

## Repressors of reprogramming

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### Abstract

Induced pluripotent stem cells (iPSCs) have been the focal point of ever increasing interest and scrutiny as they hold the promise of personalized regenerative medicine. However, creation of iPSCs is an inefficient process that requires forced expression of potentially oncogenic proteins. In order to unlock the full potential of iPSCs, both for basic and clinical research, we must broaden our search for more reliable ways of inducing pluripotency in somatic cells. This review surveys an area of reprogramming that does not receive as much focus, barriers to reprogramming, in the hope of stimulating new ideas and approaches towards

developing safer and more efficient methods of reprogramming. Better methods of iPSC creation will allow for more reliable disease modeling, better basic research into the pluripotent state and safer iPSCs that can be used in a clinical setting.

**Key words:** Reprogramming; Induced pluripotency; Stem cells

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**Core tip:** This review addresses an underappreciated aspect of cellular reprogramming, repressors of reprogramming. We review current literature focusing on inhibitors that modify cellular reprogramming.

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### INTRODUCTION

The interest in cellular reprogramming spans basic science to clinical studies with the aim to understand the pluripotent state as well as to harness its potential to generate patient-specific therapies. These goals, while lofty, seem increasingly within reach. The first evidence of somatic cellular reprogramming was provided by the experiments performed by Gurdon *et al*<sup>[1,2]</sup> which employed somatic cell nuclear transfer (SCNT) in *Xenopus laevis*. SCNT was later shown to be effective in mammals with the cloning of "Dolly" the sheep<sup>[3,4]</sup> and has recently been shown to work in humans<sup>[5,6]</sup>. SCNT requires enucleated oocytes to mediate virtually complete reprogramming of differentiated nuclei. Induced pluripotent stem cells (iPSC) were first described by Takahashi *et al*<sup>[7,8]</sup> by overexpressing *Oct4*, *Sox2*, *c-Myc* and *Klf4*-transcription factors shown to

be essential for embryonic stem cell pluripotency. The discovery that forced expression of defined factors in differentiated cells could reprogram them to a stem-cell like state has had an enormous impact on the field of stem cell research. Advances in the creation of disease-specific stem cells are allowing us to gain previously unobtainable insights into a wide variety of diseases. Understanding the mechanics of cellular reprogramming will allow a greater understanding of developmental biology but will also translate into safe, reliable patient-specific cellular therapies.

Reprogramming is often thought of as a forward-moving process that relies on exposing the differentiated nucleus to factors that induce de-differentiation. Many groups have made strides in improving the methods of enforcing the expression of reprogramming factors<sup>[9]</sup>, increasing their efficiency<sup>[10-12]</sup> and employing alternative reprogramming factors<sup>[13-15]</sup>. Problematically, iPSC and to a lesser extent even SCNT-derived stem cells have been shown to be incompletely reprogrammed, leading to possible differentiation bias or unregulated growth during later differentiation<sup>[16]</sup>. Incomplete reprogramming may also lead to unknown biases when studying disease-specific stem cells, potentially confounding results. Efficient, safe, and completely reprogrammed cells are a necessary step towards moving iPSC into the clinic. Toward this end, far less attention has been focused on eliminating endogenous factors (or their activities) which enhance cellular differentiation. Inhibition of these "negative" factors, rather than or in combination with enforcement of positive-acting factors, could lead to safer, more efficient and more completely reprogrammed iPSC. Below we summarize a number of such targets, including, as best understood, their mechanisms and their pros and cons for clinical implementation.

## TRANSCRIPTION FACTORS

After the first description of iPSC, focus was turned towards finding modifiers of reprogramming. Repression of *p53*<sup>[17]</sup>, as well as its primary target gene *p21*, or addition of mutant *p53*<sup>[18]</sup> markedly facilitates and expedites reprogramming of mouse and human cells<sup>[19-23]</sup>. These studies proposed mechanisms by which p53 inhibits iPSCs generation, including induction of cell-cycle arrest, apoptosis and senescence. By virtue of its apoptotic induction following cellular DNA damage, p53 is a master regulator of genomic integrity and serves as a major tumor suppressor<sup>[24-26]</sup>. p53, either directly or *via* pathway members, undergoes loss of function mutations in nearly every cancer. Often the cellular programs that are co-opted during the development of cancer are, in fact, reactivated features of stem cells<sup>[27,28]</sup>. Reprogramming differentiated cells to pluripotent cells involves massive genomic changes that are not unlike some of the genomic changes that occur during cancer development. Indeed, despite the ability of p53 repression to enhance reprogramming

efficiency, p53-deficient cells can yield defective iPSCs with chromosomal aberrations, genome instability and malignant transformation<sup>[18,20,29]</sup>, further highlighting the link between reprogramming and tumorigenesis. Genomic integrity is a necessity for somatic cell reprogramming in order to create viable iPSC cells as well as in maintaining cellular identity. Inhibition of p53, while an interesting target for repression because of its enhancement of reprogramming, has inherent and serious risks.

The transcription factor Bright/ARID3A regulates reprogramming through direct de-repression of key pluripotency factors Oct4, Sox2 and Nanog<sup>[30]</sup>. It has been shown to be a binding partner of Nanog<sup>[31]</sup> as well as Oct4 and Sox2<sup>[30]</sup> and is believed to function as a direct repressor of key developmental promoters. In culture, loss of Bright, either in through germline deletion, dominant negative inhibition, or shRNA mediated knockdown, is able to enhance standard reprogramming, induce pluripotency related genes and partially reprogram somatic cells to an ES-like state. Further, loss of Bright can induce complete reprogramming in the presence of LIF<sup>[30]</sup>. Bright was first discovered as a B-cell specific factor<sup>[32-34]</sup> and is critical for hematopoietic stem cell formation<sup>[35]</sup>. Bright is known to interact directly with p53<sup>[36]</sup> and is regulated by p53<sup>[37]</sup>. It is possible that part of p53's influence on reprogramming may be a function of its regulation of Bright. Loss of Bright has further been shown to inhibit senescence in culture<sup>[38]</sup>, perhaps enhancing its reprogramming abilities. Bright is a tantalizing target for further analysis but there may be still as yet unknown factors which, like Bright, have multiple functions that work towards maintaining the differentiated state of the cell.

## EPIGENETIC MODIFIERS

Epigenetic regulation of the genome is essential for proper gene expression and lineage commitment. As cells shift from a stem cell state into a lineage committed state, the genome goes through massive epigenetic changes from an "open" conformation to a "closed" conformation<sup>[39]</sup>. During differentiation, global acetylation marks, typically associated with euchromatin, are reduced and global methylation markers that are associated with condensed or heterochromatin, are increased<sup>[40]</sup>, including H3K27<sup>[41,42]</sup> and H3K9<sup>[43,44]</sup> methylation, typically associated with inactive promoters and H3K4 methylation<sup>[45,46]</sup>, which is associated with active promoter regions. Heterochromatin is tightly packed chromatin that generally inhibits gene expression. This shift in chromatin structure and epigenetic marks is a necessary part of the differentiation process, silencing of genes that are no longer expressed in that tissue type. Underlying the critical nature of these epigenetic marks, it is often incorrect expression of silenced genes that contribute to the formation of cancer<sup>[47]</sup>, demonstrating their importance in gene regulation. Reprogramming can occur when "pioneer" factors such as Oct4, Sox2,

and Klf4 bind to enhancers near silenced chromatin and can then direct the binding of other transcription factors to these regions<sup>[48]</sup>. Regions of the genome in fibroblasts, marked by H3K9me3, have been shown to be refractory to binding by Oct4, Sox2 and Klf4, however knockdown of H3K9 methylases increased reprogramming efficiency<sup>[49]</sup> and the binding of “pioneer” factors to these regions<sup>[50]</sup>. Thus, the structure and epigenetic marks of heterochromatin itself inhibits reprogramming by exogenous factors.

Stem cells often use bivalent domains to create “poised” promoters of important developmental genes<sup>[51]</sup>. These bivalent domains are characterized by the repressive H3K27me3 and the activating H3K4me3 marks, thus repressing expression of these genes but allowing for quick upregulation in response to developmental signals<sup>[52]</sup>. Bivalent domains are an important regulatory feature, allowing stem cells to repress the expression of key genes while keeping them “poised”. Epigenetic regulation of the genome is necessary for appropriate gene expression in both stem cells and differentiated cells<sup>[40,41]</sup> and thus erasure of established epigenetic marks is a necessary part of reprogramming. Several histone deacetylase inhibitors have been shown to modulate reprogramming in both SCNT and iPSC<sup>[53]</sup>. Valproic acid (VPA) is an extremely effective histone deacetylase (HDAC) inhibitor<sup>[54]</sup> and is commonly used to enhance reprogramming<sup>[55,56]</sup>. Other HDAC inhibitors have similarly been shown to enhance reprogramming in many different contexts<sup>[53]</sup>. The complexities of the histone code, in particular methylation of histones, leads to an unclear picture of how inhibitors of methylation effects reprogramming<sup>[57]</sup>. However, DNA demethylation agent 5'-Azacytidine has long been known to enhance reprogramming<sup>[58]</sup>. Repression of AID, a cytidine deaminase, inhibits reprogramming in interspecies heterokaryons<sup>[59]</sup> via promoter CpG demethylation<sup>[59,60]</sup>. Inhibition of both DNA and histone methylating components of the polycomb repressive complexes 1 and 2 (PRC1 and PRC2) have been shown to inhibit reprogramming. Repression of histone methyl transferases SUV39H1<sup>[61]</sup> and Dot1L<sup>[62]</sup> as well as YY1<sup>[63]</sup> enhanced reprogramming. Specifically, repression of the catalytic activity Dot1L reduced H3K79 methylation and increased reprogramming efficiency by 3-4 fold<sup>[49]</sup>. DNA methylation and demethylation are crucial bottlenecks during reprogramming, but because of the complexities of the signals and their importance to appropriate gene expression, in looking for effective enhancers of reprogramming, targeted inhibition of specific methylation inhibitors may give a better results.

## KINASES

The focus of understanding the mechanisms underlying reprogramming have typically concentrated on transcription factors and chromatin modifying factors. However, inhibition of select kinases has also been shown to increase reprogramming efficiency<sup>[13,64]</sup>.

Examples include the TGF- $\beta$ r1 and SRC kinases, although only inhibition of TGF- $\beta$ r1 was able to enhance reprogramming in the absence of VPA<sup>[13]</sup>. Inhibition of TGF- $\beta$ r1 allowed reprogramming to occur in the absence of Sox2 by inducing Nanog expression in intermediately reprogrammed cells that had been exposed to Oct4, Klf4 and c-Myc<sup>[13]</sup>. A small chemical screen identified additional kinase barriers to reprogramming that include the MAP p38 kinase<sup>[65]</sup>, IP3K, which catalyzes synthesis of a crucial secondary lipid messenger required for Ca<sup>+</sup> signaling<sup>[66]</sup>, and Aurora A kinase, which is essential for cell division<sup>[67]</sup>. Inhibition of each of these enhanced iPSC cell formation, indicating that factors that may have more selected specificities beyond the transcription factors that activate them may be worthy factors for inhibition.

## CONCLUSION

The potential of human embryonic stem (hESC) cells in the clinic has been understood long before science was able to deliver on that potential. The issues that may limit using hESC as a therapeutic, such as potential immune rejection and ethical concerns, has made iPSC cells a very attractive alternative approach. iPSC appear to have the same ability as hESC cells to differentiate *in vitro* and so it is hoped that they will be able to replace hESC cells therapeutically. The underlying problem is efficient creation of reliable, stable iPSC without gene integration<sup>[68-71]</sup>. While there have been massive strides made in the field of reprogramming, there is still a lack of clinically useful methods for producing iPSC that have been shown to be safe in the long term<sup>[16,72-74]</sup>. Future work must expand on creating more robust reprogramming protocols, rather than staying limited to established reprogramming methods. We must look at all the proteins and microRNAs in the cell, not just known transcription factors. We must look at both enhancers of pluripotency and repressors of pluripotency as possible targets. The field has advanced enormously in only a few short years; however in order to fully manipulate cell fate, we must be more inclusive when searching for cellular reprogramming factors and broaden our understanding of how cell fate is determined.

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