study demonstrated *in vivo* survival and function of human iDA neurons in three PD animal models. Thus, in 6-OHDA-lesioned mice and rats, the cells showed robust survival, improvements in tests of akinesia and complete restoration of amphetamine-induced rotation behavior. The excellent cell survival, function and scalability demonstrated by transplantation into parkinsonian monkeys, reveal a significant potential for implementation of cell therapies in PD patients.

In a direct approach using a combination of the TFs ASCLI, NURR1 and LMX1A, functional iDA neurons from mouse and human fibroblasts were generated without passing through a progenitor cell stage **(Caiazzo *et al.*, 2011)**. This study also indicated that DA neurons can be generated from the cells of patients with PD. More recently, the efficiency of reprogramming human cells to iDA neurons via the ASCL1/NURR1/LMX1A combination was greatly improved by addition of the small noncoding miR-124 plus a cocktail of neurotrophic factors and small molecules **(Jiang *et al.*, 2015)**. The efficiency was additionally improved to derive TH+/TUJ1+ neurons from human fetal fibroblasts, with the iDA neurons so generated expressing the DA markers AADC, ALDH, DAT, VMAT2, PITX3, NURR1, FOXA2 and EN 1. The highest conversion efficiency (60% yield) was achieved with human fetal fibroblasts but dropped progressively as the age of the fibroblast donor increased, being 16%, 11% and 8% when the donor age in years, was 0 (*i.e.*, newborn), 31 and 96, respectively **(Jiang *et al.*, 2015)**.

Another study demonstrated that a combination of the TFs, ASCLI and NURR1, along with neurotrophic factors like SHH and FGF8B, transdifferentiated embryonic mouse fibroblasts to iNs, including DA neurons and pan-neuronal cells **(Oh *et al.*, 2014)**. A combination of the nine genes *ascl1*, *ngn2*, *hes1*, *id1*, *pax6*, *brn2*, *myc*, *klf4* and *sox2*, was shown to reprogram mouse fetal and postnatal fibroblasts into iNs, 10% of which expressed TH as well as EN1 and PITX3 **(Sheng *et al.*, 2012b)**. Differentiation of rat embryonic fibroblast-derived iNPCs into functional DA neurons was enhanced by overexpression of NURR1 and FOXA2, yielding up to 40% TH+ cells **(Lim *et al.*, 2015)**. The generation of 90% TH+/TUJ1 neurons from adult mouse fibroblast-derived iNPCs generated by overexpression of *brn2*, *sox2* and *foxa2*, with or without exposure to SHH and FGF8, was also reported **(Tian *et al.*, 2015)**.

As to studies with induced neuronal cells in animal models of PD, it has been reported that iPSCs can be induced to differentiate into DA neurons and be subsequently implanted into the brain of rat models of PD where they are able to improve behavior **(Politis and Lindvall, 2012)**. These DA neurons have been reported to synaptically integrate into fetal brain and function into the adult brain after transplantation in a rat model of PD **(Wernig *et al.*, 2008)**. In another study employing parkinsonian cynomolgus monkeys (CM), iPSC-derived midbrain DA neurons survived for up to 2 years following autologous transplantation. In one of the animals with a highly successful protocol, unilateral engraftment of CM-iPSCs-derived midbrain DA neurons induced a progressive improvement of motor function contralateral to the side of DA neuron transplantation, and increased motor activity, without a need for immunosuppression. Postmortem analyses showed robust survival of midbrain-like DA neurons and extensive outgrowth into the transplanted putamen **(Hallett *et al.*, 2015; Kriks *et al.*, 2011)**.

Another study reported that iNSCs per se have therapeutic effects in a PD mouse model **(Wu *et al*., 2015)**. Furthermore, the study revealed that iNSCs engineered to overexpress the TF LMX1A (iNSC-LMX1A), can further enhance the therapeutic efficacy of iNSCs when transplanted into a mouse model of PD. It was found that iNSC-LMX1A give rise to an increased yield of DA neurons and higher levels of dopamine *in vitro*, when compared with control iNSCs that express GFP (iNSCs-GFP). When iNSCs-GFP and iNSCs-LMX1A were transplanted into PD mice, both groups showed decreased ipsilateral rotations; yet, mice that received iNSCs-LMX1A exhibited better recovery than mice that received iNSCs-GFP. This study demonstrated for the first time that iNSCs directly converted from somatic cells exert therapeutic effects when transplanted into mouse PD models. Considering that LMX1A plays a central role in the specification of DA neurons **(Deng *et al.*, 2011; Andersson *et al.*, 2006)**, the

above results suggest that this TF could be employed as an enhancing factor for differentiation of iNSCs into DA neurons. More recently, a fully defined feeder- and xenogeneic-free protocol for the generation of ventral midbrain dopamine (vmDA) neurons from hPSCs has been reported. It utilizes two reporter knock-in lines (LMX1A-eGFP and PITX3-eGFP) for *in vitro* and *in vivo* tracking. Across multiple embryonic and induced hPSC lines, this protocol consistently increased both the yield and proportion of vmDA neural progenitors (OTX2/FOXA2/LMX1A) and neurons (FOXA2/TH/PITX3) that display classic vmDA metabolic and electrophysiologic properties **(Niclis *et al.*, 2017)**.

It should be pointed out however, that NSCs seem more likely to differentiate into glia than into functional neurons after transplantation **(Holmin *et al.*, 1997; Johansson *et al.*, 1999)**, which is a disadvantage for neuron-replacement therapy for PD and other neurodegenerative diseases. An interesting alternative is offered by the fact that DA progenitors (DAPs) can be generated under appropriately modified environmental conditions by a PDR strategy similar to that used to generate iNSCs **(Kim *et al.*, 2011b)**. Although these DAPs are more committed than NSCs, the former are still not terminally differentiated neurons. More recently, Kim *et al.* **(2014)** showed that mouse fibroblasts can be directly reprogrammed into midbrain DAPs by transient (5 days) expression of the four Yamanaka genes and subsequent exposure (for 8 days) to SHH and FGF8. Within thirteen days, self-renewing and functional induced DAPs were generated. Interestingly, the inhibition of both the tyrosine kinase Jak and the glycogen-synthase kinase Gsk3β notably enhanced the reprogramming efficiency. This study demonstrated the feasibility of inducing a specific cell type, switching from fibroblasts to specific DAPs through a PDR strategy.

Although direct reprogramming approaches hold much promise for the treatment of PD, so far direct reprogramming of human cells to authentic ventral midbrain dopaminergic

(vmDA) neurons has not been documented. In contrast, a number of alternative strategies exist, not to be reviewed here, to generate human vmDA neurons by patterning embryoid bodies expressing the TF PAX6 or by floor-plate patterning **(Playne and Connor, 2017)**.

5.2 Therapeutic potential of cell reprogramming for AD

Stem cell therapy for AD has been attempted with adult cells and ESCs as well as with iPSC **(Lee *et al.*, 2016)**. Here, we will focus on the potential of induced neural cells for the treatment of AD in patients and AD animal models.

Since the initial stages of AD are characterized by an early loss of basal forebrain cholinergic neurons (BFCN), cholinergic neuron replacement appears as a relevant therapeutic goal. Within this context, it is of interest that BFCN can be consistently derived from human ESCs **(Bissonnette *et al.*, 2011)** and possibly from human iPSCs. It has been also demonstrated that functional glutamatergic neurons can be generated by reprogramming skin fibroblasts from normal individuals and familiar AD patients by overexpression of a larger set of forebrain transcription regulators (BRN2, MYT1L, ZIC1, OLIG2 and ASCL1) in the presence of neuronal survival factors **(Qiang *et al.*, 2011)**. In a different strategy, two small molecules, forskolin and dorsomorphin were used to enable the TF Neurogenin 2 (NGN2) to directly convert human fetal lung fibroblasts into cholinergic neurons with a high level of purity (>90%) and efficiency (up to 99% of NGN2-expressing cells), thus bypassing any pluripotency stage. The induced human cholinergic neurons displayed mature electrophysiologic properties and exhibited motor neuron-like features. Inclusion of an additional TF, SOX11, allowed an efficient conversion of postnatal and adult skin fibroblasts from healthy individuals and AD patients into cholinergic neurons **(Liu *et al.*, 2013)**. Using a transgene-free approach, Hu *et al.* **(2015)** were able to convert human fibroblasts from normal and AD

subjects into iNs. The authors used a chemical cocktail of seven small molecules, referred to as VCRFSGY, namely, valproic acid, CHIR99021, Repsox, Forskolin, SP600125 (an inhibitor of the N-terminal kinase JNK), GO6983, (a protein-kinase-C inhibitor, G) and Y-27632 (ROCK inhibitor, Y; *cf.* Section 4.1) which directly converted fibroblasts into neurons bypassing the neural progenitor stage. The chemically-induced human neurons resembled hiPSC-derived neurons and human iNs with respect to morphology, gene expression profiles, and electrophysiologic properties. This approach provides an alternative strategy for modeling neurological diseases and for regenerative medicine. Another promising type of cells for the treatment of AD are the neural restricted progenitor (NRP), which represents a type of transitional intermediate lying between the multipotent NPC and terminally differentiated neuron generated during normal neurogenesis. Neural restricted progenitors have the ability of self-renewal and migration in the nervous system **(Mayer-Proschel *et al.*, 1997; Yang *et al.*, 2000)** and can differentiate into neurons rather than glial cells *in vivo* and *in vitro* **(Mo *et al.*, 2007; Mujtaba *et al.*, 1999)**. When injected into the rodent subventricular zone, NRPs can migrate extensively, integrate into different regions of the brain and differentiate into various neuronal subtypes, thus contributing to brain plasticity and repair **(Yang *et al.*, 2000)**. Although NRPs appear to be well-suited for cell therapy of AD, it is difficult to obtain highly purified NRPs from normal nervous tissue **(Mayer-Proschel *et al.*, 1997; Kalyani *et al.*, 1998)**, which prevents further studies and applications that require relatively large number of cells. Hope has now arisen, however that this limitation may be overcome by cell reprogramming. In effect, it was recently demonstrated that by using only three defined TFs -SOX2, c-MYC and either BRN2 or BRN4- human fetal fibroblasts can be converted into human induced NRPs (hiNRPs) **(Zou *et al.*, 2014)**. These hiNRPs display self-renewal capacity and exhibit distinct neuronal characteristics including the expression of multiple neuronal markers, a

neuronal genome-wide transcriptional profile and neuron-like morphology. These hiNRPs can be differentiated into various terminal neuron subtypes with functional membrane properties, but not into glial cells. Thus, a direct and highly efficient conversion of somatic cells into hiNRPs may provide a new source of cells for neuronal development studies as well as for cell replacement therapy of AD patients.

Cell reprogramming is also being used for disease modeling in AD patient-derived iPSCs **(Zhang *et al.*, 2016b)**. Familial AD patient-derived iPSCs were first established by Yagi *et al.* **(2011)** who confirmed that the production of highly toxic Aβ42 peptide is enhanced in all patient-specific iPSC lines. In addition, iPSC-derived neurons carrying mutations respond sharply to γ-secretase modulators and inhibitors, indicating that neurons derived from patient-specific iPSCs hold a significant potential in AD drug discovery. In a gene expression study in iPSC-derived neurons from a 82-year-old sporadic AD patient, significant gene expression changes were observed between primary cells and induced neurons **(Hossini *et al.*, 2015)**. In another study, iPSC-derived neurons were generated from individuals with sporadic or familial AD, the latter caused by duplication of the gene encoding the amyloiod-precursor protein APP, and were shown to produce significantly higher Aβ40 levels. The study also demonstrated other key pathologic features of AD-iPSC-derived neurons, including an increase of phosphorylated tau and of its kinase (GSK) activity **(Israel *et al.*, 2012)**. Evidence has also been found that neurons derived from AD iPSCs exhibit increased vulnerability to glutamate-mediated cell death **(Duan *et al.*, 2014)** and that the accumulation of amyloid beta (Aβ) oligomers induce oxidative stress leading to apoptosis **(Kondo *et al*., 2013; Nishitsuji *et al.*, 2009)**. Neural cells derived from a sporadic AD patient and from another patient carrying the pathogenic APP-E693D mutation, produced intracellular accumulation of Aβ oligomers **(Kondo *et al*., 2013)**.

When human iPSC-derived macrophage-like cells (iPSC-ML) expressing the Aβ proteolitic enzyme Neprilysin-2 (NEP2) and the Fc-receptor-fused form of a single chain antibody specific to Aβ (termed iPS-ML/NEP2 cells), were administered intracerebrally to 5XFAD mice, a model of AD, a significant reduction in the level of Aβ in the brain interstitial fluid was observed. The authors conclude that iPSC-ML/NEP2 cells may be a potential therapeutic agent in the treatment of AD **(Takamatsu *et al.*, 2014)**. In an interesting study, human APP transgenic mice, which display progressive amyloid β deposition and at 8 weeks of age show significant deficits in spatial memory, were bilaterally transplanted in the hippocampus with neuronal precursors of cholinergic neuron phenotype that were derived from human iPSCs. After receiving the cell implants, the transgenic APP mice displayed a significant improvement in spatial memory performance as compared with untreated counterparts. Forty-five days after neural precursor cell transplantation choline acetyltransferase positive cholinergic human neurons and GABA vesicle-transporter-positive GABAergic human neurons were detected in the hippocampus of the APP mice **(Fujiwara *et al.*, 2013)**.

6. CONCLUDING REMARKS

Neurodegenerative diseases are intractable neurologic disorders that constitute a problem of growing medical and economic impact. Within this context, the development of novel therapeutic approaches like regenerative medicine may open new avenues for the treatment of brain disorders. In recent years, cell reprogramming has emerged as a powerful technology that promises to make possible the implementation of regenerative medicine for, up to now, incurable neurodegenerative diseases. It seems therefore plausible to hypothesize that in the not-too-distant future a mature cell reprogramming technology will provide effective means for treating these devastating

brain pathologies. A different path to deal with age-related brain disorders comes from the emerging evidence suggesting that rejuvenating a full organism by partial cell reprogramming may be achievable in the near future. Such an approach promises to provide humankind with the ultimate therapeutic intervention for age-related (and other) brain pathologies, rejuvenation.

The data reviewed here reveal that neural cell generation *in vitro* and to a lesser extent, *in vivo*, are fast growing fields of research whose ultimate goal is to establish effective protocols to produce substantial stocks of patient-derived neural cells, particularly, iNSC and iNPC that can be safely used for the treatment of PD, AD and other brain pathologies. While the use of direct reprogramming strategies is gaining ground, iPSC technology still remains as the main source of induced neural cells. Although the use of neural cells derived from somatic cells from the patient remains as an attractive possibility for implementing personalized regenerative medicine in the brain, the time taken to generate patient-derived neural cells from somatic cells constitutes a significant limitation for the routinely use of this approach in AD and PD. An alternative strategy that is being considered in reference to brain and other pathologies is the creation of banks of induced cells, which like blood banks, would be far more practical and cost-effective than personalized approaches.

Declaration of Interest

None of the authors has any actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations.

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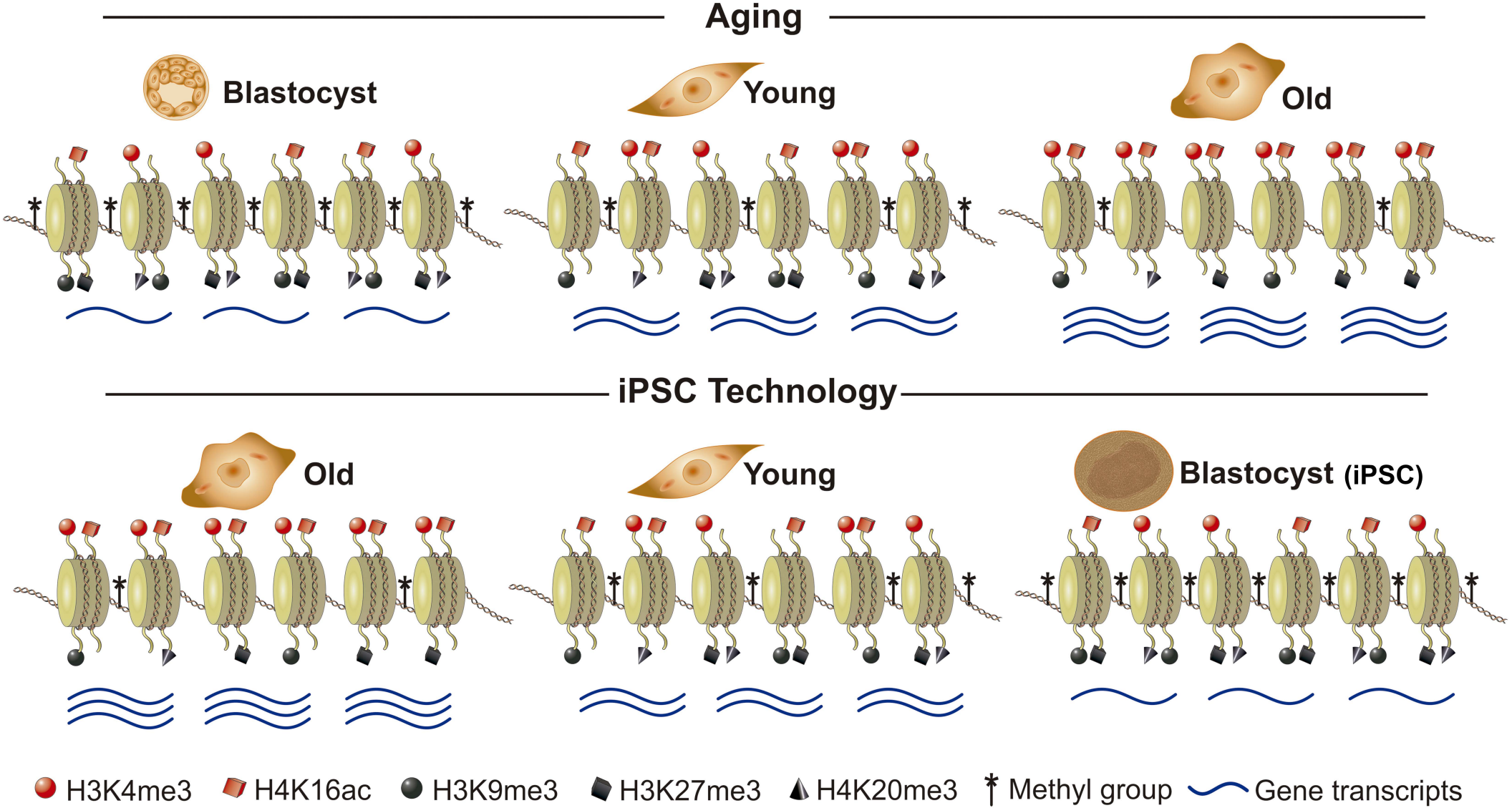
FIGURE LEGENDS

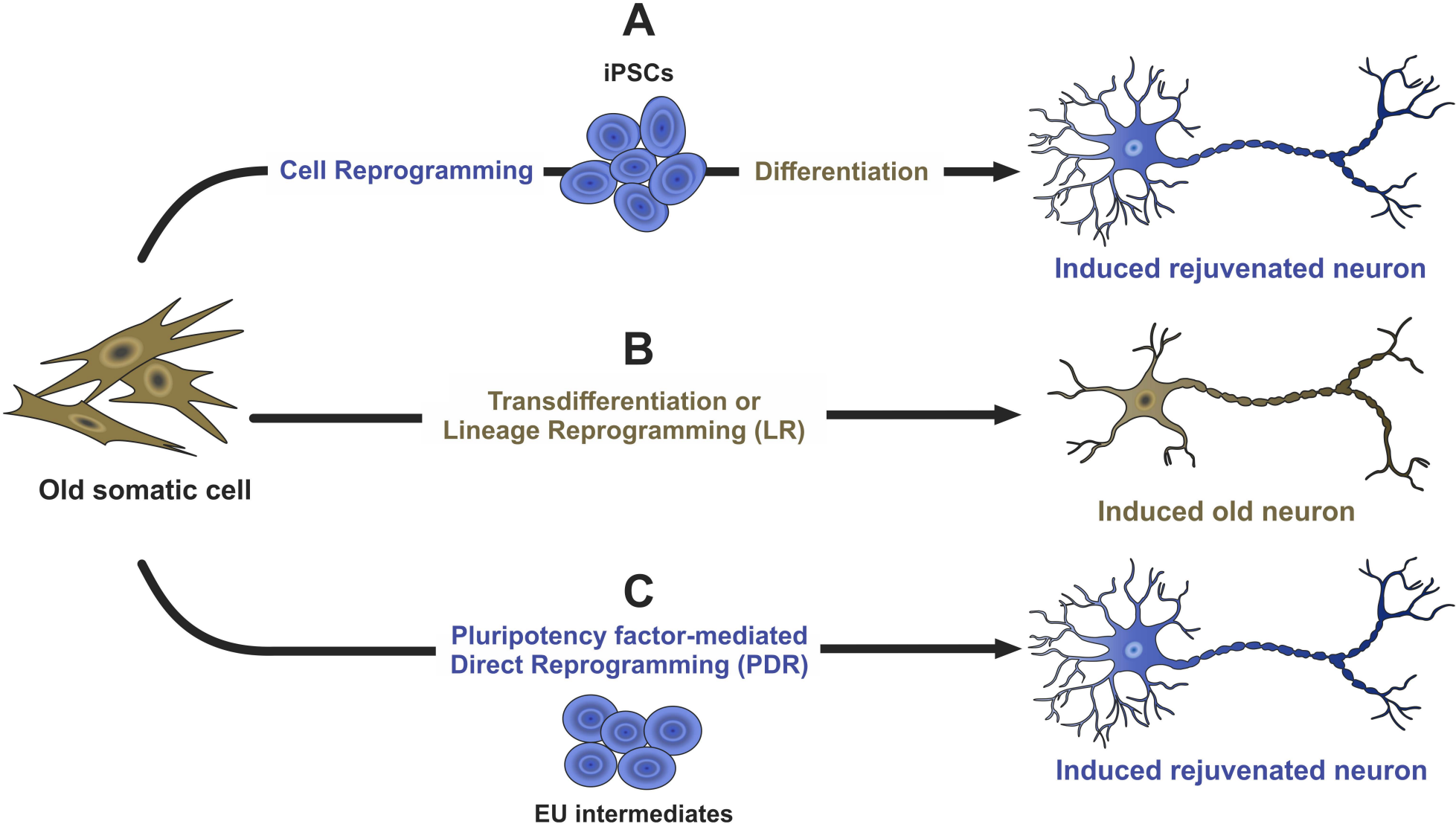
Figure1. **Diagrammatic representation of the epigenetic changes that occur in chromatin during aging and induced–pluripotent-stem-cell** (iPSC) **generation.** The upper diagram represents some of the progressive changes in histone H3 and H4 methylation and acetylation as well as DNA methylation during normal aging. The lower diagram represents the chronologic changes that may occur on the same epigenetic marks during pluripotency-factor induced rejuvenation/dedifferentiation. Red symbols represent chromatin activating marks whereas black symbols correspond to chromatin repressor marks. Stemmed stars represent DNA methylation marks. Blue wavy lines represent gene transcripts (RNAs). The key below the figure describes the above-mentioned symbols.

Figure 2. Diagrammatic representation of the different cell reprogramming methods currently being used. Diagram A illustrates the conventional **iPSC-mediated** protocol of cell reprogramming where somatic cells are first dedifferentiated to iPSC which in turn are differentiated into the induced target somatic cell. **Diagram B** represents the reprogramming procedures termed **lineage reprogramming** also called **transdifferentiation**. These procedures are based on the overexpression of cell-type specific genes and bypass the pluripotency stage. Unlike iPSC-mediated strategies, transdifferentiation does not seem to erase epigenetic marks of aging. **Diagram C** outlines direct reprograming strategies called **pluripotency-mediated direct reprogramming (PDR)** which are based on the transient overexpression of the four Yamanaka genes in somatic cells that become pluripotent intermediates **(PI)** which are responsive to specific differentiation cocktails that can take them to the desired induced somatic cell type. The rejuvenated cells are represented in blue color, the nonrejuvenated cells in brown.

Figure 3. Diagrammatic summary of the cell reprogramming reports on the generation of induced neurons (iNs), induced neural progenitor cells (iNPCs) and

induced neural stem cells (iNSCs). Diagram A summarizes the iPSC-mediated strategies, **Diagram B** represents the LR approaches, **Diagram C** illustrates PDR strategies and **Diagram D** recapitulates approaches using pluripotency genes (PDR) plus lineage-specific transcription factors. Somatic cell types of origin include skin and lung fibroblasts, glia, hepatocytes and Sertoli cells and are shown in brown color. Pluripotent and rejuvenated cells as well as the pluripotent intermediates (PIs) and iPSCs are indicated in blue. Induced neurons (iNs) are either rejuvenated or nonrejuvenated according to the reprogramming strategy used. Thus, the brown and blue portions of the iN shown in the figure represent the proportion of rejuvenated and nonrejuvenatd cells resulting from the different reprogramming stategies used to generate them. Brown reference columns correspond to integrative *in vitro* cell reprogramming approaches reported in the indicated year. Red columns indicate references to integration-free approaches. Green columns list *in vivo* approaches reported. The numbered references in the figure are:(1) Takahashi and Yamanaka, 2006; (2) Vierbuchen *et al.*, 2010; (3) Pang *et al.*, 2011; (4) Ambasudhan *et al.*, 2011; (5) Marro *et al.*, 2011; (6) Sheng *et al.*, 2012; (7) Kim *et al.*, 2011b; (8) Thier *et al.*, 2012; (9) Ring *et al.*, 2012; (10) Mitchell *et al.*, 2014; (11) Meyer *et al.*, 2015; (12) Lujan *et al.*, 2012; (13) Han *et al.*, 2012; (14) Kim *et al.*, 2016; (15) Capetian *et al.*, 2016; (16) Corti *et al.*, 2012; (17) Niu *et al.*, 2013; (18) Su *et al.*, 2014; (19) Heins *et al.*, 2002; (20) Berninger *et al.*, 2007; (21) Heinrich *et al.*, 2010; (22) Torper *et al.*, 2013; (23) Gu *et al.*, 2014.


Aging
Blastocyst
Young
Old
iPSC Technology
Old
Young
Blastocyst (iPSC)
H3K4me3
H4K16ac
H3K9me3
H3K27me3
H4K20me3
Methyl group
Gene transcripts


A
iPSCs
Cell Reprogramming
Differentiation
Induced rejuvenated neuron
B
Transdifferentiation or
Lineage Reprogramming (LR)
Old somatic cell
Induced old neuron
C
Pluripotency factor-mediated
Direct Reprogramming (PDR)
EU intermediates
Induced rejuvenated neuron

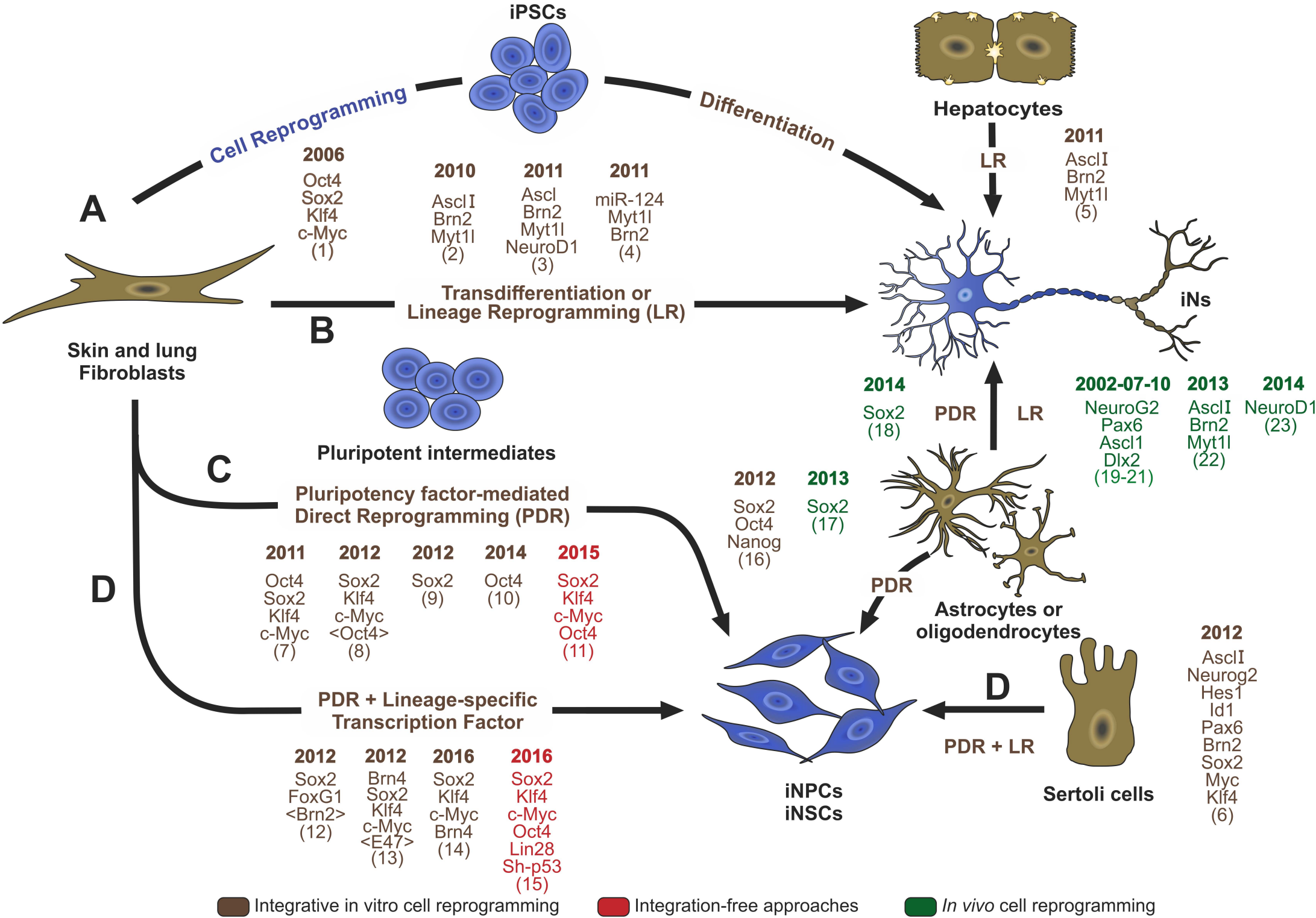

iPSCs
Cell Reprogramming
Differentiation
Hepatocytes
LR
2011
AscI
Brn2
Myt1l
(5)
A
2006
Oct4
Sox2
Klf4
c-Myc
(1)
2010
AscI
Brn2
Myt1l
(2)
2011
Ascl
Brn2
Myt1l
NeuroD1
(3)
2011
miR-124
Myt1l
Brn2
(4)
Transdifferentiation or
Lineage Reprogramming (LR)
iNs
B
Skin and lung
Fibroblasts
Pluripotent intermediates
2014
Sox2
(18)
PDR
LR
2002-07-10
NeuroG2
Pax6
Ascl1
Dlx2
(19-21)
2013
AscI
Brn2
Myt1l
(22)
2014
NeuroD1
(23)
C
Pluripotency factor-mediated
Direct Reprogramming (PDR)
2012
Sox2
Oct4
Nanog
(16)
2013
Sox2
(17)
2011
Oct4
Sox2
Klf4
c-Myc
(7)
2012
Sox2
Klf4
c-Myc
<Oct4>
(8)
2012
Sox2
(9)
2014
Oct4
(10)
2015
Sox2
Klf4
c-Myc
Oct4
(11)
PDR
Astrocytes or
oligodendrocytes
D
PDR + Lineage-specific
Transcription Factor
D
PDR + LR
2012
AscI
Neurog2
Hes1
Id1
Pax6
Brn2
Sox2
Myc
Klf4
(6)
2012
Sox2
FoxG1
<Brn2>
(12)
2012
Brn4
Sox2
Klf4
c-Myc
<E47>
(13)
2016
Sox2
Klf4
c-Myc
Brn4
(14)
2016
Sox2
Klf4
c-Myc
Oct4
Lin28
Sh-p53
(15)
iNPCs
iNSCs
Sertoli cells
Integrative in vitro cell reprogramming
Integration-free approaches
In vivo cell reprogramming

Table 1. Cell reprogramming studies related to PD

Approach	TF used	*In vitro* / *in vivo* Results	References
LR	Ascl1, Brn2, and Myt1l	Conversion of human embryonic and postnatal fibroblasts to DA neurons.	Pfisterer *et al*., 2011
iPSC	hiPSC generated by direct delivery of arginine-tagged OKSM proteins (Pro-1 and Pro-2)	hiPSC were differentiated into NPC and DA neurons by exposure to bFGF and SHH and FGF8.	Rhee *et al*., 2011
LR	Mash1, Ngn2, Sox2, Nurr1, and Pitx3	Reprogramming of human fibroblasts into DA neuron-like cells.	Liu *et al*., 2012
LR	AsclI, Nurr1 and Lmx1a	functional DA neurons from mouse and human fibroblasts	Caiazzo *et al*., 2011
LR	ASCL1/NURR1/LMX1A+miR124 plus a cocktail of neurotrophic factors and small molecules	human fibroblasts from fetal to 91y old donors to iDA neurons	Jiang *et al*., 2015
LR	ASCLI and NURR1 plus SHH and FGF8B, transdifferentiated cells to iNs, including DA neurons.	embryonic mouse fibroblasts to iNs (DA plus pan-neuronal cells)	Oh *et al*., 2014
LR	*ascl1*, *ngn2*, *hes1*, *id1*, *pax6*, *brn2*, *myc*, *klf4* and *sox2*,	Reprogramming of mouse fetal and post-natal fibroblasts into iNs, 10% of which express TH.	Sheng *et al.*, 2013
LR	NURR1 and FOXA2	Ribroblast-derived iNPCs were differentiated into functional DA neurons.	Lim *et al.*, 2015
LR	*brn2*, *sox2*, *foxa2* and *l-myc* with or without exposure to SHH and FGF8	Generation of 90% TH+/TUJ1 neurons from adult mouse fibroblast-derived iNPCs.	Tian *et al.*, 2015
iPSC	SHH and FGF8	iPSC-derived NPC were differentiated into DA neurons and other neuron types.	Wernig *et al.*, 2008
iPSC	Retinoic acid, SSH and FGF8a	Differentiation of iPSC into ventral midbrain dopaminergic neurons.	Hallett *et al.*, 2015
LR	Pax6, Ngn2, Hes1, Id1, Ascl1, Brn2, Sox2, c-Myc to induce iNPC. SAG and FGF8, followed by BDNF and AA was subsequently used to differentiate iNPC-Lmx1a to DA neurons.	TT mouse fibriblasts were reprogrammed to iNPC which were later transduced with a Nestin-Lmx1a lentivirus and differentiated into DA neurons	Wu *et al*., 2015
PDR	OKSM genes (for 5 days) followed by SHH and FGF8 (8 days). Inhibition Jak and Gsk3β enhanced reprogramming efficiency.	Mouse fibroblasts directly reprogrammed into midbrain DA precursors (DAPs).	Kim *et al.*, 2014.

Abbreviations: hiPSC, human induced pluripotent stem cell. DA, dopamine (ergic); SSH, sonic hedgehog; FGF8, fibroblast growth factor 8; TH, tyrosine hydroxylase; iNPC, induced neural progenitor cell; DAP, dopaminergic progenitor cell.

Table 2. Cell reprogramming studies related to AD

Cell Source	Approach	TF used	*In vitro* / *in vivo* Results	Reference
skin fibroblasts from normal individuals and familiar AD patients	LR (lineage-specific TF)	Brn2, Myt1l, Zic1, Olig2 and Ascl1	functional glutamatergic neurons (or… induced neuronal cells which display morphological, electrophysiological, and gene expression profiles that typify glutamatergic forebrain neurons)	Qiang *et al.*, 2011
human fetal lung fibroblasts	Small molecules + lineage-specific TF	NGN2 + forskolin, dorsomorphin	cholinergic neurons	Liu *et al*., 2013
post natal and adult skin fibroblasts from healthy individuals and AD patients	Small molecules + lineage-specific TF	NGN2, SOX11 + forskolin, dorsomorphin	cholinergic neurons	Liu *et al*., 2013
human fetal fibroblasts	PDR + lineage-specific TF	SOX2, c-MYC, BRN2/BRN4	human induced neural restricted progenitors (NRPs)	Zou *et al.*, 2014
familial AD-patient derived iPSCs	Differentiation	-	iPSC-derived neurons carrying mutations with increased amyloid β42 secretion that respond to γ-secretase inhibitors and modulators	Yagi *et al.*, 2011 Miura *et al.*, 2009
sporadic AD patient-derived iPSCs	Differentiation	-	iPSC-derived neurons expressing p-tau and GSK3 which respond to γ-secretase inhibitors	Hossini *et al*.,2015
sporadic and familial AD patient-derived iPSCs	Differentiation	-	iPSC-derived neurons that produce significantly higher Aβ40 levels, increase of phosphorylated tau and of its kinase (GSK) activity	Israel*et al.*, 2012
sporadic and familial AD patient-derived iPSCs	Differentiation	-	iPSC-derived neurons with Aβ oligomers intracellular accumulation	Kondo *et al.*, 2013
human iPSCs	Differentiation (4*)	-	iPSC-derived macrophage-like cells (iPSC-ML) which reduced the level of Aβ added to the culture medium, and the culture supernatant of iPSC-ML alleviated the neurotoxicity of Aβ. iPSC-ML expressing NEP2 i.c.v. administered in a mouse model of AD produced a significant reduction in the level of Aβ in the brain interstitial fluid	Takamatsu *et al*., 2014
human iPSCs	Differentiation by culturing iPSCs with retinoic acid, SHH and noggin-Fc	-	neuronal precursors with cholinergic neuron phenotype transplanted into bilateral hippocampus of PDAPP mice model of AD, significantly improved spatial memory dysfunction and survived for 45 days after transplantation	Fujiwara*et al.*, 2013

References to Table s1 and 2 are located on the reference list at the end of the article.