Research Article

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# MicroRNA-199a-5p inhibition enhances the liver repopulation ability of human embryonic stem cell-derived hepatic cells

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**Background & Aims**: Current hepatic differentiation protocols for human embryonic stem cells (ESCs) require substantial improvements. MicroRNAs (miRNAs) have been reported to regulate hepatocyte cell fate during liver development, but their utility to improve hepatocyte differentiation from ESCs remains to be investigated. Therefore, our aim was to identify and to analyse hepatogenic miRNAs for their potential to improve hepatocyte differentiation from ESCs.

**Methods**: By miRNA profiling and *in vitro* screening, we identified miR-199a-5p among several potential hepatogenic miRNAs. Transplantation studies of miR-199a-5p-inhibited hepatocyte-like cells (HLCs) in the liver of immunodeficient fumarylacetoacetate hydrolase knockout mice (*Fah*$^{-/-}$/*Rag2*$^{-/-}$/*Il2rg*$^{-/-}$) were performed to assess their *in vivo* liver repopulation potential. For target determination, western blot and luciferase reporter assay were carried out.

**Results**: miRNA profiling revealed 20 conserved candidate hepatogenic miRNAs. By miRNA screening, only miR-199a-5p inhibition in HLCs was found to be able to enhance the *in vitro* hepatic differentiation of mouse as well as human ESCs. miR-199a-5p inhibition in human ESCs-derived HLCs enhanced their engraftment and repopulation capacity in the liver of *Fah*$^{-/-}$/*Rag2*$^{-/-}$/*Il2rg*$^{-/-}$ mice. Furthermore, we identified *SMARCA4* and *MST1* as novel targets of miR-199a-5p that may contribute to the improved hepatocyte generation and *in vivo* liver repopulation.

**Conclusions**: Our findings demonstrate that miR-199a-5p inhibition in ES-derived HLCs leads to improved hepatocyte differentiation. Upon transplantation, HLCs were able to engraft and repopulate the liver of *Fah*$^{-/-}$/*Rag2*$^{-/-}$/*Il2rg*$^{-/-}$ mice. Thus, our findings suggest that miRNA modulation may serve as a promising approach to generate more mature HLCs from stem cell sources for the treatment of liver diseases.





## Introduction

Currently, liver transplantation is the most reliable treatment option for patients suffering from end-stage liver diseases. However, shortage of organ donors for liver transplants is a common problem. Alternative therapeutic concepts such as cell therapies or tissue engineering also require access to large numbers of hepatocytes. Therefore, the development of novel strategies, which can provide sufficient numbers of human hepatocytes, is of the utmost importance. One attractive approach is the directed differentiation of ESCs into hepatocyte-like cells (HLCs) by mimicking *in vivo* liver development. The utility of HLCs for the study of hepatitis C virus replication and infection has been successfully shown recently [1–3]. In addition, the use of HLCs to study human drug metabolism and systemic biology is emerging [4–7]. Recent publications have outlined protocols for the generation of partially functional hepatocytes from ESCs/induced pluripotent stem (iPS) cells [8–14]. Previous attempts to obtain

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*Abbreviations:* miRNA, microRNA; SMARCA4, SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A member 4; MST1/STK4, mammalian STE20-like protein kinase 1/serine/threonine-protein kinase 4; UTR, untranslated region; siRNA, small interfering RNA; HLCs, hepatocyte-like cells; NTBC, 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione.

functional HLCs from mouse ESCs have achieved limited success *in vivo* [11,13]. However, HLCs derived from ESCs are unable to function as efficiently as hepatoblasts or primary hepatocytes upon transplantation in liver repopulation models [10]. Clearly, there is a need for improved *in vitro* hepatic differentiation protocols and a better understanding of the molecular mechanisms underlying liver development.

Recently, microRNAs (miRNAs) have emerged as key regulatory molecules in liver development [15–18]. miRNA deficiency, induced upon *Dicer1* knockdown in the liver, caused deregulated expression of foetal stage-specific genes in mice [19]. miR-122 stimulated the expression of most liver-enriched transcription factors (LETFs), including HNF6 [15]. Among other miRNAs, the miR-30 family was identified as a regulator of vertebrate bile duct development [17] and the miR-23b cluster was shown to control hepatocyte differentiation [16]. Thus, increasing evidence suggests that miRNAs regulate hepatocyte differentiation during liver development. However, the identification of specific miRNA(s) that enable the generation of transplantable HLCs remains to be investigated.

The current study was designed to identify novel hepatogenic miRNAs. By miRNA profiling and *in vitro* screening we identified miR-199a-5p, whose inhibition improves hepatic differentiation, leading to the generation of hepatic cells. Further, we show that these hepatic cells are capable of engrafting and repopulating the mouse liver.



## Materials and methods

*Hepatic differentiation of mouse and human embryonic stem cells*

Mouse embryonic stem cells (mESCs) were cultured and differentiated by the "hanging drop" method as described in our previous reports [8,20,21]. As a source of human embryonic stem cells (hESCs), we chose H9 hESCs (Wicell) in approval with the German Stem Cell Act (RKI license 1710-79-1-4-41 and -81). For the hepatic differentiation from hESCs we used the standard monolayer-based hepatic differentiation protocol [22] with few modifications to improve hepatic differentiation as described in our previous study [14].

*Screening for miRNAs involved in hepatic differentiation and development*

To find potential, hepatogenic miRNAs, murine Rosa26 cells were differentiated into HLCs by the hanging drop method. Total RNA was isolated from undifferentiated mESCs, HLCs, mouse foetal liver (FL) cells from embryonic day (E) 11.5 and E13.5, and from mouse mature hepatocytes (MHs) (3 and 24 weeks old). ESC, HLC, FL, and MH cells were all obtained from Rosa26 mice to avoid differential miRNA expression profiles that may arise due to different mouse strains [23]. miRNA profiling was performed by the Comprehensive biomarker center, Cbc, DKFZ Heidelberg, Germany. More detailed information can be found in Supplementary Materials and methods.

*miRNA transfection, quantitative real time PCR (qRT-PCR), Western blot and luciferase reporter assay*

These methods were performed as described in our previous reports [24,25].

*Statistical analysis*

Significance was determined with equal variance and two-tailed Student's *t* test. One-way ANOVA was used to determine the significance when comparing more than two groups. A $p$ value of <0.05 was considered significant. Error bars represent ± SEM. $^{*}p$ <0.05, $^{**}p$ <0.005 and $^{***}p$ <0.0005.

For additional methods, please see the Supplementary Materials and methods section.

## Results

*Identification of hepatogenic miRNAs by miRNA microarrays*

To identify miRNAs that potentially affect hepatocyte differentiation from ESCs we performed miRNA expression profiling. Specifically, RNA from four groups: mESCs, mouse hepatocyte-like cells (HLCs), mouse foetal liver (FL) and mouse mature hepatocytes (MHs) was used for miRNA microarray profiling (Fig. 1A). We found distinct subsets of differentially expressed miRNAs in HLCs compared to mature or foetal livers, as shown by hierarchical clustering of miRNA profiling results depicted in a logarithmic scale (Fig. 1B). As a next step, gene set enrichment analysis (GSEA) was used to determine whether there were concordant differences between two groups based on the enrichment of miRNAs in one group compared with the other. A pre-defined list of conserved miRNAs that included the 10 highest expressed as well as the 10 lowest expressed miRNAs in HLCs compared to MHs, was used. HLCs were separately compared with MHs (Fig. 1C), FL cells or ESCs (Supplementary Fig. 1A and B) for these miRNAs. The results indicated an enrichment of the highest expressed differentially regulated miRNAs in HLCs compared to either of the other sample groups. We then validated the miRNA-profiling results by qRT-PCR analyses. We found that the expression pattern of miR-199a-5p and miR-122-5p were similar in qRT-PCR as observed in miRNA profiling (Supplementary Fig. 1C and D). The expression of miR-122-5p and the *HCR* gene, from which miR-122 is derived, increased as mouse ESCs differentiate into HLCs (Supplementary Fig. 1C). The increase in miR-122 levels during differentiation of ESCs into HLCs was detected by qRT-PCR (Supplementary Fig. 1C) but not in hierarchical clustering of the miRNA profiling results (Fig. 1B) since array data results are shown in logarithmic scale. The expression of miR-122 and *HCR* remains low in HLCs, albeit a slight increase was detected by qRT-PCR during hepatic differentiation, requiring further improvement in differentiation. The 10 miRNAs showing the highest expression in HLCs compared to MHs, based on a cut-off value of 50-fold and a second criterion that the miRNA sequence must also be conserved in humans, were selected for loss of function studies during hepatic *in vitro* differentiation (Fig. 1C). Conversely, the 10 miRNAs showing the lowest expression in HLCs compared to MHs, based on a cut-off range of 10-fold and with a conserved sequence in humans, were selected for gain of function studies during hepatic *in vitro* differentiation (Fig. 1C).

*In vitro miRNA screening identifies miR-199a-5p as a regulator of hepatic differentiation*

To examine the effect of selected miRNAs during the differentiation of mESCs into hepatocytes, we transfected HLCs with miRNA inhibitors or miRNA mimics for the loss of function or gain of function studies, respectively. mESCs were differentiated into HLCs using the hanging drop differentiation method, based on embryoid body formation and further propagation of the outgrowing endodermal cells (Fig. 1D). An important prerequisite to examine the effect of selected miRNAs is the optimal timing of transfection during differentiation. An optimal time point should avoid dilution of miRNA inhibition or enhancement effects that may result from extensive proliferation. Therefore, we chose day 32 of the hepatic differentiation as the time point