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The functions of microRNAs in pluripotency and reprogramming

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Abstract

Pluripotent stem cells (PSCs) express a distinctive set of microRNAs (miRNAs). Many of these miRNAs have similar targeting sequences and are predicted to regulate downstream targets cooperatively. These enriched miRNAs are involved in the regulation of the unique PSC cell cycle, and there is increasing evidence that they also influence other important characteristics of PSCs, including their morphology, epigenetic profile and resistance to apoptosis. Detailed studies of miRNAs and their targets in PSCs should help to parse the regulatory networks that underlie developmental processes and cellular reprogramming.

Human PSCs (hPSCs), which include human embryonic stem (ES) cells and induced pluripotent stem cells (iPSCs), are a potential source of cells for both research and cell therapy. Their ability to self-renew and to differentiate into all the cell types in the human body makes them a promising system for modelling cellular differentiation and development, as well as a valuable resource that could lead to treatments for many diseases. But to realize the full potential of hPSCs, we must gain a better understanding of the mechanisms that regulate self-renewal and the maintenance of pluripotency. Here, we focus on a unique set of miRNAs that are highly expressed in PSCs, which provide clues about the regulatory pathways that are active in these cells. We outline current work in hPSCs, in which many disease models are being developed and tested. We also mention seminal studies performed in mice that have informed much of the mechanistic work in the reprogramming and miRNA fields today. Detailed reviews of miRNA biogenesis and function^{1,2}, and of the role of miRNAs in development³, differentiation^{3,4}, cancer⁵ and apoptosis⁶, can be found elsewhere.

miRNAs were first discovered in 1993, when regulation of the gene *lin-14* by a small RNA, *lin-4*, was reported in *Caenorhabditis elegans*⁷. It was not until seven years later that a second small RNA, *let-7*, was identified⁸. Since then, miRNAs have been discovered in virtually all plant and animal species, and their biological functions and mechanisms of action have become subjects of intense research. miRNAs are non-coding RNAs that are

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often encoded in clusters in the genome. Members of miRNA families have homologous sequences, but are not necessarily transcribed from the same genomic region. However, there are many cases in which miRNA families are found in clusters and their transcription is co-regulated. According to the latest version of the miRNA database miRBase, released in August 2012, approximately 2,042 mature miRNAs have been experimentally identified so far in human, and approximately 1,281 in mouse⁹. After miRNAs are transcribed, they are processed by a specialized set of enzymes into their short (about 22 nucleotide) mature form. Through interactions with proteins in the RNA-induced silencing complex (RISC), each miRNA can target many mRNAs in a sequence-dependent fashion¹⁰. After a messenger RNA transcript is bound by an miRNA associated with a RISC, expression of the target mRNA is downregulated, either by mRNA destabilization or by inhibition of translation¹¹.

A unique set of miRNAs is present in PSCs

miRNAs are essential for development; knockout of the RNA-processing proteins Dicer1 (a ribonuclease) and Dgcr8 (DiGeorge syndrome critical region gene 8) in mice have shown that disruption of active miRNA biogenesis is lethal during embryogenesis^{12,13}. With the aim of using them as a model for human embryonic development, hPSCs have been profiled using sequencing and microarray methods to identify miRNAs that have potential roles in differentiation and development. These studies have revealed several miRNA families that are upregulated specifically in hPSCs compared to mature differentiated cell types. These include the human (hsa)-miR-302, hsa-miR-106, hsa-miR-372, hsa-miR-17, hsa-miR-520, hsa-miR-195 and hsa-miR-200 families¹⁴⁻¹⁷. Except for the hsa-miR-520 family, all of these human miRNA families have a corresponding set of homologous miRNAs in the mouse, which are also expressed in mouse ES cells. In most cases, the mouse and human homologues follow the same nomenclature; an exception is the mouse homologue of the hsa-miR-372 family, which corresponds to the mouse (mmu)-miR-290 family. As well as the miRNA families that are enriched in hPSCs, there are also several miRNAs that are expressed at significantly lower levels in hPSCs than in differentiated cells, most notably the hsa-let-7 family^{14,15}.

For most miRNAs, the targeting of cognate mRNAs is strongly influenced by the complementarity between the short 'seed' sequence at nucleotides 2-8 on the 5' end of the miRNA and sequences contained in the 3' untranslated regions (UTRs) of the mRNAs. Interestingly, the seed sequences of many miRNAs that are highly expressed in hPSCs are closely related. Several of the pluripotency-associated hPSC miRNA families (including hsa/mmu-miR-17, hsa/mmu-miR-106, hsa/mmu-miR-302, hsa-miR-372/mmu-miR-290 and hsa-miR-520) share 6/7 or 7/7 nucleotides in the seed site¹⁵. This suggests that these miRNAs may have similar mRNA targets and regulatory functions that could be important in maintaining the unique characteristics of PSCs (see Fig. 1 for a summary of published miRNA/mRNA interactions from the murine system relevant to pluripotency, and Fig. 2 for the human system).

The accurate identification of miRNA targets will help us to understand further the functional role of miRNAs in PSCs. Although there are many bioinformatic algorithms that predict the mRNA targets of miRNAs (Box 1), the experimental verification of functional

targeting remains a relatively slow process. So far, only a handful of specific direct targets have been identified, usually using luciferase reporter assays^{18,19} (Box 1). Global pull-downs using enhanced cross-linking of the RISC-associated enzyme argonaute 2 (also known as EIF2C2) to miRNAs and potential mRNA targets have validated previously identified cell cycle and TGF- β (transforming growth factor p) signalling targets, and provided evidence for additional endogenous pathways regulated by pluripotency-associated miRNAs in mouse²⁰ and human²¹.

Roles of PSC-associated miRNAs in cell-state change

Examining the effects of miRNAs on differentiation and on experimentally induced reprogramming can provide clues about the endogenous pathways regulated by miRNAs. hPSCs have been successfully differentiated in culture into many different cell types, including cardiomyocytes, fibroblasts, hepatocytes and neurons. Recently, it has been demonstrated that specific transcription factors can be used to transform human cells from one mature cell type directly to another^{22,23}, and miRNAs have been shown to enhance the conversion of fibroblasts to neurons^{24,25} and cardiomyocytes²⁶. However, the largest body of research in cell-state conversion and the most informative evidence about the roles of pluripotency-associated miRNAs, is in cellular reprogramming of differentiated somatic cells to an iPSC state.

Reprogramming of somatic cells to iPSCs was first achieved by overexpressing a set of transcription factors consisting of octamer-binding transcription factor 4 (*Oct4*; also known as *Pou5f1*), SRY-box-containing gene 2 (*Sox2*), Kruppel-like factor 4 (*Klf4*) and myelocytomatosis oncogene (*Myc*) (collectively known as OSKM) in mouse fibroblasts cultured under ES cell conditions²⁷. Shortly afterwards, human fibroblasts were successfully reprogrammed using either the same combination of transcription factors²⁸ or a similar combination of reprogramming factors (*OCT4*, *SOX2*, Nanog homeobox (*NANOG*) and the RNA-binding protein lin-28 homologue A (*LIN28A*); collectively known as OSNL)²⁹. (For an in-depth review of our current understanding of the mechanisms of reprogramming, see ref. 30.) Although it has proved difficult to dissect the functions of individual miRNAs in PSCs, the ability of certain miRNAs to enhance or inhibit reprogramming to pluripotency has provided insights into their endogenous roles in the maintenance of pluripotency.

An early report described the conversion of human cancer cells into an ES-cell-like state by retroviral transduction of the hsa-miR-302/367 cluster³¹. Although these cells were not demonstrated to have typical PSC morphology, the report showed that the ES-like cells derived from cancer cells expressed hPSC markers and also shared similar gene expression profiles and *OCT4* DNA methylation profiles to those of hPSC lines. Subsequently, several publications have shown that members of specific pluripotency-associated miRNA families, including mmu-miR-17/92 (ref. 32), mmu-miR-106a/363 (refs 32,33), mmu-miR-106b/25 (ref. 32), mmu-miR-290/hsa-miR-372 (refs 34,35) and hsa/mmu-miR-302/367 (refs 33–35), were able to enhance reprogramming in mouse and human fibroblasts when using three of the four standard reprogramming factors (*Oct4*, *Sox2* and *Klf4*; OSK). The targets through which these miRNAs have been reported to act on the reprogramming process are shown in Figs 1 and 2.

There is an increasing body of evidence that the standard reprogramming factors act in part by trans-activating pluripotency-associated miRNAs (Figs 1 and 2). For example, using chromatin immunoprecipitation (ChIP)-sequencing assays, it was shown that Oct4, Sox2 and Nanog were bound to the promoter regions of the mmu-miR-106a/363, mmu-miR-290 and mmu-miR-302/367 clusters in mouse ES cells, and that OCT4 was bound to the same conserved promoter regions of these clusters in human ES cells³⁶. Furthermore, OCT4 and SOX2 were demonstrated to trans-activate the hsa-miR-302/367 cluster, and the hsa-miR-302 and OCT4 levels decrease in parallel during human ES cell differentiation³⁷. MYC has been shown to bind and trans-activate the hsa-miR-17 cluster in human cells³⁸, and to repress hsa-miR-34 (ref. 39) and hsa-miR-29 (ref. 40) in human cancer cells. Together, these results suggest that transcription factors and miRNAs that are highly expressed in PSCs act together during reprogramming of fibroblasts to iPSCs.

A logical corollary to the discovery that PSC-associated miRNAs can enhance reprogramming is that repression of the miRNAs expressed at lower levels in PSCs compared to differentiated cells might also promote reprogramming. Indeed, repression of mmu-miR-21 or mmu-miR-29a has been shown to enhance reprogramming by OSK (ref. 41), and repression of mmu-let-7 enhances reprogramming by OSKM (refs 41,42), at least partially through de-repression of the reprogramming factor Lin28 (ref. 42) in mouse. Interestingly, Lin28 inhibits the processing of hsa/mmu-let-7 miRNA precursors in both mouse ES cells and human cancer cells, possibly explaining the initial success of reprogramming with the OSNL factors⁴³⁻⁴⁶. Moreover, mmu-miR-34 has been shown to obstruct iPSC generation by targeting and repressing *Nanog*, *Sox2* and *Mycn*, and knockout of mmu-miR-34 facilitates reprogramming of mouse embryonic fibroblasts (MEFs) by OSK or OSKM, possibly by eliminating its pro-apoptotic effects⁴⁷.

Recent reports suggest that reprogramming of mouse and human somatic cells can be achieved using only miRNAs introduced either by lentiviral transduction or transient transfection^{48,49}. Lentiviral expression of the mmu-miR-302/367 cluster was reported to reprogram mouse and human fibroblasts with up to two orders of magnitude greater efficiency than the OSKM transcription-factor-based method⁴⁸. Reprogramming mouse or human somatic cells using direct transfection of mature miRNAs⁴⁹ (including hsa/mmu-miR-200c, hsa/mmu-miR-302s and hsa/mmu-miR-369s) was, however, far less efficient. Nevertheless, it is notable that reprogrammed cells were obtained with mature miRNAs, because at present it is the only reported method that does not use either vector-based gene transfer, or oncogenic genes, transcripts or proteins, to induce reprogramming. Although these techniques have yet to be widely reproduced, reprogramming using miRNAs alone holds great promise in the stem-cell field.

Specific roles of miRNAs in reprogramming pathways

Reprogramming involves rapid and marked changes in cellular phenotype, which typically include an increase in proliferation, transition through an epithelial phenotype, changes in DNA methylation and a decrease in apoptosis. Many of the miRNAs that are highly expressed in PSCs promote these changes. A summary of the specific regulatory interactions between miRNAs and the targets that mediate these effects is shown in Figs 1 and 2.

PSC-associated miRNAs enforce the ES cell cycle.

One well-studied pathway that changes considerably between the differentiated and undifferentiated cell state is the cell cycle. PSCs have a unique cell cycle that is essential for self-renewal. hPSCs proliferate rapidly, with an abbreviated G1 phase owing to the lack of a G1/S checkpoint, resulting in a cell-cycle length of approximately 15 h compared to approximately 24 h for more differentiated human cells^{50,51}.

The role of miRNAs in the PSC cell cycle has been primarily studied by using cells without endogenous miRNAs, which has been achieved by the genetic deletion or knockdown of specific components in the miRNA biogenesis pathway. PSCs without miRNAs have a cell-cycle defect, which was partially rescued by the re-introduction of hsa-miR-372 and hsa-miR-195 in hESCs and mmu-miR-290 family members in mouse ES cells^{19,52}. Several direct mRNA targets for the hsa-miR-372 family in the cell-cycle pathway have been identified, including cyclin D1 (CCND1)³⁷, retinoblastoma-like 2 (RBL2), cyclin-dependent kinase inhibitor 1A (CDKN1A)³⁵ and 1C (CDKN1C)⁵³. The homologous mmu-miR-290 family has been shown to target retinoblastoma 1 (Rb1), retinoblastoma-like 1 (Rbl1), cyclin-dependent kinase inhibitor 1B (Cdkn1b), large tumour suppressor 2 (Lats2)⁵², WEE1 homologue 1 (Wee1) and F-box and leucine-rich repeat protein 5 (Fbx15)⁵⁴. The majority of the hPSC-specific miRNAs are involved in accelerating the G1 to S transition. This suggests that the miRNA-mediated repression of multiple factors involved in the G1/S checkpoint contributes to the unique characteristics of the cell cycle and the self-renewal program in hPSCs.

Recent work investigating how the miRNA and target mRNA landscape changes when a PSC exits the self-renewal program and differentiates, suggests that the major switches include downregulation of the highly expressed hsa/mmu-miR-17/106/302, hsa-miR-372/520 and mmu-miR-290, and upregulation of the hsa/mmu-let-7, miRNAs. Experiments in miRNA-depleted mouse ES cells revealed that these opposing miRNA groups act on the same pathways through the direct and indirect regulation of several essential targets, including sal-like 4 (Sall4), Mycn, Myc and Lin28a (ref. 42). Given the effects these miRNA have on the cell cycle, these data from the mouse system suggest that changes in cell-cycle regulation may also be integral to the process of differentiation.

miRNAs in the mesenchymal to epithelial transition.

Reprogramming cells with a mesenchymal phenotype, such as reprogramming fibroblasts to a pluripotent state, is accompanied by a marked morphological transition from a mesenchymal to an epithelial phenotype at an intermediate point in the reprogramming process. Several miRNAs have been shown to promote the mesenchymal to epithelial transition (MET), including hsa/mmu-miR-205 and the hsa/mmu-miR-200, hsa/mmu-miR-302 and hsa/mmu-miR-372 families. Many of the initial studies on the roles of miR-200 and miR-205 on MET were performed in non-PSC systems and then subsequently related to the PSC system, whereas most of the miR-302 and miR-372 studies were performed in PSCs. This difference is probably related to the fact that miR-302 and miR-372 expression are highly specific to PSCs, whereas miR-200 and miR-205 are more widely expressed. Ectopic expression of hsa-miR-205 and the hsa-miR-200 family in canine

mesenchymal cells initiates MET, by directly targeting and repressing zinc finger E-box binding homeoBox 1 (ZEB1) and ZEB2, which results in de-repression of E-cadherin⁵⁵. Furthermore, overexpression of the mmu-miR-200 family has been shown to inhibit the epithelial-to-mesenchymal transition (EMT) by directly targeting and repressing Zeb1 and Zeb2 in mouse epithelial cells treated with TGF- β ⁵⁶. The reverse process, MET, seen in the reprogramming of MEFs using OSKM, is thought to be driven by a strong bone morphogenetic protein (BMP) response, which induces the expression of the mmu-miR-200 family and mmu-miR-205 (ref. 57). Photoactivatable-ribonucleoside-enhanced crosslinking and immuno-precipitation (PAR-CLIP) experiments in human ES cells to identify the mRNA targets of miRNAs (see Box 1) showed that hsa-miR-302/367 promotes BMP signalling by targeting the BMP inhibitors transducer of ERBB2, 2 (TOB2), DAZ-associated protein 2 (DAZAP2) and SLAIN motif family, member 1 (SLAIN1)²¹. The hsa-miR-302 and hsa-miR-372 families may promote reprogramming-associated MET by repression of TGF-p receptor 2 (TGFR2) and ras homologue family member C (RHOC)³⁵, which are known to drive the reverse process of EMT (refs 58,59). Transfection of hsa-miR-302b and hsa-miR-372 (either individually or in the context of retroviral transduction of OSK) has also been shown to result in decreased levels of the mesenchymal markers ZEB1, ZEB2, fibronectin 1 (FN1), and snail homologue 2 (SNAI2)³⁵. These results suggest that the hsa-miR-302/367 and hsa-miR-200c families promote MET during reprogramming of human fibroblasts by repressing the TGF-p pathway and de-repressing E-cadherin.

Regulation of DNA methylation by miRNAs.

hPSCs are significantly hypermethylated globally compared to mature differentiated cell types⁶⁰. As somatic cells undergo the reprogramming process, marked changes in DNA methylation occur, although some debate remains about the extent of epigenetic remodelling at certain sites in the genome^{61–63}. There is also indirect evidence that miRNAs regulate the epigenetic state in PSCs. *Dicer1*-null mouse ES cells express decreased levels of the DNA methyltransferases *Dnmt1*, *Dnmt3a* and *Dnmt3b*, resulting in defects in DNA methylation. The introduction of mimics of members of the mmu-miR-290 cluster into these *Dicer1*-null mouse ES cells resulted in repression of *Rbl2* and subsequent de-repression of *Dnmt3a* and *Dnmt3b* (refs 64,65). Repression of the DNA-binding protein genes methyl-CpG binding protein 2 (*MECP2*) and methyl-CpG binding domain protein 2 (*MBD2*) in human fibroblasts has also been observed with experimental expression of hsa-miR-302 and hsa-miR-372 (ref. 35). However, the only direct evidence for modulation of human DNMT proteins by miRNAs is a report showing that the hsa-miR-29 family members directly repress both DNMT3A and DNMT3B in human lung cancer cells⁶⁶. It is possible that miRNAs and methylation status work in concert to silence and activate the necessary genes required for the transcriptional network seen in the pluripotent state.

Effects of miRNAs on apoptosis.

Recently, the mmu-miR-295 cluster was linked to survival of mouse ES cells after DNA damage through direct targeting of caspase 2 (*Casp2*) and etoposide-induced 2.4 mRNA (*Ei24*), a TP53 target⁶⁷. Furthermore, members of the hsa/mmu-miR-34 family are induced on activation of TP53, and have been shown to promote apoptosis (reviewed in ref. 68). These results are consistent with reports that inhibition of TP53 using either deletion of the

Tp53 gene in mice or by short hairpin RNAs (shRNAs) in human cell lines enhances reprogramming to the pluripotent state, allowing for the generation of human iPSCs using only two of the reprogramming transcription factors, *OCT4* and *SOX2* (refs 69–71). Human TP53 targets have also been shown to be repressed by pluripotency-associated miRNAs present in cancer cells. Specifically, hsa-miR-17/106 has an anti-apoptotic effect through regulation of CDKN1A (ref. 72) in cervical cancer cells. Other studies in human cancer cells have also shown that hsa-miR-17 and hsa-miR-106 inhibit apoptosis via repression of BCL-2-like 11 (*BCL2L11*)^{73–74} and the TGF- β pathway⁷⁵. It is likely that pluripotency-associated miRNAs have similar targets in hPSCs; however, roles for these miRNAs in the regulation of apoptosis in hPSCs have not yet been directly shown.

Conclusion

Over the past few years, it has become apparent that the distinctive pattern of miRNA expression seen in PSCs contributes to many of the unique phenotypic properties of these cells. Three main experimental approaches have been used to study miRNAs in PSCs. First, the reintroduction of miRNAs to PSCs that have had their miRNA biogenesis machinery disabled by knockout or knockdown of Dicer or Dgcr8. Second, the identification of miRNAs that can enhance reprogramming when transfected or overexpressed in somatic cells. Third, the immunoprecipitation of mRNAs cross-linked to protein components of the RISC complex. These approaches have increased our understanding of the functional roles of miRNAs in undifferentiated PSCs and reprogramming. In particular, it is clear that a subset of upregulated miRNAs in PSCs target multiple G1/S checkpoint genes to promote the establishment and maintenance of the unusually rapid PSC cell cycle.

As more sophisticated methods for manipulating individual miRNAs in the native PSC cellular environment are developed, additional insights into the interaction of miRNAs with the complex networks that maintain cellular identity will be revealed. The introduction or depletion of miRNAs involved in regulating cell-state transitions may prove useful in inducing reprogramming or differentiation along a particular lineage. In tandem with current PSC differentiation protocols, it may also be possible to accelerate differentiation processes by the depletion of highly differentially expressed miRNAs in PSCs. Manipulation of miRNAs might also be used to improve the stability of a specific differentiated cell type. Our knowledge of the roles of miRNAs in regulating cellular phenotypes may expand the utility of hPSCs for understanding human disease, in drug development pathways, and for cell therapy.

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References

1. Bartel DP MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 116, 281–297 (2004). [PubMed: 14744438]

2. Winter J, Jung S, Keller S, Gregory RI & Diederichs S Many roads to maturity: microRNA biogenesis pathways and their regulation. *Nat. Cell Biol* 11, 228–234 (2009) [PubMed: 19255566]
3. Zhao Y & Srivastava D A developmental view of microRNA function. *Trends Biochem. Sci* 32, 189–197 (2007). [PubMed: 17350266]
4. Ivey KN & Srivastava D MicroRNAs as regulators of differentiation and cell fate decisions. *Cell Stem Cell* 7, 36–41 (2010) [PubMed: 20621048]
5. Esquela-Kerscher A & Slack FJ Oncomirs—microRNAs with a role in cancer. *Nat. Rev. Cancer* 6, 259–269 (2006). [PubMed: 16557279]
6. Lima RT et al. MicroRNA regulation of core apoptosis pathways in cancer. *Eur. J. Cancer* 47, 163–174 (2011).
7. Lee RC, Feinbaum RL & Ambros V The *C. elegans heterochronic gene lin-4* encodes small RNAs with antisense complementarity to *lin-14*. *Cell* 75, 843–854 (1993). [PubMed: 8252621]
8. Reinhart BJ et al. The 21-nucleotide *let-7* RNA regulates developmental timing in *Caenorhabditis elegans*. *Nature* 403, 901–906 (2000). [PubMed: 10706289]
9. Kozomara A & Griffiths-Jones S miRBase: integrating microRNA annotation and deep-sequencing data. *Nucleic Acids Res.* 39, D152–D157 (2011). [PubMed: 21037258]
10. Selbach M et al. Widespread changes in protein synthesis induced by microRNAs. *Nature* 455, 58–63 (2008). [PubMed: 18668040]
11. Huntzinger E & Izaurralde E Gene silencing by microRNAs: contributions of translational repression and mRNA decay. *Nat. Rev. Genet* 12, 99–110 (2011). [PubMed: 21245828]
12. Bernstein E et al. Dicer is essential for mouse development. *Nat. Genet* 35, 215–217 (2003). [PubMed: 14528307]
13. Wang Y, Medvid R, Melton C, Jaenisch R & Blelloch R DGCR8 is essential for microRNA biogenesis and silencing of embryonic stem cell self-renewal. *Nat. Genet* 39, 380–385 (2007). [PubMed: 17259983]
14. Bar M et al. MicroRNA discovery and profiling in human embryonic stem cells by deep sequencing of small RNA libraries. *Stem Cells* 26, 2496–2505 (2008). [PubMed: 18583537]
15. Laurent LC et al. Comprehensive microRNA profiling reveals a unique human embryonic stem cell signature dominated by a single seed sequence. *Stem Cells* 26, 1506–1516 (2008). [PubMed: 18403753]
16. Morin RD et al. Application of massively parallel sequencing to microRNA profiling and discovery in human embryonic stem cells. *Genome Res.* 18, 610–621 (2008). [PubMed: 18285502]
17. Suh MR et al. Human embryonic stem cells express a unique set of microRNAs. *Dev. Biol* 270, 488–498 (2004). [PubMed: 15183728]
18. Barroso-delJesus A et al. The Nodal inhibitor Lefty is negatively modulated by the microRNA miR-302 in human embryonic stem cells. *FASEB J* 25, 1497–1508 (2011). [PubMed: 21266536]
19. Qi J et al. MicroRNAs regulate human embryonic stem cell division. *Cell Cycle* 8, 3729–3741 (2009). [PubMed: 19823043]
20. Leung AK et al. Genome-wide identification of Ago2 binding sites from mouse embryonic stem cells with and without mature microRNAs. *Nat. Struct. Mol. Biol* 18, 237–244 (2011). [PubMed: 21258322]
21. Lipchina I et al. Genome-wide identification of microRNA targets in human ES cells reveals a role for miR-302 in modulating BMP response. *Genes Dev.* 25, 2173–2186 (2011). [PubMed: 22012620]
22. Ieda M et al. Direct reprogramming of fibroblasts into functional cardiomyocytes by defined factors. *Cell* 142, 375–386 (2010). [PubMed: 20691899]
23. Vierbuchen T et al. Direct conversion of fibroblasts to functional neurons by defined factors. *Nature* 463, 1035–1041 (2010). [PubMed: 20107439]
24. Ambasudhan R et al. Direct reprogramming of adult human fibroblasts to functional neurons under defined conditions. *Cell Stem Cell* 9, 113–118 (2011). [PubMed: 21802386]
25. Yoo AS et al. MicroRNA-mediated conversion of human fibroblasts to neurons. *Nature* 476, 228–231 (2011). [PubMed: 21753754]

26. Jayawardena TM et al. MicroRNA-mediated *in vitro* and *in vivo* direct reprogramming of cardiac fibroblasts to cardiomyocytes. *Circ. Res* 110, 1465–1473 (2012). [PubMed: 22539765]
27. Takahashi K & Yamanaka S Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 126, 663–676 (2006). [PubMed: 16904174]
28. Takahashi K et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 131, 861–872 (2007). [PubMed: 18035408]
29. Yu J et al. Induced pluripotent stem cell lines derived from human somatic cells. *Science* 318, 1917–1920 (2007). [PubMed: 18029452]
30. Plath K & Lowry WE Progress in understanding reprogramming to the induced pluripotent state. *Nat. Rev. Genet* 12, 253–265 (2011). [PubMed: 21415849]
31. Lin SL et al. Mir-302 reprograms human skin cancer cells into a pluripotent ES-cell-like state. *RNA* 14, 2115–2124 (2008). [PubMed: 18755840]
32. Li Z, Yang CS, Nakashima K & Rana TM Small RNA-mediated regulation of IPS cell generation. *EMBO J.* 30, 823–834 (2011). [PubMed: 21285944]
33. Liao B et al. MicroRNA cluster 302–367 enhances somatic cell reprogramming by accelerating a mesenchymal-to-epithelial transition. *J. Biol. Chem* 286, 17359–17364 (2011). [PubMed: 21454525]
34. Judson RL, Babiarz JE, Venere M & Blelloch R Embryonic stem cell-specific microRNAs promote induced pluripotency. *Nat. Biotechnol* 27, 459–461 (2009). [PubMed: 19363475]
35. Subramanyam D et al. Multiple targets of miR-302 and miR-372 promote reprogramming of human fibroblasts to induced pluripotent stem cells. *Nat. Biotechnol* 29, 443–448 (2011). [PubMed: 21490602]
36. Marson A et al. Connecting microRNA genes to the core transcriptional regulatory circuitry of embryonic stem cells. *Cell* 134, 521–533 (2008). [PubMed: 18692474]
37. Card DA et al. Oct4/Sox2-regulated miR-302 targets cyclin D1 in human embryonic stem cells. *Mol. Cell. Biol* 28, 6426–6438 (2008). [PubMed: 18710938]
38. O'Donnell KA, Wentzel EA, Zeller KI, Dang CV & Mendell JT c-Myc-regulated microRNAs modulate E2F1 expression. *Nature* 435, 839–843 (2005). [PubMed: 15944709]
39. Craig VJ et al. Myc-mediated repression of microRNA-34a promotes high-grade transformation of B-cell lymphoma by dysregulation of FoxP1. *Blood* 117, 6227–6236 (2011). [PubMed: 21460242]
40. Mott JL et al. Transcriptional suppression of mir-29b-1/mir-29a promoter by c-Myc, hedgehog, and NF-k B. *J. Cell Biochem* 110, 1155–1164 (2010). [PubMed: 20564213]
41. Yang CS, Li Z & Rana TM MicroRNAs modulate IPS cell generation. *RNA* 17, 1451–1460(2011). [PubMed: 21693621]
42. Melton C, Judson RL & Blelloch R Opposing microRNA families regulate selfrenewal in mouse embryonic stem cells. *Nature* 463, 621–626 (2010) [PubMed: 20054295]
43. Heo I et al. Lin28 mediates the terminal uridylation of let-7 precursor microRNA. *Mol. Cell* 32, 276–284 (2008). [PubMed: 18951094]
44. Hagan JP, Piskounova E & Gregory RI Lin28 recruits the TUTase Zcchc11 to inhibit let-7 maturation in mouse embryonic stem cells. *Nat. Struct. Mol. Biol* 16, 1021–1025 (2009). [PubMed: 19713958]
45. Viswanathan SR, Daley GQ & Gregory RI Selective blockade of microRNA processing by Lin28. *Science* 320, 97–100 (2008). [PubMed: 18292307]
46. Piskounova E et al. Determinants of microRNA processing inhibition by the developmentally regulated RNA-binding protein Lin28. *J. Biol. Chem* 283, 21310–21314 (2008). [PubMed: 18550544]
47. Choi YJ et al. miR-34 miRNAs provide a barrier for somatic cell reprogramming. *Nat. Cell Biol* 13, 1353–1360 (2011). [PubMed: 22020437]
48. Anokye-Danso F et al. Highly efficient miRNA-mediated reprogramming of mouse and human somatic cells to pluripotency. *Cell Stem Cell* 8, 376–388 (2011). [PubMed: 21474102]
49. Miyoshi N et al. Reprogramming of mouse and human cells to pluripotency using mature microRNAs. *Cell Stem Cell* 8, 633–638 (2011). [PubMed: 21620789]

50. Becker KA et al. Self-renewal of human embryonic stem cells is supported by a shortened G1 cell cycle phase. *J. Cell. Physiol* 209, 883–893 (2006). [PubMed: 16972248]
51. Jones-Rhoades MW, Bartel DP & Bartel B MicroRNAs and their regulatory roles in plants. *Annu. Rev. Plant Biol* 57, 19–53 (2006). [PubMed: 16669754]
52. Wang Y et al. Embryonic stem cell-specific microRNAs regulate the G 1-S transition and promote rapid proliferation. *Nat. Genet* 40, 1478–1483 (2008). [PubMed: 18978791]
53. Sengupta S et al. MicroRNA 92b controls the G1/S checkpoint gene *p57* in human embryonic stem cells. *Stem Cells* 27, 1524–1528 (2009). [PubMed: 19544458]
54. Lichner Z et al. The miR –290–295 cluster promotes pluripotency maintenance by regulating cell cycle phase distribution in mouse embryonic stem cells. *Differentiation* 81, 11–24 (2011). [PubMed: 20864249]
55. Gregory PA et al. The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1. *Nat. Cell Biol* 10, 593–601 (2008). [PubMed: 18376396]
56. Korpala M, Lee ES, Hu G & Kang Y The miR-200 family inhibits epithelial-mesenchymal transition and cancer cell migration by direct targeting of E-cadherin transcriptional repressors ZEB1 and ZEB2. *J. Biol. Chem* 283, 14910–14914 (2008). [PubMed: 18411277]
57. Samavarchi-Tehrani P et al. Functional genomics reveals a BMP-driven mesenchymal-to-epithelial transition in the initiation of somatic cell reprogramming. *Cell Stem Cell* 7, 64–77 (2010). [PubMed: 20621051]
58. Bellovin DI et al. Reciprocal regulation of RhoA and RhoC characterizes the EMT and identifies RhoC as a prognostic marker of colon carcinoma. *Oncogene* 25, 6959–6967 (2006). [PubMed: 16715134]
59. Xu J, Lamouille S & Derynck R TGF- β -induced epithelial to mesenchymal transition. *Cell Res* 19, 156–172 (2009). [PubMed: 19153598]
60. Laurent L et al. Dynamic changes in the human methylome during differentiation. *Genome Res.* 20, 320–331 (2010). [PubMed: 20133333]
61. Lister R et al. Hotspots of aberrant epigenomic reprogramming in human induced pluripotent stem cells. *Nature* 471, 68–73 (2011). [PubMed: 21289626]
62. Ohi Y et al. Incomplete DNA methylation underlies a transcriptional memory of somatic cells in human IPS cells. *Nat. Cell Biol* 13, 541–549 (2011). [PubMed: 21499256]
63. Nazor KL et al. Recurrent variations in DNA methylation in human pluripotent stem cells and their differentiated derivatives. *Cell Stem Cell* 10, 620–634 (2012) [PubMed: 22560082]
64. Sinkkonen L et al. MicroRNAs control *de novo* DNA methylation through regulation of transcriptional repressors in mouse embryonic stem cells. *Nat. Struct. Mol. Biol* 15, 259–267 (2008). [PubMed: 18311153]
65. Benetti R et al. A mammalian microRNA cluster controls DNA methylation and telomere recombination via Rbl2-dependent regulation of DNA methyltransferases. *Nature structural & molecular biology* 15, 268–279 (2008).
66. Fabbri M et al. MicroRNA-29 family reverts aberrant methylation in lung cancer by targeting DNA methyltransferases 3A and 3B. *Proc. Natl Acad. Sci. USA* 104, 15805–15810 (2007). [PubMed: 17890317]
67. Zheng GX et al. A latent pro-survival function for the mir-290–295 cluster in mouse embryonic stem cells. *PLoS Genet.* 7, e1002054 (2011). [PubMed: 21573140]
68. Hermeking H The miR-34 family in cancer and apoptosis. *Cell Death Differ.* 17, 193–199 (2010). [PubMed: 19461653]
69. Hong H et al. Suppression of induced pluripotent stem cell generation by the p 53–p21 pathway. *Nature* 460, 1132–1135 (2009). [PubMed: 19668191]
70. Kawamura T et al. Linking the p53 tumour suppressor pathway to somatic cell reprogramming. *Nature* 460, 1140–1144 (2009). [PubMed: 19668186]
71. Zhao Y et al. Two supporting factors greatly improve the efficiency of human iPSC generation. *Cell Stem Cell* 3, 475–479 (2008). [PubMed: 18983962]
72. Gibcus JH et al. MiR-17/106b seed family regulates p21 in Hodgkin’s lymphoma. *J. Pathol* 225, 609–617 (2011). [PubMed: 21953646]

73. Ho J et al. The pro-apoptotic protein Bim is a microRNA target in kidney progenitors. *J. Am. Soc. Nephrol* 22, 1053–1063 (2011). [PubMed: 21546576]
74. Matsubara H et al. Apoptosis induction by antisense oligonucleotides against miR-17-5p and miR-20a in lung cancers overexpressing miR-17-92. *Oncogene* 26, 6099–6105 (2007). [PubMed: 17384677]
75. Petrocca F, Vecchione A & Croce CM Emerging role of *miR-106b-25/miR-17-92* clusters in the control of transforming growth factor β signaling. *Cancer Res.* 68, 8191–8194 (2008). [PubMed: 18922889]
76. Ebert MS, Neilson JR & Sharp PA MicroRNA sponges: competitive inhibitors of small RNAs in mammalian cells. *Nat. Methods* 4, 721–726 (2007). [PubMed: 17694064]
77. Baek D et al. The impact of microRNAs on protein output. *Nature* 455, 64–71 (2008). [PubMed: 18668037]
78. Licatalosi DD et al. HITS-C LIP yields genome-wide insights into brain alternative RNA processing. *Nature* 456, 464–469 (2008). [PubMed: 18978773]
79. Hafner M et al. Transcriptome-wide identification of RNA-binding protein and microRNA target sites by PAR-CLIP. *Cell* 141, 129–141 (2010). [PubMed: 20371350]
80. Orom UA & Lund AH Isolation of microRNA targets using biotinylated synthetic microRNAs. *Methods* 43, 162–165 (2007). [PubMed: 17889804]
81. Nonne N, Am eyar-Zazoua M, Souidi M & Harel-Bellan A Tandem affinity purification of miRNA target mRNAs (TAP-Tar). *Nucleic Acids Res.* 38, e20 (2010). [PubMed: 19955234]
82. Lewis BP, Burge CB & Bartel DP Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell* 120, 15–20 (2005). [PubMed: 15652477]
83. Miranda KC et al. A pattern-based method for the identification of microRNA binding sites and their corresponding heteroduplexes. *Cell* 126, 1203–1217 (2006) [PubMed: 16990141]
84. John B et al. Human microRNA targets. *PLoS Biol.* 2, e363 (2004). [PubMed: 15502875]
85. Kiriakidou M et al. A combined computational-experimental approach predicts human microRNA targets. *Genes Dev* 18, 1165–1178 (2004). [PubMed: 15131085]
86. Rehmsmeier M, Steffen P, Hochsmann M & Giegerich R Fast and effective prediction of microRNA/target duplexes. *RNA* 10, 1507–1517 (2004). [PubMed: 15383676]
87. Krek A et al. Combinatorial microRNA target predictions. *Nat. Genet* 37, 495–500 (2005). [PubMed: 15806104]
88. Huang JC et al. Using expression profiling data to identify human microRNA targets. *Nat. Methods* 4, 1045–1049 (2007). [PubMed: 18026111]
89. Wang X & El Naqa IM Prediction of both conserved and nonconserved microRNA targets in animals. *Bioinformatics* 24, 325–332 (2008). [PubMed: 18048393]
90. Shirdel EA, Xie W, Mak TW & Jurisica I NAViGaTing the microneome—using multiple microRNA prediction databases to identify signalling pathway-associated microRNAs. *PLoS One* 6, e17429 (2011). [PubMed: 21364759]
91. Lee H et al. BioVLAB-M MIA: a cloud environment for microRNA and mRNA integrated analysis (MMIA) on Amazon EC2. *IEEE Trans. Nanobioscience* 11, 266–272 (2012). [PubMed: 22987133]
92. Nam S et al. MicroRNA and mRNA integrated analysis (MMIA): a web tool for examining biological functions of microRNA expression. *Nucleic Acids Res.* 37, W356–W362 (2009). [PubMed: 19420067]

Box 1 Methods for identifying miRNA/mRNA target interactions

Because the primary function of miRNAs is thought to be the repression of target mRNAs, the biological effects of a given miRNA depend on the functions of its targets. Empirical methods used to detect the physical and functional interactions between miRNAs and mRNAs are labour intensive and must be interpreted with care, as the results can be influenced by the cellular context and the specific characteristics the experimental methods. Consequently, bioinformatic tools are often used to predict potential miRNA and mRNA targeting interactions. In metazoans, miRNA targeting of mRNAs is mediated by partial complementarity, with the degree of complementarity correlating only imperfectly with the strength of the functional interaction, making such predictions challenging. Some algorithms predict whether a given miRNA has the potential to target a given mRNA according to specific criteria, while others use paired mRNA and miRNA expression data to train machine-learning algorithms, and still others incorporate both outputs from target prediction methods and experiment-specific paired mRNA/miRNA expression data to enrich for the functional targeting active in a particular experiment.

Empirical methods.

The results can be influenced by the cellular context, including the levels of endogenous miRNAs and their mRNA targets. Many studies have been performed using cells in which miRNA biogenesis is deficient to eliminate endogenous miRNAs^{19–52}.

Overexpression/knockdown of individual miRNAs.

The levels of selected miRNAs can be manipulated in a stable or transient manner. The levels of target mRNAs and/or the encoded proteins can be measured (for example, see refs 10,77). It can be difficult to ensure that observed effects are direct miRNA/mRNA targeting, rather than secondary effects.

Manipulation	Method	Notes
Stable overexpression	Lentiviral vectors expressing pri-miRNA sequences.	Requires intact miRNA biogenesis for processing of the exogenous pri-miRNAs.
Stable knockdown	Lentiviral vectors expressing 'sponge' sequences ⁷⁶ (tandem repeats of target sequences) to sequester endogenous miRNAs.	miRNA sponges generally result in knockdown of miRNAs with similar seed sequences.
Transient overexpression	Transfection of synthetic miRNA mimics.	
Transient knockdown	Transfection of synthetic miRNA inhibitors to knockdown endogenous miRNAs.	Synthetic miRNA inhibitors are more specific than miRNA sponges, but can have off-target effects.

Luciferase reporter assays.

These assays typically involve co-transfection of a selected miRNA mimic and a reporter plasmid containing a candidate 3' UTR target sequence inserted downstream of the firefly luciferase gene. This technique allows for mutagenesis of the target sequence to

assess the specificity of the miRNA/mRNA interaction, and to demonstrate that the targeting is due to direct miRNA/mRNA interactions.

Detection of physical associations between miRNAs and mRNAs.

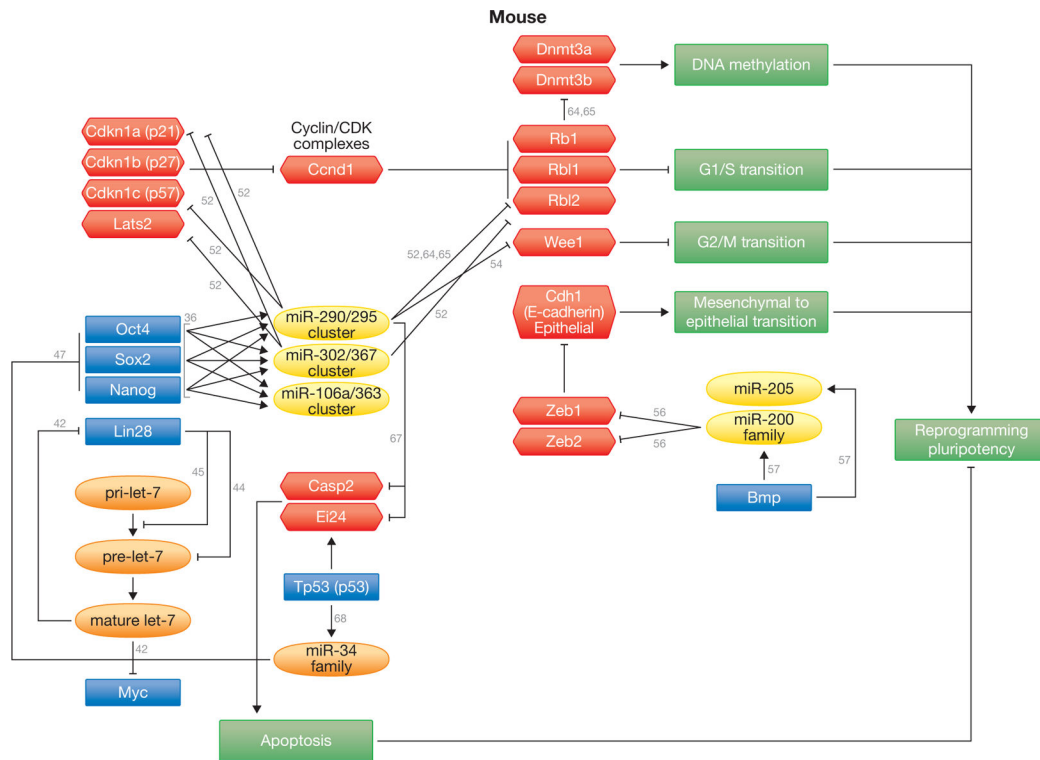
These methods are based on the assumption that the mRNAs found in complexes containing RISC-associated proteins are potential regulatory targets.

Method	Notes	Reference
HITS-CLIP	Involves pull-down of ultraviolet-crosslinked ribonucleoprotein complexes by anti-EIF2C2 antibodies, followed by deep sequencing of both the associated miRNAs and mRNAs. Because direct miRNA/mRNA interactions are not identified, data from this technique can be analysed in conjunction with miRNA/mRNA target predictions and mRNA expression data from miRNA knockdown or overexpression experiments to infer which mRNAs are the targets of the miRNA of interest.	78
PAR-CLIP	Involves the incorporation of a ribonucleoside conjugated to a photoactivatable moiety, which improves the efficiency of crosslinking. After this unique step, PAR-CLIP follows the same process as HITS-CLIP.	79
Biotinylated miRNA pull-down	A specific biotinylated miRNA mimic is transfected into cells. Ribonucleoprotein complexes containing these biotinylated miRNAs are then pulled-down using streptavidin beads, and the associated mRNAs are analysed by deep sequencing.	80
Tandem affinity purification of miRNA target mRNAs (TAP-Tar)	To decrease the high number of false-positive miRNA-to-mRNA interactions that can be seen with other methods, a specific biotinylated miRNA mimic is transfected, and then the ribonucleoprotein complexes are selected first by immunoprecipitation using an anti-AGO antibody, and second by pull-down using streptavidin beads.	81

Bioinformatic methods.

Bioinformatic methods can be divided into predictive algorithms, machine-learning algorithms that use empirical data for training, and integrative methods.

Algorithms	Notes	References
TargetScan	Prediction based on complementarity between the miRNA seed and mRNA sequences, and conservation.	82
RNA22	Pattern-based algorithm to predict miRNA/mRNA pairing.	83
miRanda	Prediction based on complementarity between the miRNA and mRNA sequences.	84
DIANA MicroT, RNAHybrid, PicTar	Thermodynamic prediction.	85–87
GenMiR++	Training with paired miRNA/mRNA data using Bayesian inference.	88
MiRTarget2	Training with paired miRNA/mRNA data using a support vector machine.	89
mirDIP/NAViGaTOR	The integration of predictions from multiple published algorithms and the building of interaction networks.	90
MMIA	Combination of predicted interactions and paired miRNA/mRNA data	91,92

**Figure 1.**

Summary of the published interactions between pluripotency-associated miRNAs and their target mRNAs from experiments in the murine system. The diagram illustrates the regulatory interactions between pluripotency-associated miRNAs and their targets in the cell cycle, as well as those influencing the mesenchymal to epithelial transition, DNA methylation and apoptosis pathways. These, in turn, influence the reprogramming of somatic cells to pluripotency. The interactions shown are taken from studies on murine cells. miRNAs that are upregulated in pluripotency are indicated in yellow, miRNAs that are downregulated in pluripotency are in orange, proteins that regulate miRNAs are in blue, and transcripts that are regulated by miRNAs are in red. Downstream functional effects are in green. References supporting regulatory interactions are indicated next to the lines.

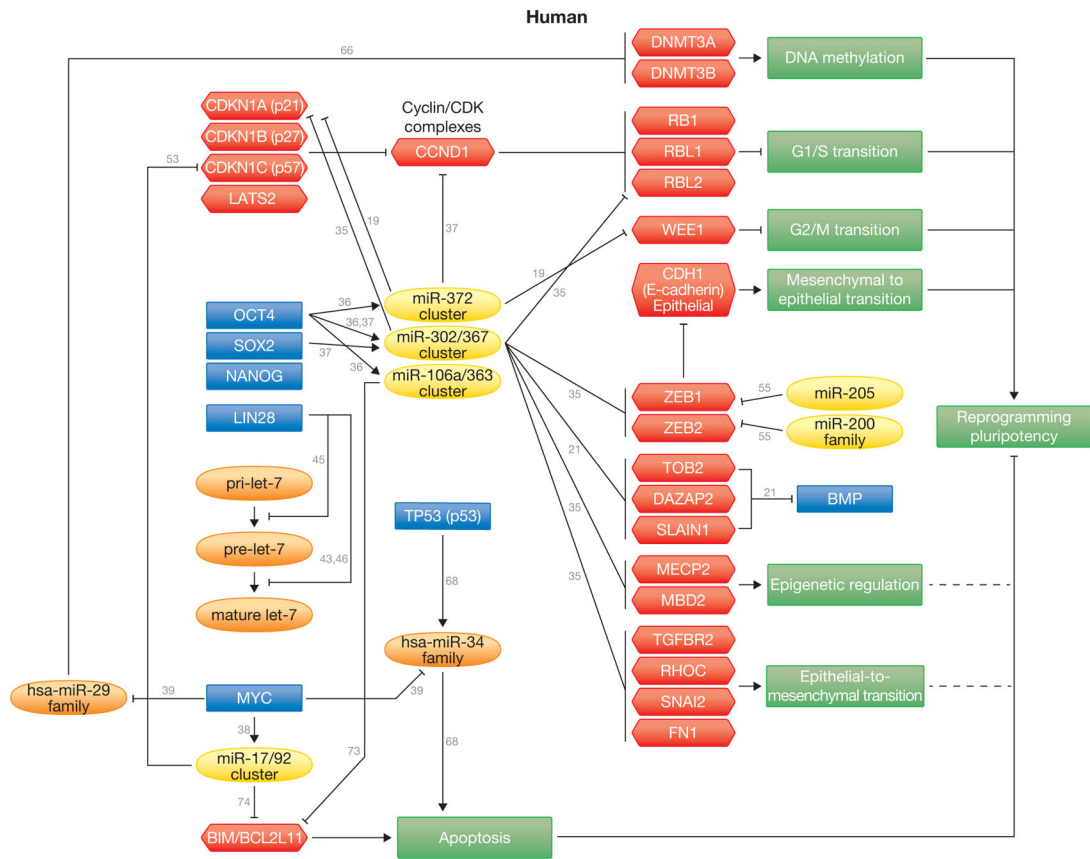


Figure 2. Summary of the published interactions between pluripotency-associated miRNAs and their target mRNAs from experiments in the human system. The diagram illustrates the regulatory interactions between pluripotency-associated miRNAs and their targets in the cell cycle, MET, DNA methylation and apoptosis pathways, which, in turn, influence the reprogramming of somatic cells to pluripotency. Colour coding is as in Fig. 1. References supporting regulatory interactions are indicated next to the lines.