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Differentiation in stem/progenitor cells along fetal or adult hepatic stages requires transcriptional regulators independently of oscillations in microRNA expression

Sriram Bandi^{a,b,1}, Sanchit Gupta^{b,2}, Tatyana Tchaikovskaya^{a,b}, and Sanjeev Gupta^{a,b,c,d,e,*}

^aDepartment of Medicine, Albert Einstein College of Medicine, Bronx, NY, United States

^bMarion Bessin Liver Research Center, Albert Einstein College of Medicine, Bronx, NY, United States

^cDiabetes Center, Albert Einstein College of Medicine, Bronx, NY, United States

^dThe Irwin S. and Sylvia Chanin Institute for Cancer Research, Albert Einstein College of Medicine, Bronx, NY, United States

^eThe Ruth L. and David S. Gottesman Institute for Stem Cell and Regenerative Medicine Research, Albert Einstein College of Medicine, Bronx, NY, United States

Abstract

Understanding mechanisms in lineage differentiation is critical for organ development, pathophysiology and oncogenesis. To determine whether microRNAs (miRNA) may serve as drivers or adjuncts in hepatic differentiation, we studied human embryonic stem cell-derived hepatocytes and primary hepatocytes representing fetal or adult stages. Model systems were used for hepatic lineage advancement or regression under culture conditions with molecular assays. Profiles of miRNA in primary fetal and adult hepatocytes shared similarities and distinctions from pluripotent stem cells or stem cell-derived early fetal-like hepatocytes. During phenotypic regression in fetal or adult hepatocytes, miRNA profiles oscillated to regain stemness-associated features that had not been extinguished in stem cell-derived fetal-like hepatocytes. These oscillations in stemness-associated features were not altered in fetal-like hepatocytes by inhibitory mimics for dominantly-expressed miRNA, such as hsa-miR-99b, -100, -214 and -221/222. The stem cell-derived fetal-like hepatocytes were permissive for miRNA characterizing mature

*Correspondence to: Albert Einstein College of Medicine, Ullmann Building, Room 625, 1300 Morris Park Avenue, Bronx, NY 10461, United States. sriram.band@gmail.com (S. Bandi).

¹Present address: Technical Operations, Cell Therapy Process Development, Sangamo Therapeutics, 501 Canal Boulevard, Richmond, CA 94804, United States.

²Present address: Division of Gastroenterology, Hepatology and Endoscopy, Brigham and Women's Hospital, 45 Francis St, Boston, MA 02115, United States.

Author contributions

SB performed experiments, acquired and interpreted data; Sanchit G analyzed data and interpreted results, TT analyzed data and interpreted results. Corresponding author designed study, obtained funding, analyzed and interpreted data; all authors contributed to preparing and approving manuscript.

Conflict of interest statement

The authors declare no conflicts of interest exist.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.yexcr.2018.06.004>.

hepatocytes, including mimics for hsa-miR-122, -126, -192, -194 and -26b, although transfections of the latter did not advance hepatic differentiation. Examination of genome-wide mRNA expression profiles in stem cell-derived or primary fetal hepatocytes indicated targets of highly abundant miRNA regulated general processes, e.g., cell survival, growth and proliferation, functional maintenance, etc., without directing cell differentiation. Among upstream regulators of gene networks in stem cell-derived hepatocytes included HNF4A, SNAI1, and others, which affect transcriptional circuits directing lineage development or maintenance. Therefore, miRNA expression oscillated in response to microenvironmental conditions, whereas lineage-specific transcriptional regulators, such as HNF4A, were necessary for directing hepatic differentiation. This knowledge will be helpful for understanding the contribution of stem cells in pathophysiological states and oncogenesis, as well as for applications of stem cell-derived hepatocytes.

Keywords

Gene expression; Hepatocyte nuclear factor-4; Liver; Oncogenesis; Pathology

1. Introduction

The role of microRNAs (miRNA) in stemness and differentiation is of general interest, including for tissue-derived or pluripotent stem cells (PSC) [1]. Although regulatory miRNA have been noted in PSC-derived epithelial, mesenchymal or hematopoietic lineages [2–10], as well as cancer stem cells [11–13], their roles in differentiation are controversial. The possibilities are that miRNA may regulate networks of crucial genes or proteins through post-transcriptional mechanisms during differentiation versus serving constitutive roles in cellular maintenance, survival, proliferation, etc., during tissue homeostasis or adaptive processes. For instance, the concept of expression-level dominance (ELD) was proposed for miRNA in species of subgenomes incorporating genetic modifications, e.g., DNA methylation states, where miRNA targeted mRNA or protein-encoding genes during development and also stress or injury responses [14]. As miRNA expression persisted across generations in interspecies hybrids, such ELD was considered to be stably maintained. Remarkably, constitutive expression of miRNA similarly regulated genomes in mammalian cells, e.g., in case of hepatitis B virus (HBV), with dominant-negative factors guiding viral replication in permissive/nonpermissive cell fusions [15]. Later, these were found to concern miRNA networks [16,17].

Evidences for effects of miRNA on cell differentiation have been gathered in many studies [2,3,5,9,18,19]. In the liver, miRNA deficiencies due to Dicer1 knockdown lead to dysregulated fetal gene expression [20]. Also, gene expression regulation by miRNA pioneers hepatic functions, e.g., hsa-miR-122 [21], which also supports hepatitis C virus replication [22]. Studies showed hsa-miR-30 [23], -23b [24], 122 [25], or -194 advanced hepatic differentiation [19], but hsa-miR-302, which affects pluripotency in PSC [9], transdifferentiated hepatocytes to pancreatic islet-like cells [10]. Elsewhere, miRNA silencing benefited cell differentiation, e.g., hsa-miR-221 downregulation for advancing

osteogenic [26], and -let-7f silencing alongside -miR-122 expression for hepatic differentiation in stem cells [27].

We considered that if miRNA were determinants of hepatic differentiation states, it should be possible to achieve alterations in cell fates in loss- or gain-of-function studies. Therefore, we examined human embryonic stem cells (hESC), hESC-derived hepatocytes and primary fetal or adult hepatocytes to obtain model systems for transitions along pluripotency and lineage maturation. This offered opportunities for subtractive analysis of miRNA content followed by applications of specific candidates-of-interest for hepatic differentiation. Although each miRNA may regulate numerous genes, discrepant nature of predicted versus actual miRNA targets has also been apparent [28]. This consideration prompted us to simultaneously probe genome-wide mRNA expression datasets for substantiating our results. These studies revealed oscillations in expression of miRNA during alterations in cell differentiation states with significant roles in molecular and cellular processes. However, hepatic differentiation of cells was independent of miRNA and required alternative mechanisms, especially those involving regulatory transcription factor networks.

2. Materials and methods

2.1. Human embryonic stem cells and fetal cells

Institutional Review Board and Embryonic Stem Cell Research Oversight Committee of Albert Einstein College of Medicine approved studies. WA-01 hESC were from WiCell (Madison, WI). Fetal human livers of 19–24 weeks gestation were from Human Fetal Tissue Repository at Einstein.

2.2. Cell culture

The Pluripotent Stem Cell Core at Einstein maintained WA-01 hESC (WiCell Foundation, Madison, WI) on irradiated feeder cells in DMEM/F12 medium with 20% knock-out serum replacer (KSR), 2mM L-glutamine, 0.1 mM MEM nonessential amino acids (NEAA), 1% penicillin-streptomycin (Invitrogen Corp., Carlsbad, CA), and 4 ng/ml basic FGF (R&D Systems, Minneapolis, MN) (complete medium). hESC-MEC were isolated from hESC cultured in DMEM with 10% FBS, as described previously [29,30]. FH-PP were sorted immunomagnetically for EpCAM and cultured to generate FH-P3, as described previously [31]. Cryo-preserved AH-PP were from Incara Pharmaceuticals (Raleigh-Durham, NC), and cultured as described previously [32].

2.3. Transfection of miRNA in cells

The hESC-MEC cells were cultured to 20–30% confluency in dishes and transfected by lipid-based siPORTNeoFX agent according to manufacturer (Ambion). The miRNA mimics and inhibitors were from Dharmacon (Lafayette, CO).

2.4. Expression of miRNA

Total cellular RNA was isolated by Trizol reagent. Expression profiling was performed by LC Sciences (MRA-1001 arrays, miRHuman_9.1_070207; Dallas, TX). Arrays contained 470 probes for mature miRNAs with seven repeats and 53 controls based on Sanger

miRBase (Release 9.1). For data analysis, background was subtracted by regression-based mapping on 5–25% of lowest intensity points excluding blanks. Spots with signals below $3\times$ background incorporating spot analysis parameters were excluded. Transcripts with < 500 signal intensity constituted below qRT-PCR detection thresholds and were excluded. For clustering, signal intensity was transformed to \log_2 by denominator of 500 across data matrix with intensity (at least one probe > 500) and variation filtering by Cluster3 software (Stanford University, Palo Alto, CA). Heat maps were constructed by setting medians to zero (JavaTree1.1.6r2 software), as recommended [33].

qRT-PCR used QuantiMir RT Kit for small RNA quantitation (System Biosciences, Mountain View, CA). Briefly, RNAs were anchor-tailed with poly-A-polymerase, followed by oligo dT annealing, with RT for 60 min at 42 °C. SYBR Green assays used forward primers from sense strands of mature miRNA and universal reverse primer (System Biosciences) in Realplex Mastercycler (Eppendorf, Hamburg, Germany). Cycle thresholds (Ct) were normalized with control RNU-43 RNA (Applied Biosystems Inc., Foster City, CA). Relative gene expression used 2^{-Ct} method.

2.5. Cellular mRNA expression

Total cellular RNAs were extracted by TRIzol Reagent (Invitrogen), cleaned by RNeasy (Qiagen Sciences, Germantown, MD), with removal of contaminating DNAs by DNase I (Invitrogen), and then reverse-transcription by Omniscript RT kit (Qiagen). Oligonucleotide primers were synthesized commercially for albumin, α -fetoprotein, cytokeratin-19, vimentin and α -smooth muscle actin (Supplementary Table 1). Platinum PCR SuperMix (Invitrogen) was used with annealing at $94^{\circ}\text{C} \times 5$ min, and 35 cycles of $94^{\circ}\text{C} \times 30$ s, $55^{\circ}\text{C} \times 30$ s, $72^{\circ}\text{C} \times 45$ s and 72°C for 10 min. PCR products were resolved in 1% agarose gels containing ethidium bromide.

Global mRNA expression was profiled by U133 2.0 Plus oligonucleotide arrays (Affymetrix, ThermoFisher Scientific, Waltham, MA) with initial data analysis as described previously [31]. Clustering of mRNA expression profiles by cell types used Affymetrix Transcription Analysis Console, version 4 (TAC). Secondary analysis used Ingenuity Pathway Analysis (IPA) (Qiagen Inc., Redwood City, CA). Annotations of miRNA targets, canonical pathways, upstream transcriptional regulators (TR) and mechanistic networks used IPA. Gene expression datasets have been deposited in NCBI's Gene Expression Omnibus [34], and are accessible through GEO Series accession numbers GSE115410, GSE108047 and GSE108048 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSExxx>).

2.6. Cellular protein expression

Cells were stained histochemically for hepatic and biliary markers, i.e., glucose-6-phosphatase (G-6-P), gamma glutamyltranspeptidase (GGT) and dipeptidylpeptidase IV (DPPIV), as described previously [30,35].

2.7. Statistical methods

All experiments were in triplicate and repeated for reproducibility. Data are shown as means \pm SEM where appropriate. Significance of differences was analyzed by *t*-tests, Chi-square,

analysis of variance (ANOVA) with posthoc tests, or Pearson correlation coefficients by GraphPad Prism7 (GraphPad Software, La Jolla, CA). $P < 0.05$ was considered significant.

3. Results

The cell types in this study, hESC, primary fetal human hepatocytes (FH) sorted for epithelial cell adhesion molecule, EpCAM, primary adult human hepatocytes (AH), and their derivatives provided hepatic differentiation stages, as follows (Fig. 1): a) PSC were represented by undifferentiated hESC capable of forming teratomas [30]; b) primary EpCAM+ FH from mid-gestation livers (FH-PP or FH-Ep-PP) [36], possessed stem/progenitor properties, including multiple epithelial and mesenchymal markers, with capacity to generate additional lineages [31,35,37]; c) primary AH from healthy donor livers (AH-PP) represented hepatic maturity [32]; d) spontaneously originating meso-endodermal cells in cultured hESC provided early fetal hepatic-stage (hESC-MEC), as detailed previously [30]; e) culture of FH-PP over three passages introduced lineage regression (FH-P3 or FH-Ep-P3) with resemblance to hESC-MEC [30,31]; and f) AH-PP in primary culture (AH-P0) offered another comparison.

3.1. Profiles of miRNA oscillated with alterations in stemness or differentiation states

From 470 annotated miRNA in arrays, we determined approximately 200 miRNA were expressed above qRT-PCR detection thresholds in cell types. This was verified by qRT-PCR in randomly selected cases (Supplementary Fig. 1).

Presence of hsa-miR-302a, -b, -c, -d, and -200c in hESC versus either FH-PP or AH-PP seemed appropriate as these marked pluripotency [9,38–40] (Table 1). However, hESC and FH-PP shared multiple miRNA. In FH-PP and AH-PP, miRNA characterizing hepatocytes were abundant: hsa-miR-122 [21], -26b, -126, -192, -194 and -let-7 members. However, differences in FH-PP and AH included abundances of hsa-miR-30b and -30c in former and of -miR-223, -215 and -148a in latter. Therefore, FH-PP exhibited aspects of stem cells and also of hepatocytes, which was consistent with their ability to generate additional lineages, e.g., chondrocytes and osteocytes or endothelial cells [36,37].

For miRNA regulation in multipotency or early fetal hepatic stages, we compared hESC-MEC and FH-P3 and noted that 17 of 20 (85%) top 20miRNA were shared (Table 2). In these cell types, abundances of hsa-miR-125b, -29a, -100, -221, -222 differed from FH-PP, AH-PP or hESC. These cell type-specific differences correlated with global miRNA expression profiles (Supplementary Fig. 2). The miRNA in hESC correlated negatively with other cell types indicating divergences in pluripotency and differentiation states. The miRNA in FH-PP versus AH-PP and also in hESC-MEC versus FH-P3 correlated significantly. Hierarchical clustering of 129 miRNA present in at least one cell type each indicated variations were related to pluripotency or differentiation in hESC-MEC, FH-P3 and AH-P0 (Supplementary Fig. 3). In AH-PP after culture, which alters hepatic gene expression [16,17], miRNA profile resembled that in FH-P3 (Table 3), including abundant hsa-miR-638, -26a and -15b. Since these miRNA were also present in hESC, this rapid return of their expression in mature hepatocytes seemed remarkable.

Mapping of chromosomal loci revealed miRNA expressed in cell types were located broadly in genome although this distribution pattern significantly varied (Supplementary Fig. 4). Thus, locus-specific regulation of miRNA expression could have contributed in expression of their targets.

3.2. Induced regulation of miRNA in intermediate stage of pluripotency and differentiation

As lineage advancement requires loss of stemness drivers, gain of maturity drivers, or both, we examined by synthetic reporters whether miRNA expression was dynamic or fixed. The hESC-MEC constituted an excellent paradigm for early fetal-like hepatic transition beyond pluripotency. To establish transduction efficiency of synthetic constructs, we used aCy3-labeled control miRNA sequence: this was incorporated by 90–100% of hESC-MEC at 10–100 nM with expression at high levels over at least 10–14 days (Supplementary Fig. 5).

Suggested by the efficacy of hsa-miR-122 in hepatic differentiation with or without additional miRNA [25,27], first we examined whether synthetic inhibitors would neutralize stemness-related miRNA to advance hsa-miR-122-dependent differentiation. We chose inhibitors for hsa-miR-222, –100, –221, –99a and –214, due to their abundances in hESC-MEC and FH-P3 (Table 2, Fig. 2). Also, previously these miRNA induced alternative nonhepatic fates in stem/progenitor cells [2–5,7,26,41,42]. This set of inhibitors for hsa-miR-222, –100, –221, –99a and –214 was designated the “miR-steminhib” group.

Transfection of hESC-MEC with 20 or 80 nM each of miR-steminhib mimics based on relative miRNA abundances in stem cells was nontoxic. Simultaneous transduction of cells with hsa-miR-122a mimic to 120 nM was also nontoxic, allowing its expression to levels in FH-PP. Thus transcriptional context of hESC-MEC was permissive for hepatic gene expression, including for miRNA. However, suppression of hsa-miR-100, –214, –221, –222 or –99a alone or with co-expression of –122a mimic had no effects on cell morphology to consider hepatic differentiation advanced (not shown).

We then examined gain-of-function after verifying absence of hsa-miR-122, –126, –192, –194 and –26b in hESC-MEC (Fig. 3A). Mimics for these miRNA were designated “miR-hepatic” group. Transfections using 120 nM of hsa-miR-122a mimic and 20 nM of other mimics approximated their relative levels in FH-PP or AH-PP. Each miR-hepatic member was expressed in hESC-MEC (Fig. 3B).

To verify transduced mimics regulated genes, we identified growth differentiation factor-10 (GDF10) and protocadherin-p2 (PCDHB2) as suitable hsa-miR-122 targets, since these were expressed in hESC-MEC. The miR-122a mimic with or without other miR-hepatic group members decreased GDF10 and PCDHB2 mRNA levels to indicate appropriate functionality (Fig. 4).

In response to miR-hepatic group transfections, morphology of hESC-MEC did not change after three or 12 days. However, albumin (Alb) mRNA level increased; and α -fetoprotein (AFP) mRNA level decreased (Fig. 5A). Also, expression of mesenchymal markers, vimentin and α -smooth muscle actin (SMA) decreased. Expression of neither cytokeratin

(CK)-19, a biliary marker, nor hepatobiliary enzymes, G6P, GGT or DPPIV, changed (Fig. 5B).

3.3. Regulation of miRNA targets in cells

To deepen insights into limited hepatic differentiation in hESC-MEC despite miR-hepatic mimics, we queried Affymetrix mRNA datasets for miRNA targets. This was according to IPA incorporating TargetScan and TarBase algorithms.

Of 17,335 targets predicted for top 20 miRNA in hESC-MEC, 673 (3.9%) targets were noted in 407 mRNAs expressed below RT-PCR detection limits after stemness mRNAs from hESC dataset were subtracted for higher stringency. In case of 5 stemness miRNA represented by miR-steminhib group, 5748 targets were predicted, of which 220 (3.8%) targets in 191 mRNAs were actually identified in hESC-MEC.

Similarly, of 18,499 targets predicted for top 20 miRNA in FH-PP, we identified 1260 targets (6.8%) in 647 nonexpressed mRNA after subtracting hESC-MEC datasets for stringency. By contrast, 2370 miRNA targets were predicted for 5 hepatic miRNA represented by miR-hepatic group, and 177 (7.5%) targets in 163 mRNA were identified in FH-PP cells after subtracting from hESC-MEC.

Canonical ontologies in comparison of miRNA targets in hESC-MEC and FH-PP revealed diverse processes (Supplementary Table 2). The miR-steminhib or miR-hepatic group targets were fewer. Top molecular and cellular functions of targeted mRNAs in hESC-MEC and FH-PP, concerned gene expression, cell growth and proliferation, cell morphology, cell movement, cell death and survival, or cellular function and maintenance, but not differentiation (Fig. 6A–D). Upstream TR driving gene networks were mined by IPA (Supplementary Table 3). For their functional relevance, 15,223 expressed mRNAs identified by Affymetrix TAC were studied in hESC-MEC, FH-PP and AH-PP: 6915 (45.5%) were common to all; 3538 (23%) were unique to FH-PP; 4770 (31%) were unique to AH-PP. The top gene network identified by IPA in mRNA for hESC-MEC concerned HNF4A – a major driver of hepatic gene expression (Supplementary Table 4). TR networks also included cell survival, proliferation or epithelial-mesenchymal transition, e.g., by Snail (SNAI) [43]. HNF4A mRNA network targets in hESC-MEC and FH-PP with reference of AH-PP (n 362) were annotated (Supplementary Table 5). In hESC-MEC, > 90% HNF4A-regulated mRNAs were absent versus either FH-PP or AH-PP, $p < 0.001$ (Fig. 6E). HNF4A network was most active in AH-PP (Fig. 6F).

In hESC-MEC and FH-PP (compared to AH-PP) least overlapping and largest mechanistic TR networks were of HNF4A and HNF1 (downstream to it) (Table 4). Cell cycle-regulated networks were prominent in AH-PP, including c-MYC, TP53 and others. As AH-PP are in G0/G1 compared with FH-PP or hESC-MEC [31,36,44], this was consistent.

Cell type clustering using mRNA profiles by Affymetrix TAC supported proposed lineage relationships (Fig. 7A). The signals for HNF4A mRNA (consensus probe 230914_at) in hESC-MEC and FH-P3 were much lower than FH-PP and AH-PP (Fig. 7B). SNAI1 mRNA was not expressed. While HNF4A mRNA may be targeted by miRNA (hsa-miR-24, -34a or

–449a [45,46]), miR-24 was expressed in hESC-MEC, FH-PP and AH-PP, but –34a and –449a were absent in all cell types. MiRNA targeting SNAIL were either absent in all cell types (hsa-miR-133, –153) [47,48] or seemed irrelevant due to presence in FH-PP and AH-PP (–30a, 30d, –22) [49–51]. Mechanistic mapping by IPA of HNF4A mRNA network revealed this most favored hepatic gene expression in AH-PP (Fig. 7C). Contrarily, in AH-PP, c-MYC and TP53 mRNA networks were less permissive for proliferation (Fig. 7D, E). The significance of these mechanistic networks was further validated by this.

4. Discussion

Oscillations in miRNA expression during hepatic lineage progression or regression in hESC-derived and primary FH or AH indicated these were related to developmental stages. Coordinated regulation of pluripotency-associated miRNA had been previously noted in hESC, e.g., hsa-miR-302 members [9,38–40]. Similarly, regulation of several -let7 family members had been previously noted: As features of hESC-derived mesenchymal stem cells (MSC) [52]; and here in early fetal stage represented by hESC-MEC or FH-P3 or after regression in AH-P0. Since various miRNA, e.g., hsa-miR-122, which characterize hepatocytes [21], were well-expressed in AH-PP or FH-PP, this verified usefulness of these cell types for stage-specific lineage events and processes.

The ubiquity of multiple miRNA irrespective of pluripotency or lineage differentiation stages suggested that these were not determinants of differentiation. This possibility was emphasized by recrudescence of miRNA after phenotypic regression in cultured hESC-MEC, FH-P3 and AH-P0 - including those expressed well in hESC, and reported in hESC-derived MSC previously [52]. Indeed, 11 of top 20 miRNA in hESC-MEC were actually shared with those hESC-derived MSC, including hsa-miR-125, –21, –199, –29, –100, –221 and -let-7 members (7a, 7f, 7i, 7d and 7e). This lineage-independence of miRNA expression was reinforced with most cases of top 20 miRNA in FH-P3 and many in AH-P0. Nonetheless, abundances of hsa-miR-21, –7a, –7f and –7d in AH-PP indicated these miRNA did not drive hepatic phenotype and cast doubt on the ability of other miRNA to do so.

The transcriptional context of hESC-MEC was permissive for hepatic gene expression. This permissiveness extended to hsa-miR-122, –126, –192, –194, and –26b mimics, including –122 mimic transfected in conjunction with mimics to inhibit multiple stemness miRNA. However, forced expression of miR-hepatic group did not achieve further differentiation, contrasting with prior hepatic differentiation studies [25]. We verified efficient incorporation and high-level expression of miRNA in cells. Also, the period of up to 12 days was adequate for cell differentiation. The assays including Alb and AFP mRNA switches characterizing transition from fetal to adult hepatocytes [36], mesenchymal functions reported by vimentin and α -SMA mRNAs as a feature of fetal but not adult hepatocytes [30,31], and G-6-P, GGT and DPPIV enzyme activities typical of adult hepatocytes [30,31,36] were also appropriate. Therefore, we surmised that miRNA were ineffective for further differentiation in hESC-MEC exemplifying fetal-like hepatic stage [31,44].

Importantly, in hESC-MEC, mRNA targets of miRNA categorized general processes, including gene expression, cell growth and proliferation, morphology, movement, assembly,

etc. The repertoire of mRNAs targeted by miRNA in hESC-MEC did not directly relate to differentiation. We did note potential role of upstream TRs with HNF4A at the top of this list and others directing gene expression or networks in cell survival, growth and proliferation, etc. As HNF4A network concerns hepatic functions, it was significant this was largely down-regulated in hESC-MEC; that component was also downregulated in FH-PP, likely because of their immaturity.

Deficiency of HNF4A mRNA itself in hESC-MEC could have arisen from miRNA targeting, gene silencing by DNA methylation, or epistatic regulation involving other TR or elements. Whereas studies of 3'-UTR and coding regions of HNF4A identified targets for hsa-miR-24, -34a and -449a [45,46], these miRNA were either absent in hESC-MEC (miR-34a, -449a) or were present in FH-PP and AH-PP (miR-24), thereby excluding this mechanism.

Although hsa-miR-let7 family was speculated to regulate HNF4A [52], this was unlikely since these -let7 members were present in FH-PP and AH-PP without interference in HNF4A expression. Differences in DNA methylation within chromosomal sites in or near genes, including HNF4A, affect gene expression in human pancreatic islets [53]. Hepatic endoderm specification or commitment is also characterized at promoter sites by DNA methylation: increases during differentiation; decreases following differentiation [54]. This process is driven by DNMT1 enzyme – the act of culture in AH rapidly silenced HNF4A via promoter methylation [55]. Regression of hepatic differentiation through TR is emphasized by our results. Interactions in regulatory epistatic circuits noted in stable liver cell lines, e.g., SNAIL, which is involved in epithelial-mesenchymal transitions and induces hsa-miR-200a - b -c, or -34a, led to repression of HNF4A transcriptionally and, in turn, SNAIL was counter-repressed by HNF4A via silencing of -200c and -34a [43]. We noted TR in mRNA networks included SNAIL although this was expressed in neither hESC-MEC nor FH-PP. Moreover, hsa-miR-200a -b or -c and -34a were absent from hESC-MEC and FH-PP. Although hsa-miR-200b was expressed at low levels in AH, -200a, -c or -34a were absent from AH, AH-P0 or FH-P3. This suggests cell lines may not inform gene regulation in primary hepatocytes.

From translational perspectives, inducing or maintaining hepatic differentiation in stem cell-derived hepatocytes or natural hepatocytes is critical for drug development or toxicology tools, disease models, stem cell biology, cell/gene therapy, etc. Previously, cell culture altered an array of miRNA networks in human hepatocytes, which could not be overcome for HBV model development [16,17]. In hESC-MEC, greater expression of stemness-associated miRNA altered cellular and molecular processes, TR networks actually restricted differentiation. This role of TR was substantiated by generating hepatocytes with overexpression in bone marrow-derived MSC of HNF4A and FOXA3 [56,57]. Similarly, HNF4A overexpression restored gene expression in damaged rat hepatocytes [58]. This is in agreement with regulation of HNF4A mRNA targets being independent of DNA methylation [55]. For hepatic differentiation in stem cells, most attractive strategies will concern induction of HNF4A or other TR native to cells. This will also be relevant for overcoming alterations by liver injury of differentiation in putative stem cells during hepatic failure or oncogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Abbreviations:

AH	adult hepatocyte
Alb	albumin
AFP	alpha-fetoprotein
CK	cytokeratin
DDPIV	dipeptidylpeptidase IV
ELD	expression level dominance
EpCAM	epithelial cell adhesion molecule
FH	fetal hepatocyte
GDF	growth differentiation factor
GGT	gamma-glutamyltranspeptidase
HBV	hepatitis B virus
hESC	human embryonic stem cells
hESC-MEC	human embryonic stem cell-derived mesodermal-endodermal cells
HNF	hepatocyte nuclear factor
IPA	Ingenuity pathway analysis
miRNA	microRNA
MSC	mesenchymal stem cells
PCDH	protocadherin
PSC	pluripotent stem cells
SMA	smooth muscle actin
SNAI	snail
TAC	Affymetrix Transcription Analysis Console, version 4

TR transcriptional regulator

References

- [1]. Greve TS, Judson RL, Blueloch R, microRNA control of mouse and human pluripotent stem cell behavior, *Annu. Rev. Cell Dev. Biol.* 29 (2013) 213–239. [PubMed: 23875649]
- [2]. Zhou X, Wang J, Sun H, et al., MicroRNA-99a regulates early chondrogenic differentiation of rat mesenchymal stem cells by targeting the BMPR2 gene, *Cell Tissue Res.* 366 (1) (2016) 143–153. [PubMed: 27177866]
- [3]. Emmrich S, Rasche M, Schoning J, et al., miR-99a/100~125b tricistrons regulate hematopoietic stem and progenitor cell homeostasis by shifting the balance between TGFbeta and Wnt signaling, *Genes Dev.* 28 (8) (2014) 858–874. [PubMed: 24736844]
- [4]. Coppola A, Romito A, Borel C, et al., Cardiomyogenesis is controlled by the miR-99a/let-7c cluster and epigenetic modifications, *Stem Cell Res.* 12 (2) (2014) 323–337. [PubMed: 24365598]
- [5]. Zeng Y, Qu X, Li H, et al., MicroRNA-100 regulates osteogenic differentiation of human adipose-derived mesenchymal stem cells by targeting BMPR2, *FEBS Lett.* 586 (16) (2012) 2375–2381. [PubMed: 22684006]
- [6]. Petrelli A, Carollo R, Cargnelutti M, et al., By promoting cell differentiation, miR-100 sensitizes basal-like breast cancer stem cells to hormonal therapy, *Oncotarget* 6 (4) (2015) 2315–2330. [PubMed: 25537513]
- [7]. Shu P, Fu H, Zhao X, et al., MicroRNA-214 modulates neural progenitor cell differentiation by targeting Quaking during cerebral cortex development, *Sci. Rep.* 7 (1) (2017) 8014. [PubMed: 28808337]
- [8]. Yoshizuka M, Nakasa T, Kawanishi Y, et al., Inhibition of microRNA-222 expression accelerates bone healing with enhancement of osteogenesis, chondrogenesis, and angiogenesis in a rat refractory fracture model, *J. Orthop. Sci.: Off. J. Jpn. Orthop. Assoc.* 21 (6) (2016) 852–858.
- [9]. Li HL, Wei JF, Fan LY, et al., miR-302 regulates pluripotency, teratoma formation and differentiation in stem cells via an AKT1/OCT4-dependent manner, *Cell Death Dis.* 7 (2016) e2078. [PubMed: 26821070]
- [10]. Lu J, Dong H, Lin L, Wang Q, Huang L, Tan J, miRNA-302 facilitates reprogramming of human adult hepatocytes into pancreatic islets-like cells in combination with a chemical defined media, *Biochem. Biophys. Res. Commun.* 453 (3) (2014) 405–410. [PubMed: 25268319]
- [11]. Roscigno G, Quintavalle C, Donnarumma E, et al., MiR-221 promotes stemness of breast cancer cells by targeting DNMT3b, *Oncotarget* 7 (1) (2016) 580–592. [PubMed: 26556862]
- [12]. Zhang J, Jin H, Liu H, et al., MiRNA-99a directly regulates AGO2 through translational repression in hepatocellular carcinoma, *Oncogenesis* 3 (2014) e97. [PubMed: 24732044]
- [13]. Zhou HC, Fang JH, Luo X, et al., Downregulation of microRNA-100 enhances the ICMT-Rac1 signaling and promotes metastasis of hepatocellular carcinoma cells, *Oncotarget* 5 (23) (2014) 12177–12188. [PubMed: 25361001]
- [14]. Zhou Y, Ren L, Xiao J, et al., Global transcriptional and miRNA insights into bases of heterosis in hybridization of Cyprinidae, *Sci. Rep.* 5 (2015) 13847. [PubMed: 26346824]
- [15]. Ott M, Ma Q, Li B, Gagandeep S, Rogler LE, Gupta S, Regulation of hepatitis B virus expression in progenitor and differentiated cell types: evidence for negative transcriptional control in nonpermissive cells, *Gene Expr.* 8 (3) (1999) 175–186. [PubMed: 10634319]
- [16]. Kumar M, Sharma Y, Bandi S, Gupta S, Endogenous antiviral microRNAs determine permissiveness for hepatitis B virus replication in cultured human fetal and adult hepatocytes, *J. Med. Virol.* 87 (7) (2015) 1168–1183. [PubMed: 25690916]
- [17]. Park SO, Kumar M, Gupta S, TGF-beta and iron differently alter HBV replication in human hepatocytes through TGF-beta/BMP signaling and cellular microRNA expression, *PLoS One* 7 (6) (2012) e39276.
- [18]. Huang F, Fang ZF, Hu XQ, Tang L, Zhou SH, Huang JP, Overexpression of miR-126 promotes the differentiation of mesenchymal stem cells toward endothelial cells via activation of PI3K/Akt

- and MAPK/ERK pathways and release of paracrine factors, *Biol. Chem.* 394 (9) (2013) 1223–1233. [PubMed: 23729621]
- [19]. Jung KH, McCarthy RL, Zhou C, Uprety N, Barton MC, Beretta L, MicroRNA regulates hepatocytic differentiation of progenitor cells by targeting YAP1, *Stem Cells* 34 (5) (2016) 1284–1296. [PubMed: 26731713]
- [20]. Sekine S, Ogawa R, Ito R, et al., Disruption of Dicer1 induces dysregulated fetal gene expression and promotes hepatocarcinogenesis, *Gastroenterology* 136 (7) (2009) 2304–2315 (e2301–2304). [PubMed: 19272382]
- [21]. Laudadio I, Manfroid I, Achouri Y, et al., A feedback loop between the liver-enriched transcription factor network and miR-122 controls hepatocyte differentiation, *Gastroenterology* 142 (1) (2012) 119–129. [PubMed: 21920465]
- [22]. Liu F, Shimakami T, Murai K, et al., Efficient suppression of hepatitis C virus replication by combination treatment with miR-122 antagonism and direct-acting antivirals in cell culture systems, *Sci. Rep.* 6 (2016) 30939. [PubMed: 27484655]
- [23]. Hand NJ, Master ZR, Eauclore SF, Weinblatt DE, Matthews RP, Friedman JR, The microRNA-30 family is required for vertebrate hepatobiliary development, *Gastroenterology* 136 (3) (2009) 1081–1090. [PubMed: 19185580]
- [24]. Rogler CE, Levoci L, Ader T, et al., MicroRNA-23b cluster microRNAs regulate transforming growth factor-beta/bone morphogenetic protein signaling and liver stem cell differentiation by targeting Smads, *Hepatology* 50 (2) (2009) 575–584. [PubMed: 19582816]
- [25]. Doddapaneni R, Chawla YK, Das A, Kalra JK, Ghosh S, Chakraborti A, Overexpression of microRNA-122 enhances in vitro hepatic differentiation of fetal liver-derived stem/progenitor cells, *J. Cell Biochem.* 114 (7) (2013) 1575–1583. [PubMed: 23334867]
- [26]. Bakhshandeh B, Hafizi M, Ghaemi N, Soleimani M, Down-regulation of miRNA-221 triggers osteogenic differentiation in human stem cells, *Biotechnol. Lett.* 34 (8) (2012) 1579–1587. [PubMed: 22547036]
- [27]. Davoodian N, Lotfi AS, Soleimani M, Ghaneialvar H, The combination of miR-122 overexpression and Let-7f silencing induces hepatic differentiation of adipose tissue-derived stem cells, *Cell Biol. Int.* (2017).
- [28]. Seitz H, Issues in current microRNA target identification methods, *RNA Biol.* 14 (7) (2017) 831–834. [PubMed: 28430005]
- [29]. Olivier EN, Rybicki AC, Bouhassira EE, Differentiation of human embryonic stem cells into bipotent mesenchymal stem cells, *Stem Cells* 24 (8) (2006) 1914–1922. [PubMed: 16644919]
- [30]. Bandi S, Cheng K, Joseph B, Gupta S, Spontaneous origin from human embryonic stem cells of liver cells displaying conjoint meso-endodermal phenotype with hepatic functions, *J. Cell Sci.* (2012).
- [31]. Inada M, Follenzi A, Cheng K, et al., Phenotype reversion in fetal human liver epithelial cells identifies the role of an intermediate meso-endodermal stage before hepatic maturation, *J. Cell Sci.* 121 (Pt 7) (2008) 1002–1013. [PubMed: 18319302]
- [32]. Cho JJ, Joseph B, Sappal BS, et al., Analysis of the functional integrity of cryopreserved human liver cells including xenografting in immunodeficient mice to address suitability for clinical applications, *Liver Int.* 24 (4) (2004) 361–370. [PubMed: 15287860]
- [33]. Saldanha AJ, Java treeview-extensible visualization of microarray data, *Bioinformatics* 20 (17) (2004) 3246–3248. [PubMed: 15180930]
- [34]. Barrett T, Wilhite SE, Ledoux P, et al., NCBI GEO: archive for functional genomics data sets-update, *Nucleic Acids Res.* 41 (Database issue) (2013) D991–D995. [PubMed: 23193258]
- [35]. Malhi H, Irani AN, Gagandeep S, Gupta S, Isolation of human progenitor liver epithelial cells with extensive replication capacity and differentiation into mature hepatocytes, *J. Cell Sci.* 115 (Pt 13) (2002) 2679–2688. [PubMed: 12077359]
- [36]. Inada M, Benten D, Cheng K, et al., Stage-specific regulation of adhesion molecule expression segregates epithelial stem/progenitor cells in fetal and adult human livers, *Hepatol. Int.* 2 (1) (2008) 50–62. [PubMed: 19669279]

- [37]. Dan YY, Riehle KJ, Lazaro C, et al., Isolation of multipotent progenitor cells from human fetal liver capable of differentiating into liver and mesenchymal lineages, *Proc. Natl. Acad. Sci. USA* 103 (26) (2006) 9912–9917. [PubMed: 16782807]
- [38]. Suh MR, Lee Y, Kim JY, et al., Human embryonic stem cells express a unique set of microRNAs, *Dev. Biol.* 270 (2) (2004) 488–498. [PubMed: 15183728]
- [39]. Lakshmipathy U, Love B, Goff LA, et al., MicroRNA expression pattern of undifferentiated and differentiated human embryonic stem cells, *Stem Cells Dev.* 16 (6) (2007) 1003–1016. [PubMed: 18004940]
- [40]. Anokye-Danso F, Trivedi CM, Jühr D, et al., Highly efficient miRNA-mediated reprogramming of mouse and human somatic cells to pluripotency, *Cell Stem Cell* 8 (4) (2011) 376–388. [PubMed: 21474102]
- [41]. Wu Y, Li Z, Yang M, et al., MicroRNA-214 regulates smooth muscle cell differentiation from stem cells by targeting RNA-binding protein QKI, *Oncotarget* 8 (12) (2017) 19866–19878. [PubMed: 28186995]
- [42]. Yang QE, Racicot KE, Kaucher AV, Oatley MJ, Oatley JM, MicroRNAs 221 and 222 regulate the undifferentiated state in mammalian male germ cells, *Development* 140 (2) (2013) 280–290. [PubMed: 23221369]
- [43]. Garibaldi F, Cicchini C, Conigliaro A, et al., An epistatic mini-circuitry between the transcription factors Snail and HNF4alpha controls liver stem cell and hepatocyte features exhorting opposite regulation on stemness-inhibiting microRNAs, *Cell Death Differ.* 19 (6) (2012) 937–946. [PubMed: 22139130]
- [44]. Bandi S, Cheng K, Joseph B, Gupta S, Spontaneous origin from human embryonic stem cells of liver cells displaying conjoint meso-endodermal phenotype with hepatic functions, *J. Cell Sci.* 125 (Pt 5) (2012) 1274–1283. [PubMed: 22349702]
- [45]. Takagi S, Nakajima M, Kida K, Yamaura Y, Fukami T, Yokoi T, MicroRNAs regulate human hepatocyte nuclear factor 4alpha, modulating the expression of metabolic enzymes and cell cycle, *J. Biol. Chem.* 285 (7) (2010) 4415–4422. [PubMed: 20018894]
- [46]. Ramamoorthy A, Li L, Gaedigk A, et al., In silico and in vitro identification of microRNAs that regulate hepatic nuclear factor 4alpha expression, *Drug Metab. Dispos.: Biol. Fate Chem.* 40 (4) (2012) 726–733. [PubMed: 22232426]
- [47]. Muraoka N, Yamakawa H, Miyamoto K, et al., MiR-133 promotes cardiac reprogramming by directly repressing Snail and silencing fibroblast signatures, *EMBO J.* 33 (14) (2014) 1565–1581. [PubMed: 24920580]
- [48]. Xia W, Ma X, Li X, et al., miR-153 inhibits epithelial-to-mesenchymal transition in hepatocellular carcinoma by targeting Snail, *Oncol. Rep.* 34 (2) (2015) 655–662. [PubMed: 26035427]
- [49]. Zhou Q, Yang M, Lan H, Yu X, miR-30a negatively regulates TGF-beta1-induced epithelial-mesenchymal transition and peritoneal fibrosis by targeting Snail, *Am. J. Pathol.* 183 (3) (2013) 808–819. [PubMed: 23831330]
- [50]. Ye Z, Zhao L, Li J, Chen W, Li X, miR-30d Blocked Transforming Growth Factor beta1-Induced Epithelial-Mesenchymal Transition by Targeting Snail in Ovarian Cancer Cells, *Int. J. Gynecol. Cancer.: Off. J. Int. Gynecol. Cancer. Soc.* 25 (9) (2015) 1574–1581.
- [51]. Zuo QF, Cao LY, Yu T, et al., MicroRNA-22 inhibits tumor growth and metastasis in gastric cancer by directly targeting MMP14 and Snail, *Cell Death Dis.* 6 (2015) e2000. [PubMed: 26610210]
- [52]. Koh W, Sheng CT, Tan B, et al., Analysis of deep sequencing microRNA expression profile from human embryonic stem cells derived mesenchymal stem cells reveals possible role of let-7 microRNA family in downstream targeting of hepatic nuclear factor 4 alpha, *BMC Genom.* 11 (Suppl 1) (2010) S6.
- [53]. Hall E, Volkov P, Dayeh T, et al., Sex differences in the genome-wide DNA methylation pattern and impact on gene expression, microRNA levels and insulin secretion in human pancreatic islets, *Genome Biol.* 15 (12) (2014) 522. [PubMed: 25517766]

- [54]. Lewis LC, Lo PC, Foster JM, et al., Dynamics of 5-carboxylcytosine during hepatic differentiation: potential general role for active demethylation by DNA repair in lineage specification, *Epigenetics* 12 (4) (2017) 277–286. [PubMed: 28267381]
- [55]. Cheishvili D, Christiansen S, Stochinsky R, et al., DNA methylation controls un-methylated transcription start sites in the genome in trans, *Epigenomics* 9 (5) (2017) 611–633. [PubMed: 28470094]
- [56]. Hu X, Xie P, Li W, Li Z, Shan H, Direct induction of hepatocyte-like cells from immortalized human bone marrow mesenchymal stem cells by overexpression of HNF4alpha, *Biochem. Biophys. Res. Commun.* 478 (2) (2016) 791–797. [PubMed: 27501760]
- [57]. Dai K, Chen R, Ding Y, Niu Z, Fan J, Xu C, Induction of functional hepatocyte-like cells by overexpression of FOXA3 and HNF4alpha in rat bone marrow mesenchymal stem cells, *Cells Tissues Organs* 200 (2) (2014) 132–140. [PubMed: 25896100]
- [58]. Nishikawa T, Bell A, Brooks JM, et al., Resetting the transcription factor network reverses terminal chronic hepatic failure, *J. Clin. Investig.* 125 (4) (2015) 1533–1544. [PubMed: 25774505]

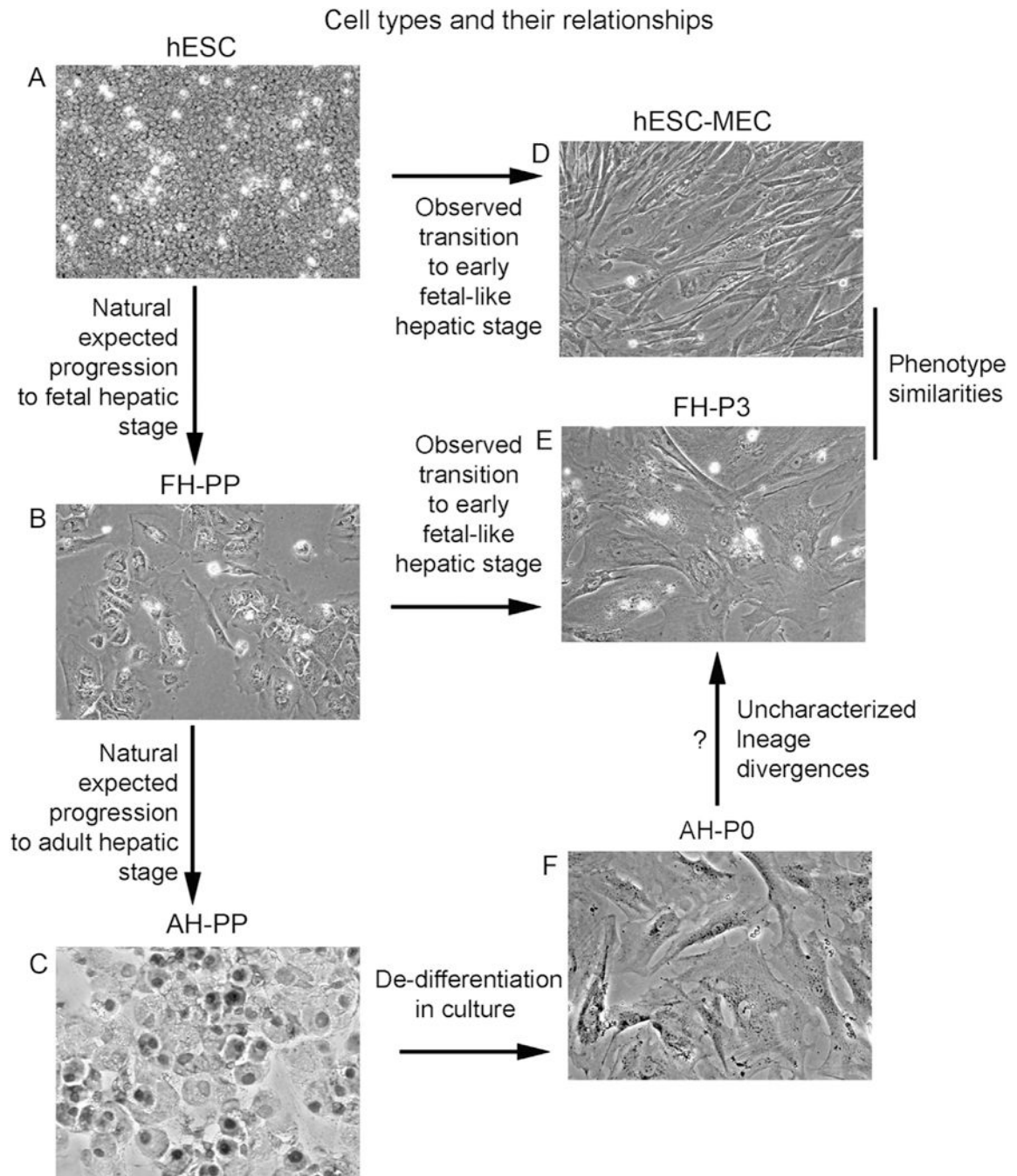


Fig. 1. Cells studied.

Phase contrast images of undifferentiated hESC (A), primary human fetal hepatocytes, FH-PP (B), primary human adult hepatocytes, AH-PP (C), spontaneously originating hESC-MEC (D), FH-PP cultured for three passages, FH-P3 (E), and AH-PP in primary culture, AH-P0 (F). The framework for proposed hepatic differentiation stages is indicated.

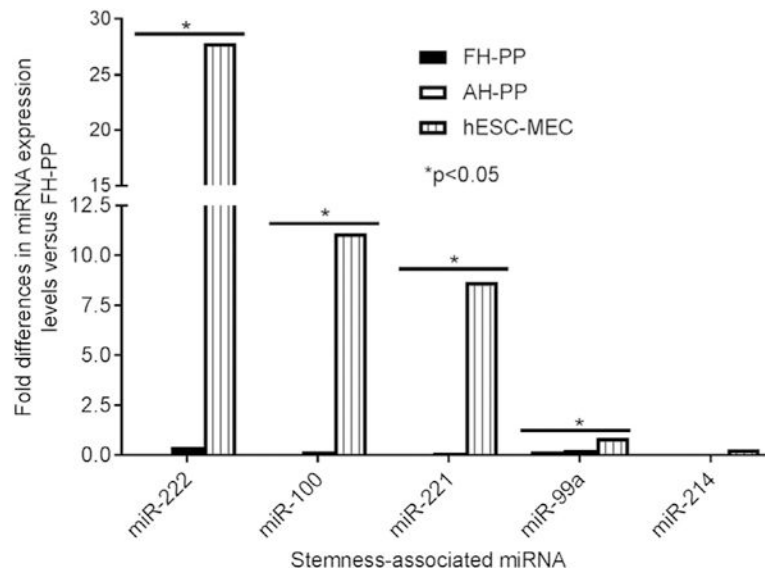


Fig. 2. Repression of stemness-associated miRNA in hESC-MEC.

Expression after 12 days in cell culture of hsa-miR-222, -100, -221, -99b and -214 by qRT-PCR. These miRNA were not expressed in FH-PP or AH-PP.

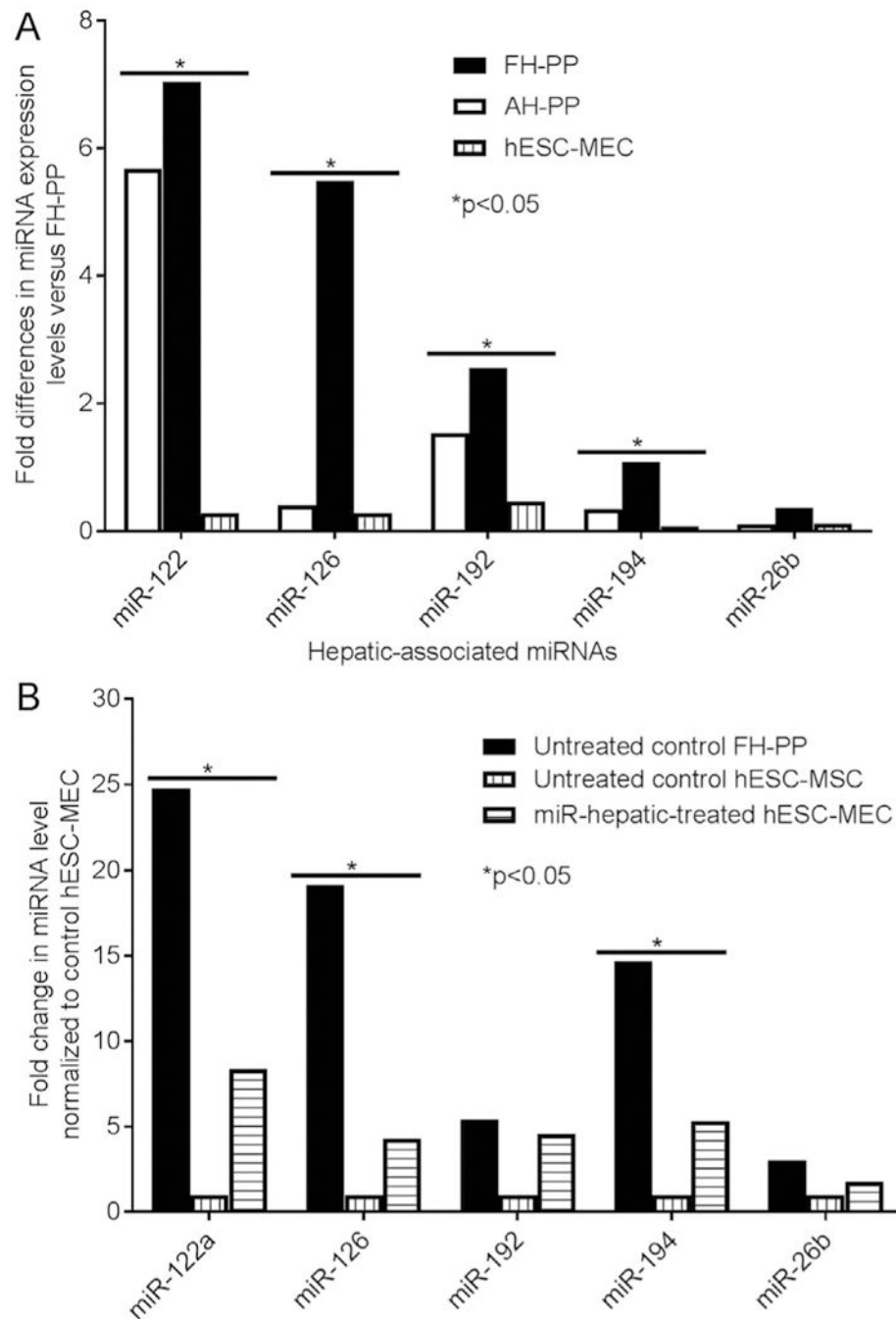


Fig. 3. Expression of hepatic-associated miRNA in hESC-MEC.

(A) Basal expression 12 days after culture of hsa-miR-122, -126, -192, -194 and -26b by qRT-PCR in FH-PP, AH-PP and hESC-MEC. (B) After 120nM hsa-miR-122a mimic and 20 nM each of -126, -192, -194 and -26b mimics, miRNA expression was observed, although below FH-PP levels.

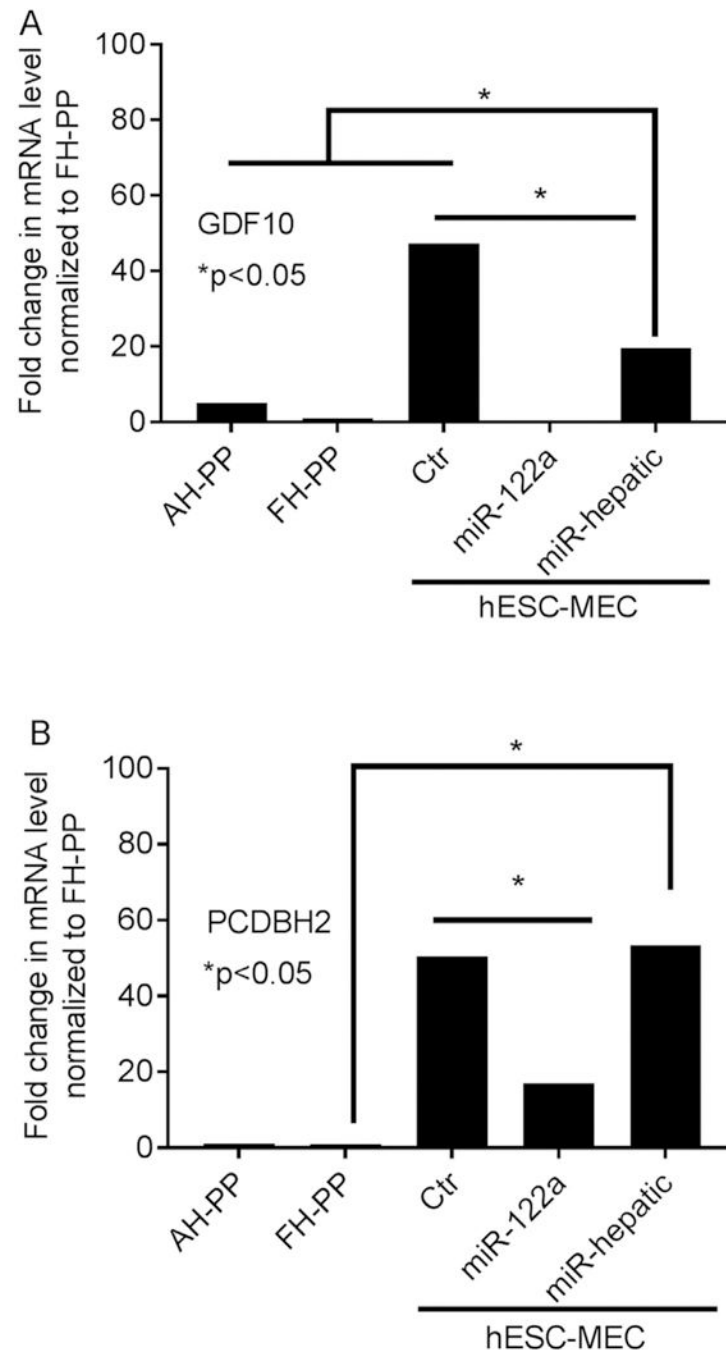


Fig. 4. Repression of target mRNAs by miRNA in hESC-MEC.

qRT-PCR for growth differentiation factor-10 (GDF10) (A) and protocadherin beta-2 (PCDHB2) (B). Introduction of either miR-122a or miR-hep group of mimics decreased mRNA expression in both cases.

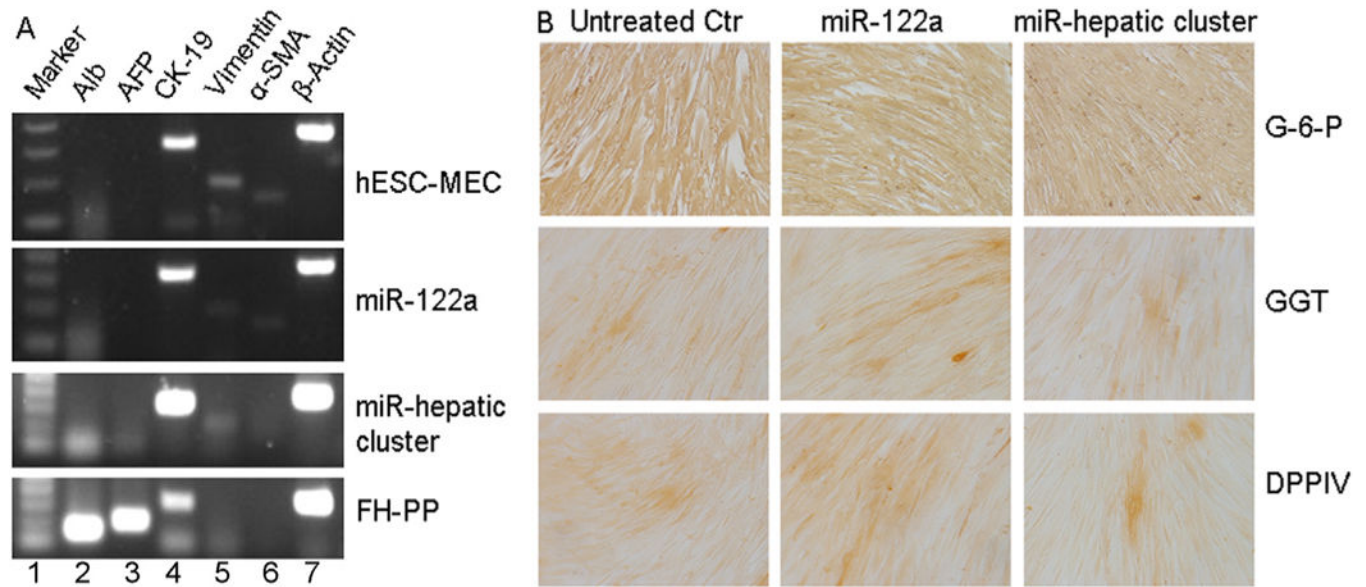


Fig. 5. Induction of hepatic differentiation in hESC-MEC by miRNA mimics.

After transfection of miR-122a alone or miR-Hep group (–122a, –126, –192, –194, –26b) gene expression was analyzed by qRT-PCR (A) or cytochemical staining (B). Slight increases were noted after miR-Hep group in expression of Alb, AFP and CK-19, although vimentin, and α -SMA were still expressed. Cytochemical staining for G-6-P, GGT and DPPIV indicated no increases after transfection of mimics.

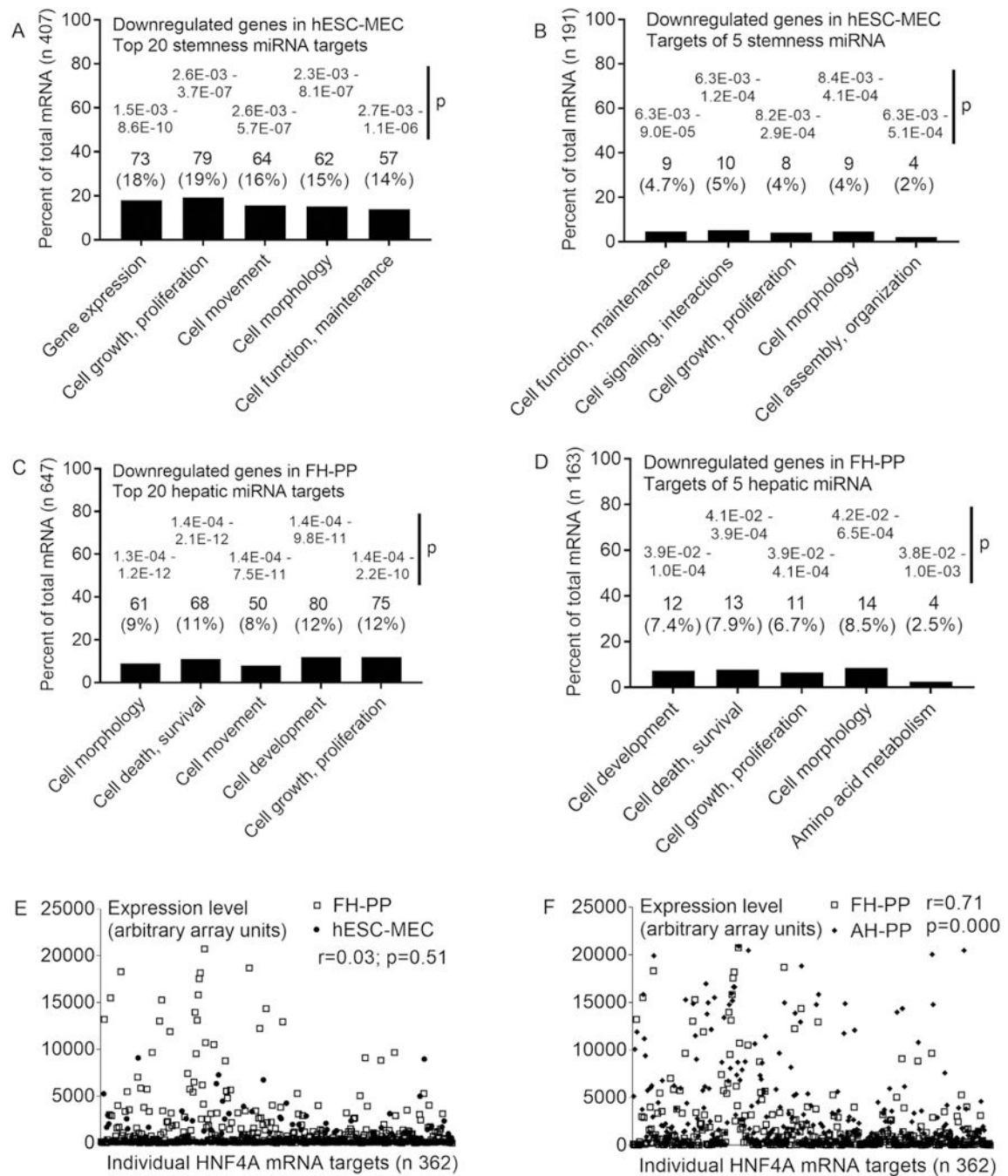


Fig. 6. Regulation of mRNAs by differentially expressed miRNA in hESC-MEC and FH-PP.

Affymetrix RNA datasets were analyzed by IPA for top 20 miRNA targets or steminhb or miR-Hep targets after subtraction with hESC (for hESC-MEC) or hESC-MEC (for FH-PP). Top cellular and molecular functions of mRNA targeted by stemness miRNA (A, B) concerned general processes. The mRNAs targeted by hepatic miRNA (C, D) also concerned general processes. The number of mRNA and their contribution to total genes per category are listed. P values are included. (E, F) HNF4A mRNA network -hESC-MEC versus FH-PP

and AH-PP versus FH-PP. This network was largely silenced in hESC-MEC (E); it was more active in AH-PP than FH-PP (F).

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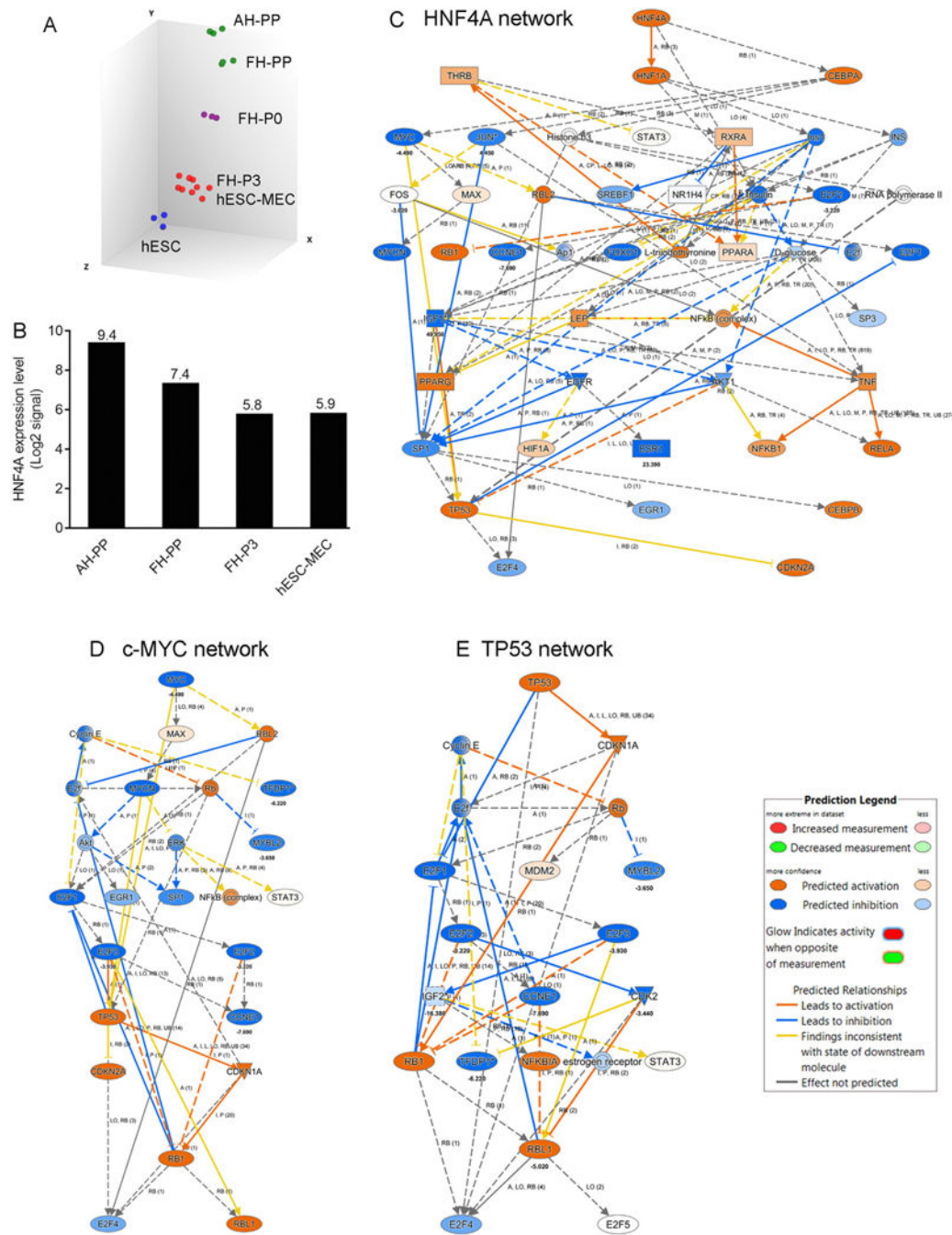


Fig. 7. Lineage progression-related differences in mRNA expression.

(A) Clustering of cell types by global gene expression profiles with Affymetrix TAC. (B) HNF4A mRNA expression (probe 230914.at). (C) Mechanistic HNF4A network in AH-PP versus FH-PP. This largely elucidated growth-inhibitory effects in AH-PP. (D, E) c-MYC and TP53 networks also indicated inhibition of cycling in AH-PP versus FH-PP. Key for network interactions is given. Network analysis is from IPA.

Table 1

Top 20 highly expressed miRNA in hESC, FH-PP and AH-PP cells.

hESC		FH-PP		AH-PP	
miRNA identity	Arbitrary expression level	miRNA identity	Arbitrary expression level	miRNA identity	Arbitrary expression level
hsa-miR-302b	15.69	hsa-miR-122a	15.96	hsa-miR-122a	16.20
hsa-miR-638	15.32	hsa-miR-638	15.45	hsa-miR-21	14.85
hsa-miR-302a*	15.14	hsa-miR-26a	14.65	hsa-miR-26a	14.27
hsa-miR-302d	15.12	hsa-miR-92	14.46	hsa-let-7a	14.10
hsa-miR-302a	14.98	hsa-miR-663	14.27	hsa-let-7f	14.02
hsa-miR-92	14.74	hsa-let-7f	13.96	hsa-miR-192	13.82
hsa-miR-663	14.55	hsa-miR-92b	13.76	hsa-let-7c	13.73
hsa-miR-20a	14.51	hsa-let-7a	13.74	hsa-miR-26b	13.55
hsa-miR-92b	14.50	hsa-miR-26b	13.49	hsa-miR-23b	13.49
hsa-miR-17-5p	14.40	hsa-miR-25	13.41	hsa-let-7g	13.29
hsa-miR-25	14.20	hsa-miR-16	13.38	hsa-let-7b	13.20
hsa-miR-20b	14.17	hsa-miR-21	13.29	hsa-let-7d	13.17
hsa-miR-302c	14.13	hsa-miR-126	12.99	hsa-miR-194	13.11
hsa-miR-106a	14.12	hsa-miR-30b	12.90	hsa-miR-23a	13.10
hsa-miR-21	14.07	hsa-miR-30c	12.87	hsa-miR-126	13.08
hsa-miR-26a	13.86	hsa-let-7g	12.86	hsa-miR-638	13.01
hsa-miR-15b	13.27	hsa-miR-192	12.84	hsa-miR-223	13.00
hsa-miR-200c	12.92	hsa-miR-23b	12.83	hsa-miR-215	12.95
hsa-miR-19b	12.78	hsa-miR-20a	12.75	hsa-miR-148a	12.81
hsa-miR-16	12.39	hsa-miR-194	12.54	hsa-miR-92	12.79

* Expression levels represent log2 of signal intensities. Bold type indicates sharing of stem cell enriched miRNAs. White type indicates hepatocyte-enriched miRNA

Table 2

Top 20 highly expressed miRNA in hESC-MEC and FH-P3 cells*.

hESC-MEC		FH-P3 cells	
miRNA identity	Arbitrary expression level	miRNA identity	Arbitrary expression level
hsa-miR-125b	14.05	hsa-miR-21	15.06
hsa-miR-21	14.01	hsa-miR-125b	14.48
hsa-miR-199a ^a	13.88	hsa-miR-638	14.22
hsa-miR-29a	13.68	hsa-let-7a	14.17
hsa-miR-100	13.63	hsa-miR-23a	13.95
hsa-miR-222	13.47	hsa-let-7f	13.94
hsa-miR-221	13.43	hsa-miR-29a	13.88
hsa-miR-23a	13.33	hsa-miR-23b	13.88
hsa-let-7a	13.30	hsa-let-7c	13.61
hsa-miR-23b	13.20	hsa-miR-221	13.47
hsa-let-7f	13.11	hsa-miR-663	13.47
hsa-miR-214	13.03	hsa-let-7d	13.45
hsa-let-7i	12.99	hsa-miR-125a	13.39
hsa-miR-99a	12.96	hsa-miR-100	13.32
hsa-let-7c	12.93	hsa-let-7e	13.29
hsa-miR-26a	12.87	hsa-miR-26a	13.28
hsa-let-7d	12.80	hsa-miR-24	13.16
hsa-miR-638	12.73	hsa-let-7b	13.06
hsa-miR-125a	12.58	hsa-miR-222	12.98
hsa-let-7e	12.52	hsa-let-7i	12.86

^a Expression levels represent log2 of median signal intensities in each sample. Bold type indicates miRNA present in both samples.

Table 3

Top 20 highly expressed miRNA in AH-P0 and FH-P3 cells.*

FH-P3 cells		AH-P0 cells	
miRNA identity	Arbitrary expression level	miRNA identity	Arbitrary expression level
hsa-miR-21	15.06	hsa-miR-21	16.12
hsa-miR-125b	14.48	hsa-miR-122a	15.43
hsa-miR-638	14.22	hsa-miR-638	14.46
hsa-let-7a	14.17	hsa-let-7a	14.40
hsa-miR-23a	13.95	hsa-let-7f	14.24
hsa-let-7f	13.94	hsa-miR-23b	14.16
hsa-miR-29a	13.88	hsa-miR-23a	14.10
hsa-miR-23b	13.88	hsa-miR-26a	14.03
hsa-let-7c	13.61	hsa-let-7c	14.03
hsa-miR-221	13.47	hsa-miR-29a	13.68
hsa-miR-663	13.47	hsa-let-7b	13.50
hsa-let-7d	13.45	hsa-let-7d	13.49
hsa-miR-125a	13.39	hsa-miR-125b	13.06
hsa-miR-100	13.32	hsa-let-7g	12.88
hsa-let-7e	13.29	hsa-miR-92	12.83
hsa-miR-26a	13.28	hsa-miR-15b	12.63
hsa-miR-24	13.16	hsa-miR-126	12.60
hsa-let-7b	13.06	hsa-miR-663	12.59
hsa-miR-222	12.98	hsa-miR-24	12.50
hsa-let-7i	12.86	hsa-miR-27b	12.40

* Expression levels represent log2 of signal intensities.
 Bold type indicates miRNA present in both samples. White type indicates miRNA expressed well also in undifferentiated hESC and hESC-MEC

Table 4

Top 20 upstream transcriptional regulators in cells identified by IPA.

FH-PP vs hESC-MEC			AH-PP vs hESC-MEC			AH-PP vs FH-PP		
TR	p-value	Targets number	TR	p-value	Targets number	TR	p-value	Targets number
HNF4A	2.72E-49	219	HNF4A	1.48E-37	362	TP53	1.14E-40	268
HNF1A	1.94E-64	114	TP53	4.81E-39	301	HNF4A	7.75E-16	252
TP53	6.75E-10	106	MYC	2.5E-35	228	MYC	5.26E-28	186
MYC	2.44E-07	74	CTNNB1	3.55E-29	168	E2F1	7.37E-39	129
CEBPB	5.89E-23	64	HNF1A	3.75E-51	152	E2F4	2.66E-47	94
STAT3	1.06E-15	64	STAT3	8.1E-28	137	CTNNB1	4.45E-07	94
CTNNB1	5.05E-09	62	FOS	2.98E-27	132	NUPR1	3.15E-17	94
CEBPA	4.8E-20	62	SP1	2.15E-25	130	SP1	1.04E-13	92
SP1	1.66E-10	53	SMARCA4	1.98E-18	123	STAT3	1.95E-11	88
SMARCA4	1.06E-08	52	CEBPB	3.89E-34	122	CCND1	6.26E-27	88
FOS	4.28E-08	47	CEBPA	2.75E-30	121	CEBPB	7.41E-17	82
SREBF1	9.22E-20	46	JUN	1.05E-28	120	HNF1A	5.61E-15	82
RELA	1.23E-10	45	HTT	1.9E-07	104	FOXO3	5.01E-24	81
NFE2L2	2.58E-10	44	NFKB1A	2.59E-20	103	TCF3	1.57E-22	81
FOXO1	6.86E-10	44	CREB1	9.03E-12	101	CDKN2A	6.83E-25	79
GATA1	5.81E-19	43	FOXO1	3.6E-16	93	RB1	4.55E-17	79
FOXO2	4.59E-22	43	TP63	5.55E-19	92	CREB1	1.01E-07	77
ONECUT1	3.42E-14	41	RELA	4.59E-13	85	SMARCA4	1.65E-06	77
CREBBP	4.16E-09	40	NFE2L2	1.92E-12	83	HTT	0.000726	75
SMARCB1	1.92E-21	40	HIF1A	1.34E-12	82	FOXO1	1.47E-12	75