Small RNAs Loom Large During Reprogramming

Rupa Sridharan[1,2,3,4,5] and Kathrin Plath[1,2,3,4,5,*]
[1]David Geffen School of Medicine
[2]Department of Biological Chemistry
[3]Molecular Biology Institute
[4]Jonsson Comprehensive Cancer Center
[5]Eli and Edythe Broad Center of Regenerative Medicine and Stem Cell Research
University of California, Los Angeles, Los Angeles, CA 90095, USA
*Correspondence: kplath@mednet.ucla.edu
DOI 10.1016/j.stem.2011.05.009

In two independent *Cell Stem Cell* reports, the Morrisey and Mori groups show that human and mouse somatic cells can be reprogrammed to produce induced pluripotent stem cells by expressing microRNAs, completely eliminating the need for ectopic protein expression (Anokye-Danso et al., 2011; Miyoshi et al., 2011).

Reprogramming of somatic cells into induced pluripotent stem cells (iPSCs) can be achieved by a small set of protein factors, generally transcription factors, whose expression is either very specific to or much higher in embryonic stem cells (ESCs) than in differentiated cells. Several groups have used microRNAs (miRNAs) to promote the transcription factor-mediated reprogramming process, and now two independent groups have derived human and mouse iPSCs by adding miRNAs, in the absence of any additional protein factors (Anokye-Danso et al., 2011; Miyoshi et al., 2011) (Table 1).

miRNAs are small noncoding RNAs that can modulate messenger RNA (mRNA) expression by base pairing between a few nucleotides in the miRNA (the seed sequence) and complementary sequences within the open reading frame or the 3′ untranslated region of the target mRNA, leading to destabilization of the mRNA or inhibition of protein synthesis. They are synthesized from longer precursors into mature ~22-mers by the action of the microprocessor and Dicer enzymatic machineries. Multiple miRNAs are often found in clusters in the genome and expressed in a cell type-specific manner, similar to transcription factors. Importantly, each miRNA can target and suppress hundreds of messenger RNAs (mRNAs); hence expression of a single miRNA could dramatically change the expression profile and identity of a cell. Since reprogramming to pluripotency requires precisely such a dramatic change in transcriptional profile, Morrisey and colleagues followed up on this idea and demonstrated in a recent issue of *Cell Stem Cell* that mouse and human iPSCs can be generated with high efficiency from fibroblasts, by expressing five miRNAs that are normally highly expressed in ESCs (Anokye-Danso et al., 2011). The study in this issue by Mori and colleagues further advances the notion that overexpression of miRNAs is sufficient for reprogramming by deriving bonafide mouse and human iPSCs by simply transfecting a small set of mature miRNA (Miyoshi et al., 2011; Table 1).

As part of an effort to develop reprogramming methods that either allow reprogramming factor expression without the need for genomic integration of foreign DNA or that replace their function with other molecules, the Morrisey study convincingly demonstrates that lentiviral expression of the mir-302–mir-367 cluster in fibroblasts induces iPSCs (Anokye-Danso et al., 2011). While it is astonishing that a few ESC-specific miRNAs are sufficient for iPSC generation, it is also remarkable that iPSCs are obtained with very high frequency. Even though difficult to directly compare, miRNA-based reprogramming appears to be two orders of magnitude more efficient than transcription factor-mediated reprogramming, when using similar viral titers. Fully reprogrammed colonies appeared within 6–8 days with mouse embryonic fibroblasts and 15 days with human fibroblasts, indicating that the process is also relatively fast. miRNA derived-iPSCs (mi-iPSCs) are similar to ESCs in standard tests for pluripotency, including contribution to adult chimeras for mouse mi-iPSCs and differentiation into the three germ layers in the context of teratomas for human mi-iPSCS. Efficient silencing of the miRNA-expressing lentiviral cassette in the reprogrammed cells confirms a stable change in cell fate.

The relatively high efficiency of miRNA-based reprogramming should make it possible to generate mi-iPSCs by simply transfecting mature, double-stranded miRNAs. A similar idea has allowed the generation of transcription factor-derived iPSCs by transfection of mRNAs coding for the reprogramming transcription factors, but requires a technically challenging protocol, which may make clinical applications difficult (Warren et al., 2010). Remarkably, the Mori group has now succeeded in reprogramming human and mouse adipose stromal cells, which are multipotent, by repeatedly transfecting a cocktail of seven miRNAs belonging to the mir-302, mir-200, and mir-369 families (Miyoshi et al., 2011). However, compared to the viral delivery of miRNA precursors (Anokye-Danso et al., 2011), the efficiency is considerably lower and decreases further when fibroblasts are targeted as the starting cell type. Given that the Mori group transfected mature miRNAs only four times within the first 8 days of reprogramming, the efficiency of this strategy might improve with repeated transfection over the course of reprogramming. Furthermore, it is possible that the need for each of the miRNA families may differ during the course of reprogramming and that sequential delivery paradigms, dictated by when a particular miRNA is most effective, could be developed. Since, different miRNAs are used in both studies, one could test various combinations of these in reprogramming.

Table 1. Summary of iPSC Reprogramming Experiments with miRNAs

Starting Cell	Transcription Factors Used	miRNAs Employed	miRNA Delivery	Remarks	miRNA Targets Tested	References
Mouse embryonic fibroblasts	inducible OSMK[a] secondary system	mmu-mir-200b or mmu-mir-200c	single transfection of miRNA mimics	enhancement of reprogramming, promotion of MET		Samavarchi-Tehrani et al., 2010
Mouse embryonic fibroblasts	retroviral OSK or OSMK	mmu-mir-106b or mmu-mir-93 or mmu-mir-106a or mmu-mir-17	repeated transfection of miRNA mimics	enhancement of faithful reprogramming, acceleration of MET	p21, Tgf-βR2	Li et al., 2011
Mouse embryonic fibroblasts	retroviral OSK or OSMK	mmu-mir-106a/18b/20b/19b/92a/363	retroviral expression of entire cluster	enhancement of faithful reprogramming, mir-106a and -20b have strongest effect		Liao et al., 2011
Mouse embryonic fibroblasts	retroviral OSK or OSMK	mmu-mir-302b/302c/302a/302d/367	retroviral expression of entire cluster	strong enhancement of faithful reprogramming, mir-367 not required for this effect, promotion of MET	Tgf-βR2	Liao et al., 2011
Mouse embryonic fibroblasts	retroviral OSK or OSKM	mmu-mir-291-3p or mmu-mir-294 or mmu-mir-295 or mmu-mir-302d	repeated transfection of miRNA mimics	enhancement of faithful reprogramming, but only with OSK		Judson et al., 2009
Mouse embryonic fibroblasts	none	mmu-mir-302b/302c/302a/302d/367	lentiviral expression of entire cluster	faithful reprogramming with high frequency, HDAC2 inhibition required		Anokye-Danso et al., 2011
Mouse adipose stromal cells	none	mmu-mir-200c + mmu-mir-302a,b,c,d + mmu-mir-369-3p,-5p	repeated transfection of miRNA mimics	faithful reprogramming		Miyoshi et al., 2011
Human fibroblasts	retroviral OSK or OSMK	hsa-mir-302b or hsa-mir-372 or mmu-mir-294	repeated transfection of miRNA mimics	enhancement of faithful reprogramming, acceleration of MET	p21, RBL2, MeCP2, TGF-βR2, RHOC and others	Subramanyam et al., 2011
Human skin cancer cells	none	hsa-mir-302a/b/c/d	retroviral expression of polycistronic cassette	some evidence of faithful reprogramming, but incomplete characterization		Lin et al., 2008
Human hair follicle cells	none	hsa-mir-302a/b/c/d	electroporation of polycistronic cassette	some evidence of faithful reprogramming, but incomplete characterization		Lin et al., 2011
Human fibroblasts	none	mmu-mir-302b/302c/302a/302d/367	lentivral expression of entire cluster	faithful reprogramming with high frequency		Anokye-Danso et al., 2011
Human adipose stromal cells (and dermal fibroblasts)	none	hsa-mir-200c + hsa-mir-302a,b,c,d + hsa-mir-369-3p,-5p	repeated transfection of miRNA mimics	faithful reprogramming		Miyoshi et al., 2011

[a] O, Oct4; S, Sox2; M, cMyc; K, Klf4.

It remains to be seen whether these miRNAs guide the transition to pluripotency through the same steps as Oct4, Sox2, Klf4, and cMyc. Interestingly, pluripotency gene activation coincides with the appearance of mi-iPSC colonies (Anokye-Danso et al., 2011), suggesting that, as in transcription factor-mediated reprogramming, the activation of the core pluripotency network is the last step, perhaps reflecting the generality of the process. Given that miRNAs can act through hundreds of targets, and that both the Morrissey and Mori protocols utilize miRNA families with different seed sequences and thereby different mRNA targets, it may be challenging to unravel the detailed mechanisms by which miRNAs induce pluripotency. In support of this notion, the Morrisey study demonstrates that, when expressed without mir-367, the mir-302 family can induce the expression of the pluripotency markers *Nanog*, *Sox2*, and *Zfp42*, but not of *Oct4* (Anokye-Danso et al., 2011). It remains unclear whether mir-367 expression on its own can activate the *Oct4* locus. Interestingly, the mir-302s have previously been suggested to be sufficient for reprogramming to the iPSC state, although iPSC isolation had not been well described and efficiency was not reported (Lin et al., 2008, 2011). These earlier studies required ectopic miRNA expression above the ESC level, suggesting that the specific response to miRNAs could also depend on their cellular concentration. Intriguingly, a similar notion may be true for transcription factor-induced reprogramming, where Oct4 levels appear to affect the reprogramming outcome.

Clues to the function of miRNAs in reprogramming also come from studies which have used some of the above miRNAs to enhance reprogramming induced by Oct4, Sox2, cMyc, and Klf4 (Table 1). A recurring target of these reprogramming-enhancing miRNAs is the TGF-β signaling pathway (Li et al., 2011; Liao et al., 2011; Samavarchi-Tehrani et al., 2010; Subramanyam et al., 2011). Inhibition of this pathway accelerates the mesenchymal-to-epithelial transition that occurs when fibroblasts reprogram. Other targets include chromatin regulators, such as MeCP2 and the histone demethylases Aof2 and Aof1, although the latter have been confirmed only by luciferase reporter assays (Lin et al., 2011; Subramanyam et al., 2011). Furthermore, reprogramming of mouse fibroblasts with the mir-302–367 cluster is completely dependent on the presence of the histone deacetylase (HDAC) inhibitor valproic acid (VPA) (Anokye-Danso et al., 2011). HDAC2 is a known target of VPA, and the authors demonstrate that fibroblasts lacking HDAC2 can be reprogrammed by mir-302–367 with high efficiency, independently of VPA. It may be that reprogramming of human fibroblasts does not depend on VPA because these cells already exhibit low levels of HDAC2. Generally, it appears that reprogramming-enhancing miRNAs counteract pathways that limit reprogramming, as the cell-cycle inhibitor p21, which functions as a barrier to transcription factor-induced reprogramming, is also suppressed by some of these miRNAs (Li et al., 2011; Subramanyam et al., 2011) (Table 1). Together, these observations suggest that transitioning to an ESC-like cell cycle, while inhibiting TGF-β signaling and allowing chromatin changes, are all key to reprogramming.

However, suppression of each putative target only partially recapitulates the improvement in reprogramming due to miRNAs. Thus, this picture is likely incomplete, and many targets of these miRNAs in reprogramming remain unknown. In addition, the mRNA targets may vary during the reprogramming process, with changing cell identity. It is also surprising that miRNAs, which suppress mRNAs, can mediate reprogramming, given that transcription factor-mediated reprogramming, in contrast, functions to activate many genes, particularly those involved in pluripotency. Interestingly, mir-369-3, used in the Mori study, is one of the very few miRNAs reported to activate protein translation upon cell-cycle arrest (Vasudevan et al., 2007).

The application of reprogramming methods to regenerative therapy will require transient and nonintegrative means of delivering the effectors, such as the synthetic, mature miRNAs described here. However, given the ongoing debate regarding the extent of molecular and functional similarities between transcription factor-derived iPSCs and ESCs and the potential for genomic instability during the reprogramming process, mi-iPSCs will require close scrutiny prior to therapeutic use.

Finally, several groups have directly converted one adult cell type to another, without traversing the pluripotent state. The Morrisey and Mori studies suggest that it should also be possible to enhance or mediate these lineage conversion events with miRNAs. Screening cell type-specific miRNAs for transdifferentiation activities, rather than focusing on transcription factors, may be a useful endeavor.

REFERENCES

Anokye-Danso, F., Trivedi, C.M., Juhr, D., Gupta, M., Cui, Z., Tian, Y., Zhang, Y., Yang, W., Gruber, P.J., Epstein, J.A., and Morrisey, E.E. (2011). Cell Stem Cell *8*, 376–388.

Judson, R.L., Babiarz, J.E., Venere, M., and Blelloch, R. (2009). Nat. Biotechnol. *27*, 459–461.

Li, Z., Yang, C.S., Nakashima, K., and Rana, T.M. (2011). EMBO J. *30*, 823–834.

Liao, B., Bao, X., Liu, L., Feng, S., Zovoilis, A., Liu, W., Xue, Y., Cai, J., Guo, X., Qin, B., et al. (2011). J. Biol. Chem. *286*, 17359–17364.

Lin, S.L., Chang, D.C., Chang-Lin, S., Lin, C.H., Wu, D.T., Chen, D.T., and Ying, S.Y. (2008). RNA *14*, 2115–2124.

Lin, S.L., Chang, D.C., Lin, C.H., Ying, S.Y., Leu, D., and Wu, D.T. (2011). Nucleic Acids Res. *39*, 1054–1065.

Miyoshi, N., Ishii, H., Nagano, H., Haraguchi, N., Dewi, D.L., Kano, Y., Nishikawa, S., Tanemura, M., Mimori, K., Tanaka, F., et al. (2011). Cell Stem Cell *8*, this issue, 633–638.

Samavarchi-Tehrani, P., Golipour, A., David, L., Sung, H.K., Beyer, T.A., Datti, A., Woltjen, K., Nagy, A., and Wrana, J.L. (2010). Cell Stem Cell *7*, 64–77.

Subramanyam, D., Lamouille, S., Judson, R.L., Liu, J.Y., Bucay, N., Derynck, R., and Blelloch, R. (2011). Nat. Biotechnol. *29*, 443–448.

Vasudevan, S., Tong, Y., and Steitz, J.A. (2007). Science *318*, 1931–1934.

Warren, L., Manos, P.D., Ahfeldt, T., Loh, Y.H., Li, H., Lau, F., Ebina, W., Mandal, P.K., Smith, Z.D., Meissner, A., et al. (2010). Cell Stem Cell *7*, 618–630.