Published in final edited form as:

Methods Mol Biol. 2013 ; 936: 247–256. doi:10.1007/978-1-62703-083-0_19.

MicroRNA Expression Profiling of Human Induced Pluripotent and Embryonic Stem Cells

Amit Sharma² and Joseph C. Wu^{1,2,3}

¹Department of Medicine, Division of Cardiology, Stanford University School of Medicine, Stanford, CA 94305, USA

²Department of Radiology, Stanford University School of Medicine, Stanford, CA 94305, USA

³Institute of Stem Cell Biology & Regenerative Medicine, Stanford University School of Medicine, Stanford, CA 94305, USA

Summary

Clinical implications of induced pluripotent stem (iPS) cell technology are enormous for personalized medicine. However, extensive use of viral approach for ectopic expression of reprogramming factors is a major hurdle in realization of its true potential. Non-viral methods for making iPS cells, although plausible, are impractical because of high cost. MicroRNAs are important cellular modulators that have been shown to rival transcription factors and are important players in embryonic development. We have generated distinct ‘microRNA-omes’ signature of iPS cells that remain in a near embryonic stem (ES) cell state and distinct from differentiated cells. Recent advances in the microRNA field and experimentally validated microRNAs warrant a review in experimental protocols for microRNA expression profile.

Keywords

ES cells; iPS cells; microRNA; microarray

1. Introduction

One of the most clinically relevant finding of the past decade is the ability to reprogram a terminally differentiated cell to an ES cell-like state. Yamanaka et al utilized ectopic expression of transcription factors Oct4, Sox2, Klf4 and cMyc in terminally differentiated somatic cells to achieve pluripotency [1]. Later, Thompson and colleagues published NANOG and LIN28 along with OCT4 and SOX2 to make iPS cell lines [2]. Despite rapid advancements in the field, two major hurdles in clinical application of iPS cells remaining are the inability to avoid stable genomic integration of viral vectors used to overexpress ‘pluripotency factors’ and the residual epigenetic signature from the cells used to derive iPS cells [3]. Efforts have been made to address these issues with limited success, and all of these approaches have their shortcomings. For instance, one promising approach is to use full-length mRNAs for ‘pluripotency factors’ [4]. However, this approach is still too expensive at present. Another approach is to use piggyback vectors in reprogramming, but this also has its limitations in being cumbersome [5]. Efforts are therefore underway to devise a new techniques to generate iPS cells without confounding factors to realize their true clinical

potential. However, more effective strategies of cellular reprogramming will require a better understanding of underlying changes that take place during the process of reprogramming [6].

Along with transcription factors, microRNAs have emerged as a novel class of regulators of gene expression [7]. These small RNAs are increasingly being recognized as important regulators of various biological pathways that control gene expression by either mediating target mRNA degradation or stalling protein synthesis [8, 9]. Interestingly, the involvement of various microRNA families have long been known in various developmental processes [10]. Therefore it is not surprising that microRNAs play a key role in the generation and maintenance of iPS cell-like state. We have previously shown a distinct microRNA expression profile in ES cells compared to terminally differentiated somatic cells and the similarity of 'microRNA-omes' of iPS cells to ES cells [11]. We have also assessed dynamic microRNA expression programs during cardiac differentiation of human ES cells [12]. Others have analyzed common changes in the expression of microRNAs (miRNAs) and mRNAs in different human ES cell lines during early commitment and examined the expression of key ES cell-enriched miRNAs in earlier developmental states [12]. Several microRNAs clusters such as miR-17-92, miR-106a-363, miR-302-367, and miR-200 showed a similar expression signature in ES and iPS cell lines. Recent advances in techniques for microRNA expression profiling and the ever growing list of validated microRNAs make it would be worthwhile to establish the protocols for genome-wide 'microRNA-omes' expression profiling, which will help us understand molecular events that culminate in cellular dedifferentiation.

2. Materials

2.1 Cell culture reagents

1. Dulbecco's modified eagle medium contains 4.5 g/l glucose (DMEM, Gibico)
2. Phosphate-buffered saline (PBS) without calcium and magnesium
3. Penicillin/streptomycin (Invitrogen)
4. Triple E solution (Invitrogen)
5. DMEM/F-12 Medium (Invitrogen)
6. MteSR1 media and 5x supplement (STEMCELL Technologies, Canada)
7. BD Matrigel Hesc-qualified (BD biosciences, USA)
8. Accutase solution (Sigma-Aldrich, USA)
9. miRNeasy Mini Kit (Qiagen Inc., Valencia, CA)

2.2 Fibroblast medium composition

DMEM containing 10% FBS, and 50 units and 50 mg/ml penicillin and streptomycin. To prepare 450 ml of DMEM medium, mix 50 ml of FBS and 2.5 ml of penicillin/streptomycin, and then fill up to 500 ml with DMEM. Store at 4°C up to a week.

2.2. MteSR1 media and 5x supplement

To prepare thaw 5x supplement at 4°C overnight and mix with MteSR1 media. Store at 4°C.

2.3 miRNA isolation and hybridization

- 3 miRNeasy Mini Kit (Qiagen, USA)
- 4 Microcon centrifugal filter (Millipore, Billerica, MA)

- 5 μ ParaFlo microfluidics chip (Atactic Technologies, Houston, TX)
- 6 6 x SSPE buffer (0.90 M NaCl, 60 mM Na₂HPO₄, 6 mM EDTA, pH 6.8)
- 7 Slide scanner (GenePix 4000B, Molecular Devices, Sunnyvale, CA)
- 8 Array-Pro image analysis software (Media Cybernetics, Bethesda, MD)

3.0 Methods

3.1. RNA preparation

The miRNeasy Mini Kit combines phenol/guanidine-based lysis of samples and silica membrane-based purification of total RNA. The upper, aqueous phase is extracted, and ethanol is added to provide appropriate binding conditions for all RNA molecules from 18 nucleotides (nt) upwards. A specialized protocol is provided for enrichment of miRNAs and other small RNAs (less than ~200 nt) in a separate fraction. The manufacturer's instructions should be followed to efficiently isolate the small RNA from fibroblasts, ES cells, and iPS cells.

4. MicroRNA microarray construction, hybridization, and scanning

Microarray assay can be performed by outsourcing to service provider, or by using following protocol.

1. Enrich small RNA fraction from 4–8 μ g total RNA sample, by size-fractionation using a YM-100 Microcon centrifugal filter (Millipore, Billerica, MA),
2. Extend the 3'-end of the small RNAs (<300 nt) isolated with a poly(A) tail using poly(A) polymerase.
3. Ligate an oligonucleotide tag to the poly(A) tail for later fluorescent dye staining.
4. Perform hybridization overnight on a μ ParaFlo microfluidic chip using a microcirculation pump (Atactic Technologies, Houston, TX). On the microfluidic chip, each detection probe that consisted of a chemically modified nucleotide-coding segment complementary that targets microRNA (from miRBase, (<http://microrna.sanger.ac.uk/sequences/>) or other control RNA, and a spacer segment of polyethylene glycol is used to extend the coding segment away from the substrate. The detection probes were made by *in situ* synthesis using photo-generated reagent chemistry.
5. Hybridization buffer is composed of 6 x SSPE buffer (0.90 M NaCl, 60 mM Na₂HPO₄, 6 mM EDTA, pH 6.8) containing 25% formamide at 34°C.
6. After RNA hybridization, circulate tag-conjugating Cy3 or Cy5 dyes through the microfluidic chip for dye staining.
7. Fluorescence images can then be collected using a laser scanner (GenePix 4000B, Molecular Devices, Sunnyvale, CA) and can be digitized using Array-Pro image analysis software (Media Cybernetics, Bethesda, MD).

5. MicroRNA microarray normalization and data analysis

In order to determine functionally relevant differences in microRNA expression, normalization needs to be performed to reduce system-related variations, including variations such as difference in sample amount, efficiency of labeling dyes, and signal gain that is subject to the scanner used to acquire the image.

1. The first step in data analysis is to subtract the background noise and then to normalize the signals using a LOWESS filter (locally weighted regression).
2. Background can be determined using a regression-based background mapping method.
3. The regression can be performed on 5% to 25% of the lowest intensity data points, excluding blank spots. The background matrix can then subtracted from the raw data matrix.
4. The data filtering removes miRNAs with normalized intensity values below a threshold value of 32 or a predetermined baseline value across all samples.
5. Gene centering and normalization may be used to transform the Log2 values using the mean and the standard deviation of individual genes across all samples. The t values are calculated for each miRNA between groups, and P values are computed from the theoretical t -distribution. miRNAs with P values below a critical P -value (typically 0.01) are selected for cluster analysis.
6. The clustering can be done using various methods. However, hierarchical clustering is the favorable method, with average linkage and Euclidean distance metrics.
7. We used TIGR MeV (Multiple Experimental Viewer) software from The Institute for Genomic Research to generate the clustering plot.
8. We performed principal component analysis can be performed using the R statistical package (cran.r-project.org).

Notes

RNA preparation

1. Determine the integrity of the input RNA for labeling and hybridization prior to use to increase the likelihood of a successful experiment
2. To prevent contamination of reagents by nucleases, always wear powder-free laboratory gloves, and use dedicated solutions and pipettors with nuclease-free aerosol-resistant tips.
3. Maintain a clean work area.
4. When preparing frozen reagent stock solutions for use:
 - a. Thaw the aliquot as rapidly as possible without heating above room temperature.
 - b. Mix briefly on a vortex mixer, then centrifuge for 5 to 10 seconds to drive the contents off of walls and lid.
 - c. Store on ice or in a cold block until use.
5. Total RNA extraction methods differ in numerous ways and may impact the yield. Some kits that are recommended for use are:
 - a. Qiagen miRNeasy Mini Kit - catalog number 217004
 - b. Applied Biosystem mirVana RNA Isolation Kit - catalog number AM1560
 - c. Invitrogen TRIzol Reagent (100 mL) - catalog number 15596-026.

We recommend these or any other commercial kits for the extraction of the total RNA including small RNA fraction. Do not use the size fractionation or small RNA enrichment

protocol. You must use the same total RNA extraction methods to obtain consistent results for comparative experiments. Different total RNA extraction methods may result in slightly different miRNA profiles. Extraction methods that use organic solvents such as TRIZOL may result in inaccurate quantification because organic solvent contamination from carry-over during the RNA extraction may compress the 260/230 ratio. The affected 260 measurements may result in inaccurate quantification of the total RNA.

Preparation of samples for hybridization

1. Do not leave samples in the ice water bath for more than 15 minutes. Longer incubations may adversely affect the hybridization results.
2. Do not allow the pipette tip or the hybridization solution to touch the gasket walls. Allowing liquid to touch the gasket wall greatly increases the likelihood of gasket leakage.
3. When you assemble the array slide to the gasket slide, keep the array slide parallel to the gasket slide at all times, and do not drop the array slide onto the gasket slide. Doing so increases the chances of samples mixing between gasket wells.
4. Be sure that the arrays are hybridized for at least 20 hours. Hybridization can occur for longer than 20 hours but the actual hybridization time should be consistent if the results are to be compared. Failure to maintain consistent hybridization times may adversely affect your data.
5. If you are not loading all the available positions on the hybridization rotator rack, be sure to balance the loaded hybridization chambers on the rack so that there are an equal number of empty positions on each of the four rows on the hybridization rack.
6. During washing following hybridization, care must be taken as some detergents may leave fluorescent residue on the dishes. Do not use any detergent in the washing of the staining dishes. If detergent is used, all traces must be removed by copiously rinsing with Milli-Q water.
7. As the dyes cy3 and cy5 are sensitive to ozone levels, all assays must be performed in environment with ozone levels of 50 ppb or less.
8. The scanning settings used to acquire images must be noted and maintained for all slides.

Acknowledgments

We thank funding support from Burroughs Wellcome Foundation, NIH DP2OD004437, and NIH RC1G036142 (JCW).

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Figure 1.

RT-PCR and quantitative RT-PCR analysis of selected genes. (A) RT-PCR demonstrated robust expression of the embryonic genes (OCT4, NANOG, SOX2, REX1, DNMT3B, GDF3) in human iPS cells and ES cells, but not in fetal fibroblasts. Undifferentiated state of ES and iPS cells was also confirmed by performing RT-PCR for markers of mesoderm (Brachyury, T), endoderm (AFP), and neuroectoderm (NCAM1), which were all negative. Equal loading of RNA for each sample was confirmed by Human 18S. (B) Quantitative RT-PCR analysis of the pluripotency genes OCT4, SOX2, and NANOG confirms similar expression levels of these genes in human iPS cells and ES cells. Error bars represent one standard deviation from the mean (* P value < 0.05 vs. fibroblasts; NS = Not Significant).

FIGURE 2b

FIGURE 2c

Figure 2. Heat maps and signal intensity plots of microRNA expression across fibroblasts, iPS cells, and ES cells. ANOVA analysis demonstrates statistically significant differential microRNA

regulation across the three samples. microRNAs with P values below 0.01 were selected for cluster analysis. **(A)** microRNAs upregulated in both iPS cells and ES cells compared to fibroblasts. Highlighted are the normalized signal intensity plots for the miR-302 cluster and miR-17-92 cluster, including its paralogs miR-106a-92 and miR-106b-25. **(B)** MicroRNAs exhibit opposite expression in iPS cells and ES cells. Note that the normalized signal intensities of the microRNAs are not dramatic, but still statistically significant. In contrast to the group of microRNAs upregulated in ES cells but not in iPS cells (top panel), we observed only one microRNA (miR-886-5p) that was upregulated in iPS cells but not in ES cells (bottom panel). **(C)** microRNAs are upregulated in fibroblasts but down-regulated in iPS cells and ES cells. Highlighted in the top and bottom panels are selected microRNAs known to be important in ES cell biology and/or organismal development, such as the let-7 cluster that has a well-established role in late development timing. The clustering was done using hierarchical methods with average linkage and Euclidean distance metrics. The “threshold” value denotes signal intensities <32, a cutoff used by the microarray service provider, below which quantitation of signal may be inaccurate.

Figure 3.

Schematic representation depicting microRNAs as regulators of cellular pluripotency. Various transcription factors and signaling pathways are known to induce and maintain pluripotent state. However, various microRNAs are shown to regulate these factors. The microRNA miR-145 has been shown to regulate OCT4, SOX2 and Klf4 posttranscriptionally, this microRNA is high in differentiated cells while its expression is reduced in ES and iPSC cells. Also, miR-302-367 cluster is transcriptionally modulated by OCT4 and hence is much higher in undifferentiated cells. Similarly cMYC is known to up-regulate miR-17-92 family. This family also affects TGF- β signaling is known to effect on cellular differentiation. Thus, interplay between microRNA and transcription factors may contribute in maintaining a pluripotent state.