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Cancer Informatics

Supplementary Issue: Network and Pathway Analysis of Cancer Susceptibility (A)

Profiling the microRNA Expression in Human iPS and iPS-derived Retinal Pigment Epithelium

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ABSTRACT: The purpose of this study is to characterize the microRNA (miRNA) expression profiles of induced pluripotent stem (iPS) cells and retinal pigment epithelium (RPE) derived from induced pluripotent stem cells (iPS-RPE). MiRNAs have been demonstrated to play critical roles in both maintaining pluripotency and facilitating differentiation. Gene expression networks accountable for maintenance and induction of pluripotency are linked and share components with those networks implicated in oncogenesis. Therefore, we hypothesize that miRNA expression profiling will distinguish iPS cells from their iPS-RPE progeny. To identify and analyze differentially expressed miRNAs, RPE was derived from iPS using a spontaneous differentiation method. MiRNA microarray analysis identified 155 probes that were statistically differentially expressed between iPS and iPS-RPE cells. Up-regulated miRNAs including miR-181c and miR-129–5p may play a role in promoting differentiation, while down-regulated miRNAs such as miR-367, miR-18b, and miR-20b are implicated in cell proliferation. Subsequent miRNA–target and network analysis revealed that these miRNAs are involved in cellular development, cell cycle progression, cell death, and survival. A systematic interrogation of temporal and spatial expression of iPS-RPE miRNAs and their associated target mRNAs will provide new insights into the molecular mechanisms of carcinogenesis, eye differentiation and development.

KEYWORDS: microRNA, induced pluripotent stems cells, retinal pigment epithelium, cancer

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Background

The two defining characteristics of pluripotent stem cells are unlimited replicative capacity and the potential to differentiate into any somatic cell type.[1] Using recently developed techniques, somatic cells can now be reprogrammed into induced pluripotent stem (iPS) cells by the introduction of four transcription factors, Oct4, KLF4, Sox-2, and c-Myc.[2] Like embryonic stem (ES) cells, iPS cells possess capabilities of self-renewal and pluripotency that make these cells so therapeutically promising, but are also responsible for a potential tumorigenic risk; however, iPS cells do not present the ethical dilemmas associated with ES cells. From a clinical perspective,

iPS cells provide a source of cells genetically identical to the patient; such cells will not elicit an immune response, thereby significantly improving the probability of successful therapy.[3,4] As evidence of their potential utility for tissue engineering and clinical applications, iPS cells have been used to generate cardiomyocytes, neurons, pancreatic beta cells, hepatocytes, and retinal pigment epithelium (RPE).[5–13]

The RPE, a specialized layer of pigmented epithelial cells located at the back of the retina, performs several functions that are essential to maintain visual health and function. RPE dysfunction caused by damage or disease underlies the pathology of blinding diseases such as age-related macular degeneration (AMD), Stargardt's disease, and retinitis pigmentosa (RP).[14] As currently available treatments do not effectively prevent vision loss, the best option may be to replace diseased RPE with transplanted healthy cells.[15,16] RPE derived from iPS (iPS-RPE) is a possible source of cells to replace the damaged RPE. Published studies have demonstrated that iPS-RPE are functionally and phenotypically similar to RPE harvested from retinal explants; the iPS-RPE expresses characteristic RPE proteins LRAT, CRALBP, PEDF, and RPE65; displays the classical highly pigmented hexagonal RPE morphology; and performs RPE functions such as phagocytosis,[9] retinoid processing, and secretion of 11-*cis* retinal.[17] However, the iPS-RPE must be thoroughly analyzed for function and safety before it can be used for clinical applications. Specifically, factors that promote pluripotency and tumorigenesis must be silenced, and the RPE must be fully differentiated.

Cutting-edge high-throughput technologies such as microarray, RNA-Seq, ChIP-Seq, and proteomics have enabled systematic analyses of genetic and epigenetic differences, leading to a better understanding of biological systems in a temporal/spatial-specific manner and across a wide range of subcellular, cellular, tissue, and organism scales.[18–23] For example, microarray approaches can distinguish both the transcriptional and translational signatures of closely related cells, ie, stem cells and their differentiated progeny.[24–26] Since the development of microarray technology, this technique has been optimized to allow transcriptomic analysis of not only the messenger RNA (mRNA), but also the small non-coding RNAs such as miRNAs.[27,28] MiRNAs are short, ~22-nucleotide strands of RNA that function by binding to mRNA, thus inducing either translational repression or degradation of the transcript.[29] MiRNAs are transcribed within the nucleus as long pri-miRNA transcripts, which are then processed first by the endonuclease Drosha to generate pre-miRNA. After leaving the nucleus, the pre-miRNA is further cleaved by the RNA enzyme Dicer, to produce the mature miRNA. Since their discovery in the early 1990s,[30] over 2,000 miRNAs have been identified in the human genome. Upwards of 50% of mammalian RNA may be regulated by miRNA; nearly every cellular process, including pluripotent stem cell self-renewal and cell fate specification, is controlled at some point by miRNA intervention. Several investigators have provided compelling evidence that miRNAs play a critical role in maintaining pluripotency and facilitating differentiation.[31] For instance, knockout of the RNA enzyme Dicer, required for maturation of miRNA, causes severe defects in the ability of stem cells to differentiate, suggesting that miRNA maturation is essential for stem cell differentiation *in vitro* and *in vivo*.[32,33] In addition, c-Myc, one of the four transcription factors used to reprogram somatic cells into pluripotent stem cells, has been shown to bind directly to the promoter regions of the miR-302/367 and miR-17/92 polycistronic clusters. These polycistrons are highly enriched in stem cells, but down-regulated following differentiation.[33]

Based upon these studies, we hypothesize that miRNAs play an important role during the differentiation of RPE from iPS, and that miRNA expression profiling will distinguish iPS cells from their iPS-RPE progeny. To test this hypothesis, two distinct groups of miRNAs: those involved in maintaining the pluripotency of stem cells and those expressed after the differentiation of iPS into RPE, were compared. Analysis of the expression of miRNAs during the process of differentiation and identification of their associated target mRNAs will provide new understanding of the factors that regulate pluripotency, self-renewal, and cell fate determination.

Methods

Culture and differentiation of iPS cells. Human iPS cells (IMR-90–1, WiCell Research Institute, Madison, WI, USA) were cultured on six-well plates coated with matrigel (BD Biosciences, San Jose, CA, USA) and maintained in mTeSR1 medium (Stem Cell Technologies, Vancouver, BC, Canada). Differentiation was initiated by replacing the mTeSR1 medium with differentiation medium consisting of 10% knockout serum replacement (Life Technologies, Grand Island, NY, USA), 0.1 mM β-mercaptoethanol, 0.1 mM non-essential amino acids, 2.0 mM glutamine, and 10 μg/mL gentamicin in DMEM/F12. iPS-RPE appeared around day 30 of differentiation in the form of pigmented foci. The foci were manually dissected out of the culture and trypsinized to prepare a single cell suspension. The iPS-RPE were cultured in fetal RPE media[34] with media changes every other day for 17 days until collected for RNA extraction.

RNA extraction. The iPS and iPS-RPE cells were lysed by running the samples through a Qi Shredder column (Qiagen, Valencia, CA, USA). Total RNA was extracted using the miRNA mini kit (Qiagen, Valencia, CA, USA),[35] followed by checking with a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA) for the concentration. RNA samples without organic solvent carryover, showing OD 260/230 above 1.7 were recommended to proceed for RNA quality determination. The quality of the RNA was determined with Eukaryote Total RNA Pico kit (Agilent Technologies, Santa Clara, CA) performed on a 2100 Bioanalyzer and software (Agilent Technologies) that detects 28S and 18S ribosomal RNA ratio and total RNA Integrity Number

(RIN). The RIN software algorithm allows the classification of total RNA, based on a numbering system from 1 to 10, with 1 being the most degraded and 10 being the most intact. Only samples with 28S and 18S ribosomal RNA ratio higher than 1.8 and RIN higher than 8 were used in this study.

miRNA analysis. MiRNA expression analysis was performed using the Agilent Human miRNA v16 microarrays in 8×60 K format according to the manufacturer's protocol (Agilent Technologies, Santa Clara, CA, USA). Each microarray provides genome-wide coverage of well-characterized miRNAs and candidates primarily selected from the miRBase database.[36] The Human miRNA 8×60K microarrays contain eight arrays, each with ~56,000 probes, representing 3,523 unique probe sequences for 1,205 human miRNAs, which cover approximately 64% of the miRNA repertoire in the human genome, and 142 miRNAs from other species. The microarrays were scanned using an Agilent Technologies Scanner G2505C (Agilent Technologies) and then miRNA expression data for analysis were generated.

Microarray data processing and analysis. Quantile normalization was performed on the expression value (MATLAB/Bioinformatics Toolbox, MathWorks, Natick, MA, USA). After removing the control probes, median values for each unique probe were calculated and values lower than 1 were set to 1 for the convenience of log2-transform. Duplicated microarrays were averaged to represent each experiment condition. Differential miRNA expressions were determined by performing two-sample *t* test for each probe. The significantly differentially expressed miRNAs were selected with *P*-values less than 0.05 and log2 fold-change greater than 1 (two-fold change). The heatmaps of differentially expressed miRNAs for each comparison were generated with the *z*-transformed expression value across samples as marked in color scale (red represents overexpression and green underexpression).

Functional enrichment analysis, pathway and network analysis, and miRNA target analysis. Functional pathway and network analyses of differentially expressed miRNAs were performed using Ingenuity Pathway Analysis (IPA) (Ingenuity® Systems, Redwood City, CA, USA). The Ingenuity Knowledge Base, a repository of biological and chemical interactions, was used as a reference set.

Functional enrichment analysis. The functional analysis module in IPA was used to identify over-represented molecular and cellular functions of differentially expressed miRNAs. The probability that each biological function assigned to the data set was due to chance alone was estimated, and a false discovery rate (FDR) <0.05 was used in multiple hypothesis testing to correct for multiple comparisons and to minimize false positives among significantly enriched functions. Gene ontology and KEGG pathway enrichment analysis was performed using the DAVID functional analysis tool.[37] Bonferroni, Benjamini–Hochberg, and FDR were used for multiple test correction.

Canonical pathway analysis. Over-represented canonical signaling and metabolic pathways in the input data were determined based on two parameters: (1) The ratio of the number of molecules from the focus miRNA set that map to a given pathway divided by the total number of molecules that map to the canonical pathway, and (2) a *P*-value calculated by Fisher's exact test that determines the probability that the association between the focus loci and the canonical pathway is explained by chance alone.

Network analysis. Network analysis used focus miRNAs as "seeds" to infer de novo interaction networks. Direct or indirect interactions between focus loci and other molecules were inferred based on experimentally observed relationships supported by at least one reference from the literature. Additional molecules from the Ingenuity Knowledge Base were added to the network to fill or join smaller networks. The network score was based on the hypergeometric distribution and calculated with the right-tailed Fisher's exact test. A higher score indicates a lower probability of finding the observed number of focus molecules in a given network by chance.

MiRNA Target Filter was used to explore experimentally validated miRNA–mRNA interactions based on relevant biological information and expression information from TarBase[38] and miRecords.[39] TargetScan was used to predict novel miRNA–mRNA interactions.[40]

Results and Discussion

The genome-wide miRNA expression profiles for iPS and iPS-RPE cells were revealed by miRNA microarray analysis. We derived the iPS-RPE and demonstrated that they displayed the classical highly pigmented hexagonal RPE morphology (Fig. 1), expressed RPE proteins, and performed functions of retinoid processing and secretion of 11-*cis* retinal.[17] In order to capture development-stage specific expression patterns, we cultured iPS-RPE for 17 days (Samples #5, #6, #42, #13, #23, and #44). Hierarchical clustering clearly showed that iPS-RPE segregated from the iPS cells (Samples #6A, 7, and 51) (Fig. 2).

Distinct miRNA profiles between iPS and iPS-RPE: promoting differentiation and inhibiting proliferation. The differentiation from iPS to iPS-RPE is a complex, orchestrated process. MiRNAs form an important regulatory layer that contributes to this lineage-specific cell fate transition. Our miRNA microarray analysis identified 155 probes that were statistically differentially expressed (fold change >2, and *P*-value <0.05, see Supplementary Table 1 for a complete list). They corresponded to 113 unique miRNAs, showing two dynamically regulated patterns.

The first class included 53 up-regulated probes corresponding to 42 unique miRNAs. Six miRNAs were previously reported to be up-regulated during the differentiation of human ES cells into RPE, including miR-181c, miR-100, miR-22, miR-222, miR-23a, and miR-26.[41,42] The highest fold-change (31-fold) was observed in miR-181c in iPS-RPE. MiR-181c is known for its tissue specificity, with preferential expression in the retina and brain.[43] MiR-181c was

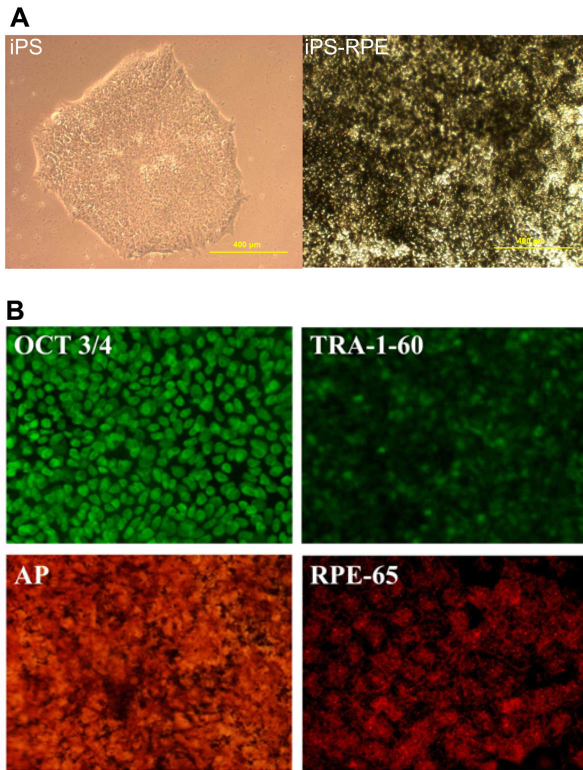

Figure 1. (**A**) Brightfield images of iPS and iPS-RPE. Brightfield images (magnification 100×) of iPS prior to differentiation (left) and RPE derived from iPS (right) (magnification 100×). iPS-RPE display classical RPE morphology of hexagonal shape and pigmentation. (**B**) iPS express pluripotent markers OCT3/4, TRA-1-60, and alkaline phosphatase (AP) (magnification 200×). iPS-RPE express RPE-specific marker RPE-65 (magnification 400×).

found to promote skeletal muscle differentiation by targeting a homeobox protein HOX-A11.[44] Its high expression in iPS-RPE suggested its role in activating and reinforcing differentiation of RPE. Thirty-six more miRNAs were newly identified as up-regulated in iPS-RPE (Table 1). The expression of miR-129–5p was elevated 28-fold in iPS-RPE. Like miR-181c, miR-129–5p is expressed in the brain tissue.[45] Growing evidence suggested that it is antiproliferative; its validated targets include cell cycle regulator cyclin-dependent kinase (CDK6),[46] eukaryotic translation initiation factor 2C3 (EIF2C3), and calmodulin binding transcription activator 1 (CAMTA1).[47,48] Other up-regulated miRNAs in iPS-RPE, such as miR-24, have been implicated in either inhibiting cell proliferation or promoting differentiation in various cell types.[41,49]

The second class of 102 probes were down-regulated, corresponding to 71 unique miRNAs. Eight miRNAs were found to be down-regulated during differentiation of RPE from human ES cells,[42,50] including miR-130a, miR15-b, miR-17, miR-18a, miR-25, miR302c, miR302d, and miR-363. Among them, miR302c and miR302d, members of the miR302 cluster, are known to promote self-renewal in human ES cells, which are directly regulated by pluripotency factors Oct4/Sox-2 and

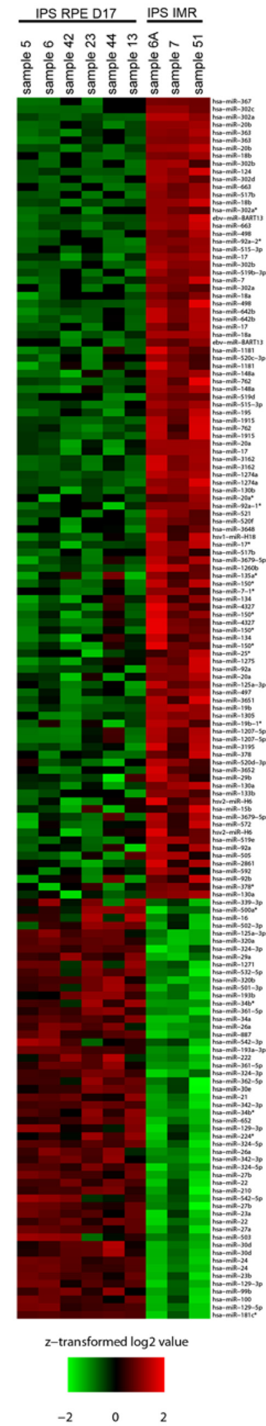

Figure 2. Hierarchical clustering of miRNA expression profiles between iPS and iPS-RPE.

subsequently target the critical cell cycle regulator cyclin D1.[8] Our expression profiling revealed an additional 63 miRNAs that were repressed during the RPE differentiation process (Table 2). The most reduced expression fold change (3,757-fold) was observed in miR-367, which is a validated pluripotency inducer. It was reported that miR-367 and miR-302, in combination with inhibition of the chromatin remodeling factor histone deacetylase (HDAC2), were able to reprogram somatic

Table 1. Representative miRNAs that were up-regulated during the differentiation from iPS to iPS-RPE.

PROBE ID	miRNA	LOG2RATIO (FOLD CHANGE)	*P*-VALUE
A_25_P00013319	hsa-miR-181c*	4.96	0.026
A_25_P00013881	hsa-miR-129–5p	4.83	0.004
A_25_P00010474	hsa-miR-100	4.76	0.028
A_25_P00010597	hsa-miR-99b	4.34	0.005
A_25_P00012226 A_25_P00012225	hsa-miR-129–3p	4.27 2.52	0.008 0.025
A_25_P00010881	hsa-miR-23b	4.24	0.025
A_25_P00010676 A_25_P00010677	hsa-miR-24	4.21 4.14	0.002 0.005
A_25_P00010682 A_25_P00010683	hsa-miR-30d	3.92 3.76	0.002 0
A_25_P00014885	hsa-miR-503	3.75	0.007
A_25_P00014821	hsa-miR-27a	3.68	0.026

Note: Probe ID as defined by Agilent Human miRNA v16 microarray.

Table 2. Representative miRNAs that were down-regulated during the differentiation from iPS cells to iPS-RPE.

PROBE ID	miRNA	LOG2RATIO (FOLD CHANGE)	*P*-VALUE
A_25_P00010984	hsa-miR-367	−11.87	0
A_25_P00010536	hsa-miR-302c	−10.14	0.002
A_25_P00010982 A_25_P00010983	hsa-miR-302a	−9.47 −4.07	0 0.006
A_25_P00010615 A_25_P00010614	hsa-miR-20b	−9.29 −8.29	0 0
A_25_P00010953 A_25_P00010954	hsa-miR-363	−8.44 −8.35	0 0
A_25_P00012431 A_25_P00014859	hsa-miR-18b	−7.85 −6.04	0 0.001
A_25_P00014840	hsa-miR-124	−6.98	0.004
A_25_P00010162	hsa-miR-302d	−6.45	0.007

Note: Probe ID as defined by Agilent Human miRNA v16 microarray.

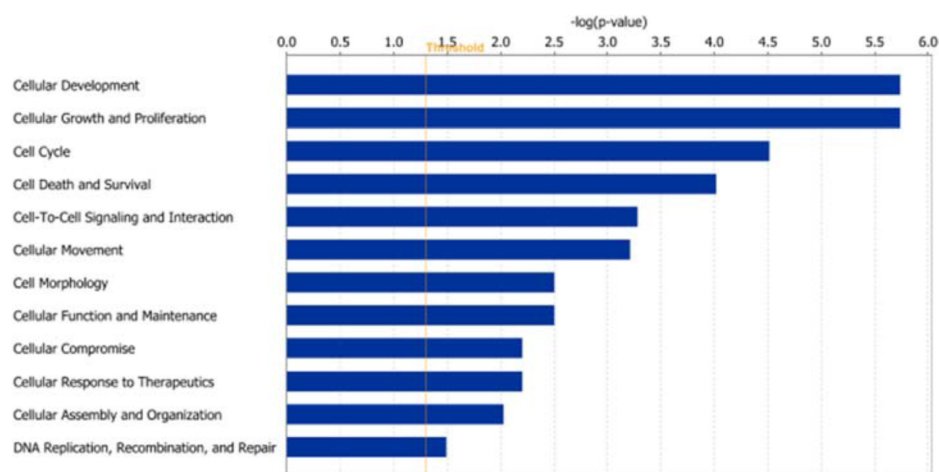

Figure 3. The molecular and cellular functions that were over-represented in the differentially expressed miRNAs during differentiation from iPS to iPS-RPE.

cells into pluripotency without any exogenous transcription factors.[51] It is therefore not surprising that miR-367 was shut off during RPE differentiation. Similarly, other highly repressed miRNAs such as miR-18b, miR-20b, and miR-17 are involved in cell proliferation and cell adhesion.[52,53]

Enrichment analysis confirmed that the most over-represented functional classes in the set of up- and down-regulated miRNAs were associated with cell fate determination including cellular development, cellular growth and proliferation, cell cycle, cell death, and survival (Fig. 3). These miRNAs were also involved in other fundamental processes such as cellular movement, assembly and organization, DNA replication, recombination, and repair. In addition, 20 miRNAs were reported to regulate cell-to-cell signaling and interactions. For example, inhibition of miR-16 in human laryngeal carcinoma cells could suppress cell motility as well as enhance cellular adhesion.[54] Similarly, miR-24 was thought to play an important role in actin adhesion, which controls epithelial stratification and cytoskeleton remodelling.[41]

Differentially expressed miRNAs are involved in sophisticated cellular networks. To achieve a systems-level understanding of miRNA dynamics and its impact on iPS-RPE differentiation, we performed pathway and network analysis. Not surprisingly, no canonical pathways were over-represented in differentially expressed miRNAs; this is likely due to the fact that the biochemical and molecular functions

Table 3. Representative targets of differentially expressed miRNAs and their biological functions.

miRNA	TARGETS	BIOLOGICAL PROCESSES ASSOCIATED WITH TARGETS
miR-30c-5p (and other miRNAs w/seed GUAAACA)	AP2A1, BCL6, CTGF, F2, GNAI2, JUN, LMNB2, MYO10, NPR3, PTPRK, SLC38A1, TNFRSF10B, TP53, WNT5A	Protein transport, T-helper cell differentiation, cell adhesion, blood homeostasis, inflammation, wound healing, transcriptional regulation, cell fate and embryogenesis
miR-34a-5p (and other miRNAs w/seed GGCAGUG)	AXIN2, BCL2, CDK6, CREB1, E2F3, E2F5, HDAC1, MAP2 K2, MYC, NOTCH1, NOTCH2, SIRT1, WNT1	Wnt/β-catenin signaling, regulate apoptosis, synchronization of circadian rhythmicity, differentiation of adipose cells, breast cancer regulation, cell cycle regulation, MAPK signaling, epithelial adherens junction signaling
miR-124–3p (and other miRNAs w/seed AAGGCAC)	AK2, AP1M2, ARAF, BDNF, CAV1, CDK2, CDK4, CDK6, DFFB, E2F5, EGR1, ELF4, FOXA2, GSN, MAPK14, SMAD5	AMPK signaling, acute myeloid leukemia signaling, axonal growth, cell cycle regulation, apoptosis signaling, cell proliferation, mitogenesis, innate immunity
miR-129–5p (miRNAs w/seed UUUUUGC)	AGO3, BMPR2, CAMTA1, ETV6, FNDC3B, GALNT1, PDS5A, SOX4, TNPO1, TP53INP1, ZFP91	RNA interference, bone formation, embryogenesis, transcriptional regulation, adipogenesis, glycosylation, DNA repair, embryonic development, protein transport
miR-133a-3p (and other miRNAs w/seed UUGGUCC)	BCL2L2, CASP9, CDC42, CDK13, CTGF, IGF1R, MCL1, NELFA, PTPRK, RB1CC1, RHOA, RUNX2, SRF, STK3	Apoptosis, cell cycle regulation, chondrocyte proliferation, differentiation, cell adhesion, tumor growth, transcriptional regulation, cell migration
miR-16–5p (and other miRNAs w/seed AGCAGCA)	ANLN, ATF6, BCL2, BDNF, CCNF, EGFR, EIF4E, FGF2, FGF7, FGFR1, HMGA1, IGF2R, JUN, KIF23, MYB, VEGFA	Cytokinesis, ER stress response, cell cycle progression, translation, cell division, cell migration, metastatic progression, angiogenesis, vasculogenesis, endothelial cell growth
miR-17–5p (and other miRNAs w/seed AAAGUGC)	BCL2,CDKN1A, E2F2, IL8, ITCH, JAK1, MEF2D, RAF1, Ras, RB1, RBL2, S1PR1, Sos, STAT3, TGFBR2, TP63	Apoptosis, cell cycle progression, inflammatory response, erythroid and lymphoid cell differentiation, IFNα/β/γ/ signaling, muscle development, neuronal differentiation and survival
miR-21–5p (and other miRNAs w/seed AGCUUAU)	APAF1, BMPR2, BTG2, CDKN1A, FAS, IL6R, JAG1, NFIB, PELI1, PTEN, RECK, SERPINB5, SOD3, SOX5, TGFBR2, TNF	Apoptosis, cell cycle regulation, immune response, cell growth, transcriptional regulation, cancer progression, oxidative stress response, embryonic development
miR-221–3p (and other miRNAs w/seed GCUACAU)	BBC3, BCL2L11, BMF, BNIP3L, CDKN1B, CDKN1C, DIRAS3, ESR1, FOS, FOXO3, ICAM1, KIT, MMP1, TIMP3	Apoptosis, cell cycle regulation, growth suppression, sexual development, cell proliferation, differentiation, stem cell maintenance, gametogenesis, mast cell development, migration
miR-23a-3p (and other miRNAs w/seed UCACAUU)	ATAT1, CXCL12, FBXO32, HES1, IL6R, LMNB1, MDH2, MET, NOTCH1, PLAU, SEPT3, SMAD3, SMAD4, SMAD5	Microtubule destabilization and dynamics, embryogenesis, immune surveillance, inflammation response, tissue homeostasis, and tumor growth and metastasis
miR-291a-3p (and other miRNAs w/seed AAGUGCU)	ADAM9, APP, KIF23, LEFTY1, LEFTY2, MICA, MYBL1	Fertilization, muscle development, neurogenesis, cell mobility, transcriptional regulation, cytokinesis
	SEPT2, STK4, TP63, UBXN1, USP12, VEGFA, VPS26A	Left–right axis determination, cytoskeleton organization

of the majority of miRNAs are yet to be discovered and the crosstalk between miRNAs and their upstream regulators/downstream effectors is yet to be elucidated.

An important step toward a better understanding of miRNA regulatory networks is to identify their target mRNAs. The IPA miRNA Target Filter using TarBase[38] and miRecords[39] revealed that 846 mRNAs were experimentally validated targets of 40 differentially expressed miRNAs (Supplementary Table 2). Enrichment analysis showed that these genes are mainly involved in cell death and survival ($P = 5.36E-72$), cellular development ($P = 7.91E-69$), organismal survival ($P = 3.48E-64$), cellular growth and proliferation ($P = 3.56E-63$), and gene expression regulation ($P = 1.32E-47$).

Notably, a large number of target genes are oncogenes, tumor suppressors, or transcriptional regulators (Table 3). KEGG pathway analysis using DAVID Bioinformatics Resource[37] revealed that 86 genes were involved in pathways related to cancer ($P = 6.23E-30$). Figure 4 shows a molecular network associated with miRNA and tumor suppressor gene TP53, in which miRNA post-transcriptional regulation acts as an important mechanism for TP53 signaling, where miRNAs can serve both as regulators and the effectors of TP53.

It is therefore important to systematically assess the potential roles of miRNAs in iPS-derived RPEs in carcinogenesis.[55,56] Several up-regulated miRNAs in iPS-RPE are

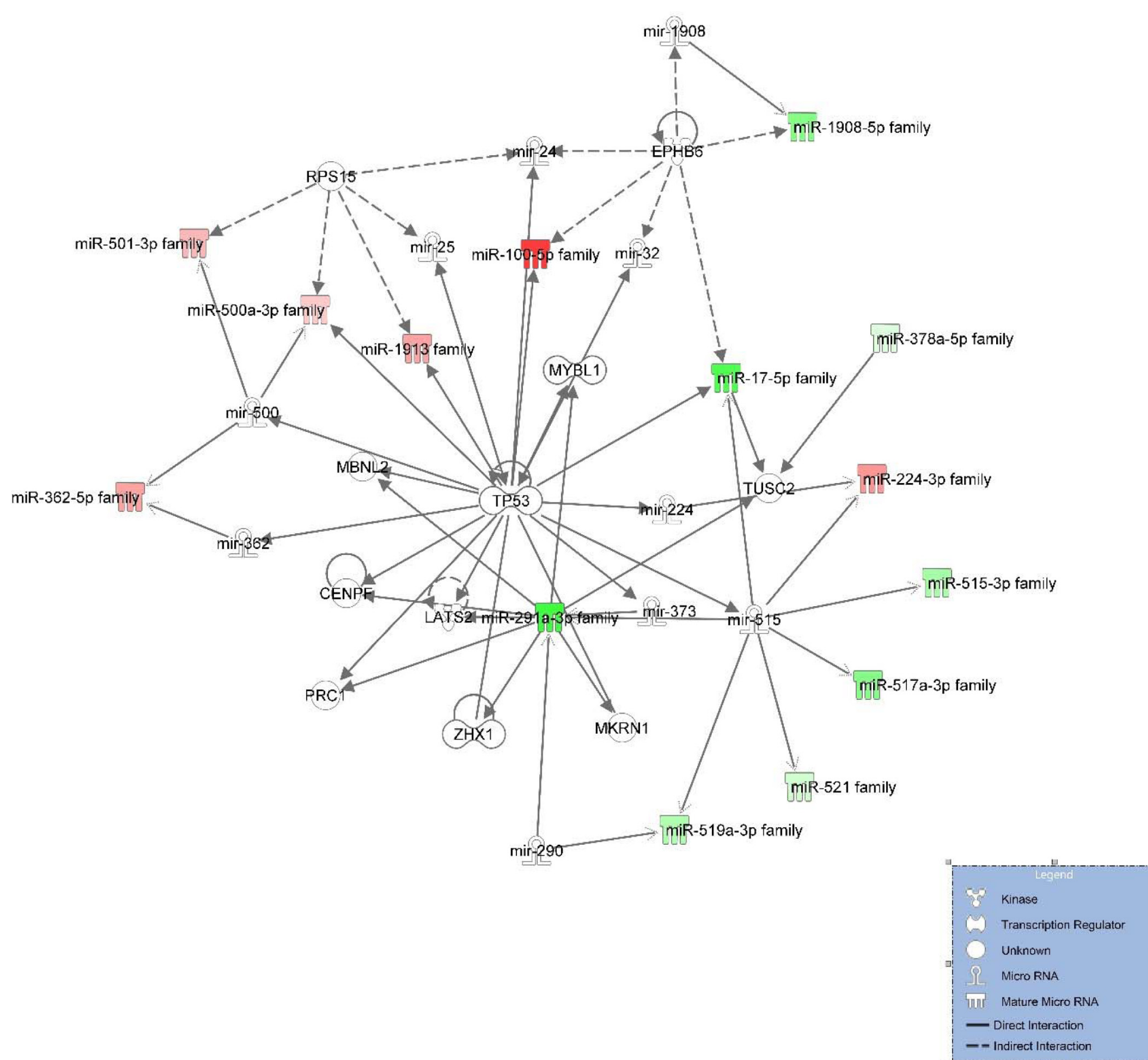

Figure 4. A molecular network associated with miRNA and tumor suppressor gene TP53.

Note: Red and green shaded nodes represent up- and down-regulated miRNAs, respectively. Solid lines show direct interaction (binding/physical contact), and dashed lines show indirect interaction supported by the literature. The annotations for the following miRNA families are: miR-100–5p (and other miRNAs w/seed ACCCGUA), miR-17–5p (and other miRNAs w/seed AAAGUGC), miR-1908–5p (and other miRNAs w/seed GGCGGGG), miR-1913 (and other miRNAs w/seed CUGCCCC), miR-224–3p (and other miRNAs w/seed AAAUGGU), miR-291a-3p (and other miRNAs w/seed AAGUGCU), miR-362–5p (and other miRNAs w/seed AUCCUUG), miR-378a-5p (miRNAs w/seed UCCUGAC), miR-500a-3p (miRNAs w/seed UGCACCU), miR-501–3p (and other miRNAs w/seed AUGCACC), miR-515–3p (and other miRNAs w/seed AGUGCCU), miR-517a-3p (and other miRNAs w/seed UCGUGCA), miR-519a-3p (and other miRNAs w/seed AAGUGCA), and miR-521 (miRNAs w/seed ACGCACU).

tumor suppressors. For example, miR-34, which is directly regulated by TP53, acts as a strong tumor suppressor by inhibiting cell proliferation, epithelial-mesenchymal transition (EMT), metastasis, and invasion. MiR-34 is commonly silenced in a variety of cancer types.[57] The expression of miR-34 was amplified by five-fold in iPS-RPE, suggesting the low proliferation potential in these fully differentiated cells. Similarly, miR-16, a suppressor miRNA that targets a number of oncogenes such as BCL2, JUN, and EGFR, was more abundant in iPS-RPE than in the iPS cells. By contrast, a number of down-regulated miRNAs in iPS-RPE are oncogenic miRNAs. The miR-17–92 cluster is found to be highly activated in solid tumors; one possible activation mechanism is through c-Myc transactivation.[58] The members of this cluster, miR-20b, miR-92, miR-17, miR-18a, and miR-19b, were either silenced or repressed in iPS-RPE cells by 626-fold, 25-fold, 19-fold, 16-fold, and 3-fold, respectively.

Further integrated miRNA–mRNA target analysis identified two inter-connected cancer-related networks: (1) The first network is related to cancer, organismal injury and abnormalities, and reproductive system disease (Fig. 5). The targets with high connectivity included: (a) the Smad2/3 complex, which is essential for the transforming growth factor beta (TGF-β) signaling pathway that regulates cell proliferation, differentiation, and apoptosis. A small RNA-Seq assay showed that SMAD can directly induce miR-92 in mouse ES cells.[59] MiR-92 is a proven oncogenic miRNA and its aberrant expression has been reported in various cancers such as hepatocellular carcinoma.[60] Our microarray analysis confirmed that the oncogenic miR-92 was significantly repressed in iPS-RPE; (b) vascular endothelial growth factor (VEGF), which plays a pivotal

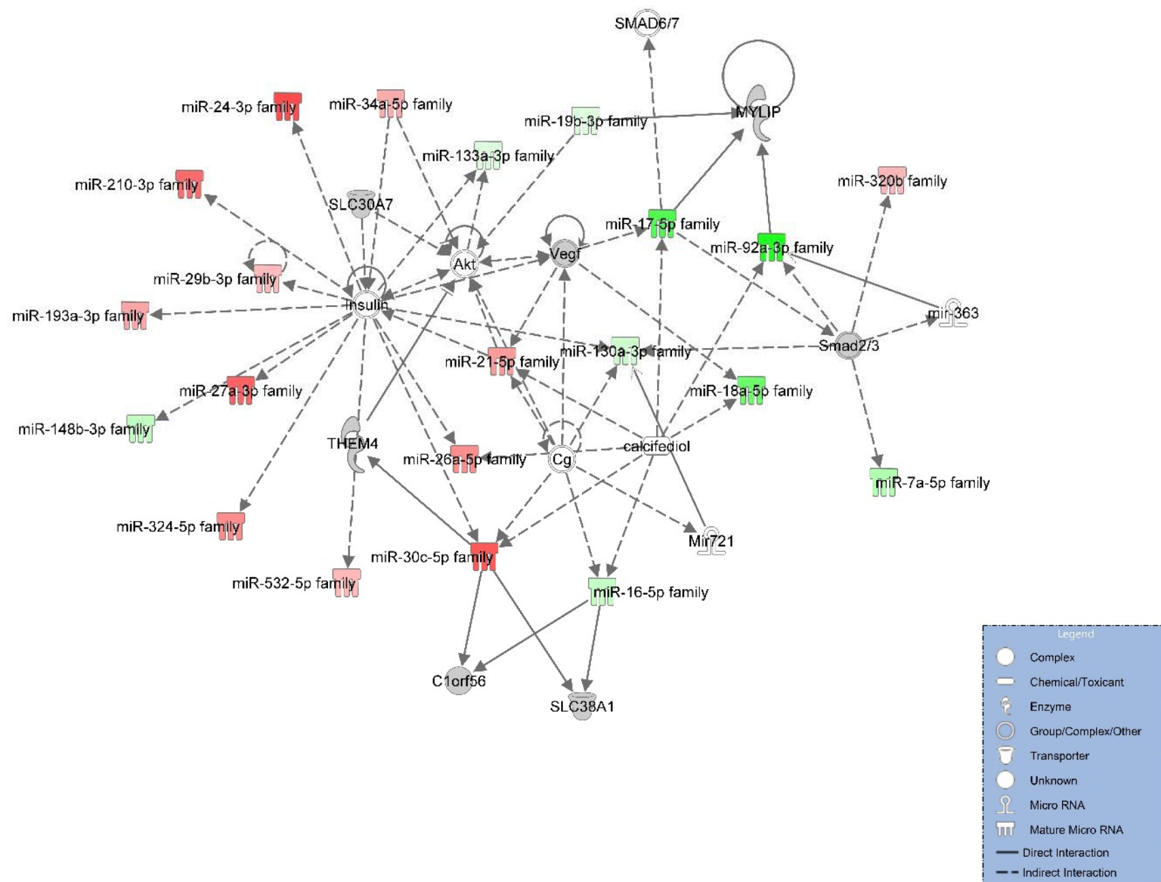

Figure 5. A molecular network associated with organismal injury and abnormalities, and reproductive system disease.

Note: Red and green shaded nodes represent up- and down-regulated miRNAs, respectively. Solid lines show direct interaction (binding/physical contact), and dashed lines show indirect interaction supported by the literature. The annotations for the following miRNA families are: miR-130a-3p (and other miRNAs w/seed AGUGCAA), miR-133a-3p (and other miRNAs w/seed UUGGUCC), miR-148b-3p (and other miRNAs w/seed CAGUGCA), miR-16–5p (and other miRNAs w/seed AGCAGCA), miR-17–5p (and other miRNAs w/seed AAAGUGC), miR-18a-5p (and other miRNAs w/seed AAGGUGC), miR-193a-3p (and other miRNAs w/seed ACUGGCC), miR-19b-3p (and other miRNAs w/seed GUGCAAA), miR-21–5p (and other miRNAs w/seed AGCUUAU), miR-210–3p (miRNAs w/seed UGUGCGU), miR-24–3p (and other miRNAs w/seed GGCUCAG), miR-26a-5p (and other miRNAs w/seed UCAAGUA), miR-27a-3p (and other miRNAs w/seed UCACAGU), miR-29b-3p (and other miRNAs w/seed AGCACCA), miR-30c-5p (and other miRNAs w/seed GUAAACA), miR-320b (and other miRNAs w/seed AAAGCUG), miR-324–5p (miRNAs w/seed GCAUCCC), miR-34a-5p (and other miRNAs w/seed GGCAGUG), miR-532–5p (and other miRNAs w/seed AUGCCUU), miR-7a-5p (and other miRNAs w/seed GGAAGAC), and miR-92a-3p (and other miRNAs w/seed AUUGCAC).

role in angiogenesis. VEGF was shown to induce miR-17 and miR-18 in pathological angiogenesis and tumors.[61] Both oncogenic miRNAs were down-regulated in iPS-RPE cells. (2) The second network is related to cancer, gastrointestinal disease, and hepatic system disease (Fig. 6). Tumor suppressor miR-100 was implicated in acute lymphoblastic leukemia (ALL) and child adrenocortical tumors. It was found to promote apoptosis by targeting the insulin-like growth factor 1 receptor (IGF1R)-mTOR signaling pathway.[62] The expression of miR-100 was enhanced by 27-fold in iPS-RPE cells, showing a strong antiproliferative effect. Similarly, miR-23 was also induced in iPS-RPE cells (by 18-fold); its tumor suppressor function is not exerted mainly through the regulation of cell cycle, but through the regulation of cell motility. MiR-23 targets a number of cytoskeleton proteins such as PLAU by enhancing cell-to-cell adhesion and inhibiting cell migration and metastasis.[63] Other targets in this network, such as mitogen-activated protein kinase 1 (MAPK1), mitogen-activated protein kinase kinase kinase 12 (MAP3K12), and cyclin-dependent kinase inhibitor 1B (CDKN1B), play integral roles in cell cycle progression and differentiation. Overall, the miRNA signature in these cancer-related networks seemed to operate to stabilize a differentiated state in iPS-RPE.

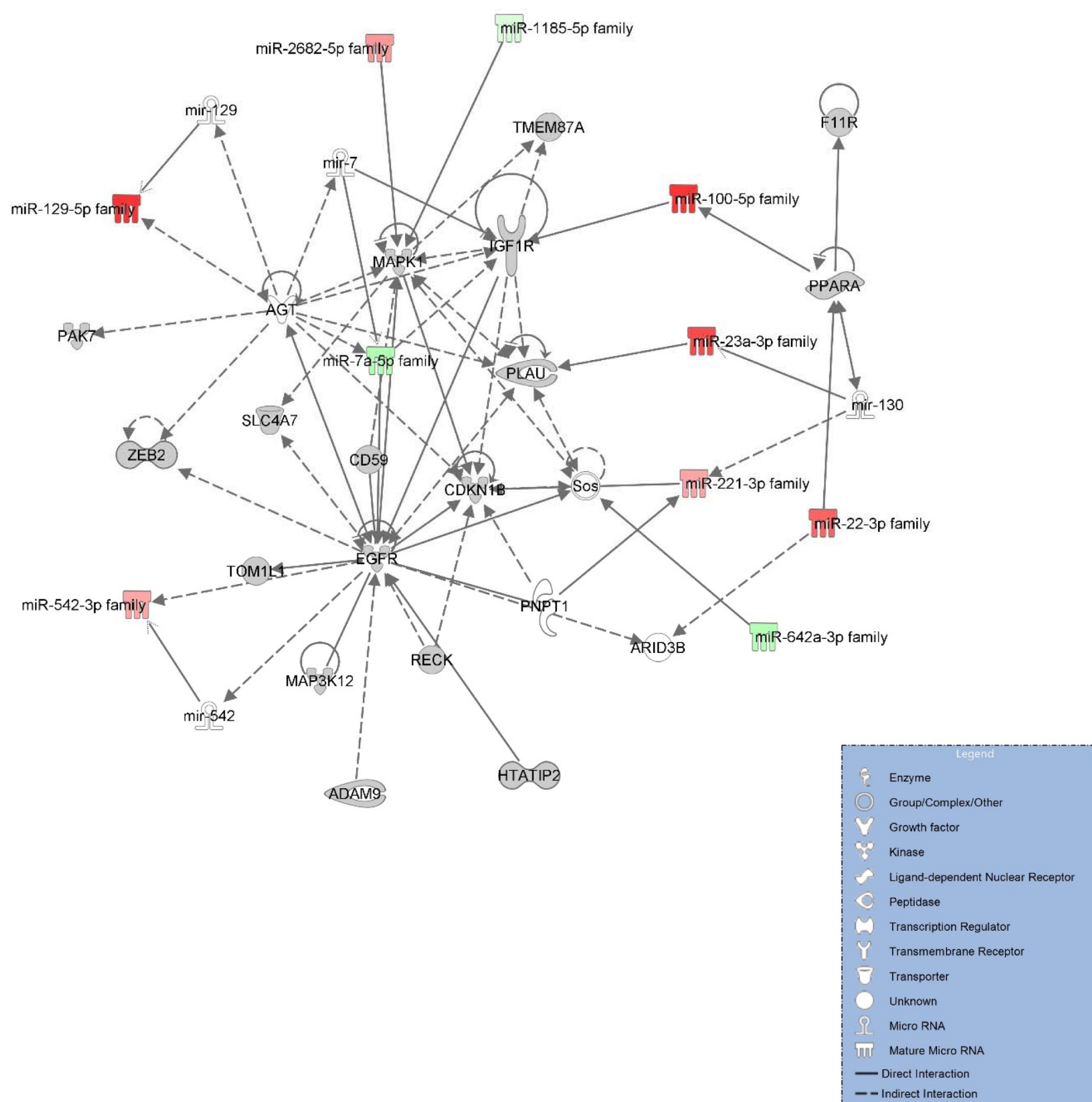

Figure 6. A molecular network associated with cancer, gastrointestinal disease, and hepatic system disease.

Note: Red and green shaded nodes represent up- and down-regulated miRNAs, respectively. Solid lines show direct interaction (binding/physical contact), and dashed lines show indirect interaction supported by the literature. The annotations for the following miRNA families are: miR-100–5p (and other miRNAs w/seed ACCCGUA), miR-1185–5p (and other miRNAs w/seed GAGGAUA), miR-129–5p (miRNAs w/seed UUUUUGC), miR-22–3p (miRNAs w/seed AGCUGCC), miR-221–3p (and other miRNAs w/seed GCUACAU), miR-23a-3p (and other miRNAs w/seed UCACAUU), miR-2682–5p (and other miRNAs w/seed AGGCAGU), miR-542–3p (miRNAs w/seed GUGACAG), miR-642a-3p (and other miRNAs w/seed GACACAU), and miR-7a-5p (and other miRNAs w/seed GGAAGAC).

Concluding Remarks

MiRNA profiling of iPS and iPS-RPE cells revealed distinct molecular signatures of post-transcriptional regulation during the differentiation process. These differentially expressed miRNAs may invoke complex miRNA–target interaction networks that coordinate a cascade of signaling responses required to suppress cell proliferation, reinforce differentiation, thereby reducing carcinogenesis. A systematic interrogation of temporal and spatial expression of iPS-RPE miRNAs and their associated target mRNAs will provide new insights into the molecular mechanisms of carcinogenesis, eye differentiation and development.

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Author Contributions

Conceived and designed the experiments: HCW, YW, JSG. Analyzed the data: HIHC, WAG, HC, RRK, HCW, YW. Wrote the first draft of the manuscript: HCW, YW, WAG, HIHC. Contributed to the writing of the manuscript: JSG, HC. Agree with manuscript results and conclusions: HCW, WAG, RRK, JSG, HIHC, HC, YW. Jointly developed the structure and arguments for the paper: HCW, YW. Made critical revisions and approved final version: HCW, WG, RRK, JSG, HIHC, HC, YW. All authors reviewed and approved of the final manuscript.

Supplementary Files

Supplementary Table 1. Differentially expressed microRNA probes between iPS-RPE and iPS cells. Fold change of 2 and P = value <0.05 were used as cutoff values.

Supplementary Table 2. Differentially expressed microRNAs and their target genes.

REFERENCES

1. Yu Z, Li Y, Fan H, Liu Z, Pestell RG. miRNAs regulate stem cell self-renewal and differentiation. *Front Genet*. 2012;3:191.
2. Yamanaka S, Takahashi K. Induction of pluripotent stem cells from mouse fibroblast cultures. *Tanpakushitsu Kakusan Koso*. 2006;51(15):2346–51.
3. de Almeida PE, Ransohoff JD, Nahid A, Wu JC. Immunogenicity of pluripotent stem cells and their derivatives. *Circ Res*. 2013;112(3):549–61.
4. Romano G, Morales F, Marino IR, Giordano A. A commentary on iPS cells: potential applications in autologous transplantation, study of illnesses and drug screening. *J Cell Physiol*. 2014;229(2):148–52.
5. Alipio Z, Liao W, Roemer EJ, et al. Reversal of hyperglycemia in diabetic mouse models using induced pluripotent stem (iPS)-derived pancreatic beta-like cells. *Proc Natl Acad Sci U S A*. 2010;107(30):13426–31.
6. Bharti K, Miller SS, Arnheiter H. The new paradigm: retinal pigment epithelium cells generated from embryonic or induced pluripotent stem cells. *Pigment Cell Melanoma Res*. 2011;24(1):21–34.
7. Buchholz DE, Hikita ST, Rowland TJ, et al. Derivation of functional retinal pigmented epithelium from induced pluripotent stem cells. *Stem Cells*. 2009;27(10):2427–34.
8. Card DA, Hebbar PB, Li L, et al. Oct4/Sox2-regulated miR-302 targets cyclin D1 in human embryonic stem cells. *Mol Cell Biol*. 2008;28(20):6426–38.
9. Kokkinaki M, Sahibzada N, Golestaneh N. Human induced pluripotent stem-derived retinal pigment epithelium (RPE) cells exhibit ion transport, membrane potential, polarized vascular endothelial growth factor secretion, and gene expression pattern similar to native RPE. *Stem Cells*. 2011;29(5):825–35.
10. Lee KS, Zhou W, Scott-McKean JJ, et al. Human sensory neurons derived from induced pluripotent stem cells support varicella-zoster virus infection. *PLoS One*. 2012;7(12):e53010.
11. Pera MF. Stem cells. A new year and a new era. *Nature*. 2008;451(7175):135–6.
12. Subba Rao M, Sasikala M, Nageshwar Reddy D. Thinking outside the liver: induced pluripotent stem cells for hepatic applications. *World J Gastroenterol*. 2013;19(22):3385–96.
13. Yoshida Y, Yamanaka S. iPS cells: a source of cardiac regeneration. *J Mol Cell Cardiol*. 2011;50(2):327–32.
14. Cour la M, Tezel T. *The Retinal Pigment Epithelium*. Amsterdam: Elsevier; 2006.
15. Carr AJ, Vugler AA, Hikita ST, et al. Protective effects of human iPS-derived retinal pigment epithelium cell transplantation in the retinal dystrophic rat. *PLoS One*. 2009;4(12):e8152.
16. Schwartz SD, Hubschman JP, Heilwell G, et al. Embryonic stem cell trials for macular degeneration: a preliminary report. *Lancet*. 2012;379(9817):713–20.
17. Muñiz A, Greene WA, Plamper ML, et al. Retinoid uptake, processing, and secretion in human iPS-RPE support the visual cycle. *Invest Ophthalmol Vis Sci*. 2014;55(1):198–209.
18. Schena M, Shalon D, Davis RW, Brown PO. Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science*. 1995;270(5235):467–70.
19. Chu Y, Corey DR. RNA sequencing: platform selection, experimental design, and data interpretation. *Nucleic Acid Ther*. 2012;22(4):271–4.
20. van Dijk EL, Auger H, Jaszczyszyn Y, Thermes C. Ten years of next-generation sequencing technology. *Trends Genet*. 2014;30(9):418–26.
21. Blow MJ, McCulley DJ, Li Z, et al. ChIP-Seq identification of weakly conserved heart enhancers. *Nat Genet*. 2010;42(9):806–10.
22. Bailey T, Krajewski P, Ladunga I, et al. Practical guidelines for the comprehensive analysis of ChIP-seq data. *PLoS Comput Biol*. 2013;9(11):e1003326.
23. Wilkins MR, Pasquali C, Appel RD, et al. From proteins to proteomes: large scale protein identification by two-dimensional electrophoresis and amino acid analysis. *Biotechnology (N Y)*. 1996;14(1):61–5.
24. Chang HY, Thomson JA, Chen X. Microarray analysis of stem cells and differentiation. *Methods Enzymol*. 2006;420:225–54.
25. Gobaa S, Hoehnel S, Roccio M, Negro A, Kobel S, Lutolf MP. Artificial niche microarrays for probing single stem cell fate in high throughput. *Nat Methods*. 2011;8(11):949–55.
26. Lamba DA, Reh TA. Microarray characterization of human embryonic stem cell – derived retinal cultures. *Invest Ophthalmol Vis Sci*. 2011;52(7):4897–906.
27. Liu CG, Calin GA, Volinia S, Croce CM. MicroRNA expression profiling using microarrays. *Nat Protoc*. 2008;3(4):563–78.
28. Wu D, Hu Y, Tong S, Williams BR, Smyth GK, Gantier MP. The use of miRNA microarrays for the analysis of cancer samples with global miRNA decrease. *RNA*. 2013;19(7):876–88.
29. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell*. 2004;116(2):281–97.
30. Lee RC, Feinbaum RL, Ambros V. The *C. elegans* heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell*. 1993;75(5):843–54.
31. Gangaraju VK, Lin H. MicroRNAs: key regulators of stem cells. *Nat Rev Mol Cell Biol*. 2009;10(2):116–25.
32. Kanellopoulou C, Muljo SA, Kung AL, et al. Dicer-deficient mouse embryonic stem cells are defective in differentiation and centromeric silencing. *Genes Dev*. 2005;19(4):489–501.
33. Luningschror P, Hauser S, Kaltschmidt B, Kaltschmidt C. MicroRNAs in pluripotency, reprogramming and cell fate induction. *Biochim Biophys Acta*. 2013;1833(8):1894–903.
34. Maminishkis A, Chen S, Jalickee S, et al. Confluent monolayers of cultured human fetal retinal pigment epithelium exhibit morphology and physiology of native tissue. *Invest Ophthalmol Vis Sci*. 2006;47(8):3612–24.
35. Qiagen. miRNeasy Mini Handbook. 2013. Valencia, CA. Qiagen.
36. Kozomara A, Griffiths-Jones S. miRBase: annotating high confidence microRNAs using deep sequencing data. *Nucleic Acids Res*. 2014;42:D68–73.
37. Huang da W, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc*. 2009;4(1):44–57.
38. Vergoulis T, Vlachos IS, Alexiou P, et al. TarBase 6.0: capturing the exponential growth of miRNA targets with experimental support. *Nucleic Acids Res*. 2012;40:D222–9.
39. Xiao F, Zuo Z, Cai G, Kang S, Gao X, Li T. miRecords: an integrated resource for microRNA-target interactions. *Nucleic Acids Res*. 2009;37:D105–10.
40. Lewis BP, Burge CB, Bartel DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell*. 2005;120(1):15–20.
41. Amelio I, Lena AM, Viticchiè G, et al. miR-24 triggers epidermal differentiation by controlling actin adhesion and cell migration. *J Cell Biol*. 2012;199(2):347–63.
42. Liu Z, Jiang R, Yuan S, et al. Integrated analysis of DNA methylation and RNA transcriptome during in vitro differentiation of human pluripotent stem cells into retinal pigment epithelial cells. *PLoS One*. 2014;9(3):e91416.

43. Ryan DG, Oliveira-Fernandes M, Lavker RM. MicroRNAs of the mammalian eye display distinct and overlapping tissue specificity. *Mol Vis*. 2006;12:1175–84.
44. Naguibneva I, Ameyar-Zazoua M, Polesskaya A, et al. The microRNA miR-181 targets the homeobox protein Hox-A11 during mammalian myoblast differentiation. *Nat Cell Biol*. 2006;8(3):278–84.
45. Lagos-Quintana M, Rauhut R, Yalcin A, Meyer J, Lendeckel W, Tuschl T. Identification of tissue-specific microRNAs from mouse. *Curr Biol*. 2002;12(9):735–9.
46. Wu J, Qian J, Li C, et al. miR-129 regulates cell proliferation by downregulating Cdk6 expression. *Cell Cycle*. 2010;9(9):1809–18.
47. Liao R, Sun J, Zhang L, et al. MicroRNAs play a role in the development of human hematopoietic stem cells. *J Cell Biochem*. 2008;104(3):805–17.
48. Nakatani K, Nishioka J, Itakura T, et al. Cell cycle-dependent transcriptional regulation of calmodulinbinding transcription activator 1 in neuroblastoma cells. *Int J Oncol*. 2004;24(6):1407–12.
49. Lal A, Navarro F, Maher CA, et al. miR-24 inhibits cell proliferation by targeting E2F2, MYC, and other cell-cycle genes via binding to "seedless" 3'UTR microRNA recognition elements. *Mol Cell*. 2009;35(5):610–25.
50. Hu G, Huang K, Yu J, et al. Identification of miRNA signatures during the differentiation of hESCs into retinal pigment epithelial cells. *PLoS One*. 2012;7(7):e37224.
51. Anokye-Danso F, Trivedi CM, Juhr D, et al. Highly efficient miRNA-mediated reprogramming of mouse and human somatic cells to pluripotency. *Cell Stem Cell*. 2011;8(4):376–88.
52. Budde H, Schmitt S, Fitzner D, Opitz L, Salinas-Riester G, Simons M. Control of oligodendroglial cell number by the miR-17–92 cluster. *Development*. 2010;137(13):2127–32.
53. Murakami Y, Tamori A, Itami S, et al. The expression level of miR-18b in hepatocellular carcinoma is associated with the grade of malignancy and prognosis. *BMC Cancer*. 2013;13:99.
54. Wu H, Liu T, Wang R, et al. MicroRNA-16 targets zyxin and promotes cell motility in human laryngeal carcinoma cell line HEp-2. *IUBMB Life*. 2011;63(2):101–8.
55. Nana-Sinkam SP, Croce CM. Clinical applications for microRNAs in cancer. *Clin Pharmacol Ther*. 2013;93(1):98–104.
56. Zhang B, Pan X, Cobb GP, Anderson TA. microRNAs as oncogenes and tumor suppressors. *Dev Biol*. 2007;302(1):1–12.
57. Hermeking H. MicroRNAs in the p53 network: micromanagement of tumour suppression. *Nat Rev Cancer*. 2012;12(9):613–26.
58. O'Donnell KA, Wentzel EA, Zeller KI, Dang CV, Mendell JT. c-Myc-regulated microRNAs modulate E2F1 expression. *Nature*. 2005;435(7043):839–43.
59. Redshaw N, Camps C, Sharma V, et al. TGF-beta/Smad2/3 signaling directly regulates several miRNAs in mouse ES cells and early embryos. *PLoS One*. 2013;8(1):e55186.
60. Shigoka M, Tsuchida A, Matsudo T, et al. Deregulation of miR-92a expression is implicated in hepatocellular carcinoma development. *Pathol Int*. 2010;60(5):351–7.
61. Jamaluddin MS, Weakley SM, Zhang L, et al. miRNAs: roles and clinical applications in vascular disease. *Expert Rev Mol Diagn*. 2011;11(1):79–89.
62. Li XJ, Luo XQ, Han BW, Duan FT, Wei PP, Chen YQ. MicroRNA-100/99a, deregulated in acute lymphoblastic leukaemia, suppress proliferation and promote apoptosis by regulating the FKBP51 and IGF1R/mTOR signalling pathways. *Br J Cancer*. 2013;109(8):2189–98.
63. Pellegrino L, Stebbing J, Braga VM, et al. miR-23b regulates cytoskeletal remodeling, motility and metastasis by directly targeting multiple transcripts. *Nucleic Acids Res*. 2013;41(10):5400–12.