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Profiling Conserved MicroRNA Expression in Recombinant CHO Cell Lines Using Illumina Sequencing

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Abstract

MicroRNAs (miRNAs) are small, non-coding RNAs that regulate multiple aspects of cell physiology. The differential expression of conserved miRNAs in two Chinese hamster ovary (CHO) cell lines producing recombinant proteins was examined relative to the CHO-K1 cell line. A total of 190 conserved CHO miRNAs were identified through homology with known human and rodent miRNAs. More than 80% of these miRNAs showed differential expression in recombinant CHO cell lines. The small RNA sequencing data was analyzed in context of the CHO-K1 genome to examine miRNA organization and develop sequence-specific miRNA resources for CHO cells. The identification and characterization of CHO miRNAs will facilitate the use of miRNA tools in cell line engineering efforts to improve product yield and quality.

Keywords

Chinese hamster ovary cells; microRNA profiling; next-generation sequencing

MicroRNAs (miRNAs) are small RNA molecules (~ 22 nt) that can globally modify gene expression and regulate a number of cellular pathways including proliferation, metabolism, and apoptosis (Muller et al. 2008). miRNAs are involved in regulating many aspects of cellular physiology that are targeted by cell engineering strategies, and so miRNAs are another tool to improve and modify Chinese hamster ovary (CHO) cell phenotypes such as the production of biotherapeutic proteins. Currently, only two hamster miRNAs are present in miRBase, cgr-mir-21 (Gammell et al. 2007) and cgr-mir-7 (Barron et al. 2011), and the role of miRNAs in the regulation of pathways relevant to biotherapeutic production are not yet well characterized.

Several recent studies have examined the differential expression of miRNAs in engineered CHO cells (Lin et al. 2011), at various timepoints during culture (Bort et al. 2011), and under varying culture conditions to enhance productivity (Gammell et al. 2007). Additional studies employing next-generation sequencing for miRNA profiling of CHO cell lines revealed the expression of 235–350 highly conserved miRNA species (Hackl et al. 2011; Johnson et al. 2011). As the human genome is predicted to contain at least 800 miRNAs, including 400–500 conserved miRNAs (Bentwich et al. 2005), there may be additional CHO miRNAs that have not yet been identified.

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To better understand the potential effects of miRNA expression in biotherapeutic production, the differential expression of miRNAs in CHO-K1 and in two recombinant CHO cell lines producing secreted alkaline phosphatase (CHO-SEAP) and tissue plasminogen activator (CHO-tPA) was examined. Small RNA libraries were prepared for Illumina sequencing from each of the three CHO cell lines. Sequencing of these three libraries yielded 4,885,687 reads from the CHO-SEAP small RNA library, 6,887,492 reads from the CHO-K1 library, and 9,308,556 reads from the CHO-tPA library for a total of 21,081,735 reads. CHO conserved miRNAs were identified by a cross-species comparative approach and then CHO-specific information, including genomic organization and hairpin sequences, were examined by alignment to the CHO-K1 draft genome (Figure 1).

Two strategies were used to identify conserved miRNAs in the selected CHO cell lines. For each, the small RNA sequences were trimmed to remove adaptor sequences and filtered to remove low quality reads. In addition, each small RNA sequence was required to have at least 10 reads (across the three cell lines) to be classified as a conserved miRNA. These small RNAs were then aligned to human and rodent miRNAs in miRBase and the resulting alignments were used as supporting evidence for miRNA identification. The aligned small RNAs were then grouped by mature miRNA sequences to generate a list of conserved miRNAs identified by each strategy. To examine differential expression, the read counts for each miRNA was normalized to the total number of filtered and trimmed reads in each cell line. Conserved miRNAs that show ≥ 1.5 -fold change in normalized expression (NE) relative to CHO-K1 were considered differentially expressed.

In the first strategy, conserved miRNAs were identified by alignment of the small RNA sequences to the mature miRNA and miRNA* sequences in miRBase using BLAST. Recently, 350 conserved miRNA and miRNA* sequences were identified in CHO cells using this approach (Johnson et al. 2011). After filtering and trimming of the small RNA data using custom scripts, 16,361 unique small RNA sequences were produced and 6,268 (38.3%) of these small RNAs mapped to human and rodent mature miRNA sequences in miRBase. The BLAST alignments were used as supporting evidence for the identification of these miRNAs. In total, 173 conserved miRNAs were identified using BLAST similarity searching. Of these, 136 miRNAs were differentially expressed in at least one recombinant CHO cell line and 75 were differentially expressed in both CHO-SEAP and CHO-tPA cells.

In the second approach, the small RNA sequences were aligned to precursor miRNAs in miRBase, including information about mature miRNA and miRNA* regions, using the CLC Genomics Workbench small RNA analysis tools. A similar analysis, in which miRNA precursor sequences were used as a reference and a short read alignment algorithm was used to map small RNAs to the miRNA reference sequences, identified 235 conserved miRNAs in CHO cells (Hackl et al. 2011). Filtering and trimming of the small RNA data generated 9,479 unique small RNA sequences and 4,098 (43.7%) of these small RNAs mapped to human and rodent entries in miRBase. Of the 160 conserved miRNAs identified in this approach, 134 miRNAs were differentially expressed in at least one recombinant CHO cell line and 79 were differentially expressed in both CHO-SEAP and CHO-tPA cells.

Between the two approaches, a total of 190 conserved miRNAs were identified (Table SI). The majority of these (143 miRNAs) were identified in both strategies, whereas a small number of unique miRNAs were identified in only the BLAST analysis (30 miRNAs) or the CLC Genomics Workbench analysis (17 miRNAs). All but four miRNAs were expressed in all three CHO cell lines; miR-149 was not expressed in CHO-K1 but was expressed, at low levels, in both recombinant CHO cell lines and miR-152, miR-155, and miR-3068 were expressed, at low levels, only in CHO-K1 and CHO-tPA cells. A similar number of differentially expressed miRNAs were identified in the recombinant CHO cell lines relative

to expression in CHO-K1 by both analysis methods (Table I). In CHO-SEAP cells, ~ 60% of the differentially expressed miRNAs were up-regulated whereas in CHO-tPA cells, ~ 70% of the differentially expressed miRNAs were down-regulated. Previous reports of miRNA profiling in CHO cells using high-throughput sequencing identified 235–350 conserved miRNAs (Hackl et al. 2011; Johnson et al. 2011). The CHO cell lines analyzed in these past studies were grown under a wider range of culture conditions, such as adherent and suspension growth, serum-containing and serum-free media, reduced culture temperature, and sodium butyrate treatment, potentially contributing to the greater range of miRNAs observed.

More than 80% (158) of the conserved miRNAs were found to be differentially expressed in at least one recombinant CHO cell line (Figure 2). Of these, over 40% were differentially expressed in only CHO-SEAP (38 miRNAs) or CHO-tPA (27 miRNAs) cells. Nearly 50% of the differentially expressed miRNAs were up-regulated (29 miRNAs) or down-regulated (45 miRNAs) in both recombinant CHO cell lines. Finally, 19 miRNAs were up-regulated in CHO-SEAP cells but down-regulated in CHO-tPA cells. Several miRNAs showing similar expression patterns in both recombinant CHO cell lines were previously demonstrated to influence cellular pathways that are often targeted in bioprocessing. The altered expression of miRNAs such as miR-1 (Leone et al. 2011) and miR-210 (Tsuchiya et al. 2011) may promote cell cycle progression and enhance cell proliferation. Altered expression of miR-125b (Chun-Zhi et al. 2010) and miR-330 (Lee et al. 2009) may promote cell survival by suppressing apoptosis. Two miRNAs differentially regulated in both cell lines, miR-30b/miR-30d (Gaziel-Sovran et al. 2011) and miR-378 (Kahai et al. 2009), have been shown to affect expression of N-acetylgalactosaminyltransferases, which can alter protein glycosylation.

The availability of a genome sequence for CHO-K1 (Xu et al. 2011) and other CHO cell lines (Hammond et al. 2011) will facilitate developing sequence-specific resources for CHO cells, including miRNA discovery. The genomic organization of miRNAs is highly conserved among many mammals and previous work suggests that CHO miRNAs may show a similar organization (Johnson et al. 2011). Alignment of the small RNAs to the CHO-K1 genome provides information about miRNA organization and CHO precursor miRNA sequences necessary to improve miRNA tools for cell line engineering. More than 56% of the approximately 21 million reads were aligned to 3,461 unique CHO-K1 genomic scaffolds using the CLC Genomics Workbench. Alignment of the small RNAs to the CHO-K1 genome showed the mapping of reads to six members of the highly conserved miR-17-92 cluster in the CHO-K1 genome (Figure 3). Comparison of the CHO-K1 genome sequence to the mouse precursor miRNA sequence for each of these six miRNAs showed an average 95% sequence identity. This supports the high sequence identity of the conserved miRNAs identified in this analysis and shows a similar organization of CHO miRNAs compared to miRNA clustering in other mammalian genomes.

The use of high-throughput sequencing for miRNA expression profiling in CHO cells has, in the absence of a reference genome, previously relied upon alignment of small RNA sequences to miRBase for the identification of conserved miRNAs. This study shows that alignment to miRBase using both BLAST and CLC Genomics Workbench, a commercial short read aligner, produce similar miRNA expression profiling results. In addition, alignment of small RNA reads to the CHO-K1 genome not only enables identification of CHO precursor miRNA sequences but may also reveal novel CHO miRNAs. The availability of the CHO-K1 genome will facilitate developing sequence resources for CHO miRNAs and their regulatory targets for CHO cell engineering.

Materials and Methods

Cell culture

CHO-K1 (ATCC CCL-61), CHO-SEAP (Hayduk and Lee 2005), and CHO-tPA (ATCC CRL-9606) cells were maintained as adherent cultures in Iscove's Modified Dulbecco's Medium (IMDM, HyClone, Logan, UT) supplemented with 10% dialyzed fetal bovine serum (dFBS, Invitrogen, Carlsbad, CA) at 5% CO₂ and 37 °C.

Small RNA sequencing and analysis

CHO cells were harvested during the stationary phase of cell culture and small RNAs were extracted from each cell line using the mirVana miRNA Isolation kit (Ambion, Austin, TX) and cDNA libraries were prepared as previously described (Lu et al. 2007). Libraries were sequenced on an Illumina Genome Analyzer IIx and reads were filtered to remove low quality sequences and trimmed to remove adaptor sequences prior to miRNA analysis. The total number of filtered and trimmed reads for each cell line was used to normalize miRNA expression.

Small RNA sequences with a minimum of 10 reads across the three cell lines were analyzed by BLASTn search against the human and rodent mature miRNAs from miRBase release 17 (Kozomara and Griffiths-Jones 2011) and by alignment to the rodent (*Cricetulus griseus*, *Mus musculus*, and *Rattus norvegicus*) and human precursor sequences in miRBase release 17 (in that species priority order) using the CLC Genomics Workbench 4.7.2 (CLC bio, Aarhus, Denmark) to identify conserved miRNAs. The alignment results were used as evidence to identify the small RNAs as conserved miRNAs. The normalized expression (NE) of each miRNA was calculated by normalizing the miRNA read counts by the total number of filtered and trimmed reads in each cell line and multiplying by 1,000,000. Differential expression was analyzed for miRNAs showing ≥ 1.5 -fold change in NE in the recombinant CHO cells lines relative to NE in CHO-K1. Small RNAs were aligned to the CHO-K1 genome using CLC Genomics Workbench and genomic alignments were visualized using Tablet (Milne et al. 2010).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Overview of CHO miRNA analysis.

Figure 2.

Differential expression of miRNAs in recombinant CHO cell lines. The number of differentially expressed miRNAs relative to CHO-K1, identified by BLAST and/or by CLC Genomics Workbench analysis, showing up-regulated (red) or down-regulated (green) expression in CHO-SEAP and CHO-tPA cells. The 19 miRNAs found to be up-regulated in CHO-SEAP but down-regulated in CHO-tPA are listed in black.

CHO WGS scaffold3425

Small RNAs aligned to CHO WGS scaffold3425

CHO-K1	108958	CCAAACCTCAACAGGCCGGGACAAGTGAATACCAT-C-GAGAAACACAGCATTTGCACAAATCCCA	109035
mmu-mir-92a-1	80	CCAAACCTCAACAGGCCGGGACAAGTGAATACCATACAGAGAAACACAGCATTTGCACAAATCCCA	1
CHO-K1	109071	CACCACCACAGTCAGTTTTGCATGGATTTCACAGCAGAGATTATTATACAGCTGGATGCAAACTGCAAACTAACCATAGACCAAGTG	109157
mmu-mir-19b-1	87	CACCACCACAGTCAGTTTTGCATGGATTTCACAGCAGAGATTATTATACAGCTGGATGCAAACTGCAAACTAACCATAGACCAAGTG	1
CHO-K1	109216	CTGGGGCTCTCCAGCTGGCAGCACTGGAAGTGCTCGTAATGCAGTAGATAG-TGGACACTACCTGCACCTATAAGCACTTTAGTGCCACAGAAGCTGTC	109313
mmu-mir-20a	107	CTGGAGTTCTACAGCTGGCAGTACTTTAAGTGCTCGTAATGCAGTAGATG-GCTACACACTACCTGCACCTATAAGCACTTTAGTGCTACAGAAGCTGTC	10
CHO-K1	109405	GCAGGCCACCATCAGTTTTGCATAGATTTCACAACTGCATTCTTCTTGTAGTGCAAGTATGCAAACTAACAGAGGGCTGC	109486
mmu-mir-19a	82	GCAGGCCACCATCAGTTTTGCATAGATTTCACAACTACATTCTTCTTGTAGTGCAACTATGCAAACTAACAGAGGGCTGC	1
CHO-K1	109540	GACATAACTTCTTATGCCAGAAGGAGCACTTAGGGCAGTAGATGCTAGTCTACTTCACTATCTGCACCTAGACCTTAGAACAAAAAGCAC	109632
mmu-mir-18a	96	GACATAACTTCTTATGCCAGAAGGAGCACTTAGGGCAGTAGATGCTAGTCTACTTCACTATCTGCACCTAGACCTTAGAACAAAAAGCAC	4
CHO-K1	109697	GTCACCATATATGCCCACAGTG-CCTGCACTGCAGTAGATGTGCATATCACTACCTGCACCTGTAAGCACTTTGACATTATCCTGAC	109780
mmu-mir-17	84	GTCAGCATAATGCTACAAGTGCCCT-CACTGCAGTAGATGCACATCACTACCTGCACCTGTAAGCACTTTGACATTATCCTGAC	1

Figure 3.

Alignment of the small RNA reads to the CHO-K1 genome. Small RNA reads mapping to the CHO-K1 WGS scaffold3425 (accession JH001979.1, lower panel) are shown relative to genomic annotation from NCBI Map Viewer (upper panel). Detailed alignments of mouse miRNAs from the miR-17-92 cluster to the CHO-K1 genome are shown below. Conserved bases are displayed in black and sequence differences are highlighted in red. Mature miRNA and miRNA* sequences for the mouse miRNAs are underlined.

Table I

Differential expression of conserved miRNAs in recombinant CHO cells relative to expression in CHO-K1 cells.

Alignment	CHO-SEAP	CHO-tPA
BLAST	↑ 64	↑ 27
	↓ 46	↓ 74
CLC Genomics Workbench	↑ 68	↑ 27
	↓ 44	↓ 74

↑ up-regulated ≥ 1.5 -fold, ↓ down-regulated ≥ 1.5 -fold.