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Comparison of microarray and quantitative real-time PCR methods for measuring microRNA levels in MSC cultures

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Abstract

The capacity for self-renewal and the multi-lineage potential of mesenchymal stromal cells (MSC) offers a therapeutic promise for regenerative medicine. MicroRNAs (miRNAs) are small non-coding RNAs that play a key regulatory role during differentiation both at the level of post-translational modulation and epigenetic control. Studies on MSCs have just begun to identify miRNA profiles in MSC and differentiated MSC. While several methods are available for miRNA exploration, microarrays and quantitative real-time PCR (qPCR) are the most common. Since there are several microarray and qPCR platforms available for miRNA detection, it is valuable to explore how these methods compare. We used the NCode Multi-Species miRNA microarray (Invitrogen) and the TaqMan Human microRNA array (Applied Biosystems) to compare microRNA expression in undifferentiated MSCs and MSCs differentiated into early osteoblasts. We show that while there is a somewhat low correlation between these two methods, there was a subset of miRNA measurements that did correlate.

Keywords

Mesenchymal stem cell; microRNA; microarray; MSC differentiation; quantitative PCR

1. Introduction

Adult mesenchymal stromal cells (MSC) are undifferentiated multi-potent cells isolated from various tissues including bone marrow, fetal tissue, peripheral blood, and adipose tissue (1–5). The capacity of MSC for self-renewal and their ability to differentiate into osteoblasts, chondrocytes, adipocytes, or fibroblasts makes these cells attractive for therapeutic transplantation. However, the multiple derivations of MSCs and their ability to generate multiple lineages will require characterizing these MSC sub-populations by their unique genetic profiles. This has been done with mRNA microarrays (6, 7) but miRNA profiles are often simpler and exhibit more drastic differences upon differentiation (8).

MicroRNAs (miRNA) are 17 to 24 nucleotide long, single-stranded, non-coding RNAs that are genome-encoded and regulate multiple cellular processes. MiRNAs can modulate gene expression by affecting transcription rate (9) mRNA stability (10), or mRNA translation into functional proteins (11). Several studies have identified populations of miRNAs that are

unique to mouse embryonic stem (ES) cells (12, 13), mouse or human embryonic carcinoma cells (14), and human ES cells (13, 15, 16). Likewise, MSCs exhibit unique miRNA expression profiles (8, 17, 18). What these studies reveal is that miRNAs may be used as diagnostic tools to discriminate between cell types as well as to uncover differentiation mechanisms. As diagnostic indicators, groups or individual miRNAs regulate cardiac differentiation (13), ESC self-renewal (19), osteogenic differentiation (20), myogenesis (21, 22), neural differentiation (14, 23, 24), and cancer (25). Such groups of miRNAs as in the case of ESC self-renewal serve as regulators of cell cycle (26, 27). Also, the presence of miRNAs in stem cells and their disappearance during differentiation suggest roles in restricting cell differentiation. In MSCs, specific populations of miRNAs are regulated during osteogenic differentiation and these miRNAs have been shown to be responsive to extracellular signals (8). Thus, there is a need for further examination of miRNA expression profiles in MSC populations.

Methods used to investigate miRNA expression include microarrays, northern blotting, small RNA library sequencing, in situ hybridization, deep sequencing and quantitative real-time PCR (qPCR). The decision of which method to choose depends on the level of data one needs to extract or the number of samples to be processed. For identifying which groups of miRNAs are being expressed, microarrays are probably the most logical method, while qPCR is often used to validate those results or to screen fewer miRNAs across larger numbers of culture conditions. However, improvements to these methods have made it possible to detect the expression of multiple miRNAs by qPCR using a microfluidics-based array of qPCR reactions. The two methods outlined here use either microarrays and qPCR to investigate miRNA expression in undifferentiated and differentiated MSCs. For the microarray platform, we used the NCode Multi-Species miRNA microarray system (Invitrogen Corporation) which requires poly(A) tailing of the miRNAs followed by tagging the tail with a DNA polymer attached to fluorescence dye molecules. Although, there are disadvantages to using microarrays, such as high variability in the signal-to-noise ratio, likely due to differences in labeling and the difficulty of specifically hybridizing such short probes, there are several advantages to using the NCode platform including increased sensitivity of the labeling reaction due to the multiple Alexa Fluor[®] molecules present on the DNA polymer and the inclusion of positive control probes. To minimize dye bias effects, it is also recommended to perform dye-swap hybridization, a technique used in the below described protocol. An alternative to microarrays is a PCR-based approach. To demonstrate such an approach, we employed the TaqMan Human microRNA Array (Applied Biosystems). This technology requires reverse transcription of miRNAs from total RNA with up to 380 stem-loop reverse transcription primers followed by the option to pre-amplify the cDNA and PCR amplification by real-time PCR analysis. Utilizing both the NCode platform and the TaqMan platform, we demonstrate the ability to identify miRNAs present in undifferentiated and differentiated MSCs and compare the microarray-detected miRNA expression to that of qPCR-detected miRNA expression.

2. Materials

2.1 MSC cell cultures and differentiation

1. MSC medium: DMEM/F12 low osmolality (Invitrogen, Carlsbad, CA) supplemented with 10% FBS (HyClone, Odgen, UT), 1X GlutaMAX (Invitrogen), 1X MEM Nonessential amino acids (Invitrogen), 10 mM HEPES (Invitrogen), and 0.5 µg/ml Gentamycin (Invitrogen). MSC medium can be stored at 4°C for up to 4 weeks.
2. Osteoblast medium: MSC medium supplemented with 10 mM Glycerol-2-phosphate (Sigma-Aldrich, St. Louis, MO), 50 µM L-ascorbic acid-2-phosphate (Sigma-Aldrich), and 100 nM Dexamethasone (Sigma Aldrich). Osteoblast medium can be stored at 4°C for up to 4 weeks.
3. Trizol (Invitrogen).
4. NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Waltham, MA).

2.2 microRNA Microarrays

1. Total RNA sample containing small RNA fraction
2. NCode™ Multi-species miRNA microarray (Invitrogen)
3. Glass Coverslips or LifterSlips™ (Thermo Scientific)
4. Wash buffers: 2X SSC/0.2% SDS, 2X SSC, and 0.2X SSC
5. 1 mM Tris-HCl, pH8.0
6. Hybex microarray incubation system (SciGene, Sunnyvale, CA) or alternative hybridization chamber
7. NCode™ Rapid miRNA labeling System (Invitrogen)
8. Microarray scanner such as the GenePix 4000B (Molecular Devices, Sunnyvale, CA)
9. Wash containers for submerging slide rack
10. Vacuum concentrator
11. Slide box

2.3 TaqMan® microRNA qPCR

1. Total RNA sample containing small RNA fraction
2. Megaplex™ RT primers for Human Pool A and Human Pool B (Applied Biosystems, Carlsbad, CA)
3. TaqMan® Human MicroRNA Array A and Array B (Applied Biosystems)
4. TaqMan® MicroRNA Reverse Transcription kit (Applied Biosystems)

5. TaqMan® Universal PCR Master mix, No AmpErase® UNG, 2X (Applied Biosystems)
6. 7900HT Fast Real-Time PCR system with the 7900HT TaqMan® Low Density Array unit (Applied Biosystems)

3. Methods

3.1 MSC differentiation and total RNA isolation

1. MSCs (8) were first expanded in MSC medium on 10 cm plasma treated culture plates and replenished with fresh media every 2 days. After reaching 80% confluence, cells were passaged onto two, 6-well plates at 10,000 cells/cm². Following a recovery period of 24 hours, three wells were harvested with 1 ml of Trizol and stored at -80°C while a second set of three wells was selected for differentiation. The medium was changed to osteoblast differentiation medium and replenished every three days. On the 7th day, osteoblasts were harvested with 1 ml of Trizol and the cell lysis was stored at -80°C.
2. Total RNA was isolated following the Trizol method (Invitrogen) and quantified using a NanoDrop ND-1000 Spectrophotometer (*see* Note 1).

3.2 NCode™ Microarray hybridization

1. A total of 1 µg of total RNA in a volume of no more than 9 µl is required for each experimental condition (two undifferentiated MSC samples and two differentiated MSC samples) (*see* Note 2 and Note 3).
2. Poly(A) tailing of the RNA is carried out using the NCode™ Rapid miRNA Labeling System. Prepare the ATP dilution with 1 mM Tris-HCl, pH 8.0 for using total RNA according to manufacturer's recommendations prior to beginning the poly(A) tailing procedure but do not add it to the total RNA until the procedure calls for the diluted ATP.
3. Dilute the NCode™ multi-species miRNA microarray control according to the manufacturer's recommendations. Add 1 µl of the diluted NCode™ multi-species miRNA microarray control to each of the four total RNA samples and bring up the volume to 10 µl.
4. Add the 10X miRNA reaction buffer, 25 mM MnCl₂, diluted ATP, and poly(A) polymerase to the 10 µl RNA as indicated in the manufacturer's protocol.

¹If RNA yield is of concern (< 100 ng), RNA may be precipitated by adding Linear Acrylamide (Applied Biosystems, Carlsbad, CA) to a final concentration of 10 µg/µl prior to ethanol precipitation. A ratio of the absorbance at 260 and 280 (A₂₆₀/A₂₈₀) and a ratio of the absorbance at 260 and 230 (A₂₆₀/A₂₃₀) need to be > 2.0 when measured at pH 8.0. If either ratio is less than 1.8, additional cleanup steps are required to remove any contaminants that remain from the RNA isolation. To determine the integrity of total RNA, the samples may be analyzed by gel electrophoresis or on the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). If the Bioanalyzer is used, a RNA Integrity Number (RIN) of > 8.0 is recommended.

²Since environmental ozone levels can have an effect on fluorescence intensity, ensure ozone levels are as low as 2 ppb prior to beginning hybridization unless the microarray laboratory has been installed with an adequate air filtering system (28, 29). A dye-flip experiment is performed to minimize any dye-effects. Thus, two arrays and four samples (two control samples and two test samples) are required for each hybridization reaction. For experiments with additional replicates, linear modeling may be performed (30).

³If the total RNA is too dilute, the RNA may be concentrated using a vacuum concentrator. However, be careful not to over-dry the RNA or it will not dissolve properly.

5. Mix, centrifuge, and incubate the four reactions at 37°C for 15 minutes (*see* Note 4).
6. Briefly centrifuge the 15 µl of poly(A) tailed RNA and add 4 µl of the ligation fluorescent mix to the corresponding RNA (*see* Note 5).
7. Add 2 µl of T4 DNA ligase to the reaction.
8. Mix and briefly centrifuge the reaction (*see* Note 6).
9. Incubate the reactions for 30 minutes at room temperature under low-light conditions (*see* Note 7).
10. Add 2.5 µl of Stop solution to each reaction. Mix and briefly centrifuge the reactions.
11. Combine the appropriate control sample (labeled with the first fluorescent dye) with its corresponding test sample (labeled with the second fluorescent dye) to yield two ligation reactions with a total volume of 47 µl each.
12. Concentrate the two reactions with a vacuum concentrator to half the volume (~23.5 µl) (*see* Note 8).
13. Add 5 µl of 10% BSA to each concentrated ligation reaction.
14. Add 28.5 µl of 2X pre-warmed hybridization buffer (*see* Note 9).
15. Incubate this hybridization mix for 10 minutes at 65°C (*see* Note 10). Protect the reactions from light.
16. Add 57 µl of the hybridization mix to the array, cover with a glass coverslip, and hybridize overnight at 53°C in a dark, humidified chamber (*see* Note 11 and Note 12).
17. Warm up 2X SSC/0.2% SDS wash buffer to 52°C.
18. Remove the coverslip by immersing the slide in pre-warmed 2X SSC/0.2% SDS for 2 min or until the coverslip floats off (*see* Note 13).
19. Wash slide in pre-warmed 2XSSC/0.2%SDS at 52°C for 15 min.

⁴While the poly(A) tailing reaction is incubating, store all poly(A) tailing components and begin to thaw the materials required for ligation. Perform the ligation reaction under low light conditions.

⁵One group of samples, a control (undifferentiated MSC) and a test sample (differentiated MSC), is labeled with one fluorescent dye. The second group of samples is labeled with the second fluorescent dye.

⁶The NCode™ dye normalization control is not necessary since a dye-flip experiment is being conducted.

⁷Inspect the slide to ensure it is free of dust or lint particles. If necessary, gently clean the slide with a stream of air.

⁸Pre-heat the 2X hybridization buffer at 53°C until the buffer is completely dissolved.

⁹The 2X hybridization buffer is viscous so pipette it slowly.

¹⁰Maintain the ligation reactions at 65°C until ready to load the reactions onto the array slides.

¹¹To load a slide there are two options, one option is to place the glass coverslip on the array and then align the pipette tip at the center of the glass coverslip and slowly pipette the liquid by allowing it to flow throughout the slide. The second option is to apply the hybridization mix first and then gently lower the coverslip onto the slide.

¹²If using the Hybex microarray incubation system (SciGene) only one chamber (which fits 4 slides) is required. Unscrew the lid of the microarray incubation chamber from its base, soak the filter paper within the chamber with 1ml of water, allow the water to soak throughout the filter paper and remove any excess water from the chamber. After loading the slide, close the chamber tightly. Load the chambers into the incubation system according to the manufacturer's recommendations.

¹³If the slide dried during hybridization it will take longer than 2 min for the coverslip to float off. Do not allow the slides to dry in between the wash steps.

20. Wash slide in 2X SSC for 15 min at room temperature.
21. Wash slide in 0.2X SSC for 15 min at room temperature.
22. Quickly transfer the slide to a centrifuge and spin at 1100 rpm for 3 min (*see* Note 14).
23. Scan and analyze the array on a microarray scanner (*see* Note 15).

3.3 TaqMan® qPCR

1. Set up two 0.2 ml tubes with undifferentiated MSC total RNA and two 0.2 ml tubes with differentiated MSC total RNA. Each tube should contain 3 µl of total RNA (350 ng to 1000 ng).
2. Set up one RT master mix with Megaplex™ RT Primers Pool A, enough for two samples and one RT master mix with Megaplex™ RT Primers Pool B for two samples according to manufacturer's protocol (*see* Note 16).
3. Add 4.5 µl of the appropriate RT Master Mix to each 3 µl of total RNA.
4. Mix and briefly centrifuge contents.
5. Incubate each reaction on ice for 5 min.
6. Perform reverse transcription by setting up a thermal cycling run according to the manufacturer's protocol.
7. If necessary, thaw the Megaplex™ RT product on ice. Add 444 µl of nuclease-free water to each RT product. Mix and briefly centrifuge each reaction.
8. Prior to setting up the PCR reactions, mix the 2X TaqMan® Universal PCR Master Mix, No AmpErase® UNG. To each Megaplex™ RT product, add 450 µl of the Universal PCR Master Mix, mix and briefly centrifuge.
9. Allow each TaqMan® MicroRNA array to reach room temperature (two of Array A and two of Array B). Aliquot 100 µl of RT product into each fill reservoir of the array. Repeat this step for the remaining three arrays.
10. Centrifuge the arrays to distribute the RT product into the reaction wells of the array according to the manufacturer's instructions.
11. Seal the array according to the manufacturer's instructions and trim the fill reservoirs from the array.
12. Load and run each array using the relative quantification mode and the 384 well TaqMan® Low Density Array thermal cycling conditions (*see* Note 17).

¹⁴The slide may be dried in a 50 ml centrifuge tube by orienting the slide so that any label is at the bottom of the tube. If a swinging bucket centrifuge is available, orient the slides so that they are parallel to the swinging bucket. The slide may be stored in a slide box until ready to analyze the array.

¹⁵Set PMT settings accordingly to control spots available on the array and then proceed with gridding of the array.

¹⁶One sample of undifferentiated MSC and one sample of differentiated MSC are labeled with Megaplex™ RT Primers Pool A. The second sample of undifferentiated MSC and sample of differentiated MSC are labeled with Megaplex™ RT Primers Pool B.

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¹⁷To analyze the data, load the SDS files for the samples labeled with the Megaplex™ RT primers Pool A into the SDS RQ manager software provided by Applied Biosystems. In the analysis settings, set the detector settings to a manual C_t and a threshold of 0.2 and select an automatic baseline. Also select the mammalian U6 probe as the endogenous control detector and the control sample (undifferentiated MSC) as the calibrator. Analyze all probes and export data. Repeat these steps for Pool B.

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Figure 1. Correlation of NCode™ miRNA microarray and TaqMan® miRNA array measurements for the 70 miRNAs detected by both assays is dependent on expression level
 Out of 454 miRNA probes present on the NCode arrays, 93 miRNA measurements showed a positive mean intensity above background. Results are plotted as the log₂ fold change between MSC and nascent osteoblasts. Out of these 93 miRNAs, 70 were present in both the NCode and TaqMan arrays. (A) Comparison between the two miRNA platforms using all 70 probes indicated poor correlation between the two methods (Spearman's rho=0.295). (B) However, using only the qPCR results filtered for a higher level of expression (a C_t or cycle threshold value of less than 30) produced increased correlation (Spearman's rho=0.528). (C)

In contrast, when qPCR results were filtered for low levels of expression (C_t value of 30 or more cycles), there was less of an association between the two methods (Spearman's $\rho=0.068$). Results indicate that fold-change measurements are somewhat similar between the two methods at higher levels of expression but that the methods disagree at lower expression levels. This is likely due to the greater sensitivity of qPCR.

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