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Authors
Lin, Changsheng
Yu, Chen
Ding, Sheng

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Toward directed reprogramming through exogenous factors

Changsheng Lin[1], **Chen Yu**[1], and **Sheng Ding**
Gladstone Institute of Cardiovascular Disease, Department of Pharmaceutical Chemistry, University of California, San Francisco, 1650 Owens Street, San Francisco, CA 94158, USA

Abstract

Direct reprogramming of one cell type into another provides unprecedented opportunities to study fundamental biology, model disease, and develop regenerative medicine. Different paradigms of reprogramming strategies with different sets of factors have been developed to generate various cell types, including induced pluripotent stem cells, neuronal or neural precursor cells, cardiomyocyte-like cells, endothelial cells, and hepatocyte-like cells. Various exogenous factors, especially small molecules modulating signaling, cellular state, and transcription, have been identified to enhance and enable reprogramming. With an increased understanding of reprogramming mechanisms and discovery of new molecules, it is conceivable that reprogramming can be achieved in a more directed and deterministic manner under entirely chemically defined conditions.

Introduction

Cell fate is controlled by both extrinsic factors (e.g., signaling molecules) and intrinsic factors (e.g., endogenous transcription factors). It has been shown that activation of the LIF-STAT3 and BMP-SMAD signaling pathways are essential for the maintenance of murine embryonic stem cells [1]. Transcription factors (TFs) downstream of the signaling pathways orchestrate with cell type-specific TFs, including Oct4, Sox2 and Nanog that form an auto-regulatory loop, to govern cell fate [1]. Consistent with such mechanism, studies of TF–mediated reprogramming demonstrated that cell fates can be manipulated by exogenous TFs as well. For example, fibroblasts can be induced into pluripotent stem cells (iPSCs) by the Yamanaka factors (Oct4/Sox2/Klf4/c-Myc), or converted to neuronal cells by Brn2/Ascl1/Myt1l [2,3]. Mounting evidence demonstrates that extrinsic factors can functionally mimic reprogramming TFs and/or enhance reprogramming process to facilitate cell fate switching. Here, we review these important extrinsic drivers for somatic cell reprogramming.

Somatic cell reprogramming is regulated by multiple signaling pathways

A successful iPSC reprogramming is to re-establish the intrinsic pluripotency transcriptional network in somatic cells. This network, in which Oct4 plays a pivotal role, involves dozens of pluripotency-associated factors and basal TFs [4].

Several signaling pathways have been reported to regulate the pluripotency of ESCs, indicating that they target certain components of the pluripotency transcriptional network in ESCs. Changes in the chromatin state of pluripotency genes, when driven by transduced factors or other regulators during reprogramming, may allow these signaling pathways to re-establish the pluripotency transcriptional network (Figure 1). We begin this review with a description of some of these key signaling molecules.

Corresponding author: Ding, Sheng (sheng.ding@gladstone.ucsf.edu).
[1]These authors contributed equally to this work.

MEK-Erk

Inhibition of MEK and glycogen synthase kinase–3 (GSK-3) by small molecule inhibitors PD0325901 and CHIR99021 (2i) completely eliminated spontaneous differentiation of ESCs in the absence of essential pluripotency signaling pathway activation [5]. During reprogramming, PD0325901 was shown to stabilize and help to select fully reprogrammed iPSCs [6]. Another study suggested that at the late stage of reprogramming, the 2i/LIF condition promoted the transition from partially reprogrammed cells to full iPSCs [7].

Wnt-GSK-3

Marson et al. showed that Wnt3a-conditioned medium increased reprogramming efficiency in mouse embryonic fibroblasts (MEFs) with ectopic expression of Oct4/Sox2/Klf4 [8]. Similarly, it was demonstrated that CHIR99021 improved the reprogramming in the absence of c-Myc and Sox2 [9]. A Wnt downstream regulator, Tcf3, was reported to occupy the promoter regions of key pluripotency genes, such as Oct4, Nanog and Sox2, to repress their expression [10]. Thus, the positive effects of Wnt pathway in reprogramming may be majorly mediated by reduced Tcf3 activity.

LIF-Stat3

Yang *et al.* demonstrated that LIF-Stat3 activation increased somatic cell reprogramming efficiency using a system that excluded the possibility of interference by two other LIF-downstream pathways, PI3K-Akt and MEK-Erk [11]. These findings suggest that the role of LIF-Stat3 is to facilitate the transition from incompletely reprogrammed cells (that are Oct4 negative and express retroviral transgenes) into fully reprogrammed iPSCs.

PI3K-Akt

The role of the PI3K-Akt pathway in the reprogramming process has not been fully elucidated. Nakamura *et al.* showed that activation of Akt promoted reprogramming after cell fusion of ESCs with thymocytes or MEFs [12]. In contrast, it also arrested transition from the two-cell to eight-cell stage after nuclear transfer [12].

Other pathways

Regulation of other pathways, such as the cyclic AMP, Hippo/Yap and Src family kinase pathways, was also reported to increase reprogramming efficiency or functionally replace certain Yamanaka factors [13–16].

Mechanisms that facilitate somatic reprogramming by regulating cellular states

Several mechanisms have been reported to facilitate the reprogramming process without direct activation of pluripotency genes (Figure 1). However, it appears in many cases that the more somatic cells are similar to pluripotent cells, the easier it will be to convert them to pluripotent cells. It is thus plausible that these additional mechanisms facilitate the shift from a somatic to a pluripotent cellular state.

Mesenchymal-to-epithelial transition and TGFβ pathway

During the reprogramming process, fibroblasts lose mesenchymal characteristics and obtain epithelial features, suggesting that the MET process is critical during reprogramming. This is consistent with findings showing that when the TGF pathway, which positively regulates the epithelial-to-mesenchymal transition (EMT, a reverse process of MET), was blocked by inhibitor of TGF receptor, there was a large increase in iPSC generation [17]. Furthermore,

the addition of a specific TGF receptor inhibitor could replace Sox2 in reprogramming [13]. Two follow-up studies provided molecular and functional evidence that the MET is necessary for reprogramming [18•,19•].

Metabolic shift

It is evident that, compared with somatic cells, many stem cells (including ESCs) rely more heavily on aerobic glycolysis to support their proliferation [20]. Thus, the transition from oxidative phosphorylation to glycolysis was suggested to be a barrier of somatic reprogramming. The findings showing that hypoxic conditions improved reprogramming support this notion [21]. It was found that PS48, an activator of 3' phosphoinositide-dependent kinase 1, helped to generate human iPSC with ectopic expression of a single TF (OCT4) by facilitating the metabolic conversion to glycolysis [22]. On the other hand, 2-deoxyglucose, a general inhibitor of glycolysis, greatly impaired iPSC generation [23]. Moreover, the glycolysis transition preceded pluripotency gene expression during reprogramming [23], suggesting that it acts at an early stage.

Anti-senescence pathways

Up-regulation of senescence control genes, including p53, p16^{INK4a}, and p21, was observed as an early event in reprogramming of fibroblasts by the Yamanaka factors [24]. Considering that somatic cells have limited proliferative potential while iPSCs have unlimited capacity for self-renewal, it is likely that cellular senescence is a barrier to reprogramming. This notion is consistent with the observation that fibroblasts from older mice had lower reprogramming efficiency [25]. Several groups pinpointed the p53-p21 pathway as a critical barrier to reprogramming [26]. They showed that knock-down of p53 in human or mouse cells greatly increased iPSC generation.

Somatic cell reprogramming controlled by gene expression regulators

As specific gene expression is central to cell identity, there is no doubt that regulators of gene expression, such as transcription factors, nuclear receptors, epigenetic modifiers and microRNAs, have direct and strong effects on cell fate determination.

Master transcription factors (TFs)

Reprogramming studies have demonstrated that combinations of different cell type-specific TFs could be applied to reprogram somatic cells directly into a variety of cell types, including iPSCs, neuronal cells, cardiomyocyte-like cells, hepatocyte-like cells, and endothelial cells, that are similar to their naturally existing counterparts [2,3,27–31]. In addition, different reprogramming paradigms have been developed. For example, applying transient expression of iPSC factors can reset fibroblasts toward plastic intermediates, which can be redirected by lineage-specific signaling molecules to generate cardiac, neural, or endothelial progenitor cells without passing through the pluripotent state [29,32,33•]. In contrast, neural precursor cells could also be generated using neural-specific TFs, such as Sox2 alone [34].

Nuclear receptors

Nuclear receptors are transcription factors that can directly bind to DNA and regulate specific gene expression in a ligand-dependent or -independent manner. Like extensively studied master TFs for pluripotency, some nuclear receptors were found to play critical roles in iPSC reprogramming as well as the maintenance of pluripotency. In addition to the well-known core auto-regulatory loop of Oct4-Sox2-Nanog [35], the nuclear receptor Esrrb could form another regulatory circuit with Tbx3 and Tcl1 for the maintenance of ESCs [36]. Furthermore, it was shown that Esrrb, along with Oct4 and Sox2, could convert MEFs to

iPSCs [37]. In this process, Esrrb forms a complex with Oct4 and Sox2, and synergistically up-regulates the expression of pluripotency genes in MEFs. Remarkably, with the help of other Yamanaka factors (Klf4/Sox2/c-Myc), another orphan nuclear receptor, Nr5a2, was able to substitute for Oct4 in iPSC generation [38]. In addition, Nr5a2 could greatly enhance iPSC reprogramming in conjunction with activation of another nuclear receptor, RARa/g [39]. The finding that the RARa agonist (CD437) and RARg agonist (AM580) dramatically increased reprogramming efficiency further supports the notion that nuclear receptors play important roles in regulating somatic cell reprogramming.

Epigenetic factors

Many studies demonstrated that small-molecule epigenetic modifiers could significantly influence reprogramming process and even substitute for certain reprogramming transcription factors (Figure 2). BIX01294, an inhibitor of G9a histone methyltransferase (HMTase), was shown to enable reprogramming of neural precursor cells or fibroblasts transduced with only two TFs, Oct4 and Klf4 [6]. Besides well-known HDAC inhibitors (e.g., VPA, NaB) that have been demonstrated to facilitate reprogramming in various contexts [40,41], Parnate, an inhibitor of histone demethylase LSD1, was shown to enhance iPSC reprogramming as well [9]. Interestingly, the well-known antioxidant compound vitamin C was recently shown to enhance reprogramming by modulating the activity of the histone demethylases Jhdm1a/1b [42]. These findings highlight the dynamic changes of histone modifications in reprogramming.

Recent mechanistic studies of iPSC reprogramming further illustrated how epigenetic changes are orchestrated in the early and late stages of reprogramming. Koche *et al.* showed that activated chromatin marks (e.g., H3K4 methylation) were targeted to promoters of pluripotency and developmentally regulated genes (e.g., Fgf4 and Lin28) prior to transcriptional activation during the early phase of iPSC reprogramming [43]. It was also reported that two epigenetic factors, Parp1 and Tet2, were recruited to pluripotency loci (e.g., Nanog and Esrrb) and established early epigenetic marks by converting 5-methylcytosine (5mC) into 5-hydroxymethylcytosine (5hmC) during reprogramming [44]. Interestingly, it was reported that along with Klf4, Sox2, and c-Myc, another Tet family protein Tet1 could enable somatic cell reprogramming in the absence of the key transcription factor Oct4 or nuclear receptors Esrrb and Nr5a2 [45], highlighting the important role of DNA demethylation (through hydroxymethylation) in reprogramming. Furthermore, other specific histone modifications were identified to occur in reprogramming. For example, inhibition of the H3K79 histone methyltransferase DOT1L (e.g., by a small molecule inhibitor) and the H3K9 methyltransferase Setdb1 (e.g., by RNAi) was shown to enhance iPSC generation [46,47]. These findings indicate that histone methylations of both H3K79 and H3K9 are barriers to iPSC reprogramming.

In addition, a recent study provided additional details of certain epigenetic changes during reprogramming [48••]. As Thy1 (a fibroblast marker) is linearly down-regulated and SSEA1 and Oct4 are linearly up-regulated during reprogramming, the reprogramming process in this study was roughly divided into three stages: early (day 3, Thy1-), intermediate (day 6–9, SSEA1+), and late (day 12, Oct4+). To determine certain epigenetic profiles in the different stages of reprogramming, ChIP-seq analyses were performed using antibodies against H3K4me3 (an active histone mark) and H3K27me3 (a repressive histone mark) in cells undergoing reprogramming. It was found that the genes carrying H3K4me3 marks were activated early or gradually (e.g. Fbx15, Cdc25c), whereas genes that were activated late (e.g. Oct4, Nanog) were often either unmarked with H3K4me3 or marked with both H3K4me3 and K3K27me3 in fibroblasts. It was also found that the demethylation of DNA did not happen until the late stage of reprogramming.

MicroRNAs

It was demonstrated that some mouse ESC–specific, cell-cycle-regulating (ESCC) microRNAs, including miR-291-3p, miR-294, and miR-295, could substitute c-Myc and enhance iPSC reprogramming with Oct4/Sox2/Klf4 [49]. Moreover, Subramanyam *et al.* showed that human ESCC miRNA orthologs hsa-miR-302b and hsa-miR-372 promoted human somatic cell reprogramming through multiple targets, including cell cycle regulators, epigenetic modifiers, and MET regulators [50]. In addition to iPSC generation, microRNAs were also shown as powerful regulator for lineage-specific reprogramming. It was reported that miR-9* and miR-124 were found to directly induce human fibroblasts into neurons with NeuroD2, Ascl1, and Myt1l [51•]. It was also demonstrated that miR-124 in conjunction with Brn2 and Mytl1 could convert human adult fibroblasts into mature neurons, suggesting that miR-124 plays an important role in neuronal specification [52•]. This finding also was supported by recent studies in which knocking down a single RNA-binding, polypyrimidine-tract-binding (PTB) protein could generate mature neurons from mouse fibroblasts via the action of miR-124 [53].

Perspectives

Among these exogenously delivered factors, small molecules and microRNAs, which can be chemically synthesized and do not modify target cell genome, have emerged as powerful tools to manipulate cell fate. While microRNAs offer the advantage of specifically targeting a large number of genes, small molecules provide precise temporal and tunable control over protein function, including rapid and reversible activation and inhibition. With an increased understanding of reprogramming mechanisms and discovery of new molecules, it is conceivable that reprogramming can be achieved in a more efficient and deterministic manner under entirely chemically defined conditions.

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• of special interest

•• of outstanding interest

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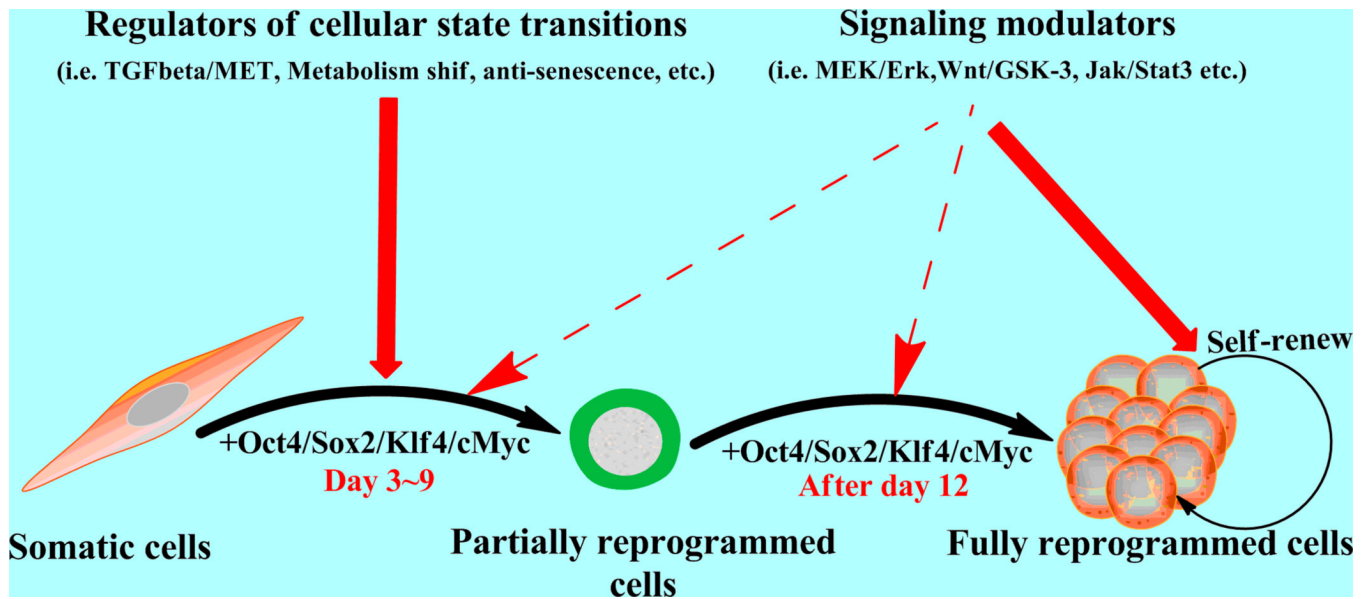
Figure 1. Important roles of signaling modulators and cellular state regulators in somatic cell reprogramming
Signaling modulators help to re-establish the pluripotency transcriptional network during reprogramming triggered by exogenous key transcription factors, whereas cellular state regulators can facilitate the shift from a somatic to a pluripotent cellular state.
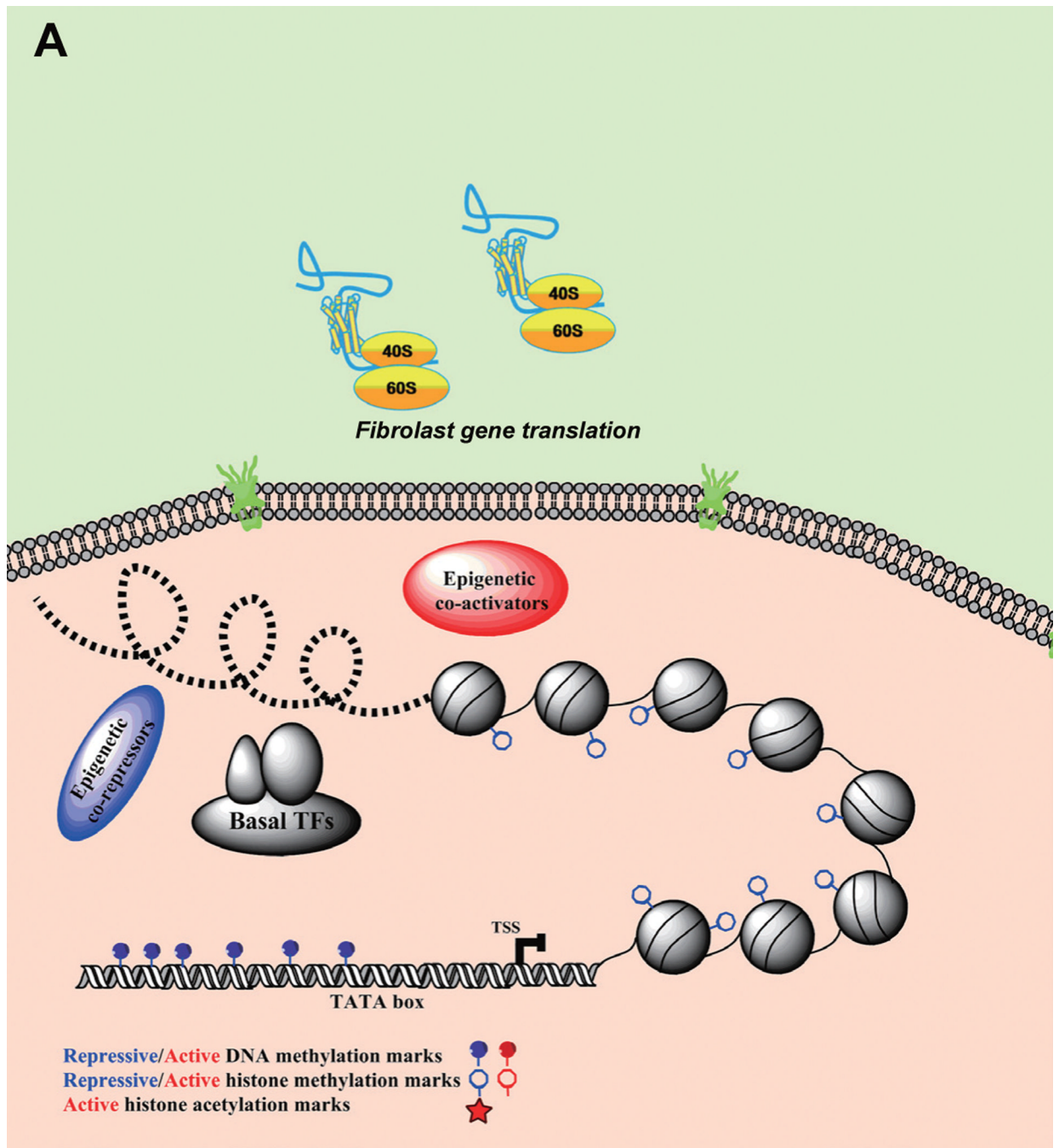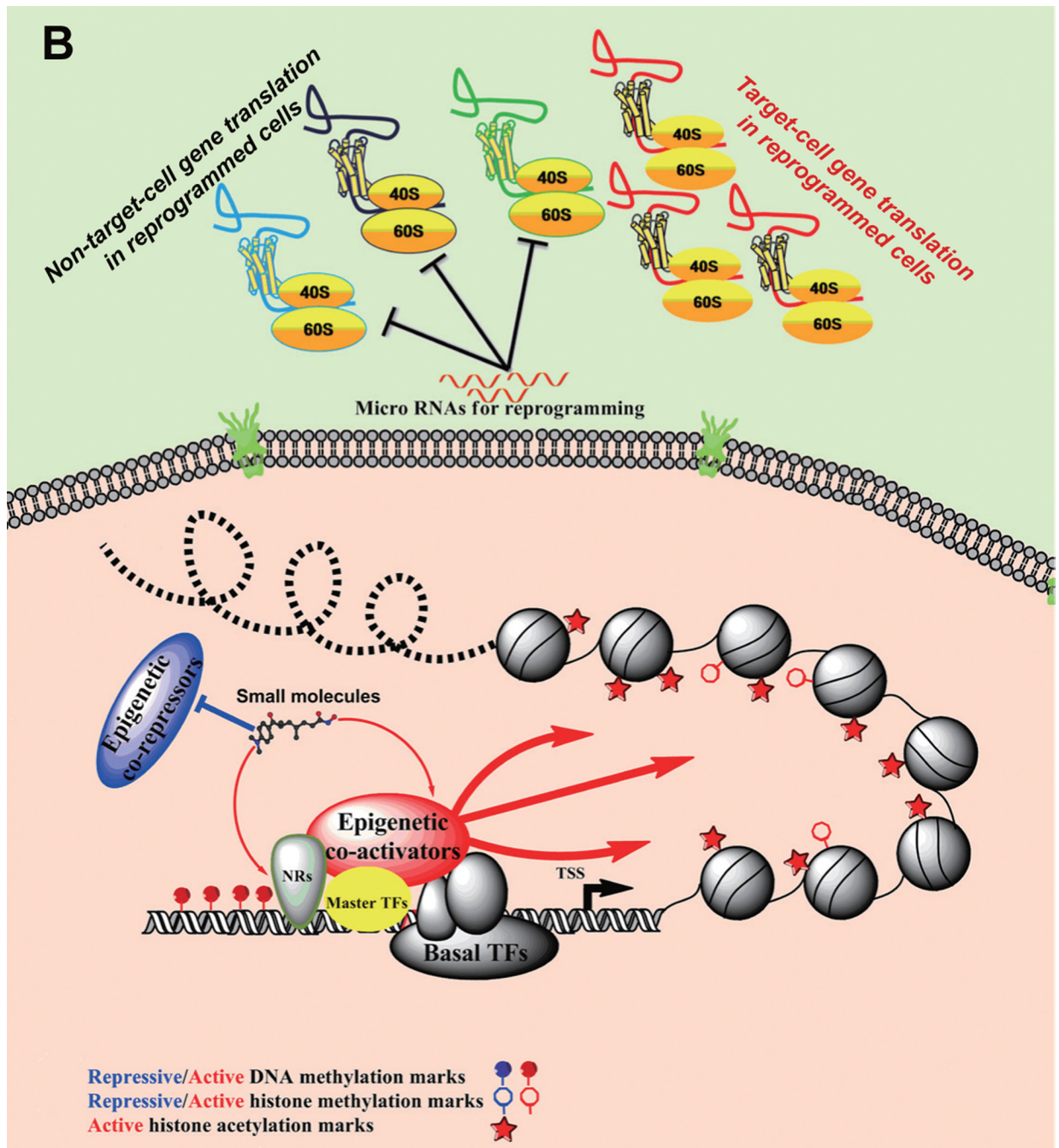
Figure 2. Target-cell genes activated by gene expression regulators and small molecules
(A) Showing transcriptional silence on target-cell gene loci (i.e. pluripotency genes, neural genes, etc.) in fibroblasts; (B) These genes are activated by exogenous transcriptional regulators, including master transcription factors (TFs), nuclear receptors (NRs), epigenetic co-activator, and related small molecules which can functionally mimic/substitute other exogenous regulators. In addition, microRNAs for reprogramming usually form an inhibitory network to block the path towards other cell fates and thereby facilitate a specific reprogramming process.