Modern Alchemy: Cellular Reprogramming and Transdifferentiation

*Summer A. Helmi \(^1\), Derrick E. Rancourt \(^1\)

\(^1\) Department of Biochemistry and Molecular Biology, University of Calgary, Canada

\(^2\) Department of Oral Biology, Faculty of Dentistry, Mansoura University, Egypt.

ABSTRACT

The early conversion of a terminally differentiated somatic cell nucleus to totipotency by nuclear transfer and the consequent development of a whole organism pioneered the field of cellular reprogramming. Since then, several studies have demonstrated that the forced expression of lineage-specific transcription factors in adult cell types can convert cell fate from one lineage to another. The consequent breakthrough of generating induced pluripotent stem cells by reprogramming adult fibroblasts to pluripotency using the transduction of the Yamanaka factors (Oct4, Sox2, Klf4 and c-Myc), opened new horizons in the fields of regenerative and personalized medicine. But the problems of reprogramming efficiency and tumor formation associated with iPSCs called for the development of other reprogramming techniques such as direct reprogramming both in vitro and in vivo. Direct cellular reprogramming in vivo, also known as in situ Transdifferentiation, is a new method to alter cell fate within the tissue that requires replacement or repair. This can be time and cost effective and may overcome some of the disadvantages associated with other methods of cellular reprogramming. In this article, we review the history of cellular reprogramming, highlighting the recent development of in situ transdifferentiation.
INTRODUCTION

Alchemy was a protoscience that focused on the transmutation of common, base metals such as lead into desirable, precious metals such as gold. Although the ability to generate gold from lead never occurred, its tradition led to the disciplines of metallurgy, chemistry and nuclear physics. In biology, it can be argued that a modern form of alchemy has arisen through the emerging field of cellular reprogramming, wherein common, base cell types such as fibroblasts are transmutated into desirable, precious cells such as pancreatic islets or cardiomyocytes. The early experiments of nuclear transfer transformed older concepts that supported the irreversible silencing or deletion of genes during the course of differentiation \cite{1}. Following nuclear transfer, cellular reprogramming achieved wide steps; it was crowned by the generation of induced pluripotent stem cells (iPSCs) \cite{2}. This breakthrough came with the hurdles of reduced reprogramming efficiency and tumor formation. Realizing cellular reprogramming as a clinical approach led to the development of direct reprogramming, also known as Transdifferentiation \cite{3}. This review covers the history of cellular reprogramming beginning with the early experiments in somatic cell nuclear transfer and ending with the introduction of in situ Transdifferentiation.

CELLULAR REPROGRAMMING

Cellular Reprogramming via Nuclear Transfer

The ability of an oocyte to reprogram a somatic cell nucleus to totipotency and the subsequent development of a new organism by nuclear transfer changed the long believed theory that cell differentiation is the result of eliminating the genes that were silenced and keeping the genes that were expressed in the tissue \cite{1}. The early nuclear transfer experiments carried out by Briggs and King successfully resulted in normal tadpoles in the frog Rana pipiens. This was achieved by transplanting a blastula cell nucleus into an enucleated egg \cite{4}. However, in a follow-on experiment, tadpoles failed to develop normally after nuclear transfer of an endodermal cell nucleus derived from a neurula stage embryo. They concluded that as development proceeds some genes needed for normal development had either been lost or were irreversibly repressed \cite{5}.

Gurdon did similar experiments with the embryos of Xenopus laevis \cite{1}. Unlike Rana pipiens, Xenopus laevis has a shorter life cycle and can produce eggs all over the entire year by hormonal stimulation. However, its most important characteristic is the anucleolate mutation, which in the heterozygous state provided a live cell nuclear marker with which cells of a donor origin could be distinguished from those that resulted from failed recipient egg enucleation \cite{6}. Gurdon transferred the nuclei from blastula and gastrula stages to eggs that proved to be of high quality, and that led to the development of normal swimming tadpoles. However, the number of healthy tadpoles decreased as the stage of donor embryo development progressed. Using similar results produced by Briggs and King, Gurdon concluded that there is no exact relation between nuclear differentiation and tissue differentiation. He suggested that...
nuclear differentiation may be more related to the processes which take place when individual cells become differentiated into their final functional state \[7\].

Nuclear transfer experiments later succeeded in mammals. Morula stage embryos were developed after transferring labeled rabbit morula cell nuclei into enucleated rabbit eggs \[8\]. Early experiments in mouse initially failed because somatic cell nuclei were transferred into enucleated zygotes instead of unfertilized eggs. One exception was when live mice were produced by the transfer of a zygote donor nucleus into an enucleated zygote \[9\]. The success of this latter experiment was thought to be due to the same developmental state of donor and recipient cells. Afterward, donor cell nuclei from later developmental stages failed to develop \[10\]. Although all experiments performed on amphibians used unfertilized eggs, it was believed, perhaps incorrectly, that zygote cytoplasm could support development better than unfertilized egg cytoplasm. Other nuclear transfer experiments using embryonic donor cell nuclei from other mammalian species included pigs \[11\], cows \[12\] and monkeys \[13\]. In species where nuclear transfer was problematic, nuclei were transferred into egg cytoplasm and, the next day, to zygote cytoplasm \[14\].

Nuclear transfer experiments led to the idea of cloning livestock for purposes of genetic engineering. In 1996, Wilmut’s group demonstrated in sheep that an embryonic cell line nuclei could be used to generate cloned sheep via transfer when fused to an enucleated egg \[15\]. A step forward used adult mammary gland cells resulted in Dolly the sheep \[16\].

Serial cloning of mice to four and six generations in two independent lines was also claimed to be successful. Although gross behavioral parameters showed no signs of premature aging, telomeres appeared to increase slightly in length. In fact, only one cloned mouse was born in the sixth generation from more than 1,000 nuclear transfers. This mouse was eaten by its foster mother, suggesting that it was unhealthy \[17\].

Adult cloned mice were obtained from mature lymphocytes which carried differentiation-associated immune-receptor rearrangements \[18\]. Genetically labeled post-mitotic olfactory neurons were also used as nuclear donors to clone adult mice \[19, 20\]. Unfortunately, a very low number of the resulting animals produced by transferring the nuclei of adult or differentiated cells resulted in normal offspring. Developmental and physiological abnormalities were found in a large number of embryos obtained, especially in the placentas. Because many of these abnormalities were not heritable, it is hypothesized that they were not caused by deficiencies in chromosome replication, but by a failure to reprogram epigenetic characteristics of somatic cells, especially imprinted genes \[21, 22\]. In support of this argument, serial cloning of mice via pluripotent embryonic stem cells (ESCs; where ESC lines were derived from each serially cloned embryo and used as donor cells) proved to be more efficient than cloning using adult cells. Some of the produced clones further developed to fertile adults \[23, 24\].

The ability to derive ESC lines from cloned embryos made the idea of cloning humans using the justification that “therapeutic cloning” could be used to generate patient-specific ESCs in order to
generate cells and tissues that would, in theory, escape immune rejection. Advanced Cell Technology, an American company conducted an unsuccessful experiment to produce cloned human embryos. Adult cumulus cells in addition to skin fibroblasts were used as donor cells. With cumulus cells as donors, embryos did not survive past the six-cell stage, while eggs reconstituted with adult fibroblasts developed pronuclei but no cleavage was evident [25]. Another discredited study claimed that 11 human ESC lines were created by somatic cell nuclear transfer using skin fibroblasts but were later shown to be fraudulent [26]. With the advent of iPSCs that occurred shortly after this drama [27], the furor to generate patient-specific ESCs via therapeutic cloning soon subsided.

**Cellular Reprogramming via Cell Fusion**

Fusing cells to create hybrids was also proposed as another promising method for cellular reprogramming at the time. Embryonic cells were fused with adult cells to test the plasticity of terminally differentiated cells. Findings showed that the resulting hybrids always shifted towards the phenotype of the least differentiated cell forming the hybrid. Pluripotent PCC4aza1 embryonal teratocarcinoma cells were fused with adult thymocytes. The resulting hybrids formed tumors characteristic of embryonal carcinoma cells. These results suggested that the embryonal carcinoma cells reprogrammed the adult thymocytes to form tumors [28]. In an embryonic germ cell-lymphocyte hybrid, suppression of lymphocyte-specific gene expression was apparent [29]. These and other studies argued the repression of the adult somatic cell gene expression in hybrid cells containing pluripotent nuclei. For example, it is possible that repression of the somatic nucleus occurs before epigenetic modifications take place in order to suppress inappropriate gene expression while these modifications proceed [30].

Cellular fusion experiments were later directed towards the possibility of applying this approach to human cells. Human embryonic stem cells were fused with human fibroblasts. The resulting hybrid cells maintained a stable tetraploid DNA content. The hybrids also had the morphology, antigen expression and growth rate of human embryonic stem cells. Differentiation of these hybrids both in vitro and in vivo yielded cells from the three germ layers. Results also showed that the somatic genome was reprogrammed to an embryonic state. These results established that human embryonic stem cells are capable of reprogramming the somatic nuclei to pluripotency [31].

**Cellular Reprogramming using Transcription Factors**

The idea of the egg cytoplasm being able to reprogram somatic cell nuclei regardless of low efficiency opened the door for further advances in the field of somatic cell nuclear reprogramming. Mouse fibroblast cell cultures treated with 5-azacytidine or 5-aza-2'-deoxycytidine contained functional contractile striated muscle cells, adipocytes and chondrocytes capable of the producing cartilage-specific proteins [32]. In a follow-on study, a transfected DNA locus, which was believed to respond to 5-aza-2'-deoxycytidine (5-aza-c, converted fibroblasts into myoblasts. Screening done to identify the locus narrowed the search to MyoA, MyoH and MyoD transcription factors. Only transfection with MyoD cDNA converted fibroblasts to stable myoblasts [33]. The discovery of MyoD indicated that
overexpression of a key transcription factor was enough to change and override the endogenous gene expression pattern of a cell. MyoD was later used to convert chondroblasts, retinal-pigmented epithelial cells and smooth muscle cells into mononucleated myoblasts and multinucleated myotubes. These data showed that the four terminally differentiated phenotypes responded to MyoD in the same way. Moreover, this response followed the same sequence observed during normal myogenesis \[34\]. These observations generated the hypothesis that transcription factors, which determine cellular identity during development, can alter cell fate when they are ectopically expressed in certain heterologous cells.

After the discovery of MyoD, several trials were carried out to change the phenotype of one blood cell type to another. For example, splenic B cells were reprogrammed to functional macrophages through the forced expression of C/EBPα. This achievement was believed to be due to the inactivation of Pax5, known to be responsible for the commitment of progenitors to become mature B lymphocytes \[35\]. Overexpression of C/EBPα in pre-T cells induced the formation of functional macrophages, while Gata-3 addition inhibited this C/EBPα-induced lineage conversion \[36\]. Gata-1 overexpressed in progenitors committed for neutrophilic monocytes reprogrammed them into erythroid as well as basophilic and eosinophilic cells \[37\]. Conditional Pax5 deletion in mice caused mature B cells from peripheral lymphoid organs to dedifferentiate \textit{in vivo} progenitors in bone marrow, which is believed to be the cause of the increased T cell numbers in the thymus of T-cell-deficient mice \[38\].

\textbf{Induced Pluripotent Stem Cells}

Takahashi and Yamanaka first hypothesized that the transcription factors, which maintain embryonic stem cell (ESC) pluripotency have the potential to turn somatic cells back to the pluripotent state. 24 candidate transcription factors known to be involved in pluripotency of experiments were reduced to four factors, Oct3/4, Klf4, Sox2, and c-Myc (OKSM), able to reprogram mouse fibroblasts into an ESC-like state \[27\]. Yamanaka and colleagues also reported that human-induced pluripotent stem cells (iPSCs) could be reprogrammed using this same combination of factors \[39\]. Human iPSCs have pluripotent characteristics that make them capable of giving rise to more than 200 cell types \[27\]. The phenomenal success of Yamanaka and his team brought an end to nuclear transfer and cell fusion experiments, especially when directed towards generating patient-specific pluripotent cells.

The Yamanaka factors were used to derive iPSCs in multiple species including rats and rhesus monkeys \[40,41\]. Furthermore, several cell sources have been used for the generation of iPSCs such as neural cells \[42\], keratinocytes \[43\], liver cells \[44\] and terminally differentiated blood cells \[45\]. Reprogramming of human fibroblasts to iPSCs has also been achieved using a different combination of reprogramming factors. For example, Oct4, Sox2, Nanog and Lin28 \[46\].

Since the development of iPSCs, understanding chromatin changes and chromatin dynamics during the process of reprogramming became essential. Initial studies have focused on the mechanism by which the four key reprogramming transcription factors work. The state of ESC chromatin is known as “open”. In open chromatin state, heterochromatin is disperse and dynamic, indicating a hyperactive
transcriptional state. A particular network of transcription factors with epigenetic proteins that interact with DNA is necessary to maintain ESCs pluripotency. iPSCs and ESC share the same characteristics; iPSCs have to sustain the same molecular structure as ESC. Moreover, they have to overcome the epigenetic barrier of closed chromatin during the reprogramming process. Hence, the reprogramming process involves a cascade of transcription factors that interacts with chromatin modifier enzymes and histone related enzymes.

It has been suggested that the reprogramming process is initiated by the exogenous transcription factors causing epigenetic changes. These transcription factors either interact with the DNA, via histone modifiers or interact together with chromatin remodeling factors. Thus, there are known time points where the OSKM factors start the reprogramming process. Certain transcription factors are capable of interacting with DNA or with a chromatin remodeling enzyme. The interaction depends on which gene is activated. Briefly, the locus, the type of transcription factor and the context determine this molecular mechanism. Oct4 was found to be indispensable for reprogramming. In some studies, Oct4 alone is sufficient for reprogramming to take place. Furthermore, Oct4 also has a significant effect on the reprogramming process when combined with Sox2.

Oct4 and Sox2 are known to form a heterodimer that interacts with some promoters. Besides, this heterodimer was found to interact with Nanog. Nanog is a transcription factor that is not part of the reprogramming cocktail presented by Yamanaka and his colleagues. However, its importance lies in participating in the ESC regulatory circuitry together with Oct4 and Sox2 to maintain pluripotency. When Oct4 and Sox2 interact with promoters in DNA, Nanog is also involved in the interaction. Oct4 and Sox2 can activate transcription in a chromatin independent manner through interaction with other co-activators. In this context, studies showed that Oct4 and Nanog together interact with repression complexes in mouse ESCs. These complexes are often histone deacetylases such as Mta1.

c-Myc is an important participant as it recruits multiple chromatin modifiers, such as histone acetyltransferases GCN5, p300 and histone deacetylases HDACs. c-Myc allows the augmentation of methylation in H3K4me3 site and the global acetylation. During the reprogramming process, c-Myc, activates its target before other core pluripotency transcription factors are activated, which facilitates opening the chromatin for other factors. An example of c-Myc’s potential in opening chromatin is its association with Tip60-p400 a complex, which acetylates and remodels nucleosomes, respectively. p400 is a part of the Swi2/Snt2 family that is famous among the ATPase chromatin remodeling enzymes, exchanging histones H2AZ-H2B within nucleosomes. It also functions to release RNA polymerase from a paused state from only about one-third of the genes that are being actively transcribed. This activity might be enhancing the reprogramming of the cells.

Klf4 activates the transcription of Sox2, which participates in the pluripotency cascade through JAK-STAT3, phosphatidylinositol 3-kinase (PI(3)K), also known as Akt and extracellular signal-regulated kinases (Erk) pathway. LIF first reaches the receptor gp130 and sends a signal to JAK, which later
phosphorylates Stat3 to activate Klf4. Klf4 will then activate Sox2, which in sequence activates Oct3/4, thus promoting pluripotency [55].

Direct cellular reprogramming

In his famous model of the epigenetic landscape (Fig. 1), Waddington suggested that cells committed to a specific lineage couldn’t be recommitted to another lineage or change channels due to energetics [56]. Yamanaka used the same analogy of channels from Waddington, proposing that cells could be pushed back up the channel towards pluripotency. This stochastic reprogramming model also predicted that through the process of partial reprogramming, cells could be redirected to down other lineages or channels [57].

![Figure 1. Stochastic Model of Cellular Reprogramming Proposed by Yamanaka.](image)

Besides undergoing the natural process of differentiation, a cell can be pushed up to the pluripotent state, or partway to a progenitor state where it is then free to differentiate towards another lineage. Cells can also pass to senescence or apoptosis.

Transdifferentiation (Fig. 2) refers to the conversion of one terminally differentiated cell state to another without regressing to pluripotency. It is also referred to as lineage reprogramming or direct conversion.
Transdifferentiation allows avoiding the iPSCs state, which can result in faster reprogramming with less chance for tumor formation.

Figure 2: Transdifferentiation

Fibroblasts are turned into different cell types in vitro including hepatocytes, neurons, muscle cells, blood cells and melanocytes.

The first Transdifferentiation attempt was achieved before the discovery of iPSCs when MyoD was used to change the fate of fibroblasts to muscle cells. MyoD’s discovery indicated that over-expression of a key transcription factor was necessary and sufficient to change and override the endogenous gene expression pattern of a cell. However, these experiments demonstrated that changes could only be achieved within cells from the same germ layer, in this case, mesoderm.

Since then, many transcription factor cocktails have been discovered for transdifferentiating one cell type to another, beyond germ lineages. For example, 13 transcription factors responsible for cardiac differentiation were narrowed down to combining Gata4, Mef2c and Tbx5 turning mouse dermal fibroblast directly into differentiated cardiomyocytes. Similarly, 19 transcription factors deemed to be essential for neural differentiation were narrowed down to Ascl1, Brn2 and Myt11 in order to convert...
mouse fibroblasts into functional neurons \(^{[62]}\). Other transcription factor cocktails and resulting cell types are shown in Table 1.

**Table 1: Direct cellular reprogramming conducted using transcription factors**

<table>
<thead>
<tr>
<th>Cell Source</th>
<th>Transcription Factors</th>
<th>Resulting type</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse embryonic fibroblasts</td>
<td>Hnf4α plus Foxa1, Foxa2 or Foxa3</td>
<td>Hepatic like cells</td>
<td>([89])</td>
</tr>
<tr>
<td>Human fibroblasts</td>
<td>Sox10, MITF, and Pax3</td>
<td>Melanocytes</td>
<td>([90])</td>
</tr>
<tr>
<td>Mouse fibroblasts</td>
<td>Erg, Gata2, Lmo2, Rux1c, and Scl</td>
<td>Blood cells</td>
<td>([91])</td>
</tr>
<tr>
<td>Human fibroblasts</td>
<td>Klf4, c-Myc + chondrogenic factor SOX9</td>
<td>Chondroblasts</td>
<td>([92])</td>
</tr>
<tr>
<td>Human gingival fibroblasts</td>
<td>Oct4, l-Myc, Runx2 and Osterix</td>
<td>Osteoblasts</td>
<td>([93])</td>
</tr>
<tr>
<td>Human endothelial progenitor cells</td>
<td>MYOCD</td>
<td>Smooth muscle cells</td>
<td>([94])</td>
</tr>
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</table>

Another Transdifferentiation approach, proposed as an alternative to transcription factor screening, uses the OKSM factors to partially revert cells so that they can be pushed forward either using chemicals or growth factors. The OKSM factors are expressed regenerating tissues such as the zebrafish tail fin blastema. This finding suggests that partial reprogramming may be necessary for regeneration \(^{[63]}\). Thus, the presence of these pluripotency factors in a differentiated cell may have the same effect of loosening chromatin arrangement and placing the cell in a plastic position where it can be directed to become any type of cell \(^{[64]}\). However, rather than completely reverting cells this alternative Transdifferentiation strategy interferes with the acquisition of pluripotency using inhibitors.

Leukemia Inhibitory Factor (LIF) is part of the interleukin (IL)-6 family of cytokines and is considered to be a very important driver of pluripotency \(^{[65]}\). LIF controls pluripotency by targeting the JAK STAT3, JAK PI3K and JAK Erk signaling pathways. These pathways cause activation of the core circuitry of pluripotency transcription factors Oct3/4, Sox2, and Nanog \(^{[66]}\). Part of the OKSM Transdifferentiation method involves blocking the reprogramming process before reaching pluripotency. This process can be achieved through removal of LIF from the cell culture media and inactivating the JAK/STAT pathway using JAK inhibitor (JI1) during the reprogramming process.

The second part of this method uses chemicals and/or growth factors to redirect differentiation towards the desired cell lineage (Fig. 3). Successful Transdifferentiation of mouse embryonic fibroblasts into beating cardiomyocytes was first achieved using this method. This study suggested that Transdifferentiation using OSKM factors was more efficient in producing cardiomyocytes compared to using iPSCs \(^{[67]}\). Neural progenitor cells have also been successfully produced using this same platform \(^{[48]}\).
Figure 3. Direct Reprogramming using OSKM factors.

JI and the absence of LIF puts the cells in a plastic state preventing them from returning to the iPSC state.

The use of transcription factors for cellular reprogramming have some safety concerns. These concerns include mutagenesis and tumor formation. Understanding the molecular mechanisms by which transcription factors work led to the identification of their downstream molecules. Moreover, figuring out their role in lineage reprogramming, lead to one of the most promising potential solutions, which is cellular conversion using small molecules. Small molecules have multiple important advantages over using transcription factors for regulating cell fate: they can be more cost-effective and cell permeable. Their synthesis, preservation, and standardization are easier. More importantly, the effects of small molecules can be optimized by changing concentrations and combinations, providing a higher degree of control over protein function [68].

The molecular mechanism of small molecules during the process of Transdifferentiation is not yet fully understood. Some of the most common small molecules include histone deacetylase (HDAC) inhibitors. These inhibitors cause chromatin decondensation and induce a short dedifferentiation state, which changes the chromatin state [69]. 5-azacytidine (5-aza-C), a DNA methyltransferase inhibitor, is believed to play a similar role in loosening the chromatin [68]. Glycogen synthase kinase-3 inhibitor (GSK3) which is
a WNT signaling activator and TGF-β inhibitors may undergo regulating transition between mesenchymal and epithelial state to promote cell reprogramming and help convert cell fate \[^{[71]}\]. It is interesting that treatment with the same small molecule can produce different cell types. For example, cells treated with 5-aza-c with various concentrations for certain times can convert human fibroblasts into functional neuronal precursors, pancreatic cells and muscle cells \[^{[70,72]}\].

An interesting study showed that direct reprogramming of mouse embryonic fibroblasts to cardiomyocytes was possible through small molecules. A two-stage protocol was used: exposure of MEFs to a small-molecule cocktail: CRFVPTZ (C, CHIR99021; R, RepSox; F, Forskolin; V, VPA; P, Parnate; T, TTNPB; and Z, DZnep) followed by cardiac reprogramming medium containing 15% fetal bovine serum (FBS), 5% knockout serum replacement (KSR), N2 and B27 was used for the induction of the beating colonies. Matrigel-coated dishes proved to better than non-coated and gelatin coated ones \[^{[73]}\].

Micro-RNAs have also been used to transdifferentiate fibroblasts to cardiomyocytes. A combination of miRNAs 1, 133, 208, and 499 induced direct reprogramming of fibroblasts to cardiomyocyte-like cells \textit{in vitro}. Here, JI1 treatment enhanced the reprogramming efficiency by ten folds than using miRNAs alone \[^{[74]}\].

**In Situ Transdifferentiation**

The success of Transdifferentiation \textit{in vitro} has encouraged the idea of \textit{in situ} Transdifferentiation (Fig 4). This process includes the direct introduction of lineage-determining transcription factors into a target organ \textit{in vivo}. If successful, this approach can present advantages over \textit{in vitro} cellular reprogramming. Less manipulation of the reprogrammed cells can be achieved making this process more clinically efficient \[^{[75]}\].
Adenovirus-mediated transduction of mouse liver with Pdx-1 activated the expression of insulin 1 and 2 and prohormone convertase 1/3 (PC 1/3). Although hyperglycemia in diabetic mice was ameliorated, no phenotypic or morphological conversion into functional β-cells was detected [76]. A similar study using the combination of Pdx-1 and Ngn3 in adeno-associated virus (AAV) serotype 8 however failed to alleviate diabetes in streptozotocin-treated mice [77]. The same result occurred using a non-viral vector approach [78]. However, Sox9-expressing hepatic duct cells, when reprogrammed in vivo using Ngn3, Pdx-1 and Mafa secreted insulin, which restored normal glucose levels in streptozotocin-treated SCID mice.

Functional β-cells capable of producing insulin were also generated when exocrine pancreatic cells were transduced in vivo with the transcription factors Ngn3, Pdx-1 and Mafa using adenovirus [79]. Although the transdifferentiated cells could not arrange themselves into pancreatic islets, these studies demonstrated that in vivo Transdifferentiation is possible which will stimulate further studies.

Resident non-myocytes in mice hearts have been reprogrammed into cardiomyocyte-like cells in vivo by local delivery of Pdx-5 after coronary ligation to simulate myocardial infarction. Resulting cells were contractile upon electrical stimulation resembling normal ventricular cells. Reprogramming was even enhanced when Pdx-5 was accompanied by thymosin β4 in the target cells [80]. These results were
confirmed when dividing non-myocytes were converted to functional cardiomyocytes in vivo after overexpression of the transcription factors Gata4, Hand2, Mef2c and Tbx5. This study also reported that fibrosis following myocardial infarction was reduced when Tbx5 was introduced to the non-dividing myocytes [81].

Direct neural conversion in vivo was also achieved in the adult rodent brain. When transcription factors Ascl1, Brn2a, and Myt11 were over-expressed in astrocytes of the striatum, functional neurons were generated [82]. In vivo reprogramming was also achieved in the brains of mice when embryonic and early postnatal colossal projection neurons of layer II/III were reprogrammed into Layer-V/VI corticofugal projection neurons through Fezf2 overexpression. Here, reprogrammed neurons acquired the molecular properties of corticofugal projection neurons [83]. Sox2 alone was able to directly reprogram resident astrocytes into proliferative neuroblasts in the brains of adult mice [84]. A subsequent study showed that the mechanism by which the reprogramming process takes place, as astrocytes were shown to passes through proliferative intermediate progenitors to become differentiated neurons [85].

In vivo iPSCs were obtained through generating genetically engineered reprogrammable mice. In these animals, overexpression of the OSKM factors could be induced upon doxycycline injection. Unfortunately, teratomas formed in almost every organ in the bodies of these mice [86]. The in vivo generated iPSCs were assumed to be totipotent. A follow-on study proved this assumption wrong. Reprogrammable mice with an Oct4-GFP reporter gene allowing in vivo cell tracking demonstrated that teratomas derived from in vivo iPSCs were morphologically indistinguishable from ESCs. They expressed pluripotency markers, and could differentiate into tissues of all three germ layers. However, these in vivo reprogrammed iPSCs did not contribute to placental tissues when introduced into eight-cell embryos, suggesting that in vivo reprogrammed iPSCs were pluripotent and not totipotent [87].

**FUTURE RESEARCH**

Stem cell therapy has shown a remarkable outcome in treating certain diseases, such as leukemia, lymphoma, and immunodeficiency. Nonetheless, utilizing stem cells for tissue transplantation, especially iPSCs and trans-differentiated cells, has not yet been successful. Further investigation is needed to understand the reasons behind an array of factors that hamper the clinical application of somatic cell reprogramming in regenerative medicine. Some of these factors include as follows.

**Safety:** Safer cellular reprogramming processes can be achieved with a better understanding of the ability of target cells to maintain new cellular properties and function over time. Studying the behavior of transfected un-reprogrammed cells is also of great importance. Also, developing strategies to assess damage to non-targeted cells and tissues inside the body is essential. Finally, it is important to find potential alternatives to viral vectors that can be safer yet efficient in reprogramming. Library screening and rational design approaches using chemicals, RNA, and CRISPR libraries will facilitate this endeavor. Similarly, new delivery methods will help to improve efficiency.
**Microenvironment:** Lineage tracing and live imaging studies can markedly enhance our understanding of how tissues sustain appropriate cell types and numbers during homeostasis, and how they respond to different injury stimuli [91]. Moreover, investigating mechanisms within cellular niches, wherein plasticity is controlled in vivo, can cast light on new, efficient and safe reprogramming methods. This includes studying cell interaction, and the role of the extracellular matrix, and cytoskeleton in influencing cell fate. Similarly, a better understanding of developmental biology including that of model organisms will help in understanding cellular plasticity.

**Cell Communication:** Although the signaling pathways involved in dedifferentiation, Transdifferentiation, and reprogramming are not utterly understood, recent studies propose that these pathways may have a critical role in the application of stem cell therapy. The TGF-β, PI3K/Akt, Wnt/β-catenin, Activin/Nodal, and Jak-Stat signaling pathways all have a fundamental role in regulating pluripotency [92]. Understanding which signaling pathways play a crucial role during dedifferentiation, Transdifferentiation and reprogramming can facilitate the development of new strategies to control these processes both in vitro and in vivo.

**Epigenetics:** Epigenetic modifications have significant control over the cellular reprogramming process. Controlling histone modifications, for example, through studying the control of the levels of H3K9 methyltransferases and H3K9 demethylases, in addition to, DNA demethylation and chromatin remodeling can lead to a better outcome of the reprogramming process.

**Immune Modulation:** Evidence suggests that the immune system suppresses regeneration [88]. No matter how great our advances become in directed reprogramming, a better understanding of how the immune system influences cellular differentiation needs to occur before regenerative medicine can be advanced. Understanding which elements of the immune system need to be suppressed to promote cell specific differentiation will not only promote the application of in situ transdifferentiation but may also facilitate natural endogenous repair.

As a potential disruptive technology, cellular reprogramming has undergone several important advancements in the recent years. Although cellular reprogramming is not close to clinical application yet, it is certainly a promising approach in cell-based regenerative medicine.

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