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Differentiating Human Multipotent Mesenchymal Stromal Cells Regulate microRNAs: Prediction of microRNA Regulation by PDGF During Osteogenesis

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Abstract

Objective—Human multipotent mesenchymal stromal cells (MSC) have the potential to differentiate into multiple cell types, although little is known about factors that control their fate. Differentiation-specific microRNAs may play a key role in stem cell self renewal and differentiation. We propose that specific intracellular signalling pathways modulate gene expression during differentiation by regulating microRNA expression.

Methods—Illumina mRNA and NCode microRNA expression analyses were performed on MSC and their differentiated progeny. A combination of bioinformatic prediction and pathway inhibition was used to identify microRNAs associated with PDGF signalling.

Results—The pattern of microRNA expression in MSC is distinct from that in pluripotent stem cells such as human embryonic stem cells. Specific populations of microRNAs are regulated in MSC during differentiation targeted towards specific cell types. Complementary mRNA expression analysis increases the pool of markers characteristic of MSC or differentiated progeny. To identify microRNA expression patterns affected by signalling pathways, we examined the PDGF pathway found to be regulated during osteogenesis by microarray studies. A set of microRNAs bioinformatically predicted to respond to PDGF signalling was experimentally confirmed by direct PDGF inhibition.

Conclusion—Our results demonstrate that a subset of microRNAs regulated during osteogenic differentiation of MSCs is responsive to perturbation of the PDGF pathway. This approach not only identifies characteristic classes of differentiation-specific mRNAs and microRNAs, but begins to link regulated molecules with specific cellular pathways.

Introduction

Mesenchymal stem cells or multipotent mesenchymal stromal cells (MSC), though commonly isolated from adult bone marrow, have also been isolated from such diverse biological sources as bone [1], skeletal muscle, lung, deciduous teeth [2] and human umbilical cord [3], and are defined by their ability to attach to a solid surface when other cells present in crude preparations

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from these tissues do not [4]. Due to the lack of a single definitive marker, MSC are generally characterized by the presence and absence of a combination of surface antigens. It has been universally accepted that MSC lack the common hematopoietic markers such as CD45, CD34 and CD14 [5]. More recently STRO-1, CD44, CD90, CD73 and CD105 have emerged as positive markers of MSC [5-8]. The self renewal capacity of MSC, thought to be limited, is known to be enhanced under specific culture conditions such as serum concentration, cell seeding density, and inclusion of growth factors such as FGF-2 [9-11]. These various culture methods may be a major source of the heterogeneous nature of the MSC cell population. Several studies have indicated the capability of MSC to differentiate into mesenchymal lineages such as adipose tissue, bone, cartilage, tendon, muscle and hematopoietic supporting stroma [12-19]. The multilineage differentiation potential of clonally derived cells within a MSC population have been shown to be variable [5,20-25], further demonstrating the heterogeneous nature of the defined MSC population.

Nevertheless, the possibility of obtaining MSC from an autologous source and their ability to differentiate into a variety of connective tissue types makes them ideal candidates for cell therapy. The unquestioned therapeutic potential of MSC is reflected in current clinical usage for treating children with Osteogenesis imperfecta, hematopoietic recovery, and for bone tissue regeneration [26-28].

Understanding key regulatory pathways and molecules either involved in maintaining MSC in their undifferentiated state or during the process of differentiation allows for a better handle on expanding and culturing these cells in large scale for therapeutic applications. Examination of gene expression profiles has revealed dynamics between MSC, progenitor cells, and committed differentiated cells [29,30]. Recently, the importance of epigenetic regulation via microRNA in controlling stem cell activities has been recognized [31-37]. Understanding these regulatory mechanisms may help to design novel processes for production of specific therapeutic cells for transplant.

MicroRNAs (miRNA), short (17–24 nucleotides), non-coding RNAs that have been identified in various organisms including mammalian cells, are thought to play a key role in several biological processes [38-41]. It has been suggested that over 30% of protein coding genes in humans are regulated by microRNA [42]. More recently, a unique set of microRNA have been shown to be associated with embryonic stem cells [43-48]. These microRNAs are expressed at high levels in several human embryonic stem cell (ESC) and human embryonal carcinoma cell (EC) lines [49,50] and decrease upon differentiation into embryoid body (EB) for two weeks in culture [50] supporting their association with the stem cell state. Furthermore, strategies to knock down microRNA levels in ESC cells demonstrate that microRNAs are essential for differentiation, likely by suppressing stem cell self-renewal programs [44,51]. The understanding of integrated gene expression and epigenetic microRNA mechanisms should be important for the characterization of stem cells and their progeny.

Studies of gene expression patterns are valuable not only for distinguishing cell types during differentiation but also for revealing signature patterns reflecting the regulation of specific intracellular signaling pathways. Work in MSC by our collaborators recently analyzed standard microarray results to identify several such pathways active during MSC differentiation [52], including the PDGF pathway acting during osteogenesis. We sought to extend this work to predict the novel regulation of microRNA transcription as a mediator of, at least, translational repression during differentiation. Specifically, we hypothesized that specific groups of microRNAs would be regulated during MSC differentiation and that a subset of these groups could be associated with PDGF-responsive transcription factors. In order to test this hypothesis we first assessed differentiation status of several MSC isolates by cell markers or gene expression. Since variability between MSC isolates is known to be a complicating factor, we

also examined a pooled preparation of MSC. MSC and differentiation-specific microRNA expression patterns were determined and used to predict PDGF-responsive pathways. Finally, PDGF regulation of the predicted microRNAs was tested by inhibition of the PDGF pathway. These studies not only identify microRNAs indicative of MSC differentiation patterns, but also demonstrate that extracellular signals contribute to microRNA regulation during differentiation, supporting a role for microRNAs during MSC development.

Materials and Methods

Cells

MSC from four independent donors were purchased from Cambrex (at passage 2) with the tissue acquisition numbers 1678C (18y, African American, male), 1822A (22y, African American, male), 1832A (18y, African American, male), and 1271C (23y, African American, Female), and were designated as Donors 1, 2, 3, and 4, respectively. A pooled MSC sample comprised of a mixture of these four donor MSC that were mixed together in equal numbers and cryopreserved. Cells were diluted to 1 million cells per vial and frozen in MSC culture media plus 15% DMSO (*Sigma-Aldrich*) at a controlled rate to -70°C by using a cell freezer box. Cell stocks were transferred to liquid nitrogen storage after 24 h. An additional MSC line (designated as Donor 5) was obtained from Dr. Rick Cohen (Rutgers University) and Dr. Eric Hume (Cooper University Hospital, Camden, NJ). The donor was a 39 year old male.

Culture

MSC were seeded at 3000 cells per cm^2 and expanded on tissue culture treated-plastic dishes (*Corning Costar*). The MSC culture media was composed of DMEM/F12 (*Invitrogen*), 10% FBS, 1X GlutaMAX (*Invitrogen*), MEM non essential amino acids, 10 mM HEPES, and 0.5 $\mu\text{g}/\text{ml}$ gentamycin (*Invitrogen*). Cells were fed on alternate days and passaged when 80% confluence was reached. Illumina gene expression and NCode™ microRNA analysis was carried out with samples that were grown less than a month in culture (P10 or lower).

Differentiation

MSC were expanded in culture and seeded in T125 flasks for differentiation into either adipocytes or osteocytes. A fraction of these cells were stored as day 0 samples. Cells were also seeded in parallel on chamber slides for differentiation. Samples were stained or harvested for RNA isolation at 7, 14, or 22 days of differentiation.

Adipocyte differentiation was initiated by seeding MSC at a density of 20,000 cells per cm^2 in the presence of adipogenic differentiation media composed of MSC growth media with 0.5 mM Isobutyl-methylxanthine (*Sigma-Aldrich*), 10 μM bovine insulin (*Sigma-Aldrich*), 1 μM dexamethasone (*Sigma-Aldrich*) and 200 μM indomethacin (*Sigma-Aldrich*).

Osteoblast differentiation was initiated by seeding MSC at a density of 5,000 cells per cm^2 in the presence of osteogenic differentiation media composed of MSC growth media with 10 mM glycerol-2-phosphate (*Sigma-Aldrich*), 50 μM L-ascorbic acid (*Sigma-Aldrich*), and 100 nM dexamethasone (*Sigma-Aldrich*).

Chondroblast differentiation was initiated by seeding MSC at a density of 8×10^5 cells per tube in a 15 ml conical tube in 0.5 ml of chondrogenic differentiation media composed of MSC growth media with 10 ng/ml TGF- β 1 (R&D systems), 50 nM L-ascorbic acid (*Sigma-Aldrich*) and 6.25 $\mu\text{g}/\text{ml}$ bovine insulin (*Sigma-Aldrich*).

Staining

MSC were stained using antibodies specific against the surface antigens CD45, CD34, CD44, CD73 and CD105. Samples were run on FACS Aria (BD Biosciences, CA) and data analyzed using FloJo (Tree Star Inc., OR).

Differentiation samples set up in chamber slides were stained with either Oil Red O for adipocytes, Von Kossa staining for osteocytes or Alcian blue for chondrocytes. Cells in chamber slides were fixed with 100 μ l freshly prepared 4% formaldehyde/D-PBS solution made from 37% formaldehyde stock solution for 30 minutes at room temperature. The formaldehyde solution was discarded and wells rinsed three times with D-PBS.

Adipocytes—After fixation, cells were rinsed with 70% ethanol and incubated with 0.3% freshly prepared Oil Red O (*Sigma-Aldrich*) working solution (3 parts 0.5% Oil Red O with 2 parts deionized water, filtered through #42 Whatman paper) for one hour at room temperature. Cells were rinsed twice with 70% ethanol. Cells were rinsed twice with deionized water, counter stained with Dapi (*Invitrogen*, D3571) and visualized under light or fluorescence microscope.

Osteoblasts—After fixation cells were rinsed three times with D-PBS and incubated with 5% silver nitrate for 10 minutes in the dark. Cells were rinsed three times with deionized water and exposed to bright light for 15 – 60 minutes. Cells were again rinsed three times with deionized water and visualized under light microscope.

Chondroblasts—After fixation, cells were rinsed twice with D-PBS and incubated with 1% Alcian Blue solution for 30 minutes at room temperature. Cells were then rinsed with 0.1% HCl solution to remove excess stain and visualized under light microscope.

AG-370 Treatment

MSC (10,000 cells per cm^2) were seeded in 6 well plates and allowed to recover for 72 hrs. PDGF receptor kinase inhibitor AG-370 (*Sigma-Aldrich*, 2 μ M) was added to cells. After 7 days, cells were harvested in TRIzol and RNA extracted for qPCR analysis of microRNA.

mRNA Expression Analysis

Total cellular RNA was amplified and labeled for Illumina BeadArray analysis as reported earlier [53]. Labeled, amplified material (~700 ng per array) was hybridized to the Illumina Bead Chip according to the manufacturer's instructions (Illumina, Inc., San Diego, CA). Array data processing and analysis was performed using Illumina BeadStudio software, GeneSpring (Agilent) and BioConductor/R (<http://www.bioconductor.org>).

Enrichment of microRNA (miRNA)

microRNA was isolated from Trizol-extracted total RNA using the PureLink miRNA Isolation Kit (Invitrogen) according to recommended protocol. The amount of microRNA was quantified using a spectrophotometer and its purity assessed by electrophoresis on a 15% Nupage urea-TBE gel (Invitrogen) and/or by running on a Bioanalyzer Nano RNA chip (Agilent).

microRNA Expression using NCode™ Microarrays

For exploratory analysis of microRNA regulation in differentiating MSCs, samples (n=1) of pooled-donor MSCs were differentiated for 7 or 14 days following the protocols for osteoblasts, adipocytes, or chondroblasts. Undifferentiated MSCs were included as a reference. The dual-color dye swap method [54] was used to study microRNA expression differences between undifferentiated and differentiated MSCs. Five hundred nanograms of the enriched microRNA

was labeled with the FlashTag™ (Genisphere, Inc.) direct labeling system and hybridized to NCode™ multispecies microRNA arrays [55] as described earlier [56] based on a loop design, balanced with respect to array, dye, and sample. By using a balanced design, sources of unwanted variation can be minimized [57]. Arrays were scanned, aligned, and median spot intensities were obtained using a GenePix 4000B scanner (Molecular Devices, Inc.). Data analysis methods are described in the Supplemental Materials.

PDGF Pathway Transcription Factor Binding Site Predictions

To determine whether MSC-regulated microRNAs were enriched for the PDGF pathway, predicted promoter regions were obtained from microRNAs declared as significant and scanned for transcription factor (TF) binding sites from TFs predicted to be regulated by the PDGF pathway (Figure 5A). A 5 kb region upstream of each microRNA gene was arbitrarily selected from human reference genome (hg18 assembly, NCBI Build 36.1) as an estimate for the transcriptional control region. Using pathway analysis software (Pathway Architect, Stratagene), mammalian cell surface receptors in the PDGF pathway were identified and a comprehensive literature search located all downstream transcription factors known to be regulated by this pathway. Seven transcription factors were identified (Figure 5A). The 5 kb upstream region sequences were scanned with position weight matrices using Transfac Pro MATCH (version 11.2, Biobase) [58]. This process was repeated for 24 non-significant microRNAs with expression levels greater than background. These predictions were used as a representative set of “unregulated” microRNAs present in this system. The number of significant binding sites identified for this grouping of microRNAs is predicted to be characteristic of a random sample. Using a paired-sample t-test, the number of binding sites identified by Transfac MATCH was compared between significant and non-significant microRNAs. In order to predict specific microRNAs to be regulated by the PDGF pathway, Z-tests were used to compare the number of significant binding sites for each significant microRNA with a mean number of binding sites across all regulated and non-regulated microRNA upstream regions.

Quantitative real-time PCR (qPCR)

qPCR for adipogenic and osteogenic marker mRNAs was carried out using 20 ng of cDNA template prepared from DNase-treated total RNA isolated from MSC, differentiated adipocytes and osteocytes. The C_t value obtained for the gene of interest was normalized to a housekeeping gene, either $\beta 2$ microglobulin or GAPDH, as indicated, to obtain the ΔC_t values. $\Delta \Delta C_t$ values were then obtained by subtracting the ΔC_t values for each gene of interest against ΔC_t values for the control sample. Results expressed as fold change in expression relative to the control sample was calculated using the equation $RQ = 2^{-\Delta \Delta C_t}$.

NCode™ microRNA qPCR was performed and differences in microRNA expression between samples determined using the relative quantification method as described earlier [56]. Briefly, the C_t values of the samples were normalized to the C_t values of GAPDH, a housekeeping gene. The resulting values were further normalized to the corresponding undifferentiated MSC. Fold difference in gene expression of the sample from the reference undifferentiated cells was calculated using the equation $RQ = 2^{-\Delta \Delta C_t}$.

Results

Characterization of MSC

Since MSC are both variable by source and difficult to identify using simple markers, we began by testing cultures for several markers. Pooled cultures of four MSC donors (see Methods) were propagated on plastic dishes and the adherent cells further characterized for the absence and presence of surface antigens that are characteristic of MSC [59]. FACS analysis of MSC

demonstrates cells negative for the hematopoietic lineage markers CD45 and CD34 and positive for CD44, CD73 and CD105 (Figure 1A). The identity of the cells as MSC was further confirmed based on their differentiation potential. The pooled MSC cells were differentiated at early passages into adipocytes, osteoblasts and chondroblasts for 7 and 14 days. MSC from three independent donors with less than 10 passages were induced to differentiate into adipocytes or osteocytes for 22 days. Uniform differentiation of MSC into adipocytes, osteoblasts and chondroblasts were determined based on Oil Red, Von Kossa and Alcian Blue staining respectively (Figure 1B). Based on these assays, we conclude that these cells, culture methods, and differentiation methods are appropriate.

To characterize cells in more detail using gene expression patterns, we conducted mRNA expression analysis on undifferentiated MSCs as well as cells differentiated using adipocytic or osteogenic protocols used in the study. A detailed description of the analysis of gene expression results can be found in the Supplemental Materials. For this analysis we assayed individual donor cells at multiple passage numbers to identify genes that were consistently regulated. To focus on genes explaining differences between cell phenotypes (MSC, adipocytes, or osteocytes), expressed genes were tested for significant changes by cell type using the Welch's F-test with a 5% false discovery rate (FDR) [60], resulting in 1,384 genes being selected (Supplemental Table 1). The selected genes were further partitioned using post-hoc analysis (Student-Newman-Keuls) into groups that distinguish cell types. 167 genes differentiated MSC from osteocytes, and 94 genes distinguished MSC from adipocytes. Assembling these with the entire ANOVA-selected genes into a Venn diagram (Figure 1C), we identify 48 genes shared between the two post-hoc groups. 46 genes uniquely distinguish adipocytes from MSC (including ACACB, CEBPA, FASN, LIPE, MVD and PLIN—see Supplemental Table 1 for a complete list of significantly different mRNAs), while 119 genes uniquely distinguish osteocytes from MSC (including ADAM19, AOX1, AURKA, DPT, LEP, LEPR, MGP, PTHR1, and TIMP4). The 243 genes found on at least one of the post-hoc groups are marked in Supplemental Table 1 with asterisks marking membership within each of the post-hoc groups.

The raw signal intensities of known MSC, adipocyte, or osteoblast markers in all samples were examined further (Table 1). This list includes genes selected as part of the ANOVA list (marked with an asterisk) as well as those that were excluded from statistical analysis due to infrequent expression (denoted with a dagger). This table also demonstrates the variability between individual donor cultures or passage numbers (see relative SEM compared with the means). This variability is another reason why some known differentiation markers were not selected by ANOVA. Of the markers associated with MSC that were clearly higher in expression level relative to adipocytes or osteocytes were Col21A1 (mean 65-fold lower in osteocytes, 18-fold lower in adipocytes) and PDGFRA (1.8-fold and 2.6-fold lower, respectively). Adipocyte differentiated cells from all three donors showed a clear up regulation of adipocytic markers relative to their undifferentiated cells or osteocyte differentiated cells, such as FABP4, LEP, LPL, and SREBF1. Among markers previously associated with osteocytes, POSTN and BGLAP were found to have expression patterns matching expectations. Gene expression patterns clearly indicate appropriate patterns of differentiation of these two cell types, validating the cells and differentiation protocols.

PDGF pathway analysis

In a parallel study, our collaborators performed a detailed analysis of predicted pathways regulated during MSC differentiation using microarrays [52]. Their work identified, among others, genes predicted to be regulated by the platelet-derived growth factor pathway (PDGF) pathway were found to be regulated during adipocyte, chondrocyte and osteocyte differentiation. The requirement for this pathway was tested using AG-370, a Tyrphostin

inhibitor of PDGF signaling, which produced fewer osteocytes and an absence of mineralized bone nodules [52], indirectly indicating the requirement for PDGF signaling during osteocyte differentiation. Of course, the PDGF pathway is not primarily known for its role in osteogenesis and is likely only one of many pathways required for differentiation [61]. However, the presence of several PDGF-pathway genes in our ANOVA list (e.g. PDGFRB, PDGFA, PDGFD, PDGFRL, NFKB1, and STAT3, Supplemental Table 1) lends additional support to the conclusion that PDGF mediates at least a portion of the osteogenesis program. Since mRNAs specific for PDGF signaling are expressed, since MSC have been found to express PDGF receptors (at least CD140B) [62], and since PDGF signaling has been found to be required for osteogenic differentiation [52], we predict that PDGF will regulate microRNAs as part of its mechanism.

Global microRNA expression

Before identifying potential targets of the PDGF pathway, we needed to identify overall patterns of microRNA regulation. An exploratory microRNA expression study was conducted on pooled MSC samples, as well as MSCs differentiated into each of three distinct cell types. Using NCode™ microRNA microarrays, samples of undifferentiated MSCs, adipo-, osteo-, and chondrogenic differentiated MSCs were assayed to determine expression levels for all known human microRNAs. We first examined which microRNA demonstrated significant regulation across any of the four conditions. Forty-four (44) probes detecting 31 unique mature microRNAs ($p < 0.05$, FDR 5%) from the MAANOVA testing of hypothesis 1 (Supplemental Materials) were identified as differentially expressed between any of the cell types (Figure 2A). Interestingly, most of the microRNAs highly expressed in human embryonic stem cells [50] were absent in MSC while some markers associated with differentiating cells, such as miR-125, were expressed in MSC.

Pearson correlations were calculated to determine the relatedness between samples using global microRNA expression values. In this analysis, D7 and D14 adipocytes were highly correlated ($r = 0.99$), as well as D7 and D14 chondroblasts ($r = 0.91$). In addition, D14 osteoblasts were highly correlated with chondroblasts ($r = 0.90$), reflective of their shared cellular origin. Thus, samples with known biological relationships were found to be correlated based on microRNA expression patterns.

Some of the probes detected on the microRNA array were designed against non-human but similar microRNA sequences [55]. This was expected due to the similarity among paralogous microRNAs from different species as well as the inability to distinguish short RNAs having only a 1-nt or 2-nt difference [55]. Turning our attention to the 27 unique human mature microRNAs detected by these probes exclusively, a K-means cluster analysis was used to compare the expression patterns of significant microRNAs. This analysis groups microRNAs into clusters with similar levels of expression across the different samples (Figure 2B). The data were fit to four clusters ($k=4$), producing the best fit of merit compromise between number of clusters and residuals. Two microRNAs could not be consistently assigned to one of the four clusters, likely due to distributed membership in multiple clusters. Mean cluster expression patterns suggest the presence of microRNAs specific to each cellular type. As expected, microRNAs with previously defined, tissue-specific expression patterns were associated with their correct tissue types (e.g. miR-143 and miR-145 enrichment in adipocytes). Novel microRNA associations appear in this analysis as well (e.g. miR-638 and miR-663 expression exclusively in chondroblasts). This result identifies sets of microRNAs demonstrating significant regulation during the cell-type specific differentiation of MSCs, and suggests that differences across the resulting phenotypes can be associated with the differential expression of specific microRNAs.

The majority of the significantly regulated microRNAs appear to be selected primarily for their differences between the osteogenic samples and all other cell types (see clusters 1 and 2, Fig. 2B), suggesting that these microRNAs may be important for bone formation. This result drove us to test explicitly which microRNAs demonstrate significant differences between osteo-differentiated MSCs and all other cell types. The osteoblast-specific microRNAs agreed with those found in K-means clusters exhibiting increased expression in osteocytes. This confirms the identity of a subset of microRNAs which are expressed primarily in differentiating osteoblasts. Additionally, this analysis provides a target set for testing the effect of key regulatory pathways on the expression of putatively osteogenic microRNAs.

Validation of microarrays by microRNA qPCR

As validation of the NCode™ array data, qPCR was performed for a selection of the microRNAs from the significant list (Figure 3—comparisons between undifferentiated cultures and osteo-differentiated cultures). We chose to assay osteogenic differentiation after only 7 days because the microarray results predicted that specific changes would be found this early in the process and because we wished to determine whether microRNA changes would precede, and possibly predict, differentiation choices. Triplicate cultures of MSC from either the pooled MSC cultures used for microRNA arrays (Figure 3A) or from a biologically distinct donor (Donor 5, Figure 3B) were differentiated for 7 days using the osteocyte protocol. Osteoblast differentiation was confirmed by testing both sets of cultures for expression of several osteocyte-specific mRNA markers (Figure 4). The cultures were collected after 7 days of the differentiation protocol, long before most osteoblast markers are significantly increased (for example, only alkaline phosphatase, Runx2, and osteopontin are significantly increased for the pooled cultures, and alkaline phosphatase for Donor 5) although all markers exhibit trend towards increased levels in both sets of cultures, demonstrating that the differentiation culture protocol was effective. A comparison between the undifferentiated and differentiated MSCs from either donor source confirms the set of selected osteogenic microRNAs (Clusters 1 and 2 in Figure 2B; see Table 3 for cluster membership) as significantly regulated during differentiation, and supports the conclusion that these microRNAs are regulated in all MSC by demonstrating their regulation in two separate MSC preparations—one a pool of three donors and one a fourth individual donor. Furthermore, the increased levels of these microRNA markers are uniformly more informative at classifying osteocyte differentiation than the traditional mRNA markers (Figure 4), confirming our prediction that microRNA changes precede phenotype changes and may be an earlier marker for assessing differentiation in MSC.

Prediction of potential mRNA targets of MSC associated miRNA

In order to investigate the specific role the PDGF pathway may have on the regulation of microRNAs, we attempted to predict responses to this pathway via bioinformatic analysis of putative promoter regions. Having no specific evidence for the mechanism of transcriptional control of these microRNAs, we hypothesized that genomic sequences upstream of the microRNA coding sites would likely contain regulatory sites responding to PDGF-stimulated transcription factors. Certainly this hypothesis would not be expected to apply to every microRNA, since microRNAs may be expressed intronically [63], for example, but it serves to allow a generalized testing by bioinformatic modeling and pharmacologic manipulation.

A comprehensive literature search identified seven documented downstream effectors of the PDGF-pathway (Figure 5A). Position weight matrices for each of these transcription factors were obtained from the TRANSFAC v11 database [58]. As an approximation of proposed regulatory regions, five thousand base-pair (5 kb) sequences upstream of human microRNA genomic coding sites were collected and scanned for potential PDGF transcription factor binding sites. As predicted, the collection of MSC-regulated microRNA promoter sequences was enriched for the PDGF-responsive binding sites, possessing significantly more binding

site predictions than expressed but unregulated promoters, $p < 0.01$ (Table 2). Thus, the expression of MSC-regulated microRNAs is predicted, as a class, to be regulated by transcription factors within the PDGF pathway.

Next we sought to identify those individual microRNAs having significantly enriched PDGF response elements. A Z-test was used to compare the number of binding sites for each MSC-regulated microRNA against a mean number of binding sites for all 63 expressed microRNAs (Table 3). Using a significance threshold of $p < 0.05$ and a 5% FDR to correct for multiple comparisons, no individual microRNA was found to be enriched for the PDGF pathway. However, we conclude that microRNAs with the lowest probability of satisfying the null hypothesis provide an exploratory confidence list for experimental confirmation. The resulting confidence list is rank-ordered by p-value and represents our hypothesized index of microRNAs predicted to be regulated by the PDGF pathway during differentiation of MSCs (Table 3).

Confirmation of PDGF-regulated microRNAs

To test our bioinformatic predictions and to determine the effect of the PDGF-pathway inhibition on the expression of significantly regulated microRNAs, we treated pooled MSCs with the protein tyrosine kinase inhibitor Tyrphostin AG-370 during a 7 day osteogenic differentiation protocol (Figure 3A), to focus on early microRNA changes and to reduce effects of the long-term toxicity of AG-370 [52]. As hypothesized, the majority of “high-ranking” regulated microRNAs demonstrate significantly reduced expression ($p < 0.05$) in the presence of AG-370 (Figure 3A—comparisons between differentiated untreated and AG-370-treated). Those microRNAs with predicted responses falling lower on the confidence list showed less of a global reduction in expression upon treatment with the PDGF inhibitor. Pretreatment (48 hrs) with AG-370 resulted in a more complex result with several microRNAs responding to the pre-treatment by exhibiting a reduced expression level, while other microRNAs demonstrated a significant increase upon differentiation, perhaps as a result of a pre-conditioning effect (results not shown). Regardless, the direct inhibition of PDGF during osteogenic differentiation of MSCs resulted in the reduced expression of several microRNAs.

These results in Figure 3A represent the average response from the four MSC isolates that were pooled in this culture. To test whether predictions would also apply to a biologically-distinct MSC isolate, we repeated the AG-370 inhibition study with MSC derived from a single 39-year old male donor (Donor 5, Figure 3B). As expected, some microRNAs exhibited a different sensitivity to AG-370 inhibition, however, the majority of microRNAs ranking highly on the prediction list (Table 3) were similarly inhibited in each cell preparation. We conclude that PDGF mediation of microRNA regulation, whether due to a direct or indirect mechanism, is a general feature of MSC differentiation into osteoblasts and may play a role in promoting differentiation. More importantly, pathway analysis of microRNA expression patterns during MSC differentiation yields interpretable results that may be tested using standard biochemical techniques.

Discussion

Our goal was not only to identify microRNAs that are regulated during differentiation of MSC but also to predict a subset of microRNAs that could be regulated by a specific signaling pathway. As an example, we chose the PDGF signaling pathway and its regulated transcription factors (Figure 5A). By measuring the frequency of finding transcription factor binding sites (using position weight matrices from the TRANSFAC database) within 2 kb upstream of mature microRNA on the genome, and comparing frequencies between microRNAs regulated during osteogenesis with those that are not regulated, we identified a set of putatively PDGF-regulated microRNAs. To test this prediction, we inhibited PDGF signaling during osteogenic

differentiation and found that many of the predicted microRNAs are affected (Figure 3). This leads to our hypothesized regulatory network, as depicted in Figure 5B. PDGF signaling would be expected to regulate a target set of transcription factors, among other genes [52]. In addition to downstream regulation of target mRNAs, these PDGF-regulated transcription factors may also regulate microRNA transcription. The resulting microRNAs would be predicted to inhibit, for example, non-osteogenic mRNA translation, reinforcing the regulatory network. By predicting PDGF-regulated microRNAs and then confirming the involvement of PDGF in their regulation, we have laid the initial groundwork to support this novel network in osteogenic differentiation.

In general, the multilineage differentiation potential and the possibility of using an autologous source of MSC isolated from patient bone marrow makes it an appealing and promising cell therapy agent for treating various human diseases [10]. Generating sufficient amounts of cells for therapeutic application requires extensive culture of MSC in vitro starting from the bone marrow [5]. Several methods of expansion and maintenance resulting in MSC with varying differentiation potential have been reported [64-66]. To promote these cells as routine clinical agents, more detailed characterization of the molecular regulatory network level is necessary.

We characterized individual and/or pooled MSC using cell sorting of surface markers (Fig. 1A), cytochemistry of differentiated cultures (Fig. 1B), and a comprehensive screen of the gene expression and microRNA expression patterns of MSC and the changes that occur upon differentiation into adipocytes or osteocytes (Fig. 1C, Table 1, Supplemental Table 1). Samples used for expression analysis were similar in their proliferative potential, based on their population doubling time (not shown), and differentiation potential, based on morphology and staining pattern of differentiated cells. Gene expression profiling demonstrated several sources of variability among MSC isolates but it readily distinguished them from other pluripotent cell types such as ESC [67]. MSC are heterogeneous populations of cells. MSC are derived from different tissue sources and different donors, leading to a high degree of variability between isolates, as demonstrated by the correlation studies in Supplemental Figure 1. Furthermore, unlike most ESC, the same MSC cell line can vary with passage, adding to the difficulty in describing these cultures with gene expression. Demonstrating with this issue, the correlation value between one donor-derived MSC was somewhat different from another, indicating basal differences in donor MSC (e.g., R^2 of 0.912 between MSC from two donors in Supplemental Figure 1E). A similar deviation was also observed with the same donor MSC but differing by one passage with variation largely resulting from low-level expression of genes (0.804, Supplemental Figure 1F). The correlation value of MSC and differentiated progeny was much lower indicating a greater deviation in the global gene expression profile upon differentiation. In spite of the differences between the various MSC populations, we were able to select a consistent set of genes for which the change in pattern of expressed gene between undifferentiated MSC and cells differentiated to adipocytes or osteocytes tightly cluster indicating the overall similarity between the cell groups (Supplemental Figure 1B).

The role of microRNA expression as a key regulatory element in differentiation is recognized but is still poorly defined at the level of specific mechanisms. Interestingly, the pattern of microRNA expression in MSC is substantially different from pluripotent stem cells such as human embryonal carcinoma and human ESC [49,50]. Known pluripotent associated markers such as miR302a, b, c and d, and miR 200c are absent in MSC (see Table 3). Again, despite the heterogeneous nature of MSC, a group of microRNAs was identified as differentially expressed between MSCs, differentiated adipocytes, chondrocytes, and osteocytes.

Collection of both microRNA and mRNA gene expression patterns enables us to begin to examine specific cellular mechanisms or pathway regulated during MSC differentiation. As an example of this approach, we describe the predicted regulation of a set of microRNAs by

members of the platelet-derived growth factor (PDGF) pathway, which was previously identified through pathway analysis of MSC gene expression results [52]. While PDGF is not normally known as a mediator of osteogenic differentiation, the addition of AG-370 reduced the number of osteocytes and mineralization [52]. It remains possible that the inhibition of PDGF has an indirect effect on osteogenic differentiation through reduced proliferation, but this would also require activation of intracellular signaling. We were able to confirm the involvement of this specific pathway in the osteogenic differentiation of MSCs. We then successfully determined that genes in this pathway have a greater likelihood of regulating microRNAs identified as significant from the same samples, based on the content of their upstream sequences (Table 3). While we were unable to statistically predict specific microRNAs having an enriched presence of PDGF-responsive binding sites, we were able to demonstrate good concordance between an exploratory ranking (Table 3) and results of PDGF pathway inhibition using AG-370 in both pooled MSCs (Figure 3A) and a biologically distinct MSC preparation (Figure 3B). In another example of PDGF involvement with microRNAs, mir-140 was found to inhibit PDGF signaling in zebrafish, helping to control formation of palate [68], demonstrating a complementary relationship in a developmental program other than MSC. Together, these studies demonstrate how existing network information can be applied in conjunction with microRNA expression data to begin to unravel the as yet elusive mechanisms governing the regulation of these small molecules.

A second benefit to this approach is realized when we examine the target predictions using regulated mRNAs and microRNAs to generate hypotheses for future analyses. These associations are abundant across our two datasets. For example, *SLC7A5* is predicted by the RNA22 algorithm [69] to be targeted by mir-26A, which increases in adipocytes. However, *SLC7A5* mRNA increases in both adipocytes and osteoblasts, and this mRNA is not normally found in adult adipocytes but is found in bone. Perhaps the adipocyte-specific transcriptional regulation of *SLC7A5* is not yet active and the microRNA inhibition of expression shifts the cell away from bone phenotype. Another example, *STARD8*, is similarly expressed in bone but not adipose tissue. This mRNA is increased during both adipocyte and osteoblast differentiation over MSC and is predicted to be targeted by mir-31. Since mir-31 is not reduced in adipocytes, does this inhibitory mechanism prevent production of *STARD8* in adipocytes?

Transcription factors are likely to participate in differentiation mechanisms. *GATA6*, known to be involved in cardiac and lung differentiation [70], is increased in osteoblasts compared with MSC but not in adipocytes. Since mir-31 continues to be expressed in adipocytes, this may be a mechanism for inhibiting continued expression of *GATA6* as MSC differentiate into adipocytes. *ARNT2*, an aryl hydrocarbon nuclear translocator protein known to be expressed in bone stromal cells [71] is another target of mir-31 that may be inhibited in adipocytes. *ARNT2* may be de-repressed since mir-31 decreases in osteoblasts. The majority of these predictions focus on mir-31 as a likely inhibitor of the osteocyte differentiation pathway and a good candidate for knockdown in future experiments.

While variability in the microRNA expression between MSC derived from different donors is apparent both in its undifferentiated state and cells differentiated to adipocytes or osteoblast, the pattern of microRNA expression is distinct from earlier reported expression in human embryonic stem cells and their differentiated cells. Combined analysis of microRNAs and mRNAs expression in heterogeneous stem cell populations such as MSC provides a tool to identify possible microRNA regulators and their gene targets providing a rationale for further perturbation studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Characterization of MSC and differentiation protocols

A preparation of pooled MSC from four individual isolates (see Methods) was assessed for cellular markers before and after differentiation. (A) FACS analysis for the absence (CD34, CD45) or presence (CD44, CD73, CD105) of surface antigens. Shaded peaks represent antibody labelled cells while the blank peaks are Antibody isotype controls. (B) MSC cells differentiated into chondrocytes, adipocytes, osteocytes for 7 and 14 days and subsequently stained with Alcian Blue, Oil red and Von Kossa staining, respectively. (C) Comparison of post-hoc subsets of mRNAs. Individual donors (Donor 1 cultured at 7 or 8 passages and Donors 2 or 3 cultured at 10 passages) were assessed for gene expression by Illumina BeadChip arrays (see Supplemental Methods). A Venn diagram compares the overlap between 243 genes

selected as differentially expressed between MSC and osteoblasts (red) or adipocytes (green) with the 48 overlapping genes labelled in yellow. Selected examples of genes in each category are shown. These results, along with known MSC markers shown in Table 1 and the ANOVA-selected genes in Supplemental Table 1, demonstrate that each cell isolate exhibits appropriate cellular markers and validates cell sources as well as differentiation protocols.

Figure 2. Exploratory microRNA expression patterns in MSC and differentiating products

A set of pooled MSC were prepared for microRNA analysis on microarrays. (A) Hierarchically-clustered heatmap of 44 microRNA probes, corresponding to 31 unique mature microRNAs, identified by testing Hypothesis 1 at 5% FDR. The probe list includes non-human microRNA sequences, likely due to the inability of hybridization to detect single-nucleotide mismatches in all cases, as described previously [55]. However, similar probes (such as human let-7f and mouse let-7f) cluster similarly and likely detect the same mature human microRNA. Results are color-coded by Z-score-normalized expression levels according to the legend and frequency histogram shown as an inset. (B) K-means clusters of the 27 unique human microRNAs corresponding to the probes declared significant. From the list of significantly-regulated

microRNA probes (panel A), unique human mature microRNAs were extracted and clustered using $k=4$, which produced the best fit of merit compromise compared with other numbers of clusters tested. Identities of cluster members are shown in Table 3.

Figure 3. qPCR assay of microRNAs predicted to be regulated upon osteocyte differentiation or AG-370 treatment

Predictions were tested in either (A) pooled MSC cultures identical to those used in the microarrays or (B) from a biologically distinct donor (Donor 5). In each case, triplicate cultures of MSC were collected as undifferentiated (red bars), differentiated for 7 days following the osteocyte protocol (light blue), or treated with the PDGF pathway inhibitor AG-370 (dark blue) during differentiation. Results depict the mean fold-change from undifferentiated levels, $\pm$ SEM ($n=3$). To confirm the exploratory microarray results, undifferentiated cultures were compared with differentiated cultures using Student's t-test at $p < 0.05$ (*). To confirm predicted regulation by PDGF pathway intermediates, differentiated cultures were compared with

AG-370-treated cultures by Student's t-test at $p < 0.05$ (§). For each microRNA assay, the rank position in the confidence list (Table 3) is shown in parentheses.

Figure 4. Confirmation of osteocyte phenotype upon differentiation of MSC cultures

Pooled donor MSC cultures (red) or individual Donor 5 cultures (blue) were assayed for osteocyte marker mRNAs after 7 days of differentiation by qPCR. Mean $\pm$ SEM ($n=3$ cultures) values of RQ (relative quantities, based on GAPDH internal control mRNA and undifferentiated cultures using the $\Delta\Delta C_t$ method). Differences were tested using Student's t-test and are marked with an asterisk (*) as increased from undifferentiated or a dagger (‡) as decreased with AG-370 treatment from differentiated at $p < 0.05$.

Figure 5. Regulatory networks in MSC differentiation. (A) PDGF-responsive pathways leading to regulation of transcription factors

This pathway was assembled using Pathway Architect (Stratagene) to identify potentially regulated transcription factors likely to be affected by PDGF receptor stimulation. Each pathway is supported by at least one publication from the scientific literature (not shown). **(B) Proposed regulatory network.** PDGF signalling, inhibited by Tyrophostin AG-370, induces specific transcription factors (identified in panel A), in turn regulating both osteogenic target mRNA genes and a specific set of microRNAs, including let-7, miR-24, miR-125b, and miR-138. Increased levels of those microRNAs are predicted to inhibit translation of non-osteogenic target mRNAs to diminish their inhibition of osteogenesis.

Table 1

Expression patterns of known MSC differentiation markers To compare cultures of individual MSC isolates with previous gene expression studies, mean $\pm$ SEM expression levels are shown for three differentiation conditions (MSC, Adipo, Osteo) using four different cell conditions: Donor 1/Passage 7; Donor 1/Passage 8; Donor 2/Passage 7; and Donor 3/Passage 7. The values are the background-subtracted gene expression measurements taken from Illumina BeadChips. Within each group of genes arranged by cell-type specificity, genes are sorted by mean expression level. Results indicate overall expression trends agreeing with previous studies yet demonstrating variability among individual isolates. Genes shown with an asterisk (*) are present on the ANOVA list (Supplemental Table 1). Genes shown with a dagger (†) were not found on the list of genes expressed in $\geq 50\%$ of samples so were not selected by ANOVA.

Specificity	Gene Symbol	Illumina Transcript ID	MSC	Adipo	Osteo	Description	UniGene ID	Entrez Gene ID
Adipocyte	*FABP4	GI 4557578-S	400.5 $\pm$ 265.36	20,925.5 $\pm$ 4,857.31	28.3 $\pm$ 10.67	Fatty acid binding protein 4, adipocyte	Hs.391561	2167
	*LPL	GI 4557726-S	95.4 $\pm$ 36.42	10,304.8 $\pm$ 2,360.04	1.3 $\pm$ 2.57	Lipoprotein lipase	Hs.180878	4023
	*CEBPA	GI 28872793-S	119.5 $\pm$ 48.35	2,818.9 $\pm$ 542.17	48.9 $\pm$ 7.27	CCAAT/enhancer binding protein (C/EBP), α	Hs.76171	1050
	PPARG	GI 20336230-A	470.7 $\pm$ 57.79	2,788.1 $\pm$ 822.11	17.9 $\pm$ 10.35	Peroxisome proliferator-activated receptor gamma	Hs.162646	5468
	PGD	GI 40068517-S	198.0 $\pm$ 50.67	1,933.7 $\pm$ 560.00	429.3 $\pm$ 56.51	Phosphogluconate dehydrogenase	Hs.464071	5226
	*LEP	GI 4557714-S	1,746.5 $\pm$ 776.09	1,477.7 $\pm$ 485.87	10.2 $\pm$ 1.74	Leptin	Hs.194236	3952
	*SREBF1	GI 22547194-S	553.8 $\pm$ 165.11	1,004.1 $\pm$ 187.68	192.5 $\pm$ 9.39	Sterol regulatory element binding transcription factor 1	Hs.592123	6720
	POSTN	GI 5453833-S	1,871.8 $\pm$ 740.57	101.0 $\pm$ 65.00	3,206.4 $\pm$ 1,091.90	Perlecan, osteoblast specific factor	Hs.136348	10631
	BGLAP	GI 6005831-S	224.7 $\pm$ 19.29	158.0 $\pm$ 23.35	361.5 $\pm$ 45.60	Bone γ -carboxyglutamate (glu) protein (osteocalcin)	Hs.530479	11243
	*ALPL	GI 13787191-S	5.2 $\pm$ 8.86	28.8 $\pm$ 7.76	13.2 $\pm$ 7.50	Alkaline phosphatase, intestinal	Hs.37009	248
Osteocyte	*SPPI (OPN)	GI 38146097-S	419.9 $\pm$ 318.25	12.8 $\pm$ 4.85	9.7 $\pm$ 1.24	Secreted phosphoprotein 1 (osteopontin)	Hs.313	6696
	*RUNX2	GI 10863884-S	-0.6 $\pm$ 0.54	-5.6 $\pm$ 1.28	-9.6 $\pm$ 1.99	Runx-related transcription factor 2	Hs.535845	860
	VIM	GI 4507894-S	10,458.0 $\pm$ 2,159.34	12,398.5 $\pm$ 1,724.50	17,530.6 $\pm$ 3,878.75	Vimentin	Hs.642813	7431
	*ANXA2	GI 4757755-S	8,279.6 $\pm$ 1,988.74	6,004.0 $\pm$ 586.20	13,698.9 $\pm$ 2,187.90	Annexin A2	Hs.511605	302
	PDGFRA	GI 15451787-S	2,251.3 $\pm$ 434.88	877.1 $\pm$ 211.46	1,251.5 $\pm$ 260.93	Platelet-derived growth factor receptor, α polypeptide	Hs.74615	5156
	THY1	GI 24475732-S	1,875.8 $\pm$ 660.88	1,072.8 $\pm$ 383.72	2,431.3 $\pm$ 188.53	Thy-1 cell surface antigen; synonyms: CD90		
	ENG (CD105)	GI 4557554-S	736.9 $\pm$ 72.56	221.2 $\pm$ 92.75	1,765.2 $\pm$ 227.50	Endoglin	Hs.76753	2022
	FZD2	GI 5922012-S	537.1 $\pm$ 9.04	261.5 $\pm$ 81.29	1,202.5 $\pm$ 198.59	Frizzled homolog 2	Hs.142912	2535
	*CD44	GI 21361192-S	229.0 $\pm$ 32.87	220.1 $\pm$ 44.68	1,098.6 $\pm$ 134.15	CD44	Hs.502328	960
	*COL2A1	GI 18780272-S	185.0 $\pm$ 68.76	11.5 $\pm$ 11.28	-0.3 $\pm$ 4.75	Collagen, type XXI, α 1	Hs.47629	81578
Housekeeping	*BST1	GI 4757873-S	27.1 $\pm$ 8.65	-2.7 $\pm$ 5.42	343.1 $\pm$ 50.35	Bone marrow stromal cell antigen 1	Hs.169998	683
	B2M	GI 37704380-S	11,351.1 $\pm$ 1,472.97	8,040.0 $\pm$ 889.31	11,908.4 $\pm$ 1,482.55	β 2-microglobulin	Hs.534255	567

Table 2

Frequency of position weight matrix hits specific for PDGF pathway in expressed but not regulated microRNAs vs. regulated microRNAs To test whether potentially PDGF-responsive DNA sequences could be found within a genomic region 5 kb upstream of microRNAs regulated during osteogenesis, we counted the number of "hits" or sequences matching the position weight matrix (PWM) describing all variations of a known transcription factor binding site. The TRANSFAC matrix identifier is listed for each PWM matching a PDGF pathway transcription factor (summarized in Figure 5A). Two groups of results are shown for non-regulated microRNA that were found to be expressed in MSC and differentiated products and for microRNAs selected as significantly regulated (see Figure 2 and Table 3).

Transcription Factor	Matrix Identifier	Hits	Non-regulated Hits/miRNA	Hits	Regulated Hits/miRNA
AhR	V\$AHR_01	11	0.46	15	0.38
	V\$AHR_Q5	1	0.04	1	0.03
	V\$AHRARNT_01	2	0.08	7	0.18
	V\$AHRARNT_02	1	0.04	13	0.33
	V\$AHRHIF_Q6	0	0.00	17	0.44
Jun(2)/AP1	V\$AP1_01	38	1.58	83	2.13
	V\$AP1_Q2_01	9	0.38	21	0.54
	V\$AP1_Q4_01	1	0.04	6	0.15
	V\$AP1_Q6_01	7	0.29	13	0.33
	V\$APIFJ_Q2	6	0.25	11	0.28
NFKB1	V\$EBOX_Q6_01	9	0.38	26	0.67
	V\$MYC_Q2	10	0.42	44	1.13
	V\$MYC_MAX_01	0	0.00	18	0.46
	V\$MYC_MAX_02	1	0.04	4	0.10
	V\$MYC_MAX_B	1	0.04	0	0.00
STAT3	V\$NMYC_01	0	0.00	5	0.13
	V\$STAT_01	6	0.25	7	0.18
	V\$STAT_Q6	13	0.54	21	0.54
	V\$STAT3_01	7	0.29	4	0.10
	V\$STAT3_02	9	0.38	25	0.64

Table 3

Ranking of individual microRNAs by relative frequencies of transcription-factor binding sites Each unique human microRNA genomic locus (identified by precursor name) is associated with the name of the mature microRNA, the K-means cluster ID (Figure 2B), the z-score p-value, the rank of the p-value, and the Benjamin-Hochberg [60] false discovery (FDR) rate cut-off. The z-score p-value is calculated by comparing the number of PWM matches within this 5 kb region upstream of each microRNA genomic locus with the average match rate for all for regulated and non-regulated microRNA loci. The false discovery rate cut-off lists the p-value that would be within the 5% FDR level if this ranked p-value were less than the cutoff. Therefore, no individual microRNA locus was judged to be significant compared with all expressed microRNA loci. However, the resulting, rank-ordered list highlights microRNAs having a greater chance of being regulated by PDGF at the top and least likely to be regulated by PDGF at the bottom.

miRNA	Precursor	Mature miRNA	NAK-means	Cluster	P-value	Rank	(i)FDR	Cut-off
hsa-mir-24-1	hsa-mir-24	2	0.002	1	0.001			
hsa-let-7a-3	hsa-let-7a	2	0.003	2	0.003			
hsa-let-7b	hsa-let-7b	2	0.009	3	0.004			
hsa-mir-30a	hsa-mir-30a	3	0.069	4	0.005			
hsa-mir-31	hsa-mir-31	3	0.095	5	0.006			
hsa-mir-125b-2	hsa-mir-125b	2	0.129	6	0.008			
hsa-mir-138-1	hsa-mir-138	3	0.129	7	0.009			
hsa-mir-22	hsa-mir-22	1	0.129	8	0.010			
hsa-let-7c	hsa-let-7c	2	0.160	9	0.012			
hsa-let-7g	hsa-let-7g	2	0.160	10	0.013			
hsa-mir-16-1	hsa-mir-16	3	0.160	11	0.014			
hsa-mir-26a-2	hsa-mir-26a	2	0.210	12	0.015			
hsa-mir-23a	hsa-mir-23a	2	0.220	13	0.017			
hsa-mir-27a	hsa-mir-27a	2	0.220	14	0.018			
hsa-mir-27b	hsa-mir-27b	2	0.220	15	0.019			
hsa-let-7i	hsa-let-7i	2	0.260	16	0.021			
hsa-mir-26a-1	hsa-mir-26a	2	0.260	17	0.022			
hsa-mir-24-2	hsa-mir-24	2	0.270	18	0.023			
hsa-mir-638	hsa-mir-638	4	0.270	19	0.024			
hsa-let-7a-1	hsa-let-7a	2	0.320	20	0.026			
hsa-let-7a-2	hsa-let-7a	2	0.320	21	0.027			
hsa-let-7f-1	hsa-let-7f	2	0.320	22	0.028			
hsa-mir-125b-1	hsa-mir-125b	2	0.320	23	0.029			
hsa-mir-143	hsa-mir-143	1	0.320	24	0.031			
hsa-mir-16-2	hsa-mir-16	3	0.320	25	0.032			
hsa-let-7d	hsa-let-7d	2	0.330	26	0.033			
hsa-mir-23b	hsa-mir-23b	2	0.340	27	0.035			
hsa-mir-29b-2	hsa-mir-29b	2	0.340	28	0.036			
hsa-mir-21	hsa-mir-21	1	0.390	29	0.037			
hsa-mir-222	hsa-mir-222	5	0.390	30	0.038			
hsa-mir-29a	hsa-mir-29a	2	0.390	31	0.040			
hsa-mir-29b-1	hsa-mir-29b	2	0.390	32	0.041			
hsa-mir-138-2	hsa-mir-138	3	0.400	33	0.042			
hsa-mir-145	hsa-mir-145	3	0.400	34	0.044			
hsa-mir-663	hsa-mir-663	4	0.410	35	0.045			
hsa-let-7f-2	hsa-let-7f	2	0.420	36	0.046			
hsa-let-7e	hsa-let-7e	1	0.460	37	0.047			
hsa-mir-221	hsa-mir-221	2	0.460	38	0.049			
hsa-mir-320	hsa-mir-320	1	0.470	39	0.050			