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Research paper

Variability of miRNA expression during the differentiation of human embryonic stem cells into retinal pigment epithelial cells

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ABSTRACT

Embryonic stem cells (ESCs) and induced pluripotent stem cells can be induced to differentiate into retinal pigment epithelium (RPE). MiRNAs have been characterized and found playing important roles in the differentiation process of ESCs, but their length and sequence heterogeneity (isomiRs), and their non-canonical forms of miRNAs are underestimated or ignored. In this report, we found some non-canonical miRNAs (dominant isomiRs) in all differentiation stages, and 27 statistically significant editing sites were identified in 24 different miRNAs. Moreover, we found marked major-to-minor arm-switching events in 14 pre-miRNAs during the hESC to RPE cell differentiation phases. Our study for the first time reports exploring the variability of miRNA expression during the differentiation of hESCs into RPE cells and the results show that miRNA variability is a ubiquitous phenomenon in the ESC differentiation.

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1. Introduction

MicroRNAs (miRNAs) are a class of small, single stranded RNAs. They take part in many biological processes, directly or indirectly, through binding to their target mRNAs. Primary miRNAs (pri-miRNAs) are transcribed from miRNA genes and are then spliced by the nuclease Drosha to form miRNA precursors (pre-miRNAs) of 60–70 nucleotides (nt) that can fold into hairpin structures. Pre-miRNAs are transported by Exportin 5 from the nucleus to the cytoplasm, where they are processed into double-stranded RNA (5p arm/3p arm), 19–25 nt in length by Dicer, an RNase III (Bartel, 2004). The duplex is unwound by a helicase, and then one or both of the 5p or 3p arms can be selected and loaded onto the RNA inducible silencing complex (Newman and Hammond, 2010; Slezak-Prochazka et al., 2010). MiRNAs repress gene expression by binding to target mRNAs in the form of ribonucleoprotein complexes that mediate mRNA destabilization (Krol et al., 2010). The 2–7/8th site from 5′ terminus of the miRNA (the seed region) is a key to recognizing its target mRNAs (Bartel, 2004). MiRNAs can block mRNA translation or lead to target mRNA degradation by complementarily binding to the 3′-UTR of target mRNAs. MiRNAs are involved in many normal and pathological cellular and physiological processes through regulating expression of their target genes.

MiRNA diversity lies in variation of miRNA sequences and/or miRNA expression. Variability of miRNA sequences is observed in an increasing number of organisms and reveals the universality of miRNA sequence diversity (Azuma-Mukai et al., 2008; Berezikov et al., 2011; Chiang et al., 2010; Ebhardt et al., 2009; Fernandez-Valverde et al., 2010; Kuchenbauer et al., 2008; Neilsen et al., 2012; Reese et al., 2010; Ruby et al., 2007; Stark et al., 2007; Wu et al., 2009; Xia and Zhang, 2012). In the past, the most abundant miRNA sequences processed from a pre-miRNA was usually defined as the corresponding mature miRNA, while the less abundant, variant sequences produced from the same miRNA locus were not considered functional and were discarded (Landgraf et al., 2007; Ruby et al., 2006). More recently, deep sequencing technology has enabled miRNA variants to be explored in detail and many miRNA heterogeneities have been found. Indeed it is now possible to comprehensively explore sequences and expression level/pattern variations of miRNAs in organisms, tissues or cells. The sequence and expression level of the same miRNA may vary in different stages (Fernandez-Valverde et al., 2010), or tissues (Landgraf et al., 2007), or even among different species (for orthologous miRNAs) (Xia and Zhang, 2014). The diversity of miRNA sequences originate from imprecise cleavage by Drosha or Dicer, 5′ nucleotide addition (Bizuayehu et al., 2012; Ebhardt et al., 2010), 3′ nucleotide addition (Burroughs

Abbreviations: ESC, embryonic stem cell; hESC, human embryonic stem cell; RPE, retinal pigment epithelium; miRNA, microRNA; pri-miRNA, primary miRNA; pre-miRNA, miRNA precursor; isomiR, miRNA isoform; ADAR, adenosine deaminases-acting-on-RNA; A-to-I, adenosine-to-inosine; hiPSC, induced pluripotent stem cell; AMD, aged-related macular degeneration; PC, early pigmented cluster; PD, 15 day partially differentiated ES cells; SNP, single nucleotide polymorphism; GO, gene ontology.

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et al., 2010; Lu et al., 2009), nucleotide polymorphism (single nucleotide polymorphism) and RNA editing. MiRNAs of different lengths or with polymorphic sites are termed isomiRs. IsomiRs are classed into three main categories: 5′-isomiRs, 3′-isomiRs, and polymorphic isomiRs (Neilsen et al., 2012). Moreover, 5′ and 3′-isomiRs can be subclassified into templated or non-templated modifications (Neilsen et al., 2012).

Variation of miRNA expression is due to regulation by tissue-specific regulatory elements, stage-specific regulatory elements, epigenetic modification, and as yet undefined miRNA processing and degradation mechanisms. These factors result in differential expression of miRNAs and affect the preference selection of dominant miRNA arm-switching under different conditions.

Aged-related macular degeneration (AMD) is a chronic disease that results from cell death or irreversible damage in the retinal pigment epithelium (RPE), and it is the leading cause of blindness among the elderly in the developed world (Vugler et al., 2007). In the past, the disease had no specific treatment; however, recently, stem cell-derived RPE cells have been considered as ideal candidates for transplantation into the retina for the treatment of AMD (Haruta, 2005; Ramsden et al., 2013). Based on the strategy of using stem cells for cell replacement therapy, human embryonic stem cells (hESCs) or induced pluripotent stem cells (hiPSCs) can be used to produce RPE cells. To understand in detail the variations in expression and the roles of miRNAs during the differentiation of hESCs or hiPSCs, we have explored differential expression, miRNA editing and potential miRNA functions in the phases from stem cells to RPE cells. This work aimed to discover the role of miRNAs in RPE differentiation and to further the understanding of RPE cells derived from hiESCs for use in the treatment of AMD.

2. Materials and methods

2.1. Experimental data

MiRNA deep sequencing data of different hESC lines, hESC- or hiPSC-derived RPE cells at different stages, and fetal RPE cells were obtained from a previous study (Hu et al., 2012) (the accession number in the NCBI Gene Expression Omnibus for this data is GSE37686). These data include four stages of hESC to RPE cell differentiation: human ESC lines, BG01 (BG01 ESC01 and BG01 ESC02) and H9 (stage 1), 15 day partially differentiated hESCs derived from hESC lines BG01 and H9 (designated as BG01 PD and H9 PD) (stage 2), early pigmented clusters (PCs) after 30 days of differentiation (designated as H9 PC) (stage 3), and RPE derived from human stem cell lines H9 and HSF1 and human induced pluripotent stem sell line hiPS2 (designated as H9 RPE, HSF1-RPE and hiPS2 RPE respectively); in addition, there is a sample of fetal RPE (designated as fRPE). These RPEs have been cultured for over 3 months (stage 4) (Hu et al., 2012). These 10 samples and the details are also shown in Table S1.

2.2. Identification of novel miRNAs

First, cutadapt (Martin, 2011) was used to remove adapter sequences using a quality score cutoff of 20 and sequences of 18–26 nt in length were selected for further analysis. Second, known and novel miRNAs were identified by miRDeep2 (Friedlander et al., 2012). Here, a score cutoff corresponding to a prediction signal-to-noise ratio of >10 was used.

2.3. Identification of differentially expressed miRNAs

Identification of differentially expressed miRNAs among the different stages of hESC differentiation into RPE cells was performed with R (Ihaka and Gentleman, 1996) and the Bioconductor edgeR package (Robinson et al., 2010). Only BG01 ESC01 and BG01 ESC02 were treated as replicates, and these datasets in each time point being treated as a group. We only considered miRNAs with normalized read counts no less than 10 in at least half of samples at any one stage. Here a miRNA with normalized read counts of no less than 100 will be considered as a highly expressed miRNA.

2.4. Identification of isomiRs and analysis of miRNA editing

To analyze isomiRs during the course of hESC differentiation into RPE cells, we performed the analysis on the small RNA deep sequencing data after trimming adapter sequences and discarding reads mapping to more than one genomic position.

MiRNA editing sites were detected using methods described previously (Alon et al., 2012). We first filtered the reads to only retain those with a length of 18 to 26 nucleotides. As the 3′ ends of mature miRNAs can carry modifications common to other RNAs, in the form of adenylation and uridylation (Burroughs et al., 2010), the last two bases of each read were trimmed to avoid considering instances of non-templated 3′-terminal addition (Chiang et al., 2010). Then the filtered and trimmed reads were mapped to the genome (hg19) using Bowtie (Langmead et al., 2009), allowing one mismatch and only keeping reads with a single best alignment. The minimum quality score allowed in each location of the mismatch was no less than 30 (corresponding to an error rate of 0.1%). Moreover, known single nucleotide polymorphisms (SNPs) sites were removed from miRNA editing sites using the dbSNP (Sherry et al., 2001) data set (snp138) downloaded from the UCSC Genome Browser (Karolchik et al., 2003).

2.5. Arm-switching miRNAs

To determine the read counts from the 5′ and 3′ arms, the read counts of each miRNA were normalized to reads per million, which is the read number of each miRNA per million mapped reads in each library. Only pre-miRNAs with 10 or more reads produced from either arm in either of the two compared samples were considered. And the mean read counts were computed for each arm if a stage had more than one sample. Here, we used miR-5p and miR-3p to represent miRNAs (reads) produced from 5p and 3p arms of a pre-miRNA, respectively. The fold enrichment was calculated as the ratio of 5′ to 3′ arm reads and only those having fold changes no larger than 0.5 or no less than 2 in the four differentiation stages were regarded as significantly changed. And only the arm-switching miRNAs with significant change were used for further study.

2.6. Functional analysis of miRNA targets

To determine the biological function of miRNAs, we explored the function and biological pathways of their targets. MiRNA targets were predicted by TargetScan (Lewis et al., 2005) and PITA (Kertesz et al., 2007) programs. Gene ontology (GO) enrichment and KEGG pathway analysis were performed using GOstats (Falcon and Gentleman, 2007) and GeneAnswers (Feng et al., 2010, 2012) in the R statistical computing and graphics environment (Ihaka and Gentleman, 1996).

3. Results and discussion

3.1. Identification of novel miRNAs and cluster analysis of miRNA expression profiles during hESC differentiation into RPE cells

We analyzed novel and known miRNAs from cells in the four differentiation stages (Table S1) using miRDeep2 (Friedlander et al., 2012). MiRDeep2 predicted 141 potential novel miRNA hairpins, and identified 941 known miRNA hairpins, at the relatively stringent score cut-off of 4 and signal-to-noise ratio of 10.5 (Table S2). After filtering of the predicted novel miRNAs by removal of loci matching rRNA/tRNA, and keeping only novel miRNAs with significant Randfold (Bonnet et al., 2004) p-values for their corresponding pre-miRNAs, the final predicted list

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