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From microRNAs to targets: pathway discovery in cell fate transitions

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Abstract

MicroRNAs (miRNAs) are 22 nt non-coding RNAs that regulate expression of downstream targets by messenger RNA (mRNA) destabilization and translational inhibition. A large number of eukaryotic mRNAs are targeted by miRNAs, with many individual mRNAs being targeted by multiple miRNAs. Further, a single miRNA can target hundreds of mRNAs, making these small RNAs powerful regulators of cell fate decisions. Such regulation by miRNAs has been observed in the maintenance of the embryonic stem cell (ESC) cell cycle and during ESC differentiation. MiRNAs can also promote the dedifferentiation of somatic cells to induced pluripotent stem cells. During this process they target multiple downstream genes, which represent important nodes of key cellular processes. Here, we review these findings and discuss how miRNAs may be used as tools to discover novel pathways that are involved in cell fate transitions using dedifferentiation of somatic stem cells to induced pluripotent stem cells as a case study.

MicroRNA-mediated suppression of mRNAs

Details of how miRNAs recognize and downregulate their downstream mRNA targets can be found in other excellent reviews [1–3] and the topic is only briefly discussed here. MiRNAs are approximately 22nt long small RNAs that regulate their targets through incomplete nucleotide complementation. Most miRNA-mRNA targeting occurs through base-pairing between a short sequence located at the 5′ end of the miRNA, called the seed sequence, and its mRNA target. This seed sequence, ranging from nucleotide positions 2–8 in the miRNA, largely defines the miRNA's downstream targets and hence is the basis of most target prediction programs (reviewed in [1]). Exceptions to seed sequence pairing exist, but these make up a much smaller repertoire of miRNA-mRNA targeting events [4]. The exact consequence of miRNA-mRNA pairing is controversial, although the end result is both a decrease in mRNA and protein levels [5–8]. Interestingly, within cells, pairing between miRNA-mRNA can be regulated by various mechanisms including co-expression of the target and miRNA, alternative poly-adenylation leading to alternative 3′UTRs of mRNAs, and protein based enhancement or suppression of specific miRNA-mRNA pairing [9–17]. Ultimately, this minimal requirement of nucleotide complementation for miRNA-mRNA pairing results in a single miRNA suppressing hundreds of targets [1].

MicroRNA Redundancy

Studies in miRNA function have been complicated not only by the fact that a single miRNA regulates multiple targets, but also by functional redundancy among miRNAs in many, if not most, biological processes [18–20]. This redundancy results in part from miRNAs existing

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in large families sharing common seed sequences that can be co-expressed in the same cell, and hence share overlapping downstream mRNA targets [20]. Redundancy also occurs at the level of co-targeting where multiple distinct miRNAs with very different sequences commonly target a single transcript through non-overlapping sites [21].

A powerful means of overcoming this redundancy in order to study individual miRNA function in a given biological setting is to first remove all miRNAs and then reintroduce individual miRNAs mimics. Global removal of miRNAs is made possible by deleting genes encoding proteins responsible for the processing of miRNAs. The biogenesis of most miRNAs requires two essential processing steps: primary- to precursor-miRNA by the DGCR8/DROSHA complex and precursor- to mature miRNA by DICER [22,23]. Knockout alleles for all three genes encoding these proteins have been made, thus providing powerful tools with which the function of individual miRNAs can be studied [24–28].

MicroRNA functions in embryonic stem cells

Deletion of *Dgcr8* or *Dicer* in embryonic stem cells (ESCs) results in two interesting phenotypes, a proliferation defect and a block in differentiation [20,26–29]. The proliferation defect is associated with an accumulation of cells in the G1 phase of the cell cycle. In a screen conducted to identify miRNAs that could rescue this phenotype, members of the miR-290 and 302 clusters were uncovered [20]. The miR-290 cluster is highly expressed in mouse ES cells, while the 302 cluster is highly expressed in human ES cells [30,31]. The specific members of these clusters that rescue proliferation share a common seed sequence and are collectively termed the ESCC family of miRNAs for ESC cell cycle promoting miRNAs. The ESCC miRNAs target a number of important cell cycle regulators. These included the CDK inhibitor Cdkn1a, the tumor suppressor Lats2, and members of the RB family of proteins. Expression of Cdkn1a without its 3′UTR in wild-type ES cells partially recapitulates the cell cycle phenotype of *Dgcr8* knockout cells, indicating that Cdkn1a can only partially explain the effect of ESCC miRNA loss [20].

In addition to the cell cycle defect, *Dgcr8* knockout ES cells fail to downregulate pluripotency factors when cultured under differentiation-inducing conditions [28,29]. Introduction of members of the let-7 family of miRNAs can rescue this defect. Let-7 miRNAs are highly expressed in differentiated tissues and hence are well positioned to repress the self-renewal program [32]. Profiling after introducing let-7 into *Dgcr8* knockout cells combined with bioinformatic analyses reveals a large number of likely targets for this family of miRNAs [29]. These targets include multiple well-known pluripotency genes such as nMyc, Sall4, and Lin28. Interestingly, Lin28 is an inhibitor of let-7 biogenesis producing a double negative feedback loop [33]. Members of the let-7 family of miRNAs are not the only miRNAs shown to promote silencing of ESC self-renewal. For example, miR-145 and miR-134 silence self-renewal of human and mouse ESCs respectively. MiR-145 targets Oct4, Sox2 and Klf4, while miR-134 targets Nanog and LRH1, all important pluripotency genes [34,35].

Collectively these studies show that miRNAs are critical regulators of the switch between ESC self-renewal and differentiation. While a single miRNA family, the ESCC family, seems to be largely responsible for promoting ESC self-renewal, multiple miRNAs can promote differentiation [36]. Importantly, each of these miRNAs is predicted to have many downstream mRNA targets.

One microRNA – many targets

Historically, most miRNA phenotypic studies have focused on one or a small number of targets. These include classic examples such as the regulation of lin-14 by the small RNA

lin-4 and the regulation of lin-41 by let-7 in worms [37,38]. However, it is becoming increasingly apparent that regulation of a single target by a miRNA is unlikely to explain how they function in most situations. Indeed, the power of miRNAs influencing a cell fate decision is likely through their ability to alter levels of many genes and pathways simultaneously. For example, the introduction of a lineage-specific miRNA (miR-124) into HeLa cells (tumor cells of cervical origin) results in a global shift in their expression profile towards that of a cell of the neural lineage. Much of the shift can be attributed to direct targeting by the miRNA, as there is a strong enrichment of seed matches in the 3′UTRs of downregulated genes [7].

Functional experiments have corroborated these early findings. For example, a number of miR-124's targets have since been confirmed as direct targets with functional relevance to neuronal differentiation [39–41]. Another example is miR-31, which is repressed in metastatic breast cancer cells, while its targets including RhoA, Fzd3, ITGA5, M-RIP, MMP16 and RDX are upregulated [42]. Co-expression of three targets, ITGA5, RDX and Rho in breast cancer cells overcomes the block in metastasis upon overexpression of miR-31, while individual targets had only a partial effect [43]. Additional examples of miRNAs with multiple targets in common processes are provided in Table 1. However, this list is not comprehensive.

Induced pluripotency – resetting the clock

In 2006, Yamanaka and colleagues demonstrated that the introduction of four transcription factors, Oct3/4, Sox2, Klf4 and cMyc into somatic cells, resulted in their conversion into pluripotent cells, called induced pluripotent stem cells [44]. While many groups thereafter have also succeeded in creating induced pluripotent stem cells from a variety of starting cell populations, little is known about all the changes that a cell has to undergo to become pluripotent. Furthermore, reprogramming remains to date a slow and relatively inefficient process and a better understanding of downstream events after introduction of the Yamanaka factors would enable us to develop patient-specific iPS lines in a faster and safer manner. Often cells become trapped in a partially reprogrammed state following introduction of the factors [45–47]. These cells are self-renewing, but not pluripotent. The comparison of partially reprogrammed cells to fully reprogrammed cells or embryonic stem cells or to the starting population of differentiated cells has provided valuable insight into the roadblocks during the process of reprogramming [46,47]. Such comparisons have revealed that reprogramming of MEFs to iPS cells requires a genome-wide alteration of epigenetic marks. This includes the conversion of monovalent histone methylation marks such as H3K4me3 or H3K27me3 to bivalent marks at developmental genes; the reactivation of transcription of pluripotency genes and the loss of DNA methylation from a large part of the genome [48]. In support of the role of epigenetic modifications during dedifferentiation, treatment of cells with small molecule inhibitors to HDACs, DNA methyltransferases and the G9a methyltranferase have been shown to enhance the process of reprogramming [46,49–51]. Transcriptional profiling of partially reprogrammed cells indicates that while a number of genes associated with cell proliferation and DNA synthesis are upregulated, genes such as *Cdkn1a* which regulate cell cycle checkpoints are also expressed at high levels. Indeed, a number of studies have reported that knockdown of Cdkn1a, p53 or Ink4/Arf enhance the efficiency of reprogramming [52–57]. In addition to the involvement of epigenetics and cell cycle in the process of reprogramming, the conversion of cells from a mesenchymal to an epithelial state is also required [58,59]. Thus, the acquisition of pluripotency is associated with a number of changes in the state of the cell, of which only a few are known.

MicroRNAs and induced pluripotency: pathway discovery during cell fate transitions

Similar to small molecules, miRNAs can influence reprogramming. The ESCC miRNAs and the closely related miR-106 family enhance the efficiency of reprogramming [60–63]. Indeed, it was recently reported that the miR-302 cluster (consisting of miRs-302a–d and miR-367) alone could produce iPSCs from both mouse and human fibroblasts [64]. Interestingly, the promoters of the miR-290 cluster (hsa-miR-371/372/373 in human) and miR-302 clusters are bound and regulated by the original Yamanaka factors, Oct4, Sox2, and cMyc [32,60,65]. Therefore, these transcription factors are likely at least in part acting through these miRNAs to promote de-differentiation of somatic cells.

These results lead to the obvious question of what downstream targets underlie the miRNA's remarkable capacity to revert an adult cell back to an embryonic stem cell. Hints come from profiling experiments following the introduction of the ESCC miRNAs into *Dgcr8* knockout cells [29]. Similar to the miR-124 experiments in HeLa cells described above, hundreds of transcripts with seed matches in their 3′UTR are downregulated. Interestingly, there is a highly significant enrichment of seed matches in the open reading frame and 3′UTR, consistent with targeting in both regions of the transcript. In order to gain a better understanding of pathways regulated by these miRNAs during reprogramming, a subset of these targets have been further characterized [63]. This subset was selected on the basis of their known role in potentially relevant cellular processes including cell cycle regulation, epithelial-mesenchymal transition (EMT), vesicular trafficking, cell signaling and epigenetic modifications. Knockdown of any single individual target within this subset of targets only marginally increases the efficiency of reprogramming, much less so than the miRNA itself. This finding suggests that these targets/cellular processes work together to promote reprogramming. Indeed, co-suppression of two targets further enhances reprogramming. Therefore, these miRNAs uncovered cellular pathways, whose role in reprogramming was not fully appreciated and that act synergistically to promote the induction of pluripotency.

Using miRNAs as tools to dissect mechanisms of cell fate transitions

While the promiscuous nature of miRNA targeting defies simplification of their roles, we propose that they can be used as a powerful tool to uncover the multiple pathways/cellular processes that underlie cell fate decisions (Fig. 1). The approach takes advantage of the fact that the hundreds of mRNA targets have been evolutionarily selected to have a defined cellular outcome. That is, the identification of miRNAs that promote a specific outcome, combined with the identification and functional characterization of all its targets can provide a holistic picture of the events that must occur. As mentioned in this review, a number of studies performed on a smaller scale have already provided evidence for the success of such an approach. However, a full-scale study functionally testing all downstream targets has yet to be performed.

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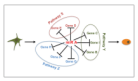

Figure 1. Pathway discovery using microRNAs

Introduction of single miRNAs that regulate cell fate decisions followed by profiling can uncover numerous target genes that are key nodes of signaling pathways.

Table 1
Individual miRNAs regulate the expression of multiple targets

Listed are a few examples of miRNAs and some select targets that are involved in cell fate transitions.

MicroRNA	Validated Targets	Context	Organism	Reference
miR-124	NeuroD1, Baf53a, Ptbp1, Scp1, Sox9, Dlx2, Jag1	Neural development	Xenopus, Mouse, Chick	[39–41,66,67]
miR-31	RhoA, Fzd3, Itga5, Mmp16, M-Rip, Rdx	Metastasis	Human	[42]
miR-19	Pten, Ppp2r5e, Prkaa1, Bim	Leukemia	Mouse	[68]
miR-21	Tiam1, Anp32a, Smarca4, P12 Cdk2ap1	Cancer	Human	[69–71]
miR-200	Zeb1, Zeb2, Ets1, Suz12, Bmi1	Cancer	Human and mouse	[72–78]
miR-294, miR-302, miR-372	Cdkn1a, Rbl2, Lats2, Tgfbr2, RhoC, Mecp1–p66, Mecp2, Aof1, Aof2, Cyclin D1	Embryonic stem cells and dedifferentiation	Mouse and human	[20,62,63,65,79–83]
Let-7	Hmga2, Ras, cMyc, NMyc, Sall4, Lin28, Trim71, Cyclin D1, Tlx	Differentiation and cancer	Mouse, human and C. elegans	[29,33,84–89]