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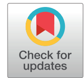

Strategies for *in vivo* reprogramming

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Reprogramming has the potential to provide specific cell types for regenerative medicine applications aiming at replacing tissues that have been lost or damaged due to degenerative diseases and injury. In this review we discuss the latest strategies and advances of *in vivo* reprogramming to convert cell identities in living organisms, including reprogramming induced by transcription factors (TFs) and CRISPR/dCas9 synthetic TFs, as well as by cell fusion and small molecules. We also provide a brief recap of reprogramming barriers, the effect of senescence on reprogramming efficiency, and strategies to deliver reprogramming factors *in vivo*. Because of the limited space, we omit dwelling on naturally occurring reprogramming phenomena such as developmentally programmed transdifferentiation found in the nematode *Caenorhabditis elegans*.

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Introduction

The dogma that differentiated cells have restricted cellular plasticity was already challenged in 1958 by John Gurdon, who cloned the frog *Xenopus laevis* using nuclear transfer [1]. This pioneering work inspired Campbell *et al.* to clone the sheep Dolly 40 years later [2]. In the meantime, in 1987, Davis *et al.* directly reprogrammed mouse fibroblasts into muscle cells, in a process also known as transdifferentiation, by ectopic overexpression of the transcription factor (TF) MyoD, while Gehring's team transdifferentiated *Drosophila* antenna into legs using ectopic overexpression of the TF *Antennapedia* [3,4•]. The emergence of a broader reprogramming research field started 2006, when Takahashi and Yamanaka published that the TF cocktail Oct3/4, Sox2, Klf4 and c-Myc (aka OSKM) reprograms somatic cells to a state of pluripotency, thereby generating 'induced pluripotent stem cells' (iPSCs) [5]. Commonly, most reprogramming procedures are performed *in vitro*, but the much-anticipated scenario of utilizing reprogramming for regenerative medicine applications raises the need for *in vivo* reprogramming.

From *in vitro* to *in vivo* reprogramming

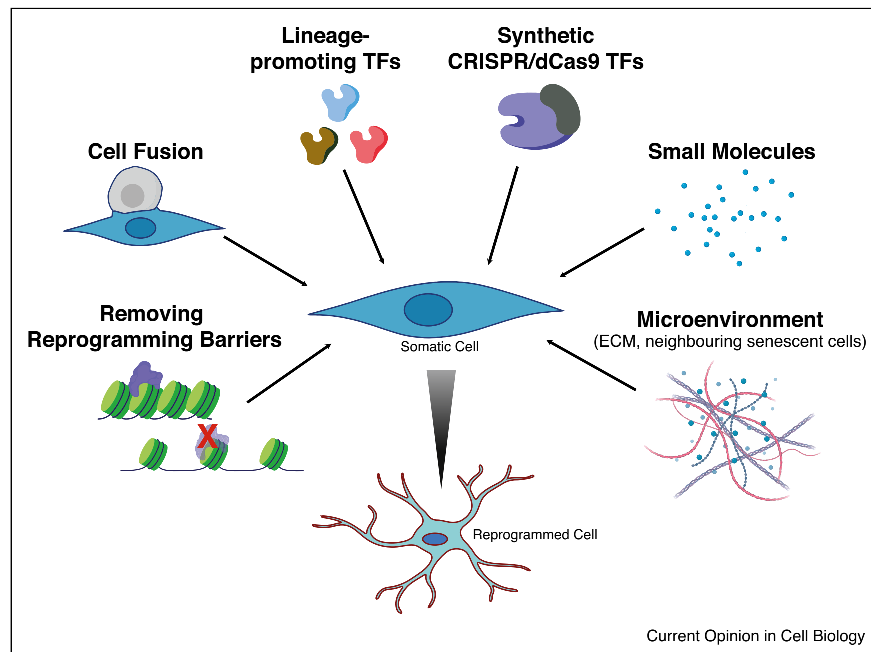
Decades of developmental biology research in various model organisms identified cell fate-inducing TFs that can be used for cellular reprogramming. Forced expression of one single TF such as MyoD (induces muscle fate) [3], or C/EBPα (induces B-cell conversion to macrophages [6], and in *Caenorhabditis elegans* the Zn-finger TF CHE-1 (germ cell reprogramming to neurons) [7,8], or the GATA TF ELT-7 (induces intestinal fate) [9], can be sufficient to induce cell fate conversion. Other types of reprogramming require combination of different TFs: OSKM reprogram differentiated cells to iPSCs [5], Ascl1 +Brn2 +Myt1L transdifferentiate fibroblasts to neurons [10], and Ngn3 +Pdx1 +Mafa directly convert pancreatic cells to insulin-producing β-cells [11]. Initially, TF-induced cellular conversion *in vivo* could be demonstrated mainly in *Drosophila* [4•] and *C. elegans* [8]. In contrast, most mammalian reprogramming procedures were performed *in vitro*, except the *in vivo* transdifferentiation of pancreatic cells to β-cells in insulin-deficient mice by viral delivery of TFs to the pancreas [11]. Subsequently, iPSC reprogramming was achieved *in vivo* by two independent groups using transgenic mice with doxycycline-inducible OSKM [12,13], and differentiated cells of murine retinas could be reprogrammed to a progenitor-like state via cell fusion [14]. While such reprogramming experiments raised the hope for generating tissues by *in vivo* reprogramming, safety concerns and the issue of limited conversion efficiency remain. *In vivo* cell fate conversion requires high efficiency in order to generate sufficient target cells, while preventing the generation of cell populations, which could give rise to tumours.

Current *in vivo* reprogramming strategies are summarized in Figure 1.

Efficiency of *in vivo* reprogramming

Differences between *in vitro* and *in vivo* reprogramming efficiencies could arise from local microenvironmental conditions. Notably, the gene expression profile of *in vivo* generated iPSCs is more similar to that of embryonic stem cells (ESCs), as *in vitro* generated ones [12].

Figure 1



Overview of current reprogramming strategies. Somatic cells can be directly reprogrammed *in vivo* by using one or several of the following methods: Removing reprogramming barriers, cell fusion, natural lineage-promoting TFs, synthetic CRISPR/dCas9 TFs, small molecules ('chemical reprogramming') and modulating the microenvironment.

Furthermore, *in vivo*-generated iPSCs harbour characteristics of totipotency as they can differentiate to trophectoderm – a feature that ESCs generally do not possess [12]. Interestingly, reprogramming efficiency could also be linked to senescence – a protective cellular mechanism, which increases with aging and upon tissue damage. In fact, OSKM overexpression *in vivo* induces tissue damage and many cells respond to this insult by becoming senescent [15]. While senescence has been described as a reprogramming barrier *in vitro* [16], it appears that senescent cells promote *in vivo* reprogramming in their direct vicinity through the secretion of various soluble factors, also known as senescence-associated secretory phenotype (SASP) [15,17]. Generally, SASP reinforces senescence, recruits immune cells, promotes tissue remodelling, and also stimulates regeneration and cellular plasticity: oncogenic-induced senescence in the liver reactivates stem cell markers in non-senescent cells [17] and injury-induced senescence enables reprogramming of Pax7+ muscle stem cells [18]. Likewise, Nanog-positive stem cells in murine lungs could only be generated by *in vivo* reprogramming upon treatment with the DNA damaging agent Bleomycin to trigger senescence [15]. Analogously to injury-triggered senescence, age-related senescence also promotes reprogramming, however, with increased teratoma formation as a by-product. It is conceivable that, besides increased senescence, also cell-autonomous fate protection mechanisms might decrease during aging [11,14]. Among SASP, interleukin 6 (IL6) seems to play a crucial role for the increased efficiency of *in vivo* reprogramming [15,18]. Many senescence-related signalling pathways are regulated by the INK4a/ARF locus, which acts as a reprogramming barrier *in vitro* [16,19], but promotes reprogramming *in vivo* [15]. In the absence of INKa/ARF, tissues fail to efficiently secrete cytokines, including IL6, resulting in reduced *in vivo* reprogramming [15]. In this context, a recent study showed that INK4a is required for OSKM-mediated senescence, while ARF is dispensable [20]. Such striking differences of *in vivo* versus *in vitro* reprogramming with respect to signalling pathways emphasize the importance of using *in vivo* models to study reprogramming. Since many degenerative diseases such as Alzheimer's or Muscular Dystrophy are age-related, the fact that cells in an aged organism might be easier to reprogram, is encouraging. However, the accompanying formation of teratomas prompts for measures to prevent detrimental side-effects during *in vivo* reprogramming.

Recent *in vivo* reprogramming examples

Reprogramming to liver cells

The liver is one of the few mammalian organs that has a natural regenerative capacity and is endogenously repaired after injury. The regenerative capacity of the liver seems to be dependent on bone marrow cell (BMC) migration and their fusion with hepatocytes [21]. This

fusion forms hybrid cells that proliferate and produce cells for liver regeneration [21]. Furthermore, ectopic expression of the TFs FOXA3, GATA4, HNF1A, and HNF4A from a lentiviral vector can convert murine myofibroblasts into hepatocyte-like cells *in vivo* (reprogrammed hepatocytes, rHeps) [22]. Recently Cheng *et al.* demonstrated that injection of FOXA3, HNF1A, and HNF4A into patient-derived tumour xenografts reprogrammed carcinoma cells into rHeps in living mice, which lost malignant phenotypes and retrieved hepatocyte-specific characteristics [23]. This intriguing example demonstrates that *in vivo* reprogramming could also serve as a therapeutic strategy for cancer treatment.

Direct conversion to neuronal cells

Another recent study showed that BMCs can fuse with neuronal cells in murine adult brains, which might be a mechanism to protect and regenerate brain tissues. As cell fusion can induce cellular reprogramming by altering cellular plasticity [24], BMCs are in the focus of many studies aiming to achieve reprogramming of different tissues *in vivo*. For instance, it was shown that transplanted BMCs into a humanized mouse model of Friedreich's ataxia could stimulate neuronal repair in the brain [25]. Furthermore, it was found that following retinal damage, endogenous BMCs migrated to the injury site and fused with Müller glia cells (MGCs), which then converted into retinal neurons [26•]. This endogenous repair process could be enhanced by perturbations of the SDF1/CXCR4 pathway, which led to higher *in vivo* reprogramming efficiencies of MGCs to neurons [26•].

Importantly, MGCs of new-born mice can also be converted to neurons by ectopic expression of the TF Ascl1 [27]. However, MGCs derived after postnatal day 16 required the addition of the histone deacetylase inhibitor trichostatin-A, indicating a more repressive chromatin state of older MGCs. Indeed, the overall chromatin state of younger MGCs appeared to be in a more permissive state as measured by an assay for transposase-accessible chromatin (ATAC-seq), thus highlighting the importance of removing epigenetic reprogramming barriers in order to increase reprogramming efficiency *in vivo* [27]. Strikingly, based on these findings, Yao *et al.* succeeded in partially restoring vision in congenitally blind mice [28••]. They first stimulated proliferation of MGCs with β-catenin and subsequently induced reprogramming by overexpressing the rod cell fate-specifying TFs Otx2, Crx, and Nrl. Four weeks later, the primary visual cortex of treated mice showed activity after light exposure, indicating that generated rod cells were functional and integrated into already existing retinal circuits [28••].

Further, a recent study demonstrates neuronal *in vivo* conversion of neuroblasts into mature myelinating oligodendrocytes by forced expression of the TFs OLIG2 and SOX10 in a demyelination mouse model. Interestingly, this reprogramming occurred also spontaneously with very low frequency in the absence of ectopic TF expression, revealing an unexpected plasticity of committed neuroblasts [29]. More recently, Matsuda *et al.* reported that the TF NeuroD1, which had previously been shown to directly reprogram astrocytes in the cortex of stab-injured mice into neurons [30], is able to transdifferentiate microglia to neurons *in vitro* and *in vivo* [31]. This potential of NeuroD1 relies on its ability to occupy bivalent chromatin domains to initiate neuronal gene expression, before suppressing microglial genes by altering the epigenome [31]. Generally, the recent success in neuronal *in vivo* reprogramming could be a future strategy to treat lesions in the adult brain.

Generation of muscle and other cell types by reprogramming

The earlier mentioned senescence-dependent *in vivo* reprogramming of Pax7+ muscle stem cells [18] is only one of several recent *in vivo* muscle reprogramming examples. For instance, cardiomyocytes could be generated from cardiac fibroblasts by ectopically overexpressing the TFs Gata4, Mef2c, and Tbx5 [32,33•], or by small-molecule compounds [34]. Furthermore, transient reprogramming by OKSM factors in skeletal muscle enhances regeneration without tumorigenic side effects [35], which also improves multiple aging symptoms by inducing rejuvenation as seen in a mouse model of progeria [36].

Another recent study by Kurita *et al.* reports the *in vivo* reprogramming of wound-resident mesenchymal cells to epithelial cells. Viral delivery of the factors DNP63A, GRHL2, TFAP2A, and MYC leads to epithelialization from the surface of cutaneous ulcers in mice [37]. Such *in vivo* reprogramming could be used to cure non-healing wounds, further highlighting the potential of cellular reprogramming for regenerative medicine.

Delivery of reprogramming factors *in vivo*

The use of genome integrating viruses for the delivery of reprogramming factors bears risks such as insertional mutagenesis, which prompt for alternative methods better suited for future clinical applications: non-integrative Sendai virus vectors were applied to deliver cardiac reprogramming factors *in vivo* to reduce fibrosis in a mouse model of myocardial infarction [32]. Also, nanoparticle-based gene carriers were used to convert fibroblasts into cardiomyocytes *in vivo* [33•], or a tissue nanotransfection device generating a focused electric field for direct cytosolic delivery of DNA *in vivo* to transdifferentiate fibroblasts into endothelial cells [38].

An alternative approach to using reprogramming TFs is the application of small molecules, which has recently been reviewed [39•]. For instance, a chemical cocktail could directly reprogram adult cardiac fibroblasts into

Table 1

Recent studies reporting *in vivo* reprogramming

Starting cell fate	Target cell fate	Reprogramming factors/ reagent	Delivery/induction	*Species* + context	Reference
Undefined	Teratoma	OCT4, SOX2, KLF4, cMYC	Inducible transgenic DNA	*Mouse* + senescence ind.	Mosteiro *et al.* [15]
Epithelial/liver cell	Stem cell-like	H-Ras^{V12}-induced senescence	Transposable DNA injection	*Mouse* + senescence ind.	Ritschka *et al.* [17]
Skin cell	Induced neuron/endothelial cell	ASCL1, BRN2, MYT1l/ETV, FOXC2, FLI1	DNA via nano-transfection	*Mouse* + injury-induced ischaemia	Gallego-Perez *et al.* [38]
Muscle stem cell	Stem cell	OCT4, SOX2, KLF4, cMYC	Inducible transgenic DNA	*Mouse* + injury induced senescence	Chiche *et al.* [18]
Hepatic fibroblast	Hepatocyte	HNF1A, HNF4A, FOXA3, GATA4	DNA via AAV delivery	*Mouse* + liver fibrosis	Song *et al.* [22]
Hepatoma cell	Hepatocyte-like cell	HNF1A, HNF4A, FOXA3	DNA via AAV delivery	*Mouse* + hepatocellular carcinoma	Cheng *et al.* [23]
Neuroblast	Myelinating oligodendrocyte	OLIG2, SOX10	DNA electro-poration	*Mouse* + induced demyelination	Waly *et al.* [29]
Microglia	Induced neuron	NeuroD1	DNA via LV delivery	*Mouse*	Matsuda *et al.* [31]
Neuron	Binucleate neuronal heterokaryon	Fusion with bone-marrow cells (BMCs)	BMC trans-plantation	*Mouse* + Friedreich's Ataxia	Kemp *et al.* [25]
Rod photo-receptor	Cone-like cell	Split dCas9-activator/ repressor	DNA via AAV delivery	*Mouse* + retinitis pigmentos	Moreno *et al.* [44•]
Müller glial cell	Retinal neuron	ASCL1, TSA	Inducible transgenic DNA	*Mouse* + NMDA-induced neuronal injury	Jorstad *et al.* [27]
Müller glial cell	Rod photoreceptor neuron	β-Catenin; OTX2, CRX, NRL	DNA via AAV delivery	*Mouse* + congenital blindness	Yao *et al.* [28••]
Müller glial cell	Amacrine neuron via reprogrammed hybrid	Fusion with bone-marrow cells (BMCs)	Intravitreal injection of NMDA	*Mouse* + NMDA-induced neuronal injury	Pesaresi *et al.* [26•]
Undefined skeletal muscle cell	Stem cell-like cell	Transient OCT4, SOX2, KLF4, cMYC	Inducible transgenic DNA	*Mouse* + surgical skeletal muscle injury	de Lázaro *et al.* [35]
Undefined cardiac cell	Cardiomyocyte-like cell	GATA4, MEF2c, TBX5	DNA on gold nanoparticles	*Mouse* + myocardial infarction	Chang *et al.* [33•]
Cardiac fibroblast	Cardiomyocyte-like cell	CRFVPTM drug cocktail	Orally and intra-peritoneal inj.	*Mouse*	Huang *et al.* [34]
Cardiac fibroblast	Cardiomyocyte-like cell	GATA4, MEF2c, TBX5	Sendai virus vectors	*Mouse* + myocardial infarction	Miyamoto *et al.* [32]
Germ cell	Neuron	CHE-1; FACT depletion	Inducible transgene	*C. elegans*	Kolundzic *et al.* [7]

Abbreviations. NMDA: *N*-methyl-D-aspartate; CRFVPTM: C – CHIR99021, R – RepSox, F– Forskolin, V – VPA, P – Parnate, T – TTNPB, M – Rolipram; TSA: histone deacetylase inhibitor Trichostatin-A; AAV: adeno-associated viral; LV: lentiviral; ind.: induced.

cardiomyocytes *in vivo*, which, in contrast to TF-based reprogramming, depends on injury-induced fibroblast activation [34].

For transient OKSM induction in skeletal muscle, Wang *et al.* used plasmids instead of viral vectors, as this approach might be safer than using genome integrating lentiviruses or retroviruses for future clinical applications [40]. Taken together, these alternative delivery strategies hold great promise for future clinical applications that rely on *in vivo* reprogramming of patients' endogenous cells to repair and regenerate tissue.

CRISPR/dCas9-based synthetic TFs for *in vivo* reprogramming

The genome-editing tool CRISPR/Cas9 is becoming increasingly popular to support or induce reprogramming. Wang *et al.* used CRISPR/Cas9 to knockout the MyoD gene in mouse myoblasts, resulting in their transdifferentiation to brown adipocytes [40]. Furthermore, a modified Cas9 that is deficient for its DNA cutting activity, but still binds DNA (deactivated Cas9 or dCas9), can be fused to transcription activators or repressors [41]. These CRISPR/dCas9-TFs can simultaneously target several genes, using different guide RNAs, to reprogram somatic cells *in vitro* into neurons [42] or iPSCs [43] and rod cells into cone cells *in vivo* [44•]. Importantly, the application of this technology for *in vivo* reprogramming requires the large size of the dCas9 gene, which further increases upon fusion to transcriptional modulators, to be taken into account. Also, the need for guide RNAs, as well as potential immunogenicity of the Cas9 protein, must be considered [45•]. Nevertheless, CRISPR/dCas9-TFs might prove to be powerful tools to induce or enhance *in vivo* reprogramming approaches.

Reprogramming inhibitory mechanisms

The efficiency of reprogramming is generally limited due to cell fate safeguarding mechanisms, which act as barriers for reprogramming [7,8]. We already mentioned the necessity of a histone deacetylase inhibitor to reprogram MGCs into neurons upon ectopic expression of Ascl1 in mice which were older than 16 days [27]. Our current knowledge of reprogramming barriers is continuing to grow (reviewed in Refs. [46,47]), in part also due to studying *in vivo* reprogramming in model organisms such as the nematode *C. elegans*. It allows investigating reprogramming barriers *in vivo* due to genetic tractability, ease of transgenesis and the feasibility of performing large-scale genetic screens [7,8]. For instance, the histone chaperones LIN-53 (RBBP4/CAF-1p48) and FACT (facilitates chromatin transcription) were initially identified in *C. elegans* as cell fate reprogramming barriers. Their mammalian counterparts were shown to block reprogramming to iPSCs and transdifferentiation to neurons in mice and human cells [7,47]. These examples demonstrate that understanding cell fate protection in model organisms can help to increase reprogramming efficiency of human cells for future regenerative medicine applications (Table 1).

Concluding remarks and perspectives

Our current knowledge of fate-specifying TFs is derived mainly from decades of classic developmental biology research. *In vitro* studies taught us how to translate this knowledge to reprogram cell fates, either back to a more pluripotent state or to another differentiated fate – even across germline layers. Importantly, recent studies revealed that some findings cannot directly be translated to an *in vivo* setting, largely due to specialized microenvironments or required processes such as senescence. While our overall understanding of inducing cellular reprogramming is rapidly growing [48], we need to better understand the global changes during these processes at the molecular level. Besides chromatin and gene expression dynamics, also metabolic processes emerge as an important layer of reprogramming checkpoints [49]. Natural transdifferentiation events provide an alternative system to study how cell fate conversion is orchestrated in a robust way. In *C. elegans*, the developmentally programmed transdifferentiation of a rectal epithelial cell to a neuron has been studied in great detail and revealed key insights into the interplay of TFs and different chromatin regulators during transdifferentiation [50]. Another recently discovered natural conversion event in *C. elegans* is a sex-dependent glial cell to neuron conversion, which happens only in sexually mature males [51]. Studying naturally occurring *in vivo* reprogramming phenomena, together with the application of single-cell transcriptome analysis during different reprogramming events, will help to dissect generalizable and specific molecular trajectories of cell fate conversion. While such insights are critical to enhance *in vivo* reprogramming, the emergence of organoid technology might further help to investigate reprogramming in an *in vivo* like system, leading to enhanced strategies for applying reprogrammed cells for tissue replacement therapies in the future.

Conflict of interest statement

Nothing declared.

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