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Comprehensive MicroRNA Profiling Reveals a Unique Human Embryonic Stem Cell Signature Dominated by a Single Seed Sequence

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INTRODUCTION

Embryonic stem cells (ESCs) possess three features that in combination set them apart from all other cell types: the ability to self-renew indefinitely, the potential to generate every differentiated cell type, and a normal genetic complement. In mice, these properties can be demonstrated by the ability of the cells to develop into whole animals by germline transmission. As a proxy for a germline assay, human embryonic stem cells (hESCs) have been shown to be capable of differentiation into all three germ layers, both in culture by embryoid body formation and *in vivo* by teratoma formation. It is our goal to characterize the regulatory processes underlying these properties of hESCs on a molecular level.

MicroRNAs (miRNAs) are small (19-25 nt) endogenous noncoding RNAs that have been shown to influence the abundance and translational efficiency of cognate mRNAs. Discovered in *C. elegans*, miRNAs are known to control critical timepoints in development of plants and lower animals. However, the roles of miRNAs in the development of higher animals are less well understood. Details of the biogenesis and mechanisms of action of

miRNAs continue to be the subjects of intense investigation [1-9].

There is evidence in mouse that miRNAs may be implicated in ESC self-renewal and differentiation. Murine ESCs with either reduced *Dicer1* or absent *Dgcr8*, enzymes necessary for miRNA processing, displayed proliferation defects. In addition, the *Dgcr8* knockouts showed accumulation of cells in G1, which may point to alterations in regulation of cell cycle in these mutant cells [10, 11]. Both mutant murine ESC lines retained expression of pluripotency markers, but were not able to differentiate normally [11, 12]. Of note, *Dicer1*-null homozygous mouse embryos appeared to be unable to produce normal ESCs [13].

Previous reports on miRNAs in ESCs include two studies describing isolation and cloning of novel miRNAs, one in murine ESCs [14] and one in hESCs [15]. These authors confirmed differential expression of a subset of the cloned miRNAs in ESCs by Northern blot. An additional four studies measured miRNA expression in murine ESCs using qRT-PCR [16, 17] and using a microarray-based platform [18, 19]. In all six studies, two clusters of miRNAs were found to be strongly expressed in ESCs

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(mir-302, mmu-mir-290/hsa-mir-371/372/373). The mir-290 cluster has also been noted to be expressed in trophoblast stem cells, suggesting that it may play a role in cellular self-renewal [14, 20].

A recent study reporting the largest miRNA cloning and sequencing effort to date included two samples of murine ESCs [21]. This study involved sequencing ~330,000 clones from 256 small RNA libraries from a wide variety of organs from human, mouse, and rat. The limited sample replication and low clone counts (only ~1000 clones per library were sequenced) make it difficult to glean statistically significant differential expression information from this dataset, but the murine ESC data are generally consistent with the miRNA expression results generated by the other methods discussed above.

The unique biology of miRNAs, as well as limitations in detection and quantitation methods for these small RNAs, has made it difficult to understand their functions in higher animals. It appears that there are likely to be more than 1000 miRNAs in animals. Overexpression experiments indicate that each miRNA can downregulate 100-200 transcripts [22]. Also, transcripts may contain multiple miRNA target sequences in their 3'-UTRs, and hence be regulated by more than one miRNA. Furthermore, there are classes of closely related, but not identical miRNAs that differ at only one or a few nucleotides.

The small size of miRNAs and the existence of closely related types create technical difficulties for detection methods. Traditional methods, such as cloning and Northern blot, are time-consuming and are limited by the low abundance of some miRNAs. Direct hybridization methods are neither sensitive nor specific enough for this application. qRT-PCR methods are sensitive, specific, and quantitative, but are impractical for profiling large numbers of genes in multiple samples.

Here, we describe the application of a novel, robust, and highly reproducible microarray method to generate global miRNA profiles of hESCs, neural stem/progenitor cells (NSCs and NPCs), mesenchymal stem cells (MSCs), and differentiated cells (including a cell line differentiated from an hESC line), and the identification of cell-type-specific differences in miRNA usage that may regulate self-renewal and pluripotence.

MATERIALS AND METHODS

Cell culture:

XE (eXtraembryonic Endoderm) cells were differentiated from WA09 cells in hESC medium [23] with 20 ng/ml bFGF on Matrigel in the absence of feeders. By immunofluorescent antibody staining and gene expression profiling, XE cells do not express ESC-specific markers and do express markers that are associated with primitive endoderm (R Gonzalez, unpublished).

XE cells were differentiated from WA09 cells in hESC medium [23] with 20 ng/ml bFGF on Matrigel in the absence of feeders. XE cells are predominantly euploid (**Supplementary Figure 8**), polygonal, flat cells that grow in monolayer and resemble fibroblasts. By immunofluorescent antibody staining and gene expression profiling, XE cells do not express ESC-specific markers (POU5F1/OCT4, LIN28, EBAF, UTF1, and ZFP42/REX) and do express markers that are associated with primitive endoderm (GATA6, DAB2, SPARC/osteonectin, PLAT, and PLAU) (R Gonzalez, unpublished). The XE cells are genotypically identical to the parent WA09 cells by SNP genotyping using the Illumina Hap550 platform. The SNP genotyping results between the XE cells and two WA09 samples were 99.994% and 99.996% identical, whereas the results between the two WA09 samples were 99.997% identical. These results are within the error of the platform. Unrelated samples are typically ~75% identical.

All other cell types were derived and propagated as described in the references listed in Table 1.

RNA purification

Total RNA, including miRNA, was purified from all cell types using the mirVANA miRNA Isolation Kit (Ambion). Total RNA quantitation was performed using a Nanodrop N-1000 spectrophotometer. RNA quality was demonstrated using the Bio-Rad Experion Automated Electrophoresis System (RNA Standard Sensitivity Kit).

DNA purification

Genomic DNA was purified from WA09 and XE cells using the DNeasy Blood & Tissue Kit (Qiagen).

Microarray quantitation of miRNA expression

Microarray-based miRNA expression profiling was performed using a novel method (Fan et al. in preparation). The method was a modification of the high throughput gene expression profiling assay, the DASL® Assay developed previously [24]. It applied a solid-phase primer extension (after target hybridization) to enhance the discrimination among homologous miRNA sequences. In addition, PCR with universal primers was used to amplify all targets prior to array hybridization.

One specific assay oligonucleotide was designed for each miRNA, consisting of three parts: at the 5' end was a universal PCR priming site; in the middle was an address sequence, complementary to a corresponding capture sequence on the array; and at the 3' end was a miRNA-specific sequence. 700 assay probes were designed, corresponding to 397 well-annotated human miRNA sequences (Sanger miRBase Release v9.0 in October, 2006, miRBase: <http://microrna.sanger.ac.uk/>) and 303 miRNAs identified recently from human and chimpanzee brain [25].

Pooled assay oligonucleotides corresponding to the 700 human miRNAs are first annealed to cDNA. An allele-specific primer extension step is then carried out; the assay oligonucleotides are extended only if their 3' bases are

complementary to their cognate sequence in the cDNA template. The extended products are then amplified by PCR using common primers, of which one is fluorescently labeled, and hybridized to a microarray bearing the complementary address sequences. The DASL process, array image processing and signal extraction was as described previously [24].

miRNA microarray data processing

Data preprocessing was performed in BeadStudio v2.0. Data from each microarray was quantiles-normalized using Expander [26]. miRNAs undetectable in all samples were removed. Technical replicates were averaged, and then biological replicates were averaged. For details on further analysis, see **Supplementary Text Part 2**.

Data analysis. For further details please see Supplementary Text Part 3.T-test

For the hESC vs. non-hESC analysis, Welch's t-test was performed with a p-value cutoff of 0.05, multiple testing correction by False Discovery Rate (implemented in GeneSpring [27]).

Consensus clustering

Consensus clustering was performed using Pearson distance and average linkage [28] (implemented in GenePattern [29, 30]). For each value of k varying from 2 to 10, 100 iterations were performed. The consensus cumulative distribution function (cdf) and delta area plots were examined, and k=6 was determined to be the model with the smallest k for which the consensus cdf plot approximated the ideal step function, with insignificant proportional increases in the delta area with increasing k's above 6 (**Supplementary Figure 3**).

miRNA grouping

miRNA grouping was performed using the CLICK algorithm [26] via the Expander software [31]. Computation of p-values to

determine significance of overlaps between miRNA groups and annotations were performed by computing the tail of the hypergeometric distribution [32].

miRNA clustering analysis

miRNAs were considered to belong to the same genomic cluster if the genomic locations of the first nucleotides of the predicted pre-miRNA hairpins are within 50 kb (as suggested previously [33]).

Seed similarity graph

miRNA seed sequences were aligned using the Needleman-Wunsch algorithm [34]. A similarity graph was constructed, where edges connected miRNA pairs with 6-7 identical positions in the alignment. The graph was subsequently clustered using CAST [35]. The clustering results were displayed using Cytoscape [36].

Consensus seed sequence identification

Consensus seed sequences for groups of miRNAs with related seed sequences upregulated in hESCs relative to non-hESCs were calculated using ClustalW [37].

Spectral Karyotyping

Cells were harvested and karyotyped [23]. Karyotyping was done using SkyPaint™ and SkyView™ software according to the manufacturer's instructions (Applied Spectral Imaging).

RESULTS

We used a novel microarray-based method (see **Materials and Methods**) to determine the expression of 397 mature human miRNAs listed in the Sanger database version 9.0, October 2006, and of 303 miRNAs recently identified in human brain [25] in 62 samples representing 26 cell lines, including hESCs, NSCs, NPCs, MSCs, and differentiated cells [38-49] (**Table 1**). There were two to four biological replicates per cell

line, and two technical replicates per biological replicate (see **Supplementary Text Part 4** for details). Raw data is given in Supplementary Table 1. After preprocessing and filtering, bioinformatic analysis techniques were applied to the data (see **Supplementary Figure 1** for diagram of experimental design).

We verified that reproducibility of technical and biological replicates was excellent, and that the reported results are robust to the number of biological replicates used (see **Supplementary Text Parts 1 and 2**, and **Supplementary Figure 2**).

miRNAs differentially expressed between hESCs and differentiated cells are spatially coregulated

We initially focused on miRNAs differentially expressed in hESCs compared to the other cell types. miRNA genes occur in the genome as independently transcribed units, in introns of coding genes, and in clusters that are transcribed as polycistrons [50, 51]. When the differential expression was plotted against the genomic location, it was apparent that a large proportion of the differentially expressed miRNAs in hESCs occurred in clusters (the most prominent clusters are shown in **Figure 1**, data plotted across all chromosomes is shown in **Supplementary Figure 3**).

The most prominent upregulated clusters are found on chromosomes 4, 13, 19 and X. The mir-302 cluster, located on chromosome 4, has been associated with murine and human ESCs [14-16]. Chromosome 19 contains two subclusters located 25 kb apart, an ESC-associated cluster consisting of hsa-mir-371/372/373 [14-16] and a large primate-specific placenta-associated cluster containing 54 miRNAs spanning 96 kb [52]. Two paralogous clusters occur on chromosome 13 (mir-17 cluster) and the X chromosome (mir-106a cluster). The chromosome 13 cluster is associated with a number of cancers [50], and has been shown to be upregulated by *MYC* and to downregulate *E2F1* [53]. Interestingly, in

mouse, *Myc* has been shown, in combination with *Sox2*, *Pou5f1/Oct4*, and *Klf4*, to be sufficient for transforming somatic cells into ESC-like cells induced pluripotent stem cells (iPS cells) capable of germline transmission [54-56].

A large bipartite cluster on chromosome 14 (11 and 46 miRNAs spanning 59 kb and 45 kb, respectively) is downregulated in hESCs. This cluster is located downstream of the reciprocally expressed imprinted *DLK1* and *GTL2/MEG3* genes. This cluster was first identified in mouse [57] and noted to be a maternally-expressed imprinted cluster, with expression controlled by an intergenic differentially-methylated region (DMR) located between the *DLK1* and *GTL2/MEG3* genes.

Identification of a large number of miRNAs not previously associated with ESCs

In order to identify hESC-specific expression of miRNAs, we extracted a list of 150 miRNAs that were significantly differentially expressed in hESCs (FDR < 0.05). Seventy-six were upregulated and seventy-four were downregulated (**Figure 2**). Previous reports have identified thirty-seven of these to be differentially regulated in murine or human ESCs [14-16].

Figure 2 is a plot of our differential expression results compared with findings from the previous reports. For the miRNAs with data available from earlier reports, the concordance in assignment of up- and down-regulation is good, particularly when the findings have been reported in more than one study. However, we have discovered novel hESC-specific differences in miRNA expression. These include the large downregulated cluster on chromosome 14 and the large upregulated cluster on chromosome 19. We have confirmed these findings by qRT-PCR (not shown).

Oncogenic miRNAs are upregulated and tumor suppressor miRNAs are downregulated in hESCs

A number of miRNAs have been associated with human cancers [58, 59]. Based on the literature, cancer related miRNAs can be categorized as oncogenic or tumor suppressor. In addition to experimental evidence pointing to the roles of these miRNAs in human cancers, these miRNAs have been shown to target mRNAs whose products have significant roles in cancer [60, 61]. Our data indicated that the oncogenic miRNAs were significantly upregulated ($p = 0.008$), and the tumor suppressor miRNAs were significantly downregulated ($p = 4.75 \times 10^{-4}$), in hESCs (**Figure 2**).

hESCs possess a distinct miRNA profile

In order to investigate the potential utility of miRNA profiling in classifying diverse cell types, we performed unsupervised consensus clustering of cell samples using an agglomerative hierarchical clustering algorithm, which showed that there were four major cell sample clusters (**Figure 3A and Supplementary Figure 4**). The hESC samples were all found in a single cluster, containing two subclusters. The neural lineage cells, on the other hand, partitioned into an adult NPC (aNPC) cluster and a fetal NSC (fNSC) cluster. The MSCs and differentiated cell types, including the fibroblasts, HUVECs, glial cells, and XE cells, formed a fourth major cluster. These four major clusters were also found when the data was analyzed using two other unsupervised clustering methods, non-negative matrix factorization (NMF) and K-means (**Supplementary Text Part 5 and Supplementary Figures 5 and 6**).

The two choriocarcinoma cell lines (BEWO and JEG3) were not closely associated with any of the four major clusters, or with each other. However, the JEG3 line shares characteristics with members of the fNSC cluster (**Figure 3**). These results demonstrate the utility of unsupervised classification in discovering differences among ostensibly closely related cell types.

miRNA expression profiles distinguish categories of cell types

To better understand the relationships between cell types, we also identified groups of miRNAs that share patterns of expression (we are using the term “grouping” here to avoid confusion with the term “miRNA cluster” used previously, that indicates spatial clustering of miRNA genes in the genome). Using an algorithm which maximizes both the within-group homogeneity and the between-group separation [26], we formed seven miRNA groups, each sharing a distinct expression pattern (**Figure 3B and 3C**). Only twenty miRNAs did not fall into one of the seven expression pattern groups (Group 0, **Figure 3C and Table 2**).

The chromosomal locations of the miRNAs in each group were nonrandom (**Supplementary Figure 7**). For instance, the groups that were differentially expressed in hESCs were enriched for members that were clustered in the genome, and depleted of members located in introns of coding genes (**Supplementary Figure 7**).

Table 2 summarizes the composition of each miRNA group, and surveys the available knowledge about the component miRNAs. miRNAs have been associated in previous studies with organs, chromosome locations, activation by specific transcription factors, and other properties. We focused on seven of these associations. Our dataset contained 208 miRNAs originally identified by deep sequencing of small RNAs from human brain (“Human brain expressed”) [25], 51 miRNAs that have been described in the literature as “CNS-related” [62-64], 13 miRNAs regulated by REST (“REST-regulated”), which is involved in repressing neural-specific genes in non-neural cells [65], 33 “Oncogenic” miRNAs [59], 25 “Tumor suppressor” miRNAs [59], 44 miRNAs in the chromosome 14 cluster, and 44 miRNAs in the chromosome 19 cluster.

Groups 1 and 2 accounted for the bulk of the differences between the aNPCs and fNSCs. Group 1 contained 184 miRNAs that were upregulated in aNPCs relative to the other cell

types. This group was significantly enriched for “Human brain expressed” miRNAs. Group 2 consisted of 106 miRNAs upregulated in fNSCs, and mildly downregulated in the aNPCs. The “CNS-related” and “REST-regulated” miRNAs were overrepresented in this group.

Group 3 contained 100 miRNAs and had a more complex profile. In general, the expression of the miRNAs in this group was correlated with the degree of differentiation, with lowest expression in the hESCs, intermediate expression in the NSCs/NPCs, and highest expression in the differentiated cells. The “Tumor suppressor” and “CNS-related” miRNAs were overrepresented in this group. Notably, most of the miRNAs in the large cluster on chromosome 14 also belonged to this group

The 70 miRNAs in Group 4 were mildly upregulated in the hESCs, and very strongly expressed in the BEWO choriocarcinoma cells. Almost all of the members of the chromosome 19 cluster were found in Group 4. Interestingly, this cluster of miRNAs has been strongly associated with the placenta [52].

Group 5 was a group of 51 miRNAs that had a profile of highest expression in the fibroblasts, MSCs, XE cells, and some of the hESC samples. A possible interpretation of this group is that it contains the miRNAs that are upregulated in fibroblast/MS-like cells. The sporadic upregulation of this group of miRNAs in some hESC samples may be due to a degree of heterogeneity in those cultures. The “Human brain-expressed” miRNAs were overexpressed in this group as well.

Group 6 contained 37 miRNAs that are uniquely upregulated in hESCs, and included all of the members of the mir-302 cluster, which has been identified as ESC-specific in prior studies [14-16].

Group 7 consisted of 26 miRNAs that were upregulated in a diverse set of cell types, including one of the glial cell lines, the

HUVECs, and the JEG3s. It was enriched in “Human brain-expressed” miRNAs.

One seed sequence dominates the population of miRNAs upregulated in hESCs

A number of miRNA target prediction models are based on the assumption that nucleotides 2-8 in the mature miRNA sequence constitute a seed sequence that contributes heavily to target mRNA specificity by Watson-Crick base-pairing [66-69]. We compared the seed sequences of hESC-upregulated miRNAs and observed that members of the mir-302 cluster (chromosome 4), the chromosome 19 cluster, the mir-17 cluster (chromosome 13), and the mir-106a cluster (X chromosome), have similar seed sequences. By grouping the miRNAs significantly upregulated (FDR<0.05) in hESCs relative to non-hESCs according to seed sequence, we identified four seed similarity clusters that share near-identical seed sequences (**Figure 4**, only clusters containing at least five miRNAs were considered). The largest cluster has a consensus miRNA seed sequence (AAGTGC) that is dramatically overrepresented in hESCs (**Figure 4A**, p-value = 1.2×10^{-14}). In fact, 18 of the 21 miRNAs containing this seed sequence were among the 76 miRNAs upregulated in hESCs.

We also examined the cognate target mRNAs for the major (**Figure 4A**) as well as the three minor seed similarity clusters (**Figure 4B-D**), as predicted by five miRNA target prediction algorithms, TargetScan [68], MirZ [70-72], PicTar [66], RNA22 [72], and Miranda [71]. With four of the prediction methods, and particularly with the methods using sequence/target sequence matching together with cross-species conservation (TargetScan, MirZ, and PicTar), the targets were significantly enriched for genes with promoters bound by the ESC-associated transcription factors *NANOG*, *SOX2*, and *POU5F1/OCT4* [73] (**Supplementary Table 1**). In many cases, the Gene Ontology categories associated with transcription were also overrepresented in the predicted mRNA targets (**Supplementary Table 1**). The zebrafish embryo expresses a very

abundant miRNA family, the mir-430 family, which contains the same consensus seed sequence as the major hESC-upregulated seed similarity cluster (**Figure 4A**). Remarkably, the targets of this cluster, as predicted by TargetScan and MirZ, were significantly enriched for homologs of mRNAs found to be upregulated in the zebrafish embryo when the mir-430 family was knocked out [74] (**Supplementary Table 1**).

DISCUSSION

Molecular profiling of hESCs in the context of a diverse collection of somatic stem cells and differentiated cells can be used to identify a unique hESC molecular signature, to identify potentially shared pathways between cell types that otherwise are quite dissimilar, and to identify candidate molecular species that warrant functional analysis. The results we report here constitute the most comprehensive determination of miRNA expression in hESCs to date. The strengths of our approach include a highly specific, reproducible, high-throughput platform for measurement of miRNA expression, and a well-curated collection of diverse cell types, including hESCs, NSCs/NPCs, MSCs, and differentiated cells. We report on a large number of novel miRNA-hESC associations, as well as additional evidence of hESC-specific expression for miRNAs previously described to be upregulated in hESCs. Our results indicate that a large proportion of miRNAs differentially expressed in hESCs occur in clusters in the genome, including two very large clusters that have not been associated previously with hESCs. One of these clusters, located in an imprinted region of chromosome 14, is of particular interest. Given the imprinted nature of this region [75] and the fact that aneuploidies and subchromosomal deletions commonly occur during propagation of hESC lines, it is clearly important to examine this region in a number of hESC lines to look for changes in methylation status and for evidence of loss-of-heterozygosity.

We have found that oncogenic miRNAs, including the mir-17 cluster, which has been genetically and functionally associated with human cancers, are overrepresented in the miRNAs upregulated in hESCs, while tumor suppressor miRNAs are overrepresented in the miRNAs downregulated in hESCs. We note that the two choriocarcinoma lines do not cluster closely with the hESC lines, or with each other. It has been observed that the profiles of different cancer cells are quite diverse [76], varying by diagnosis, stage, and prognosis (reviewed in Calin and Croce 2006 [77]). For an individual cancer type, specific oncogenic miRNAs are overexpressed (and specific tumor suppressor miRNAs are downregulated), while others are not. In contrast, the large majority of oncogenic and tumor suppressor miRNAs were found to be differentially expressed in hESCs. This suggests that while hESCs are not closely related to any one type of cancer cell, they may share some mechanisms for self-renewal with cancer cells as a class.

We observed a large set of miRNAs upregulated in hESCs, whose sequences have near-identical seeds. This overrepresentation of a class of highly similar seed sequences points to a critical role for repressing a group of cognate mRNAs in maintaining the stem cell state. The mir-430 family, which appears to be responsible for rapid clearance of maternal mRNAs in the zebrafish embryo, has a seed sequence (AAGTGCT) that matches this consensus seed sequence [74]. The recently discovered mmu-mir-467 family, ten copies of which are present in the mmu-mir-297b cluster on chromosome 2, also contains this seed sequence, and was observed to have the highest clone counts in the mouse ES cells [21].

Previous studies from this and other labs have shown that hESCs have mRNA and DNA methylation profiles that are highly uniform across different hESC preparations (even when those preparations originate from different hESC lines derived and propagated under different conditions in different laboratories), and that are distinct from the profiles from a variety of other

cell types [40, 78-80]. We have shown here that there is also a characteristic hESC miRNA profile that can be used to distinguish hESCs from all other cell types. We have also shown that in contrast to hESCs, cells that have been classified as neural stem and progenitor cells by classical marker studies can be categorized into two distinct subgroups by miRNA profiling. This is consistent with our comprehensive analysis of mRNA profiles in many of the same cell types (Mueller et al., in review).

Grouping miRNAs by coherence in expression pattern across conditions revealed several specific miRNA expression patterns that highlighted a number of subtleties in the expression of related miRNAs. As noted above, the mir-302, hsa-mir-371/372/373, and large chromosome 19 clusters are all upregulated in hESCs and have similar seed sequences, suggesting that they target a similar pool of mRNAs. However, we have seen that while the mir-302 cluster is expressed in a hESC-specific pattern, two of the other clusters upregulated in hESCs (hsa-mir-371/372/373 and the large chromosome 19 clusters) are also highly expressed in one of the trophoblast-type cell lines.

At least two regulatory mechanisms have been shown to result in coregulation of clustered miRNAs. There is evidence that two of the RNA Polymerase II-transcribed miRNA clusters differentially expression in ES cells are transcribed polycistronically. The mir-17-5p cluster was shown to have a pri-miRNA species that contained all the members of the cluster [50], while mouse counterpart to the downregulated cluster on chromosome 14 was shown to have an initiating histone mark in the promoter region and elongation histone marks throughout the remainder of the cluster [81]. In contrast, the large miRNA cluster on chromosome 19 appears to be transcribed by RNA Polymerase III, with RNA Pol III promoters present in Alu repeats occurring between each miRNA gene. Group-specific regulatory elements may be responsible for these

different expression patterns. However, identifying such regulatory sequences will be challenging, as direct experimental evidence on the miRNA composition of many of the presumably polycistronic miRNA transcripts is not available, and the locations of most miRNA transcriptional start sites are unknown.

CONCLUSION

These findings significantly extend our knowledge, and suggest potential roles for miRNAs in regulating cellular pluripotency and self-renewal. As noted above, there have been a number of reports on miRNA expression in embryonic stem cells [14-19]. Ours is the first study based on a well-replicated dataset including samples from a large collection of homogeneous, well-characterized cells run on a novel high-throughput miRNA expression platform. The non-pluripotent cells in this study encompass wide variety of cells, including somatic stem cells, tissue-derived differentiated cells, hESC-derived differentiated cells, and choriocarcinoma cells, which provide a broad context in which to place the hESC samples. The miRNA expression platform is extremely sensitive and specific, and is the most comprehensive high-throughput platform available.

We conclude that miRNA profiling can be used for robust classification of diverse cell types,

and that hESCs possess a unique miRNA signature, with the upregulated miRNAs dominated by a single seed sequence. Our results point to specific individual miRNAs and families of miRNAs that are good candidates for future functional studies into the potential roles of miRNAs in the maintenance of the undifferentiated hESC state and in hESC differentiation.

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Figure 1. Prominent clusters of miRNAs showing differential expression in hESCs compared to non-hESCs.

The log₂ ratio between the average miRNA expression in the hESC samples and in the non-hESC samples is mapped by genomic location. Points to the left of the black lines have lower relative expression in hESCs, while points to the right of the black lines have higher relative expression in hESCs. Solid blue diamonds: miRNAs differentially expressed at an FDR<0.05, empty blue diamonds: the rest of the miRNAs. The x-axis indicating the log₂ ratio is the same scale for all chromosomes; only the x-axis for chromosome 1 is shown. Highlighted clusters: chromosome 4 (mir-302 cluster), chromosome 13 (mir-17 cluster), chromosome 14 (bipartite imprinted cluster), chromosome 19 (primate-specific cluster and mir-371/372/373 cluster), X chromosome (mir-106a cluster). For the highlighted clusters, the log₂ ratios are shown on the x-axes. The images of the chromosome are from the U.S. Department of Energy Genome Programs (<http://genomics.energy.gov>). Note that t-test takes variance into account, and therefore genes with higher log₂ expression ratios do not necessarily have a more significant differential expression.

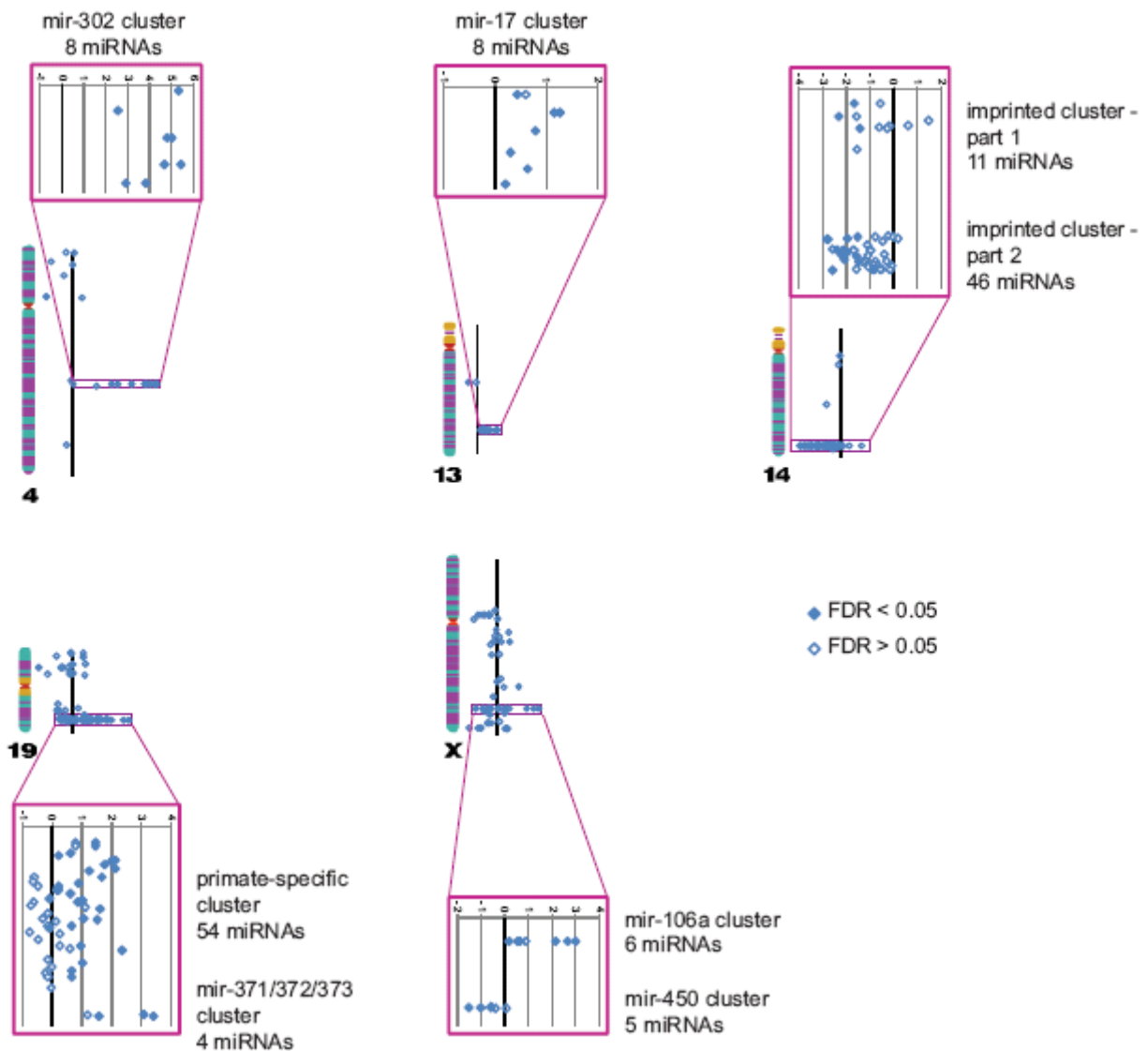


Figure 2. Data from this report compared to results from previous studies on miRNAs in ESCs or cancer, aligned by genomic location.

A. The log₂ ratio between the average miRNA expression in the hESC samples and in non-hESC samples is presented in the histogram, with the log₂ ratio indicated on the y-axis. Only miRNAs for which there is statistically significant differential expression (FDR<0.05) and/or data from previous reports are shown. A linear representation of the genome below the graph is mirrored on the x-axis. miRNAs are evenly spaced on the x-axis, so genomic distances are not scaled. Red bars: miRNAs with significant differential expression. Blue bars: miRNAs with previously published data. Data from previous studies are shown by squares and circles; the distance from the x-axis for these points is arbitrary, as previous reports were largely qualitative. Light blue squares: miRNAs previously described as both oncogenic and tumor suppressor. Dark blue squares: miRNAs previously designated as oncogenic (above the x-axis) or tumor suppressor (below the x-axis). Red, green, and lavender circles: data from hESCs by Northern blot [15], mouse ESCs by qRT-PCR [16, 17], and mouse ESCs by Northern blot [14], respectively. Circles above the x-axis have higher expression in ESCs, circles below the x-axis have lower expression in ESCs, in relation to control cells used in those reports. **B.** Table showing the number of oncogenic miRNAs upregulated, and the number of tumor suppressor miRNAs downregulated, in the hESCs. Expected values were rounded to the nearest integer. p-values are according to one-tailed t-test.

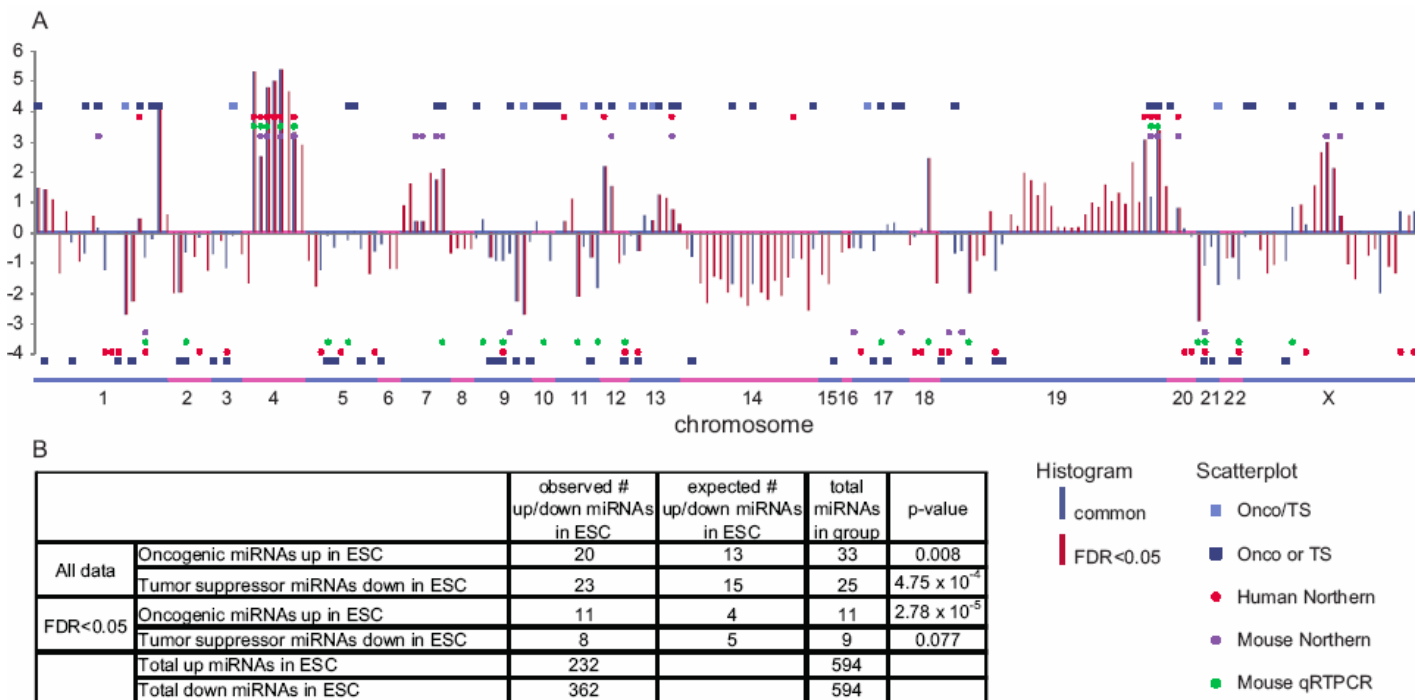


Figure 3. Consensus clustering and miRNA grouping results.

A. Consensus clustering matrix for the 26 cell lines. Results are based on 100 iterations of an unsupervised hierarchical clustering algorithm [28] (implemented in GenePattern [29, 30]) **B.** Heatmap showing expression profiles for the 26 cell lines, ordered according to the consensus clustering matrix in **A.** miRNAs in the heatmap are grouped according to the miRNA grouping results in **C.** **C.** Average expression patterns of miRNA Groups in the 26 cell lines. The order of cell lines is the same as in **A.** The number of miRNAs in each group and the homogeneity values are shown. Bars indicate \pm one standard deviation.

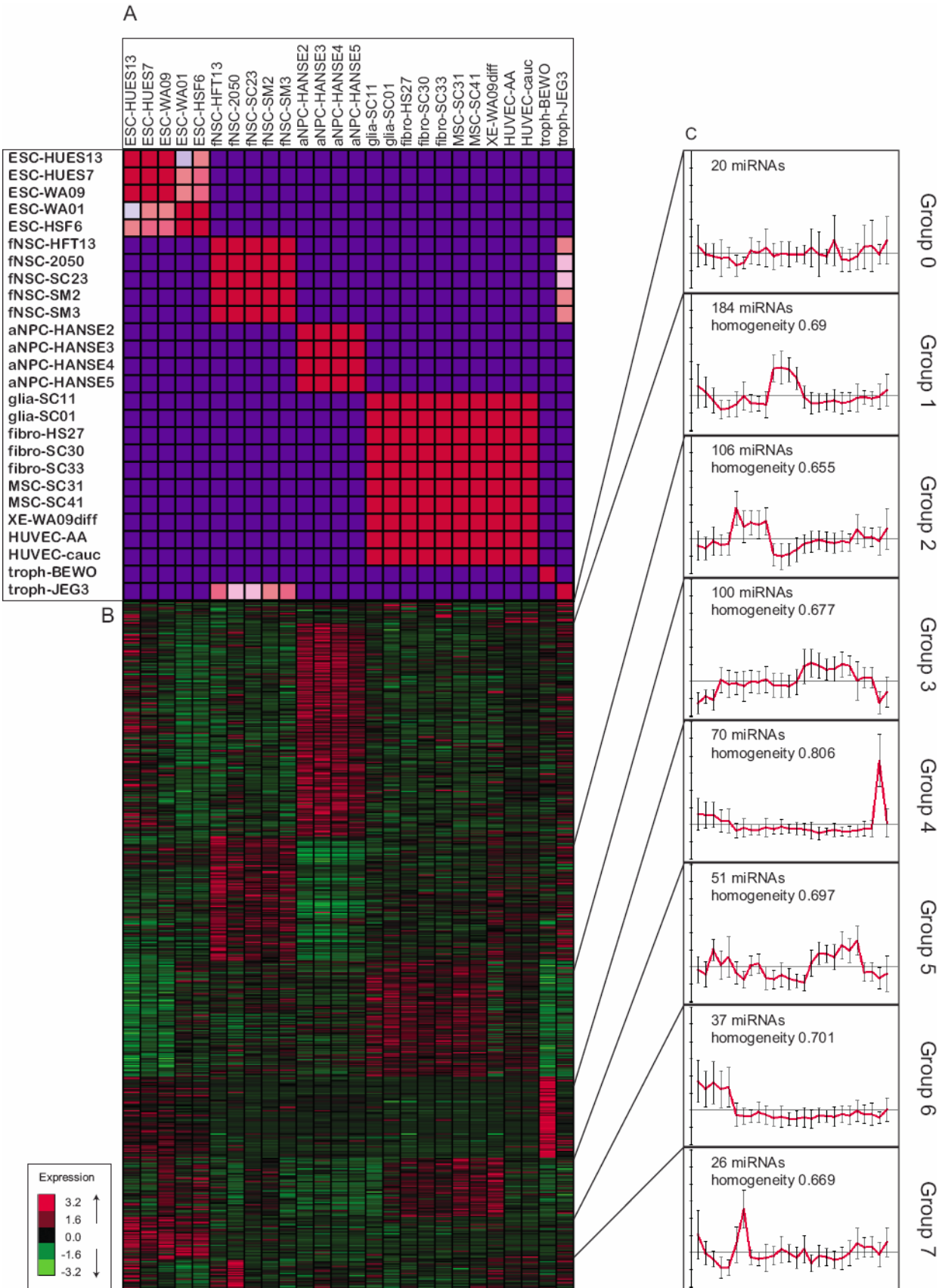


Figure 4. Seed similarity graph and consensus seed sequences. miRNAs significantly upregulated (FDR<0.05) in hESCs relative to non-hESCs are grouped by seed sequence. Nodes are labeled with the miRNA name. The average expression of the miRNAs in hESCs is represented by the log intensity on the y-axis. Red lines connect miRNAs that have identical seed sequences (mature miRNA positions 2-8). Blue lines connect miRNAs with six out of seven matches in the seed sequence. Consensus seed motifs are shown for each cluster. miRNAs that appeared in a similarity cluster with fewer than four other upregulated miRNAs are not shown (23 singleton miRNAs that do not have seed sequence similarity with any other upregulated miRNAs, five groups consisting of three miRNAs, and one group of four miRNAs). Lines (edges) between miRNAs in different similarity clusters are not shown. **A.** The major seed similarity cluster upregulated in hESCs. **B-D.** Three minor seed similarity clusters upregulated in hESCs.

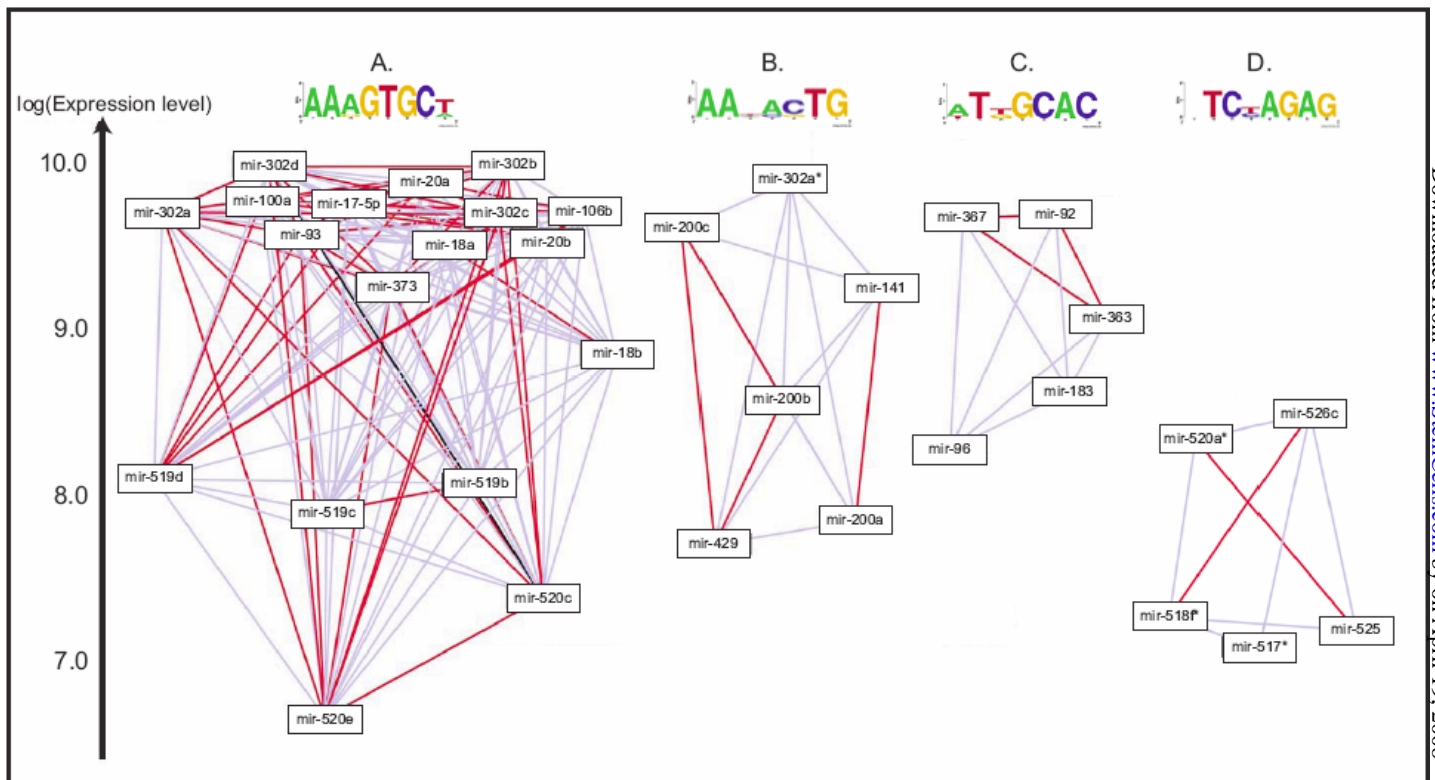


Table 1. Cell lines analyzed, description of the cell lines, number of biological replicates, contributing collaborators, and relevant citations.

Sample Name	Description	# Biological Replicates	Source	Citations
HUES7	Undifferentiated Human Embryonic Stem Cell	2	CJL	Cowan 2004
HUES13	Undifferentiated Human Embryonic Stem Cell	2	CJL	Cowan 2004
WA09	Undifferentiated Human Embryonic Stem Cell	2	LCL	Thomson 1998
WA01	Undifferentiated Human Embryonic Stem Cell	2	PHS	Thomson 1998
HSF6	Undifferentiated Human Embryonic Stem Cell	2	HSP	Abeyta 2004
SM2	Primary Fetal Neural Progenitor Cells	2	SRM*	Palmer 2001
SM3	Primary Fetal Neural Progenitor Cells	2	SRM*	Palmer 2001
HFT13	Fetal Neural Stem Cell Line	2	DRW	Sidman 2007
2050	Fetal Neural Stem Cell Line	2	DRW	Flax 1998
SC23	Neural Stem Cell Line from 28 Week Gestation	2	PHS	Palmer 2001, Schwartz 2003
HANSE2	Primary Adult Neural Progenitor Cells	3	NOS	Palmer 2001
HANSE3	Primary Adult Neural Progenitor Cells	3	NOS	Palmer 2001
HANSE4	Primary Adult Neural Progenitor Cells	3	NOS	Palmer 2001
HANSE5	Primary Adult Neural Progenitor Cells	3	NOS	Palmer 2001
SC01	Primary Glial Cell Line	2	PHS	Palmer 2001, Schwartz 2003
SC11	Primary Glial Cell Line	2	PHS	Palmer 2001, Schwartz 2003
SC30	Primary Dermal Fibroblast Cell Line	2	PHS	Sly 1969
SC31	Bone Marrow Mesenchymal Stem Cell Line, CD105+, CD34-	2	PHS	Sly 1969

SC33	Primary Dermal Fibroblast Cell Line	2	PHS	Sly 1969
SC41	Bone Marrow Mesenchymal Stem Cell Line, CD105+, CD34-	2	PHS	Sly 1969
XE	Extraembryonic Endoderm Phenotype, Differentiated from WA09	3	LCL	Gonzalez R, unpublished
HS27	Neonatal Foreskin Fibroblast	3	LCL	ATCC CRL-1634
HUVEC-AA	Primary Human Umbilical Vein Endothelial Cells, African American Patients	4	DBC	Jaffe 1973
HUVEC-Cauc	Primary Human Umbilical Vein Endothelial Cells, Caucasian Patients	4	DBC	Jaffe 1973
BEWO	Choriocarcinoma Cell Line	2	DBC	Patillo 1968
JEG3	Choriocarcinoma Cell Line	2	DBC	Kohler 1971

CL	Christina J. Lu	The Burnham Institute, UCSD Department of Reproductive Medicine
DBC	Dongbao Chen	UCSD Department of Reproductive Medicine
DRW	Dustin R. Wakeman	The Burnham Institute, UCSD Department of Biomedical Sciences
HSP	Hyun-Sook Park	Mizmedi Hospital, Seoul National University
LCL	Louise C. Laurent	The Burnham Institute, UCSD Department of Reproductive Medicine
NOS	Nils. O. Schmidt	Department of Neurosurgery, UKE Hamburg
PHS	Phillip H. Schwartz	Children's Hospital of Orange County
SRM	Scott R. McKercher	The Burnham Institute
*	tissue source:	Advanced Bioscience Resources, Inc.

Table 2. Composition of the eight miRNA groups indicated in **Figure 3**. Properties of the miRNAs in each group are described in the text. For each category of miRNA (Human brain expressed, CNS-related, etc.), the number of miRNAs with detectable expression in at least one cell type, the number observed in each group, and the number expected to be in each group, are listed. “obs” indicates observed, “exp” indicates expected. P-values are calculated according to a one-tailed test, assuming a hypergeometric distribution. The Bonferroni corrected alpha is $0.05/56 = 9 \times 10^{-4}$. Significant p-values are highlighted in red.

Groups 0-3	Number detected	Group 0: 20 miRNAs			Group 1: 184 miRNAs			Group 2: 106 miRNAs			Group 3: 100 miRNAs		
		observed	expected	p-value*	observed	expected	p-value*	observed	expected	p-value*	observed	expected	p-value*
Total	594												
Human brain expressed	208	5	7	0.006	109	64	2.52E-16	6	37	1	17	35	1
CNS-related	51	0	2	1	3	16	1	20	9	1.30E-04	51	9	4.81E-46
REST-related	13	0	0	1	1	4	1	6	2	0.016	5	2	0.05
Oncogenic	33	1	1	0.688	1	10	1	8	6	0.220	9	6	0.08
Tumor suppressor	25	2	1	0.204	3	8	1	3	4	0.855	16	4	7.77E-08
Chromosome 14 cluster	44	0	1	1	8	14	1	0	8	1	34	7	1.61E-20
Chromosome 19 cluster	44	0	1	1	1	14	1	0	8	1	0	7	1

Groups 4-7	Number detected	Group 4: 20 miRNAs			Group 5: 184 miRNAs			Group 6: 106 miRNAs			Group 7: 100 miRNAs		
		observed	expected	p-value*	observed	expected	p-value*	observed	expected	p-value*	observed	expected	p-value*
Total	594												
Human brain expressed	208	7	25	1	32	18	2.25E-05	10	13	0.893	19	9	6.00E-05
CNS-related	51	1	6	0.999	2	4	0.948	4	3	0.395	0	2	1
REST-related	13	0	2	1	1	1	0.693	0	1	1	0	1	1
Oncogenic	33	6	4	0.181	4	3	0.312	4	2	0.142	0	1	1
Tumor suppressor	25	1	3	0.959	0	2	1	0	2	1	0	1	1
Chromosome 14 cluster	44	0	5	1	1	4	0.984	1	3	0.947	0	2	1
Chromosome 19 cluster	44	42	5	4.70E-08	1	4	0.984	0	3	1	0	2	1

me 19 cluster				-43									
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*p-value: one-tailed test, hypergeometric distribution. Bonferroni corrected alpha $< 0.05/56 = 9 \times 10^{-4}$

Comprehensive MicroRNA Profiling Reveals a Unique Human Embryonic Stem Cell Signature Dominated by a Single Seed Sequence

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