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# Cellular plasticity in reprogramming, rejuvenation and tumorigenesis: a pioneer TF perspective

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## Review

Cellular plasticity in reprogramming,  
rejuvenation and tumorigenesis: a pioneer  
TF perspectiveAurélia Huyghe <sup>1</sup>, Aneta Trajkova,<sup>1</sup> and Fabrice Laval <sup>1,\*</sup>

The multistep process of *in vivo* reprogramming, mediated by the transcription factors (TFs) Oct4, Sox2, Klf4, and c-Myc (OSKM), holds great promise for the development of rejuvenating and regenerative strategies. However, most of the approaches developed so far are accompanied by a persistent risk of tumorigenicity. Here, we review the groundbreaking effects of *in vivo* reprogramming with a particular focus on rejuvenation and regeneration. We discuss how the activity of pioneer TFs generates cellular plasticity that may be critical for inducing not only reprogramming and regeneration, but also cancer initiation. Finally, we highlight how a better understanding of the uncoupled control of cellular identity, plasticity, and aging during reprogramming might pave the way to the development of rejuvenating/regenerating strategies in a nontumorigenic manner.

**Pluripotent reprogramming is a stepwise process**

The generation of induced pluripotent stem cells (iPSCs) *in vitro* with OSKM holds great promise for the development of personalized therapies [1]. Following this pioneer work, instrumental studies demonstrated that differentiated cell types can also be reprogrammed to pluripotency *in vivo*, paving the way to future applications in regenerative medicine [2,3]. However, initial attempts at *in vivo* reprogramming showed that it triggers the formation of not only benign, but also malignant tumors [3,4].

Reprogramming is a stepwise process marked by the emergence of cellular intermediate states, presenting a progressive gain of **cellular plasticity** (see [Glossary](#)) and loss of **cellular identity** followed by the activation of the pluripotent network [5,6]. During the process, the cells reach a 'point of no return' after which they will not go back to their differentiated state ([Figure 1](#)). The inability of cells to successfully overcome different obstacles, including the activation of oncogenic barriers (senescence and cell death), partly explains the low efficiency of the process [7]. A large proportion of the epigenetic features remodeled during the early phase of reprogramming are also modified during regeneration and aging. Therefore, researchers elaborated protocols of partial reprogramming in which cells begin to reprogram but do not reach the pluripotent state. These approaches led to groundbreaking effects both *in vitro* and *in vivo*, including rejuvenation [8–11], improvement of regenerative abilities in various organs [9,12,13], and restoration of vision [14]. However, partial reprogramming was reported to trigger and/or foster oncogenic transformation in certain normal and neoplastic contexts [3,4,15]. This persistent risk of tumorigenicity severely hinders the future applications of *in vivo* reprogramming-based approaches.

Oct4, Sox2, and Klf4 belong to the family of 'pioneer' TFs, a specific class of molecules able to interact with, and remodel, the nucleosome to open silent chromatin [16]. Pioneer TFs can trigger

**Highlights**

Advances in dissecting *in vivo* reprogramming (mediated by the transcription factors Oct4, Sox2, Klf4, and c-Myc) reveal that it triggers rejuvenation and improves regeneration in various cells and complex tissues, while still presenting an inherent risk of tumorigenicity.

Aging was recently found to be caused by loss of epigenetic information that can be restored by reprogramming even if the precise molecular mechanisms remain to be fully understood.

Recent findings showed that specific features, such as cellular plasticity, cellular identity, and aging, are controlled by distinct gene regulatory networks during reprogramming.

The precise action of pioneer transcription factors on the epigenome remains to be fully understood to identify alternative molecules that will pave the way toward the development of nontumorigenic reprogramming-based strategies in humans.

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a widespread reconfiguration of chromatin and TF occupancy in various contexts, including not only early development and reprogramming, but also regeneration and cancer initiation. Hence, the promiscuous binding of pioneer TFs to the genome was recently found to trigger undesired epigenetic and transcriptomic changes in certain contexts [17]. Therefore, a better understanding of the molecular events driven by pioneer TFs will help to uncouple their beneficial and detrimental actions during cell fate conversions.

Here, we review the effects of *in vivo* reprogramming with a particular focus on not only its benefits, such as rejuvenation and regeneration, but also risks, namely tumorigenesis. We highlight the key roles of pioneer TFs in triggering these effects, in particular in generating cellular plasticity during cell fate conversions, regeneration, and cancer development. We finally discuss how the regulation of specific cellular features (aging, identity, and plasticity) can be uncoupled, paving the way to the future development of *in vivo* reprogramming-based approaches in a nontumorigenic manner.

### Reprogramming *in vivo* triggers rejuvenation and improves regeneration

Reprogramming to pluripotency and generation of iPSCs is achievable *in vivo*, indicating that the microenvironment is not sufficient to constrain OSKM action [3]. In detail, OSKM ubiquitous expression triggers the formation of differentiated **teratomas** in various organs (pancreas, kidney, intestine, adipose tissue, liver, intracranial, stomach, and heart), revealing that multiple cell types are capable of reprogramming with different kinetics [3,10]. While the emergence of cellular intermediates is well documented *in vitro* [5,6,18], the molecular steps and regulators of *in vivo* reprogramming remain largely unknown. A recent analysis of pancreatic cells at day 7 of reprogramming *in vivo* showed that cellular intermediates, prone or refractory to reprogramming, also emerge *in vivo* [19].

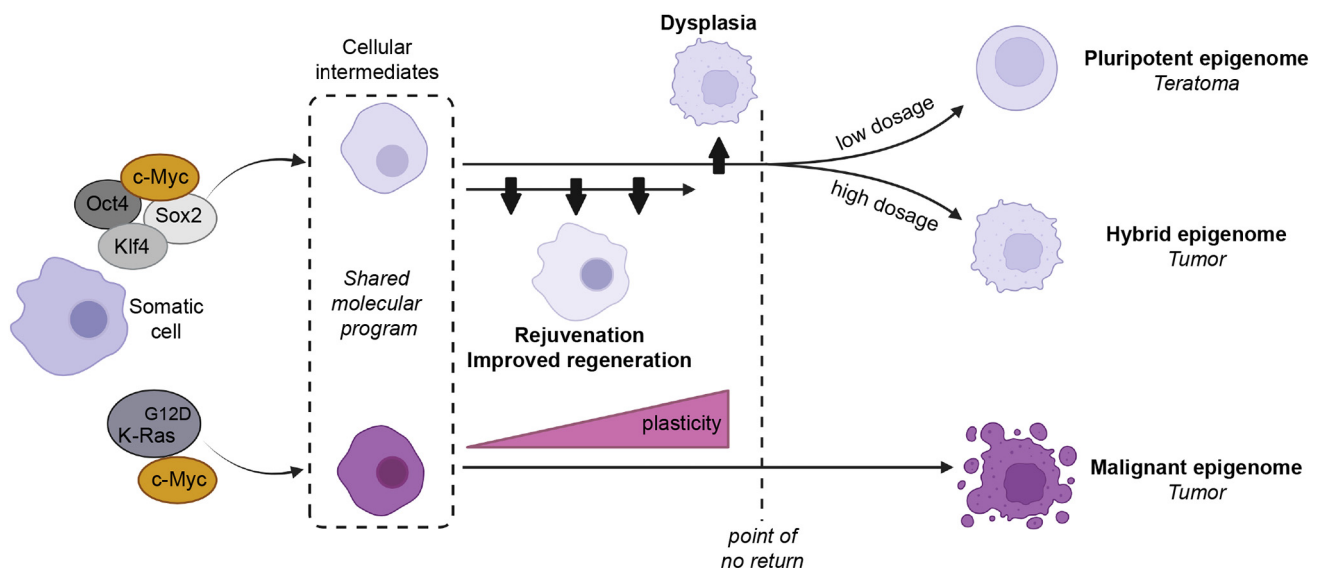
#### Glossary

**Cellular identity:** unique combination of molecular features (genomic, epigenetic, and transcriptomic) that leads to a specific phenotype and function in a cell.

**Cellular plasticity:** ability of a cell to change its identity under normal, pathological, or experimental contexts.

**Lineage infidelity:** process by which a cell acquires a mixed identity comprising the coexistence of multiple transcriptional/epigenetic programs.

**Teratoma:** benign tumors comprising differentiated derivatives from the three germ layers. Therefore, teratomas are indicative of the multilineage differentiation potential of a cell.



**Figure 1. Reprogramming can trigger not only rejuvenation but also cancer development.** A shared molecular program emerges in cellular intermediates during reprogramming (induced by Oct4, Sox2, Klf4, and c-Myc; OSKM) and oncogenic transformation (induced by an oncogenic form of K-Ras mutated on a single residue: K-Ras<sup>G12D</sup> and c-Myc) [6]. During the early phase of reprogramming, the epigenetic marks are remodeled and cellular intermediates gain in cellular plasticity. The termination of pluripotent reprogramming (cyclic or by pulse) in this time window contributes to rejuvenation and regeneration [9,11]. However, depending on the cellular model and the reprogramming protocol used, this window can lead to reversible dysplastic features in cells [4]. A ‘point of no return’ arbitrarily defines the limit after which cells accumulate irreversible epigenetic/genetic modifications. After this point, the outcome of prolonged reprogramming and oncogenic transformation is engaged. The dosage of OSKM controls the final epigenetic landscape of reprogramming. The hybrid epigenome refers to cells acquiring partial reprogrammed features [4,33]. Figure created with BioRender ([biorender.com](https://www.biorender.com)).

The refractory cluster harbors high activity of the AP-1 family of TFs, in line with the barrier role of these factors *in vitro* [6,18–20]. Senescence also promotes *in vivo* reprogramming via the paracrine secretion of a variety of molecules, including IL-6 [21]. By contrast, natural killer (NK) cells constrain the process by targeting reprogramming cells that upregulate NK-activating ligands (MULT1, ICAM1, and CD155) that bind the NKG2D receptor [22].

Different settings of partial reprogramming, by which a short exposure to OSKM engages cells in the process without reaching pluripotency, have been elaborated. Some settings showed groundbreaking effects on rejuvenation and regeneration. Many of the epigenetic marks that are remodeled during early reprogramming are also dysregulated during the aging process, including post-translational DNA methylation, modifications of histones, and chromatin remodeling [23]. In line with this, various epigenetic clocks were developed to estimate age based on the level of methylation of a restricted number of CpG sites in the genome [24–26]. Recent studies demonstrated that reprogramming (mediated by OSKM+Lin28+Nanog: OSKMLN) induces a rapid and persistent amelioration of aging hallmarks and a reversal of cellular age at the transcriptomic, epigenetic, and cellular levels in aged (60–90 years) compared with young (25–35 years) fibroblasts/endothelial cells [27]. Moreover, recent work showed that loss of epigenetic information can cause mammalian aging [23]. The authors took advantage of *I-PpoI*, an endonuclease from *Physarum polycephalum* that recognizes 20 canonical targets in the mouse genome, to generate the ICE mouse model (which has inducible changes of the epigenome). The induction of *I-PpoI* triggers the formation of double-strand breaks (DSB) that are perfectly repaired by cells. However, the repeated induction of DSBs led to the erosion of the epigenetic landscape, partial loss of cellular identity, senescence, and advancement of DNA methylation clock [24]. In that context, the adeno-associated virus (AAV) delivery of OSK significantly restored youthful features [23]. In addition, pioneer works revealed that a regimen of partial and cyclic reprogramming (mice were exposed to OSKM 2 days per week) led to the erasure of epigenetic signs of aging and to the amelioration of various aging hallmarks in the context of not only pathological, but also normal aging [9,11,28]. OSKM also improved recovery from toxin-mediated pancreas and muscle injury and also reduced scar tissue formation following cutaneous wounds. Finally, a single short OSKM pulse performed early in life was reported to modestly prevent some age-related tissue structure deteriorations and fibrosis [29].

A recent body of work also explored the effects of OSKM on the rejuvenation of specific cell types *in vivo*. Adult mammalian cardiomyocytes (CMs) are poorly proliferative and have low regenerative capability. A specific and short expression of OSKM (6–12 days) in adult CMs reprogrammed them toward a fetal state [10]. Moreover, OSKM expression, before or during myocardial infarction, improved heart repair through proliferation of pre-existing CMs. OSKM action is dependent on the developmental stage of CMs. It can sustain an immature proliferative state in neonatal CMs, while allowing the acquisition of a mitosis-competent state in adult CMs. Additionally, OSK expression in mouse retinal ganglion cells was found to restore youthful DNA methylation/transcriptomic patterns and to reverse vision loss in both a model of glaucoma and aged mice [14]. Cyclic OSKM expression also induces rejuvenation in the central nervous system. It restored adult hippocampal neuron migration in the dentate gyrus of old mice, associated with partial improvement of cognitive functions [30]. Similarly, partial reprogramming in myofibers promoted tissue regeneration in a chemically induced tibialis anterior muscle degeneration model. Mechanistically, OSKM regulate genes critical for the stem cell microenvironment, leading to stem cell proliferation [12]. Collectively, these studies show the possibility of using partial reprogramming to rejuvenate cell types and complex tissues to achieve functional recovery of organs that fail to regenerate under normal conditions. However, the

parameters of OSKM exposure (expression level and duration) required to achieve beneficial effects are cell type and/or organ specific.

### A tumorigenic risk is associated with *in vivo* reprogramming

Pluripotent reprogramming and oncogenic transformation share molecular analogies, as reviewed elsewhere [7,31,32]. A recent comparison of gene signatures of OSKM-mediated reprogramming and K-Ras-driven transformation in the pancreas revealed some shared transcriptomic features [19]. Comparison of the roadmaps of reprogramming and transformation at the single cell level in mouse embryonic fibroblasts (MEFs) demonstrated the existence of an early shared epigenetic program induced by OSKM and oncogenic K-Ras/c-Myc (Figure 1) [6].

Paralleling the exploration of these analogies, the question of the intrinsic tumorigenicity of *in vivo* reprogramming is still under debate. As mentioned previously, the full process ultimately leads to the formation of teratomas, but the development of these benign tumors simply reflects the acquisition of a multilineage differentiation potential. However, pioneer work reported the development of malignant tumors in various organs when reprogramming was induced but prematurely abrogated before reaching pluripotency (after 7 days of OSKM expression) [4,33]. The molecular mechanisms driving this tumorigenic development remain incompletely defined, but some parameters are impactful.

The first parameter to consider is the duration of OSKM exposure. An acute effect of OSKM is the formation of dysplastic lesions in multiple tissues, but dysplasia is often reversed following short exposure to OSKM (~2 days). However, prolonged exposure (~7 days) drives the subsequent formation of tumors, which persist after OSKM removal [4]. Some kidney tumors share characteristics with Wilms' tumors, the most common pediatric kidney tumor. Tumors in the pancreas harbor gene signatures related to germ cell tumors, in accordance with a 'rejuvenating' role of OSKM [33]. Transcriptomic analysis of kidney tumors showed that the embryonic stem cell (ESC)-core and ESC-Myc modules are activated at similar levels in pluripotent and tumoral cells. However, the ESC-Polycomb targets largely failed to be repressed in tumors [34]. Similarly, DNA methylation changes were partial, indicating that the abnormal growth of unsuccessfully reprogrammed cells can lead to tumor formation in which a hybrid epigenetic landscape has been stabilized (Figure 1).

In addition to the duration, the level of expression of OSKM is critical for tumorigenic risk. Low OSKM expression led to teratoma formation, whereas high levels triggered the generation of malignant tumors [33]. At the cellular level, the dosage and duration of OSKM exposure in adult CMs also influenced the development of heart tumors [10]. Short OSKM exposure (6 days) led to moderate signs of reversible cardiac remodeling, while prolonged time (21 days) triggered the development of epithelial-like neoplasms. Interestingly, mice carrying only one OSKM allele developed cardiac neoplasms after 7 weeks of OSKM exposure.

Finally, some regimens of Yamanaka factors were demonstrated to be nontumorigenic in certain contexts. For example, the exclusion of c-Myc from the cocktail was sufficient to avoid the development of cancerous lesions in the eye after prolonged expression [14]. Similarly, the long-term and cyclic induction of OSKM (2 days per week) did not lead to tumor formation. However, in these settings, the reduction of biological age was constrained to kidney and skin [11]. Altogether, these findings indicate that OSKM exposure promotes neoplasia in certain settings. Therefore, a better understanding of the molecular mechanisms associated with this tumorigenic risk could facilitate the development of safe reprogramming-based therapies.

### Reprogramming generates cellular plasticity

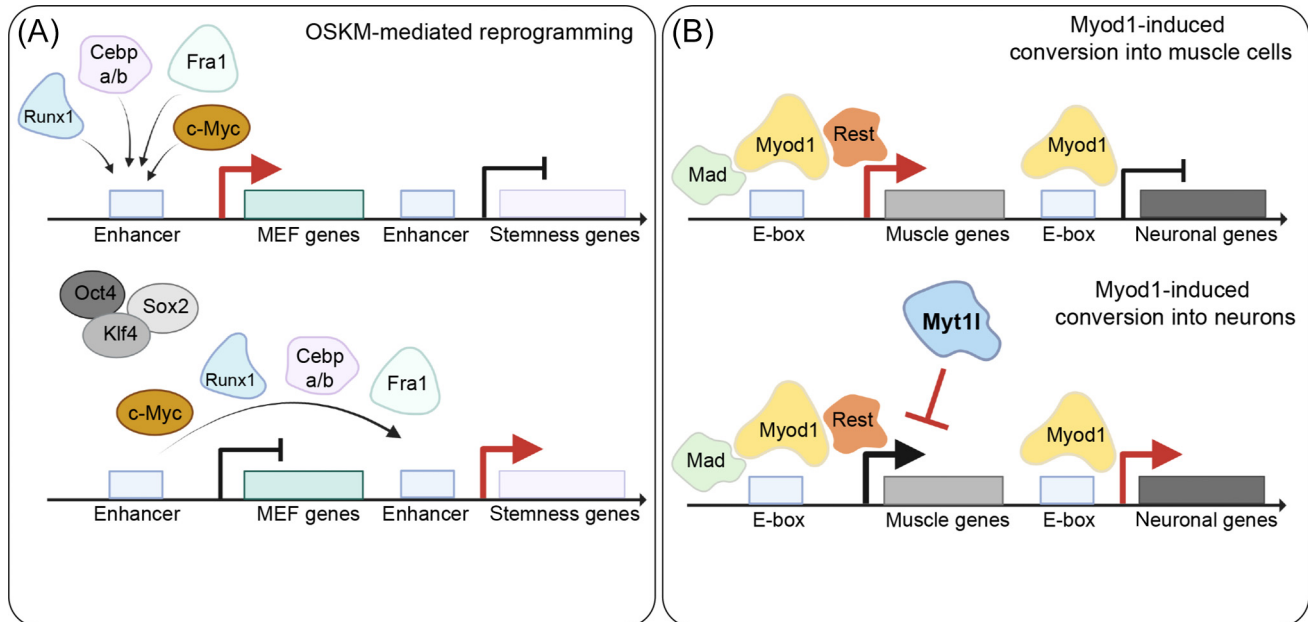
The previous section indicates that OSKM can generate epigenetic changes that lead cells to acquire undesired phenotypes, potentially leading to malignancy. In line with this, recent insights from single cell approaches showed that *in vitro* reprogramming is executed in a series of bifurcating decisions, where each point on the reconstructed trajectories corresponds to a concrete cellular decision [35,36].

The first bifurcation leads cells to assume a ‘refractory’ stromal fate or a ‘prone’ mesenchymal–epithelial transition (MET) fate that mainly gives rise to reprogrammed cells. Of note, the trajectories of reprogramming are dependent on the cell type of origin and on the cocktail of factors [37,38]. By contrast, late bifurcations were found to generate a battery of alternative cell fates, such as neural and extra-embryonic lineages in both human and mouse cells [35,36]. Interestingly, aberrant gene inductions were initially reported in pioneer bulk RNA-sequencing analysis but considered to be disorganized gene expression [39]. The advancement of the single cell technologies showed instead that they correspond to true cell fate decisions. Nonetheless, these alternative routes appear mainly absent from chemical-mediated reprogramming, indicating that the use of pioneer TFs (see next section) might be responsible for such aberrant acquisition of cellular plasticity [40]. The emergence of a cluster of ‘trophectoderm-like’ cells was recently reported during OSKM reprogramming of human dermal fibroblasts [41]. These cells were successfully stabilized into induced trophectodermal stem cells (TSCs) that resembled both molecularly and functionally TSCs derived from first-trimester placentas. Collectively, these studies show that the action of pioneer TFs is not exclusively deterministic toward a specific cell fate. Pioneer TFs also generate cellular plasticity and windows of opportunity for somatic cells to engage into alternative paths.

### Pioneer TF activity during reprogramming and transdifferentiation

TF-mediated cell fate conversions require a genome-wide epigenetic resetting that includes the opening of silent chromatin. Nucleosomes impair DNA binding of TFs, but a specific class of embryonic ‘pioneer’ TFs interact with, and remodel, the nucleosome to open silent chromatin, as reviewed elsewhere [42]. The ability of pioneer TFs to initiate transcriptional programs is instrumental for programming cell fate during development [43]. Numerous embryonic pioneer TFs are able to drive the conversion of fibroblasts toward a plethora of lineages [44]. Interestingly, their activity is dependent on different parameters, including stage-specific cofactors. During the early steps of reprogramming, OSK were found to redistribute c-Myc on the MEF genome, which may divert it from its tumorigenic action [45]. OSK also hijack MEF cofactors (Runx1, Cebpa, Cebpb, and Fra1) and epigenetic regulators (Brg1, Hdac1, and Mbd3) to initiate the inactivation of MEF enhancers [18,46]. In line with this, Oct4, Sox2, and Klf4 are not distributed similarly on the chromatin when expressed solely or in combination (Figure 2A) [18]. As another example, Ascl1 and Myod1 are two pioneer basic helix–loop–helix (bHLH) TFs that are sequence related but drive the transdifferentiation of MEFs into neural and muscular cells, respectively [17] (Figure 2B). The binding of the two TFs to MEF chromatin was found to be qualitatively similar, reflecting their sequence similarities, but the transcriptional output of their ectopic expression was strikingly distinct (muscular versus neural cells). These distinct changes in fate specification were found to be due to strong differences in the DNA-binding affinity of Ascl1 and Myod1 and to their respective interaction with cofactors. Moreover, and in line with the generation of cellular plasticity by pioneer TFs, the pro-myogenic factor Myod1 was found to bind promiscuously to neuronal genes and the pro-neuronal factor Ascl1 to myogenic ones [47]. Therefore, this promiscuous activity needs to be canalized in different ways, including by transcriptional repressors, such as Myt1l. Myt1l exerts a pro-neuronal function by repressing a variety of non-neuronal genes occasionally induced by Ascl1 [47]. Interestingly, however, when Myt1l expression is combined with the pro-myogenic factor Myod1, this latter unexpectedly promotes the emergence of





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**Figure 2. Pioneer transcription factor (TF) activity during reprogramming and transdifferentiation.** (A) Oct4, Sox2, Klf4, and c-Myc (OSKM)-mediated reprogramming. The pioneer TFs OSK trigger widespread reconfiguration of chromatin and redistribute on the chromatin c-Myc and mouse embryonic fibroblast (MEF) cofactors, such as Runx1, Cebp a/b, and Fra1 [18]. (B) Myod1-induced cell fate conversion. Myd11 converts the pro-myogenic factor Myod1 into a pro-neuronal factor by inhibiting the muscle program and exploiting Myod1 promiscuous binding to neuronal genes [47]. Figure created with BioRender (biorender.com).

neurons by binding promiscuously to neural genes. Furthermore, dissecting the action of the pioneer TF NeuroD1 during the direct conversion of mouse microglia into neurons also involves transcriptional repressors. NeuroD1 initially binds to closed bivalent chromatin regions to induce neuronal gene expression. Among NeuroD1 primary targets, the transcriptional repressors Scrt1 and Meis2 are induced to silence the microglial program [48].

Altogether, these findings highlight the ability of pioneer TFs to generate cellular plasticity especially via their promiscuous binding to chromatin. However, this phenomenon depends on the epigenome of the cell of origin, the cofactors, and the intrinsic abilities of the TF.

### Pioneer TF activity in regeneration and cancer initiation

Numerous embryonic pioneer TFs are induced in adult lineages in response to stress and/or during cancer formation, highlighting how embryonic programs might have been co-opted during evolution as stress-response options (Table 1). Under stress, pancreatic acinar cells induce the expression of the pioneer TFs Sox9 and Klf4, which are normally expressed by ductal cells. The two TFs trigger **lineage infidelity** by conferring a mixed progenitor/ductal identity by repressing some acinar enhancers and by activating ductal ones [49]. This transient process, termed acinar-to-ductal metaplasia (ADM), facilitates pancreas regeneration and is resolved by redifferentiation. In response to oncogenic K-Ras (K-Ras<sup>G12D</sup>), Sox9/Klf4-mediated ADM is also induced before the formation of pancreatic ductal adenocarcinoma [50,51]. Similarly in the lung, regeneration triggers lineage infidelity in alveolar type 2 epithelial cells, which acquire a pre-alveolar type-1 mixed cell identity [52]. K-Ras<sup>G12D</sup> also triggers the emergence of a mixed cellular state from AT2 cells during the early phase of lung tumorigenesis [53]. The ability of pioneer TFs to sense stress and trigger lineage infidelity in a regenerative context is hijacked

Table 1. Pioneer TF function in development, regeneration, and tumorigenesis

Pioneer TF	Development-related function		Stress-related function		Tumorigenic-related function	
	Description	Refs	Description	Refs	Description	Refs
Klf4	Regulates key pluripotency genes, such as <i>Nanog</i>	[70]	Contributes to cutaneous wound healing	[71]	Induces cellular identity change in pancreas cancer initiation	[51]
Sox9	Establishment and maintenance of multipotent neural stem cells	[72]	Cartilage regeneration	[73]	Promotes proliferation, metastasis, and endocrine resistance in breast cancer	[74]
	Sex determination	[75]	Renal tubule epithelial regeneration	[76]	Promotes migration, invasion, and EMT via Wnt/ $\beta$ -catenin pathway in non-small-cell lung cancer (NSCLC)	[77]
	Required during pancreas organogenesis and chondrogenesis	[78]	Skin wound re-epithelization	[79]	Initiates pancreatic ductal adenocarcinoma by acinar-to-ductal metaplasia	[50]
Klf5	Suppresses ERK signaling in mESC	[80]	Attenuates cardiomyocyte inflammation induced by stress	[81]	Promotes gastric and prostate cancer cell proliferation	[82]
	Promotes epiblast lineages while suppressing primitive endoderm lineage	[83]	Contributes to liver regeneration by inducing biliary epithelial cell proliferation	[84]	Redistributed across chromatin to regulate transition from Barrett's esophagus to esophageal adenocarcinoma	[85]
Gata6	Specifies primitive endoderm	[86]	Controls identity of sebaceous duct lineage cells for wound healing	[87]	Modulates chromatin landscape during early stage of NSCLC to promote proliferation	[88]
			Controls hair follicle regeneration	[89]	Enables self-renewal of colon adenoma stem cells	[90]
			Required for proper lung epithelial regeneration	[91]	Sustains oncogenic lineage survival in esophageal adenocarcinoma	[92]
Sox2	Essential for self-renewal and pluripotency of ESCs	[93]	Implicated in central nervous system remyelination after demyelination by activating oligodendrocyte progenitor cells differentiation	[94]	Facilitates neuroendocrine differentiation in prostate adenocarcinoma to acquire resistance toward antihormonal therapies	[95]
			Regulates skin repair, likely by controlling neural crest precursor cells	[96]	Promotes lineage plasticity and drug resistance by phenotypic shift from androgen receptor (AR)-dependent luminal epithelial cells to AR-independent basal-like cells	[97]
			Mediates salivary gland regeneration after radiation injury	[98]	Epigenetic switch between Sox2 and Sox9 regulates lung cancer cell plasticity	[99]
Gata4	Necessary for development of cardiac muscle cells	[100]	Protects against liver fibrosis and promotes regeneration	[101]	Induces migratory phenotype of hepatoblastoma cells	[102]
	Required for follicular development and normal ovarian function	[103]	Promotes myocardial regeneration	[104]	Promotes metastasis in lung adenocarcinoma	[105]

during cancer initiation. In these examples of regeneration, lineage infidelity is transient and resolved rapidly. Therefore, understanding their maintenance during cancer initiation is critical.

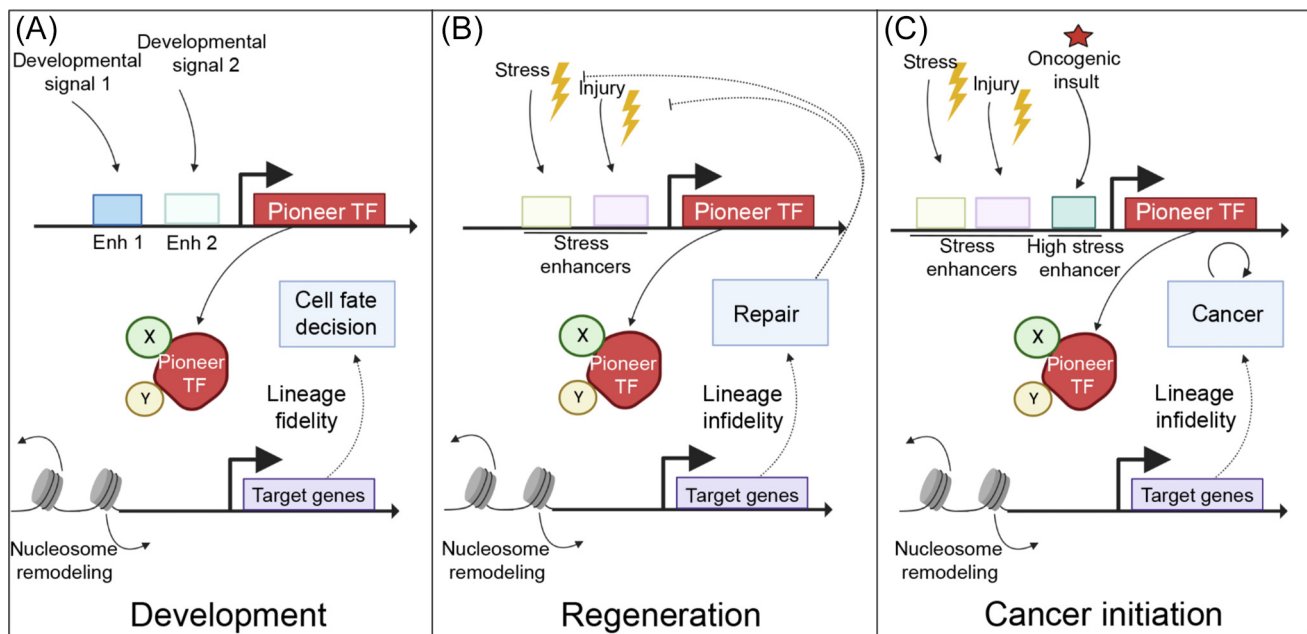
In the lung, TP53 promotes lineage infidelity and its genetic inactivation stalls the cells in this mixed state, indicating that the loss of this tumor suppressor can be directly responsible for the persistence of lineage infidelity [52]. In the skin, the pioneer TFs Klf5 and Sox9, normally expressed in a mutually exclusive manner, are expressed simultaneously during wound response. This lineage infidelity process is reinforced by the activation of specific stress-induced TFs, such as ETS2 by MAPK-mediated phosphorylation [54]. Molecularly, during both repair and tumorigenesis, 'stress-responsive' enhancers become activated and override the homeostatic enhancer landscape. However, during wound repair, the activity of those 'stress-responsive' enhancers decreases and the Klf5/Sox9 balance is restored. By contrast, in cancer initiation, a new enhancer



landscape comprising ‘high stress’ tumor-specific enhancers is proposed to lock malignant cells into a lineage infidelity state (Figure 3). These findings highlight how pioneer TF activity influences both regeneration and tumorigenesis.

### Uncoupling cellular identity, plasticity, and aging during reprogramming

The groundbreaking effects of reprogramming, and its inherent tumorigenic risk, prompted researchers to better dissect the early steps of the process. Recent work deciphered the sequence by which cellular identity, plasticity, and aging are modulated during reprogramming. Fibroblast identity repression was found to occur by distinct mechanisms depending on the reprogramming factor used (Ascl1, MyoD1, FoxA2, Sox2, or Oct4) and no general principle emerged, highlighting that different entry points exist to erase cell identity [55]. A special relationship between the germ layer of the cell of origin and the TF was evidenced, and the mesodermal factor MyoD1 was found to repress fibroblast identity most potently. Therefore, therapeutic cell conversions may best be limited to related cell types when possible. Another conclusion is that cell fate conversion does not scale gradually with increasing the expression level of reprogramming factors but rather depends on a critical expression threshold. It indicates that an optimal, cell type-specific level of reprogramming factors exists, which is sufficient for reprogramming and that may also minimize the risk of failure and/or tumorigenicity [33]. In line with this, the capture of early cellular intermediates recently allowed researchers to uncouple the regulation of cellular identity and plasticity during MEF reprogramming [6]. These early intermediates are already highly prone to generate iPSCs even if their MEF identity scores are not yet significantly decreased [35,37]. Multi-omics characterization of these rare cells revealed the existence of a partitioned regulatory network, driven by the bHLH TF Atoh8, which controls specifically cellular plasticity.



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**Figure 3. Pioneer transcription factor (TF) function during development, regeneration, and cancer initiation.** (A) During normal development, developmental signals drive the expression of pioneer TFs by specific enhancers. Pioneer TFs subsequently trigger nucleosome remodeling and induce different transcriptional programs to dictate embryonic lineage segregation and ensure lineage fidelity [42]. (B) Pioneer TF expression is transiently activated via stress and/or injury enhancers during regeneration, leading to mixed cellular identity. These transient lineage infidelity events contribute to repair and are rapidly resolved by redifferentiation [54]. (C) During tumorigenesis, activation of novel high stress enhancers by the oncogenic insult stabilizes lineage infidelity events [69]. Figure created with BioRender (biorender.com).

The effect of OSKMLN on the epigenetic clock of human fibroblasts and endothelial cells was also described to occur before the epigenetic changes of cellular identity, indicating that rejuvenation may be achievable while preserving cellular identity [8]. By contrast, when partial reprogramming is induced in primary adipogenic and mesenchymal stem cells, the somatic identity was found to be suppressed transiently [56].

OSK expression in inducible changes to the epigenome (ICE) fibroblasts restored age-associated mRNA changes and epigenetic age without impacting cellular identity [23]. By contrast, work in human fibroblasts showed that, if reprogramming were to be separated in three phases (initiation, maturation, and stabilization), rejuvenation could be pushed to the maturation phase, where the complete pluripotency program is attained [57].

In the context of pathologies, such as neurological age-associated diseases (AAD), different laboratories have used OSKM-based reprogramming methods to generate patient-derived cell types of interest, such as neurons [58]. However, such an approach is limited to model aging because the iPSC-derived neurons are reset and have erased many of the aging features required for AAD modeling [59]. As an alternative, the so-called direct conversion of fibroblasts into induced neurons maintains the age-associated features of donor cells [60,61]. Therefore, the comparison of neurons derived from fibroblasts from patients with Alzheimer's disease using reprogramming or direct conversion revealed age-dependent features, such as a pronounced instability of the mature neuronal fate [62].

### Concluding remarks

The exploration of OSKM-mediated reprogramming *in vivo* constitutes a great opportunity to identify pathways and/or molecules that control regeneration and aging for therapeutic purposes, even if major limitations persist. First, the studies of reprogramming *in vivo* are not numerous and most findings still rely on *in vitro* work. Therefore, the principles dictating early reprogramming *in vivo* should be intensively explored in the coming years (see [Outstanding questions](#) and [Figure 4](#)). In line with this, the multiple autocrine and paracrine barriers constraining *in vivo* reprogramming efficiency remain to be identified. Moreover, the molecular bases of the cell type- and organ-specific sensitivity to reprogramming also remain imperfectly understood. Results of different studies are difficult to compare because the nature of the reprogramming cocktail and the duration of exposure often differ. In terms of rejuvenation, future studies should also systemically assess the functional and physiological effects of reprogramming on cellular rejuvenation. The partial restoration of a youthful gene expression pattern is limited and may not significantly correlate with the functional reversion of an aging phenotype. Identifying which degree of transcriptomic and epigenomic reversion is needed to achieve a functional effect will be vital. Exploring the epigenetic landscape of various organs at the single cell level in response to OSKM requires the development of lineage-tracing tools. In addition, focusing research on organs and/or cell types described as refractory to reprogramming might be of interest to identify endogenous barriers. As an example, comprehensive single cell transcriptomic atlases were recently built in various tissues undergoing aging or calorie restriction (CR) [63]. CR emerged as one of the most effective interventions to rejuvenate cells and organs, delay the onset of age-associated diseases, and extend healthspan and lifespan, and its geroprotective effect was found to restore the aging-disturbed immune ecosystem in particular.

Pioneer TF activity is directly related to their ability to reprogram, regenerate, and potentially poise the cells for oncogenic transformation. These findings highlight the need for more profound studies of pioneer TF mechanisms and functioning, especially in human cells, if translational strategies are to be foreseen. As depicted in [Figure 1](#), we posit that somatic cells acquire a hybrid unstable epigenetic landscape that is initially reversible. After the point of no return, cells adopt a

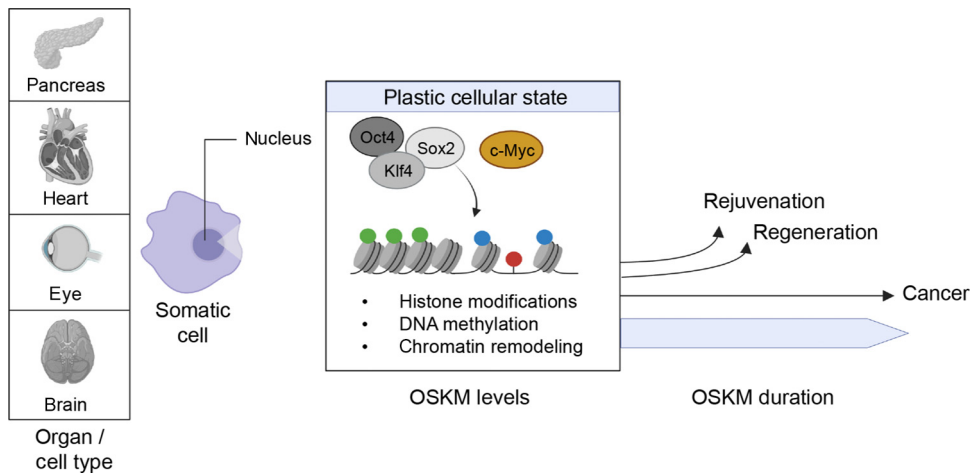
### Outstanding questions

What are the molecular and epigenetic determinants of cellular plasticity?

How can we reconcile the cell type-specific kinetics of *in vivo* reprogramming with medical needs? Would translational medicine need personalized strategies?

Can we manipulate endogenous regulators of *in vivo* reprogramming (immunity, senescence) to render the process nontumorigenic?

How do adult cells encode, store, and restore youthful epigenetic information in response to reprogramming?



**Trends in Cell Biology**

**Figure 4. Challenges of *in vivo* reprogramming research.** Oct4, Sox2, Klf4, and c-Myc (OSKM)-mediated reprogramming triggers various outcomes, including rejuvenation, regeneration, and cancer. These effects are due, at least partly, to epigenetic modifications that depend on OSKM level and duration of expression. A better definition of the action of pioneer TFs on the epigenome, and of the cellular conversions they trigger in a cell type- and organ-specific manner, is required to precisely modify cellular features *in vivo*. This knowledge might enable OSKM to be replaced by molecules able to modulate specifically cellular aging and/or cellular plasticity without promoting tumorigenesis. Figure created with BioRender ([biorender.com](https://www.biorender.com)).

reprogramming or tumorigenic trajectory. The neoplastic risks related to OSKM use should be thoroughly investigated, especially in the context of pre-neoplastic lesions to avoid any detrimental outcomes. A related question is whether, and to what extent, alternative molecules can recapitulate OSKM effect on reprogramming/rejuvenation. Recently, the multipotent factor Msx1 was found to restore a certain degree of youthful expression in myogenic cells [56]. Moreover, Nanog was also reported to confer a youthful phenotype to senescent myoblasts and to improve molecular features of skeletal muscle in a mouse model of premature aging [64]. In addition, chemical stimulation following exposure to small molecules might constitute a suitable alternative to not only induce cellular plasticity in both rodent and human cells [65], but also trigger rejuvenation [66].

Finally, a fascinating remaining question is how OSKM, or alternative molecules, trigger organismal rejuvenation. One hypothesis would be that these factors rewire globally the chromatin landscape in an embryonic manner and, therefore, abolish the epigenetic erosion caused by aging. In line with this, recent studies showed that heterochromatin loss during aging leads to long interspersed nuclear element (LINE) derepression in aged cells. In this context, LINE-1 causes heterochromatin erosion by regulating the activity of the histone-lysine *N*-methyltransferase suppression of variegation 3-9 homolog 1 (Suv39h1). Meanwhile, LINE-1 expression is high in pre-implantation embryos, in which it regulates transcription and developmental potency [67]. Depletion of LINE-1 RNA in dermal fibroblast cells from patients with different progeroid syndromes was recently found to restore heterochromatin H3K9Me3/H3K27Me3 marks and reverse DNA methylation age *in vitro* [68]. Another intriguing possibility would be that adult cells encode, store, and restore youthful epigenetic information in response to pioneer TF expression or chemicals. Based on the ‘relocalization of chromatin modifiers’ theory of aging, deciphering how OSKM trigger the reorganization of which chromatin modifiers remains to be investigated [23]. The roles of covalent modifications, DNA-binding proteins, RNA-guided chromatin-modifying factors, RNA–DNA hybrids established early in life, and bookmarking systems also remain to be investigated. Altogether, such future work will pave the way toward the development of nontumorigenic reprogramming-based strategies in

humans. These approaches should aim at replacing OSKM with molecules controlling specifically features, such as rejuvenation and/or regeneration, for human health improvement.

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### Declaration of interests

The authors declare no competing interests.

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