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The parallel universe: microRNAs and their role in chronic hepatitis, liver tissue damage and hepatocarcinogenesis

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Summary

In recent years, enormous progress has been made in identifying microRNAs (miRNAs) as important regulators of gene expression and their association with or control of various liver diseases such as fibrosis, hepatitis and hepatocellular carcinoma (HCC). Indeed, many genes encoding miRNAs as well as their targets have been described and their direct or indirect link to the respective liver diseases has been investigated in various experimental systems as well as in human tissue. Here we discuss current knowledge of miRNAs and their involvement in liver diseases, elaborating in particular on the contribution of miRNAs to hepatitis, fibrosis and HCC formation. We also debate possible prognostic, predictive and therapeutic values of respective miRNAs in liver diseases. The discovery of liver disease related miRNAs has constituted a major breakthrough in liver research and will most likely be of high relevance for future therapeutic strategies, especially when dealing with hepatitis, fibrosis and HCC.

Key words: *chronic hepatitis; hepatocellular carcinoma; fibrosis; miRNA*

Introduction

MicroRNAs (miRNAs) are endogenously expressed non-coding RNAs that are 20–24 nucleotides long and have been shown to regulate gene expression, cellular differentiation, development and disease [1]. miRNAs were first described in *C. elegans* [2] and in various eukaryotic cells except fungi, algae, and marine plants [3]. Up to now more than a thousand miRNAs have been described in humans [4–9]. miRNAs regulate various physiological processes, including the stability or translation efficiency of specific mRNAs (table 1). As individual miRNAs are able to regulate a large number of different mRNAs (encoded by 250–500 target genes), there is a strong likelihood that approximately 20–80% of transcribed human genes are regulated by miRNAs [6, 9]. The efficacy in binding and "neutralising" their targets depends on various parameters (e.g. primary sequence of the miRNA and target mRNA, three dimensional structure of the miRNA, co-factors etc.).

Since the discovery of the first miRNA *lin-4* in 1993 in *C. elegans* [2], understanding of how miRNAs work has dramatically increased. In addition to their importance in controlling physiological processes, miRNAs were also shown to play an important role in various pathologies, including diseases of the liver such as fibrosis, hepatitis or hepatocellular carcinoma (HCC) (table 1) [3, 7, 10].

Upon transcription of miRNA encoding genes (usually by RNA polymerase II), transcripts are capped with a modified nucleotide at the 5' end, polyadenylated at the 3' end and further spliced to form the primary miRNA – also called – "pri-miRNA" [11]. One pri-miRNA molecule may be constituted by 1–6 miRNA precursor transcripts with hairpin loop structures, which consist of about 70 nucleotides each. The double-stranded RNA structure of the hairpins in a pri-miRNA is recognised by the nuclear protein Pasha, which binds and activates the enzyme Drosha to form the "Microprocessor" complex [11]. Pre-miRNAs are then exported from the nucleus and further processed into mature miRNAs. Drosha and Pasha act in the cell nucleus, where processing of pri-miRNA to pre-miRNA takes place, while Dicer is involved in subsequent processing into mature miRNA in the cytoplasm [12]. Hairpin length and loop size impact on Dicer processing efficacy [13] and mature miRNAs specifically bind mRNAs through target sequences situated in the 3′- or 5′-untranslated region (UTR) being either completely or partially complementary to the respective miRNA [14]. Binding of miRNAs to their mRNA targets leads to either RNA degradation or inhibition of translation follows. Although either strand of the duplex may potentially act as a functional miRNA, only one strand is usually incorporated into the RNA-induced silencing complex (RISC) where the miRNA and its mRNA target interact [15].

The RISC constitutes a ribonucleoprotein particle that consists of one single-stranded short interfering RNA (siRNA) and the Argonaute protein, which is endonucleolytically

active [16]. The RISC is thereby capable of cleaving mRNAs complementary to the siRNA. The detailed mechanism whereby the human RISC cleaves a target RNA has recently been demonstrated [17]. It was also shown that during the course of target recognition, the RISC interacts with single-stranded RNA non-specifically within a short time frame, thereby promoting siRNA-target RNA annealing [17].

Despite the fact that some miRNAs are expressed in a broad range of different cell types and tissues, the expression of most miRNAs is strictly limited to specific organs and tissues [10, 18]. *miR-122,* for example, is only abundantly expressed in the liver and was not detected in other tissues analysed [3, 10, 19]. Furthermore, its liver-specificity is well conserved among many species. This shows that expression of particular miRNAs can be tightly regulated and is – in some instances – conserved in various species.

Extensive work has been done to elucidate the relevance of miRNAs in hepatitis, liver fibrosis and hepatocarcinogenesis, which may be the fundament for molecular based therapies (table 1) [20–22]. Hence we particularly focus on this topic in this review.

Anatomical and physiological basis of the liver

The anatomical units of the liver are the so-called "hepatic lobules". They consist of a hexagonal arrangement of hepatocyte plates radiating outward from the central vein. At the edges of the lobules portal triads are arranged in a regular distribution, harbouring a bile duct and a terminal hepatic artery branch and portal vein. Hepatocytes, Kupffer cells, liver sinusoidal endothelial cells (LSECs), hepatic stellate cells (HSCs), bile ducts, cholangiocytes, infiltrating lymphocytes, granulocytes, monocytes, migratory macrophages and dendritic cells build the cellular substrate of the liver and its blood passing through the organ [23]. The morphological subunits, the hepatic lobules, consist of liver cell plates and intervening sinusoids. Functionally the liver can be regarded as a collection of different compartments, divided into so-called "acini" and "Rappaport" zones, which control metabolic (glucose and lipid metabolism), synthetic (serum proteins and coagulation factors), catabolic, bio-transformatory (dismantling of serum proteins, hormones and transformation of contaminants), storage (glycogen, triglycerides, metals and vitamins) and removal functions (biliary components) [24, 25].

What is known about miRNAs in rodent and human liver pathologies?

In recent years various mRNAs targeted by miRNAs have been identified in the field of liver disease research [14]. Indeed, much effort has been undertaken to significantly improve our understanding of the role of miRNAs in physiological but also pathological processes such as hepatitis, liver fibrogenesis and especially hepatocarcinogenesis [26–29].

miRNAs, liver metabolism and metabolic disorders

Since the liver is the main organ involved in gluconeogenesis it is heavily implicated in production and transportation of fatty acids and cholesterol. The metabolic syndrome is frequently associated with steatohepatitis, which is an emerging precondition for HCC. Consequently, investigations have been conducted to find out whether deregulation of miRNAs can be observed in models of metabolic dysfunction. In the event that miRNAs are involved in the context of these diseases a modulate thereof would of course be of potential interest for therapeutic approaches but also for basic medical research [30, 31]. *miR-23a*, *miR-26b*, *miR-29c*, *miR-30a*, *miR-30e*, *miR-103*, *miR-125a*, *miR-140*, *miR-146*, *miR-191*, *miR-193*, *miR-195*, *miR-210*, *miR-221*, *miR-222*, *miR-223*, *miR-290*, *miR-292*, *miR-296*, *miR-365* were all described as being up-regulated in rats that spontaneously develop a diabetes-like phenotype characterised by pancreatic β-cell dysfunction, reduced insulin sensitivity and insulin resistance [32]. In the same rat model *miR-15b*, *miR-22*, *miR-27b*, *miR-100*, *miR-125b*, *miR-126*, *miR-200a*, *miR-335*, *miR-422b* were reported to be down-regulated. Thus, miRNAs were clearly reported to be deregulated in the course of metabolic dysfunction. The reported miRNAs indeed seem to be relevant for glucose and lipid metabolism as some of the above described miRNAs were also shown to be deregulated in patients suffering from diabetes. Moreover, some of those miRNAs also contribute to the pathogenesis of fatty liver development in a rat model [32]. Whether the reported deregulation of some miRNAs is the cause of the above described diseases or whether it is a consequence thereof needs to be investigated in detail for each of the miRNA candidates.

In mice, silencing of one particular miRNA – *miR-122* – reduced hepatic steatosis in a high-fat-diet model, indicating that *miR-122* is causally involved in the regulation of the hepatic lipid metabolism [33–35]. The above reported models possibly represent elegant tools to investigate the molecular mechanisms whereby particular miRNAs contribute to disease progression in humans [32]. Still, much more work is needed to gain detailed insight into the most important miRNAs involved in metabolic dysfunction of the liver and fat tissue – not infrequently, quality, type of RNA preparation and quantity of tissue specimens are limiting factors.

Involvement of miRNAs in liver fibrosis and liver regeneration

In any form of acute or chronic liver disease hepatocyte cell death occurs, being the starting point for tightly organised repair programmes and compensatory proliferation [36, 37]. The complex process of fibrogenesis, describing an excessive deposition of extracellular matrix proteins, is induced by liver cell injury and activation of various cell types, including hepatic stellate cells (HSCs). Following liver cell damage, type I and type III collagens are deposited in the portal tracts, the intermediate zone or the centre of the liver lobe. Predominantly HSCs located adjacent to endothelial cells, are believed to be responsible for fibro-

Table 1: miRNAs differentially expressed in human and murine liver tissues under different disease conditions.

miRNA	Differential expression	Gene target(s)	Functional Impact	Disease	References
miR-15b	↑	Nd	Nd	HCC	[99–101]
miR-17.5p	↑	Nd	Promotion of tumour growth, metastasis	HCC	[70]
miR-18	↑	Nd	Proliferation, growth promoting	HCC	[100, 102, 103]
miR-18a	↑	ERα	Proliferation	HCC	[99, 101, 104, 105]
miR-19a	↑	Nd	Proliferation	HCC	[100, 104, 106]
miR-21	↑	PTEN	Apoptosis, growth	Viral hepatitis, cirrhosis, HCC	[67, 99, 100, 103, 106–110]
miR-30d	↑	Gai2	Nd	HCC	[111]
miR-34a	↑	NOTCH1, c-Met	Proliferation	HCC	[67, 100, 108],
miR-93	↑	E2F1	Proliferation	HCC	[67, 100, 104, 105, 108]
miR-96	↑	Nd	Nd	HCC	[67, 110, 112]
miR-106b	↑	E2F1	Proliferation	Viral hepatitis, cirrhosis, HCC	[67, 93, 103, 113]
miR-130b	↑	Nd	Nd	Viral hepatitis, cirrhosis, HCC	[99, 100, 103]
miR-143	↑	FNDC3B	Promotion of metastasis	HCC	[114]
miR-151	↑	RhoGD1A	Nd	HCC	[100, 110, 115]
miR-181b	↑	TIMP3	Promotion of metastasis	HCC	[71]
miR-182	↑	Nd	Nd	HCC	[100, 109, 110, 112]
miR-183	↑	Nd	Apoptosis	HCC	[100, 110, 112]
miR-185	↑	Nd	Nd	HCC	[100, 116]
miR-210	↑	Nd	Nd	HCC	[67, 100, 105, 108]
miR-221	↑	P52/Kip2, p27/Kip1, Bmf, PTEN, TIMP3, DDIT4	Inhibition of apoptosis	Viral hepatitis, cirrhosis, HCC	[66, 67, 99–101, 103, 108, 110, 117–119]
miR-222	↑	PTEN	Inhibition of apoptosis	HCC	[67, 99–101, 105, 107, 108, 110, 112, 117]
miR-224	↑	API-5	Promotion of growth, proliferation, apoptosis	HCC, non-tumour tissue	[67, 99, 101, 102, 105, 107, 110, 112]
miR-301	↑	Nd	Nd	Viral hepatitis, cirrhosis, HCC	[99, 100, 103, 110, 112]
miR-374	↑	Nd	Nd	HCC	[100, 110, 112]
miR-602	↑	RASSFIA	Inhibition of apoptosis	HCC	[120]
miR-I	↓	c-Met, FoxPI, HDAC4	Inhibition of tumour growth and metastasis	HCC	[121]
let-7c	↓	c-Myc	Inhibition of cell growth and proliferation	Liver ageing, stellate cell pro-liferation, different upon HCV or HBV infection, HCC	[67, 99, 119, 122, 123]
let-7g	↓	Type I collagen a2, c-Myc	Inhibition of cell growth and proliferation	Stellate cell pro-liferation, HCC	[100, 116, 119, 122–124]
miR-23b	↓	u-PA, c-Met	Inhibition of metastasis	HCC	[125]
miR-26a	↓	Cyclin D2, Cyclin E2	Inhibition of tumour growth	HCC	[126]
miR-29	↓	Bcl-2, Mcl-1	Promotion of apoptosis	HCC	[127]
miR-101	↓	Fos, Mcl-1	Promotion of apoptosis, inhibition of cell growth	HCC, non-tumour tissue without viral hepatitis	[101, 103, 105, 109]
miR-122	↓	Cyclin G, Bcl-w, ADAM 17, ADAM 10	Tumour suppressor, promotion of apoptosis, invasion and metastasis, impact on angiogenesis and drug resistance	HCC	[99, 100, 107, 108, 116, 119, 128–131]
miR-124	↓	CDK6, VIM, SMYD3, IQGAPI	Inhibition of tumour growth and metastasis	HCC	[132]
miR-125a	↓	Nd	Nd	HCC, HBV infection	[100, 102, 108, 112]
miR-125b	↓	Nd	Inhibition of cell growth, proliferation	HCC	[101, 105, 108, 112, 116]
miR-126	↓	Nd	Nd	HCC	[100, 116, 133]
miR-139	↓	Nd	Nd	HCC, non-tumour tissue without viral hepatitis	[103, 110, 112]
miR-145	↓	Nd	Nd	Stellate cell proliferation, HCC	[99, 100, 110, 112, 119]
miR-148a	↓	Nd	Nd	HCC	[100, 109, 116]
miR-150	↓	Nd	Nd	Liver ageing, HCC	[67, 114, 118, 134]
miR-195	↓	Cyclin D1, CDK6, E2F3	Proliferation	HCC, HBV infection	[99, 100, 102, 105, 112, 119, 135]
miR-198	↓	Nd	Nd	HCC	[69]
miR-199a-5p	↓	Nd	Nd	Hepatitis, cirrhosis, HCC, non- tumour tissue	[100, 102, 103, 105, 119]

miR-199b	↓	Nd	Nd	Hepatitis, cirrhosis, HCC	[99, 103, 112, 119]
miR-200b	↓	ZEB1, ZEB2	Nd	HCC	[103, 112, 119]
miR-203	↓	ABCEI	Nd	HCC	[132]
miR-214	↓	Nd	Nd	HCC	[100, 103, 110, 112, 119]
miR-223	↓	Stathmin1	Proliferation	HCC	[99, 100, 103, 105, 119]
miR-375	↓	YAP	Inhibition of tumour growth and metastasis	HCC	[136]
Nd: not determined					

genesis and are activated by TGF-β1, angiotensin and leptin [38].

With respect to hepatic fibrogenesis, *miR-195* has been investigated intensively in various experimental models. Down-regulation of cyclin E1 and upregulation of p21 expression by *miR-195* was reported to cause interferon beta (IFNβ)-driven HSC proliferation and subsequent fibrogenesis [39]. Cyclin E1, which is critically involved in carcinogenesis in general, is expressed in the late G1 phase of the cell cycle and activates the cancer relevant kinase Cdk2 [40]. Transgenic mice constitutively overexpressing cyclin E were reported to develop malignancies. p21 is a TGF-β target which is equally well silenced in hematopoietic diseases such as leukaemia [41]. Furthermore, in primary cell culture experiments it was demonstrated that IFNβ could block cell proliferation of human HSCs, LX-2 cells, by delaying cell cycle propagation from G1 to S phase. These findings showed a miRNA-mediated effect of IFNs, subsequently driving HSC proliferation and fibrogenesis [39].

Recently, Roderburg and colleagues have systematically analysed miRNA regulation in a mouse model of carbon tetrachloride [CCl(4)]-induced hepatic fibrogenesis. Their data described a group of miRNAs specifically deregulated in livers of mice undergoing hepatic fibrosis. Interestingly, it turned out that within the panel of identified miRNAs all three members of the *miR-29* family were significantly down-regulated in livers of CCl(4)-treated mice [42]. These experimental data also correlated with data gained from human patient material. Lower expression of *miR-29* was found in livers of patients with advanced liver fibrosis. As far as the mechanism is concerned Roderburg and colleagues were able to show that downregulation of *miR-29* in murine HSC was mediated by TGFβ, TLR signalling and activation of NF-κB signalling cascade [42]. The authors therefore concluded that *miR-29* mediates the regulation of liver fibrosis involving TGF-β1- and NF-κB-dependent downregulation of *miR-29* family members within HSC. In line with this it was shown in another publication that many extracellular matrix (ECM) genes are down-regulated by *miR-29*. Thus, *miR-29* re-introduction is also proposed as a potential therapeutic agent to treat liver fibrosis [43].

However, the miRNAs described above are not the only ones implemented in affecting liver fibrosis: *miR-23b*, for example, was recently shown to play an important role in the termination of liver regeneration in rats, 120 hours after 70% partial hepatectomy [44]. One of the targets of *miR-23b* during liver regeneration turned out to be Smad3, which is well established as a crucial modulator in carcinogenesis [45]. It has been suggested that upregulation of *miR-23b* promotes cell proliferation of BRL-3A rat liver cells, corroborating the notion that *miR-23b* expression levels influence the proliferative capacity of heaptocytes. In parallel, upregulation of *miR-23b* inhibited TGF-β1-driven apoptosis. Thus, Yuan and colleagues proposed that *miR-23b* is involved in liver tissue regeneration by activating TGF-β1 and Smad3 signalling [44].

Partial hepatectomy is widely used to stimulate liver cell growth and is generally accepted as a "gold standard" in research to investigate regenerative processes in the liver [46]. After partial hepatectomy removing 50% of the liver mass, a total of 30 miRNAs were shown to be down-regulated. Predominantly those were found that are implicated in cell cycle regulation. Among them, *miR-22a, miR-26a, miR-30b, Let7f* and *Let7g* were significantly down-regulated while cell cycle regulating genes like cyclin G1 were drastically up-regulated 2 days after partial hepatectomy [47].

Further, partial hepatectomy and treatment with dimethylnitrosamine (DMN) or diethylnitrosamine (DEN), which resemble alkylating agents leading to DNA modification and resulting in mutations, is used to study the mechanisms of liver fibrosis [48]. The *rno-miR-34* family, consisting of *miR-34a, miR-34b* and *miR-34c*, was reported to be up-regulated in DMN-driven hepatic fibrosis in rats [49]. Moreover, in this model the *rno-miR-34* family members were shown to target acyl-CoA synthetase long-chain family member 1 (ASCL1), which is involved in fatty acid metabolism and therefore hepatic functions, and activates transcription [49]. As fatty liver is often associated with liver carcinogenesis, it is also interesting to note that the *miR-34* family displays pro-apoptotic and anti-proliferative properties [49], suggesting a causal relationship between *miR-34* family members and uncontrolled hepatocyte cell growth.

Liver fibrogenesis is known to be mainly induced by TGF-β1 [50]. In order to silence TGF-β1 specifically in HSCs, TGF-β1 pri-miRNA encoding plasmids were generated under the control of a GFAP promoter – ensuring expression on HSCs in liver cell culture upon overlaying wounded immortalised rat liver stellate (HSC-T6) monolayers with the respective plasmids [50]. This application might be a novel approach to the treatment of liver fibrosis in a conditional manner, since inhibition of cell proliferation and induction of apoptosis of activated immortalised HSC-T6 cells was experimentally reached *in vitro* [50].

Finally, in a recent study four human and murine miRNAs (*miR-199a*, antisense *miR-199a**, *miR-200a*, and *miR-200b*) were drastically upregulated in progressing liver fibrosis in mice that were compared to controls in a CCl(4)-induced mouse model compared to olive oil-treated animals [51]. Experimental results were correlated with hu-

man data. Progression of hepatic fibrosis in this CCl(4)-driven model was shown to be linked to and significantly correlated with over-expression of the *miR-199* and *miR-200* [51].

How miRNAs influence the hallmarks of liver cancer

The initially established 6 hallmarks of cancer [52] have recently been enlarged to 7 [53] and thereafter modified to 9 hallmarks of cancer [54]. The obvious question arose whether some of the hallmarks described are actually influenced by miRNAs. Here we aimed to dissect miRNAs for their contribution to hepatocarcinogenesis by evaluating the respective cancer hallmarks.

Sustained proliferative signalling

Self-renewal potential and differentiation capacity are known to be endogenous properties of human embryonic stem cells as well as of cancer cells [55]. A recent study by Kim and colleagues demonstrated that different subtypes of miRNAs may function in a lineage-specific manner during the process of human embryonic stem cell and hepatocyte differentiation [56]. This multipotent capacity may be highly relevant during carcinogenesis. Indeed, recent work by Li and colleagues demonstrated the involvement of miRNAs in neoplastic transformation of liver cancer stem cells [57]. It is worth mentioning that by investigating human HCC samples in an undifferentiated, high grade HCC with a poor prognosis, a stem cell-like miRNA profile, including the *miR-371-3* cluster, was reported [58]. This *miR-371-3* cluster is typically overexpressed in embryonic stem cells, and is downregulated during differentiation *in vivo* [58]. This might be of clinical relevance, since functional analysis of miRNAs in human HCC stem cells (HsCs) revealed that conserved *let-7* and *miR-181* family members were also up-regulated in HsCs [59]. Cell culture studies attempting to model carcinogenic growth patterns of hepatocytes seen in liver tissue specimens demonstrated that another miRNA, *miR-146,* is able to promote hepatocyte proliferation and colony formation as well [60].

In childhood, hepatoblastomas are the most frequently observed malignant liver tumours. *miR-492,* processed from the *keratin 19* (*KRT19*) gene in hepatoblastoma was shown to be up-regulated in metastatic hepatoblastoma [61]. Thus, elevated expression levels of *miR-492* and *KRT19* were described as frequently found in metastases of hepatoblastomas. miRNA array analysis-based data derived from *in vitro* experiments with stably *miR-492* overexpressing cell lines were confirmed in human hepatoblastoma tissue samples. *miR-492* overexpression was found to be driven by the oncogene pleomorphic adenoma gene 1 (*PLAG1*) which is frequently altered in hepatoblastomas [61], suggesting a functional link between *miR-492* and liver cancer.

Evading growth suppressors

Many tumour cells found ways to downregulate the activity of tumour suppressor genes and to modulate particular cancer-related signalling pathways [54]. In the human hepatoma cell-line HepG-2 the effect of arsenic trioxide (ATO) as a potential chemotherapeutic agent in cancer therapy was studied [62]. ATO's hypothesised modes of anti-tumourigenic action range from induction of apoptosis through its activating effects on several caspases, inhibition of cell growth through interactions with various signalling pathways, promotion of cell differentiation, and inhibition of angiogenesis by downregulating vascular endothelial growth factor (VEGF).

miR-29a was revealed as possessing synergistic effects with ATO based on the treatment of an HCC cell line (HepG-2) by inhibiting cell growth and inducing apoptosis. Thus, an additive effect of *miR-29a* and ATO was proven *in vitro*. This might enable a reduction in the amount of ATO used in clinics by reinforcing a combined therapeutic approach of ATO together with *miR-29a* in order to reduce side effects of the monotherapy by partially replacing ATO [62]. In line with this is that inhibition of *let-7* increased the chemosensitivity of HSCs to sorafenib and doxorubicin, a feature which may also be helpful in HCC treatment since sorafenib is a small molecular inhibitor of several tyrosine protein kinases which was successfully used for the treatment of HCC [63, 64].

Resisting cell death

Tumour cells developed various strategies to evade apoptosis, enabling continuous cell growth. Loss of TP53, increased expression of antiapoptotic regulators from the bcl2 family, or survival signals (e.g. Igf1/2), downregulation of pro-apoptotic factors (e.g. Puma) can be encountered as such strategies [54].

A popular example of a miRNA which is critical for HCC development due to apoptosis regulation is *miR-221.* It fulfils two contrasting roles regarding regulation of apoptosis, one pro- and one anti-apoptotic pathway. On the one hand *miR-221* was shown to be drastically upregulated in response to death receptor-mediated apoptosis, and on the other hand its ectopic expression may protect primary hepatocytes as well as hepatoma cells from apoptosis [65]. *In vivo* overexpression of *miR-221* in hepatocytes by adeno-associated virus serotype 8 (AAV8) delays FAS-triggered fulminant murine liver failure – again supporting the idea that expression of *miR-221* acts anti-apoptotically. Moreover, *miR-221* was shown to upregulate hepatic p53 upregulated modulator of apoptosis (Puma) expression, thereby increasing the expression of a pro-apoptotic member of the Bcl2 protein family. *miR-221* was even suggested as a possible therapeutic target in treating hepatitis and liver failure [65].

Enabling replicative immortality

Normal cells undergo a limited number of cell proliferation cycles and thereafter undergo controlled cell death. Tumour cells have the capacity to grow without limitation. This exclusive feature is also called "replicative immortality" [54]. In HCC overexpression of *miR-221* was shown to lead to transcriptional induction of two cyclin-dependent kinase inhibitors (CDKIs), CDKN1C/p57 and CDKN1B/p27, supporting cell proliferation of hepatocytes [66]. These two CDKNIs were indeed shown to serve as respective targets for *miR-221* in the course of HCC development in humans [67]. Analysis of benign and malignant human liver tumours revealed increased expression levels

of the gene encoding *miR-224* while expression levels of *miR-122a* and *miR-422b* were decreased [67]. Thus cell proliferation and replicative immortality of tumour cells are directly supported by *miR-224*. Furthermore, human HCC were characterised by high levels of *miR-21*, *miR-10b, miR-222,* which may therefore serve as liver tumour markers [67]. In addition, Pineau and co-workers typified miRNAs based on expression profiles in liver tissues and in 35 HCC cell lines. On the basis of these experiments 12 miRNAs (*miR-106b, miR-21, miR-210, miR-221, miR-222, miR-224, miR-34a, miR-425, miR-519a, miR-93, miR-96* and *Let-7c*) could be linked to human liver pathogenesis, ranging from normal liver integrity over cirrhosis to HCC, which explains the relevance of particular miRNAs in disease progression starting from the inducers of hepatitis, liver cirrhosis and finally HCC [67].

Inducing angiogenesis

miRNAs predominantly driving neo-angiogenesis still have to be identified as specific miRNAs and are not established yet.

miRNAS and activating invasion and metastasis

A sign of malignancy is invasive growth and tumour cell spread throughout the body. *miR-31* was described as a master regulator of metastasis of different cancer types because it not only controls metastasis-relevant genes or genes promoting proliferation, but also because it was shown to control cell cycle and apoptotic cell death [68].

Another miRNA that was shown to be linked with metastasis is *miR-492. miR-492* was first reported to be of relevance in hepatoblastomas and was also shown to be upregulated in metastatic hepatoblastoma [61]. Hence it potentially serves as a biomarker to evaluate hepatoblastoma progression.

Silencing of *miR-181* lowered the motility of hepatic cancer stem cells (HsCs) and the invasiveness of liver tumour cells. The respective tumour cells were characterised by EpCAM positivity – already known to be a liver stem cell marker – which is expressed by very aggressive HCC cells [69]. In addition, *let-7* was described as supporting tumour invasion and directly targetting SOCS-1 and caspase-3 activity [59].

High *miR-17.5p*, *miR-143* and *miR-181b* expression are known to promote tumour cell growth and metastatic processes with spread of hepatic tumour cells to extrahepatic organs [69–71]. By contrast, *miR-23b* was reported as being downregulated in HCC because under normal conditions this particular miRNA acts as a tumour suppressor [69–71].

The tumour promoting inflammatory environment: miRNAs, inflammation, tissue damage, fibrosis and HCC

It is well established that chronic hepatitis (e.g. induced by HBV of HCV) and HCC development are interconnected [72–75]. On the basis of sequencing and bioinformatics data, miRNA transcriptome deregulation was shown in hepatitis B-driven HCC development [76]. In humans the miRNAs *miR-7*, *miR-196b*, *miR-433* and *miR-511* affect the viral polymerase as well as the S gene of HBV while *miR-205* affects the X-gene of HBV, thereby making it a potentially useful therapeutic tool in HBV-induced hepatitis [76, 77]. Furthermore, *miR-345* appears to target the HBV pre-C gene and downregulation of *miR-345* facilitates the protein expression of HBV pre-C which is a precursor of HBeAg [77].

As mentioned above, *miR-122* was also shown to be crucially involved in the control of hepatitis C virus (HCV) infection, in addition to cholesterol metabolism and HCC formation, as described above [76]. HCV is a positive-sense single-stranded RNA virus which can lead to chronic infection resulting in chronic hepatitis, liver cirrhosis and, in many instances, to HCC [70]. *miR-122* was shown to be essential for HCV replication in cultured human hepatocyte cell lines (Huh7). Moreover, *miR-122* directly binds to two adjacent sites in the 5′-UTR of HCV RNA [76]. This particular binding is responsible for increased viral replicon amplification and RNA synthesis. It seems that *miR-122* predominantly has a more indirect stimulatory effect on viral RNA synthesis than a direct effect on protein synthesis [35]. Studies by Roberts and colleagues have shown that *miR-122* stimulates accumulation and translation of HCV RNA via the HCV 5`-UTR. In addition, *miR-122* appears to modulate a second replication cycle via a so far unclear mechanism [35]. One way in which *miR-122* acts on its targets would be the influence on viral RNA stability. Interestingly, treatment with IFNβ was reported to downregulate *miR-122* expression, thereby offering a potential explanation for the therapeutic effects of IFNβ [78]. However, the role of *miR-122* is controverted in this context since another group failed to observe a correlation between expression of *miR-122* and antiviral treatment with IFNβ [79]. Never-

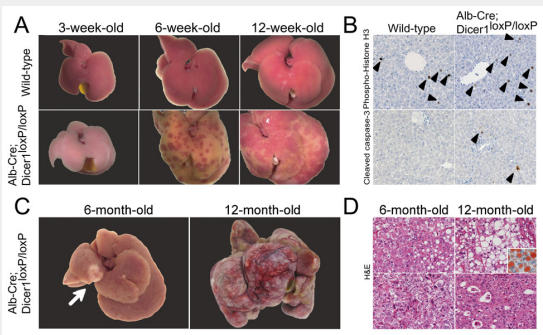

Figure 1

Disruption of Dicer1 induces dysregulated foetal gene expression and promotes hepatocarcinogenesis. (A) Macroscopy of wild-type and *Albumin-Cre;Dicer1*$^{loxP/loxP}$ livers at various time points postnatal (p.n.). At 3 weeks p.n. *Albumin-Cre;Dicer1*$^{loxP/loxP}$ mouse livers appeared pale when compared to wild-type livers. At 6 weeks, the *Albumin-Cre;Dicer1*$^{loxP/loxP}$ liver have developed a yellow colour with red areas. The normal-coloured areas had expanded at 12 weeks. (B) Immunohistochemical analysis for phospho-histone H3 and cleaved Caspase-3 in 3-week-old murine livers of *Albumin-Cre;Dicer1*$^{loxP/loxP}$ and wild-type mice. (C) HCC in *Albumin-Cre;Dicer1*$^{loxP/loxP}$ mice at as early as 6 and 12 months p.n.. One HCC nodule observed in a 6-month-old *Albumin-Cre;Dicer1*$^{loxP/loxP}$ liver (left panel, *white arrow*). At 12 months of age *Albumin-Cre;Dicer1*$^{loxP/loxP}$ livers carry multiple HCC (*right*). (D) On histological level HCC displayed mild steatosis, *top*, *left*), prominent steatosis (*top*, *right*), as indicated by oil red O staining (*inset*), poorly differentiated tumour cells with a solid growth pattern (*bottom*, *left*), and a pseudoglandular pattern (*bottom*, *right*). The figure was adapted from [83].

theless, *miR-122* was discussed as a potential target candidate controlling HCV replication. Unfortunately quantitative analyses of *miR-122* and HCV-RNA in infected human liver specimens revealed a complicated interaction mechanism since activation of HCV translation does not take place via structural transformation in the HCV internal ribosomal entry site (IRES) but is driven by Argonaute [80, 81]. This is in contrast to results in cell culture experiments as virus load did not correlate with *miR-122* levels. Subjects with lower pre-treatment *miR-122* levels displayed a low response rate to IFN therapy. However, *miR-122* was downregulated in Huh7 cells upon IFNβ treatment. Noteworthy is that pegylated IFNα does not affect *miR-122* levels in human and murine liver cells. Furthermore, in patients with HCV infections autoantibodies against a miRNA binding protein Argonaute2 (Su antigen) were found, indicating that *miR-122* levels could already predict response efficacy to IFN-based therapies [80]. A strong antiviral effect has recently been shown in HCV infected chimpanzees [35].

Furthermore, miRNAs can be deregulated concomitant with a developing inflammatory environment in the liver. *miR-125b*, *miR-146a*, *miR-155* were shown to be involved in inflammatory reactions to lipopolysaccharides (LPS), as evidenced by the fact that chronic alcohol treatment led to a time-dependent increase in *miR-155* in macrophages *in vitro*, showing a link between miRNAs and inflammatory responses [78]. Similarly, an increase in *miR-155* and TNFα production was observed in Kupffer cells in a murine model of alcoholic liver disease.

By inhibiting NF-κB signalling with MG-132 and Bay11-7082 an NF-κB mediated upregulation of *miR-155* in Kupffer cells could be confirmed [78]. NF-κB being one of the most important inflammation and cancer related signalling pathways, these data highlight the importance of miRNAs in liver carcinogenesis since the link between miRNAs and NF-κB signalling is already known for other tumours [78, 82].

Deregulating cellular energetics

Little is known of energetic changes on the cellular level upon the action of particular miRNAs. In particular, genetic and chromosomal changes leading to HCC formation are hypothesised to be due to energetic alterations [54]. Molecular alterations to liver cells resulting in HCC formation are well known [83, 14]. However, correlations between energy status and tumorigenicity are still lacking and are poorly understood.

Still, new insights have been achieved by recent experiments showing that ageing rat livers revealed increased expression of *miR-34a* and *miR-93* [14]. These miRNAs impact inversely on the expression of Mgst1 and Sirt1, which are involved in oxidative stress [14]. The latter is known to be an energy dependent process.

Genome instability and mutation

Genomic instability is found in most tumour entities. Chromosomal instability describes a high turnover of structural and numerical chromosomal changes occurring over time in tumour cells when compared to non-neoplastically transformed normal cells [54]. Additional possibilities to generate genomically instable conditions in cells are thought to occur through microsatellite instability or high numbers of base-pair mutations [84]. The latter mechanism is best studied in colorectal cancer. HCC are characterised by loss and gain of chromosome fragments, point mutations and epigenetic changes, plus extinction of gene expression alterations due to hypermethylation of the respective promoters [14, 84, 85]. Recent publications have linked aberrant miRNA processing to liver cancer progression [83], describing the development of a pro-carcinogenic environment which is most likely responsible for spontaneous HCC development. Evidence of the direct role of miRNAs in controlling genomic stability, however, remains elusive.

Dicer and experimental HCC

In most cancer types, Dicer, the key enzyme involved in miRNA processing, has been reported to be downregulated [86]. To test whether Dicer 1-dependent processing of microRNAs influences various biological processes in the liver, Sekine and colleagues depleted Dicer 1 specifically in hepatocytes. The authors describe how the conditional knockout of Dicer1 in hepatocytes leads to an efficient decrease of Dicer 1 expression at 3 weeks p.n. [83]. It is noteworthy that this caused prominent steatosis and depletion of glycogen storage [83]. Moreover, livers with Dicer1-specific depletion in hepatocytes display a gene expression profile indicative of cell growth and progenitor cell (re)-differentiation, leading to both increased hepatocyte proliferation and overwhelming apoptosis. Over time, the entire liver tissue regenerated, mainly due to progressive repopulation of Dicer1-expressing wild-type hepatocytes which had escaped Cre-mediated recombination [83]. At 1 year of age approximately 60% of the mutant mice spontaneously developed HCC that were derived from Dicer1-deficient hepatocytes (fig. 1). Thus, these data showed for the first time that processing of miRNAs through Dicer1 has a critical impact on hepatocyte survival, metabolism, and tumour suppression in the liver (fig. 1) [83]. Interestingly, when conditional deletion of Dicer in the liver was done by an alternative cre-deleter (afp-cre), which already results in embryonic downregulation of Dicer, no dramatic changes in the metabolic function of the liver were detectable [83].

Moreover, Dicer knockout mice were shown to display altered vascular development and remodelling in general [87].

Diagnostic and prognostic miRNAs in HCC and potential therapeutic implications

From a diagnostic point of view miRNAs may be beneficial as they offer additional information which can be used in combination with methods used hitherto, such as histopathology or analysis of serum transaminase levels (fig. 2). miRNA expression in different cancer types showed that miRNAs are frequently downregulated independently of the type of neoplasms [88]. In HCC, some miRNAs were linked to specific clinico-pathological findings and risk of metastasis and tumour recurrence [89–91]. *miR-96*,

miR-139-5p and *miR-142-3p* seem to be of predictive value in evaluating recurrence rates after HCC resection [91].
Downregulation of *miR-145* and *miR-199b* as well as upregulation of *miR-224* were often detected in premalignant dysplastic nodules. These changes still remained at later stages of HCC development [92]. Low expression levels of *miR-26* correlated with short survival but better response to IFN-based treatment in patients with HCC [93]. *miR-145* represents an early HBV-associated hepatocarcinogenic event, being significantly higher in HBV infected patients than in patients positive for HCV RNA [92]. Therefore it might serve as an early diagnostic HCC marker.
In line with the Milan criteria for orthotopic liver transplantation, miRNAs may be used in future as a predictor for recurrence after resection in patients with HCC [91].
Serum markers are widely used for detection of various disease-related parameters [94]. Even for HCC circulating miRNAs were regarded as potential biomarkers [95]. Consequently, serum miRNA profiling may also serve as a biomarker not only for viral infections (e.g., HBV or HCV infection), but also for the development of HCC. *miR-375* and *miR-92a,* for example,were reported to be upregulated in sera of patients infected with HBV as well as in patients suffering from HBV-induced HCC [85]. In addition to well established serum markers such as α-fetoprotein (AFP), lens culinaris agglutinin-reactive AFP (AFP-L3%), and des-g-carboxyprothrombin (DCP), analysis of *miR-16* levels in serum samples improves sensitivity and specificity for HCC detection [96]. It was recently reported that in blood samples of 532 Chinese patients with liver cirrhosis, a *miR-196a2* variant was associated with increased susceptibility to HCC [97].
Anti-miRNA based therapies have been experimentally tested in different cancer types. A miRNA controlled adenovirus mediated anticancer treatment was recently shown to work without affecting endogenous miRNA activity [98].
Targeted downregulation of *miR-122* might offer new therapeutic approaches for the treatment of HCC, given the fact that *miR-122* influences cell cycle control and the expression of metalloproteinases, both representing essential milestones in the progression of cancer [79, 81].

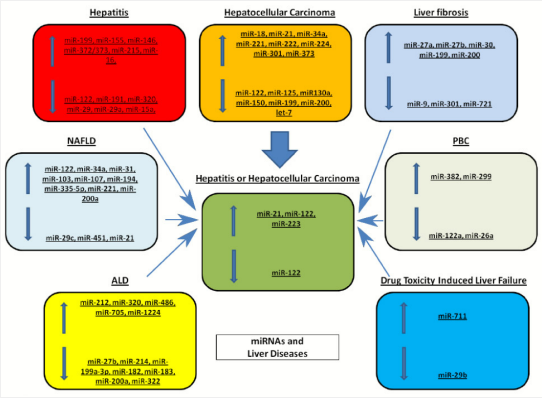

Figure 2

Schematic representation of miRNA expression and its deregulation in various liver diseases. This includes hepatitis, hepatocellular carcinoma (HCC), liver fibrosis, nonalcoholic fatty liver disease (NAFLD), hepatitis and HCC, primary bilary cirhossis (PBC), alcoholic liver disease (ALD), and drug toxicity induced liver injury. Arrows pointing upwards indicate upregulation. Arrows pointing downwards indicate downregulation.

Conclusions

HCC has many underlying causes and there is increasing evidence for an important role of miRNAs in the pathogenesis of HCC of different aetiologies. Much work has already been done to clarify the role of particular miRNAs in liver pathogenesis, especially in hepatocarcinogenesis. On the basis of the molecular understanding, efforts are under way to define new prognostic and predictive HCC markers – directly from tissue but also in sera.
There is an urgent need for therapies in the liver diseases described above, definitely including liver fibrosis and liver cancer as the diseases that should be treated very efficiently in the future. miRNA-based therapeutic approaches may become a very promising alternative to current approaches in clinical oncology in the near future, but such approaches will need to be tested not only in various murine models but also macaques, before they can enter early clinical testing.

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Figures (large format)

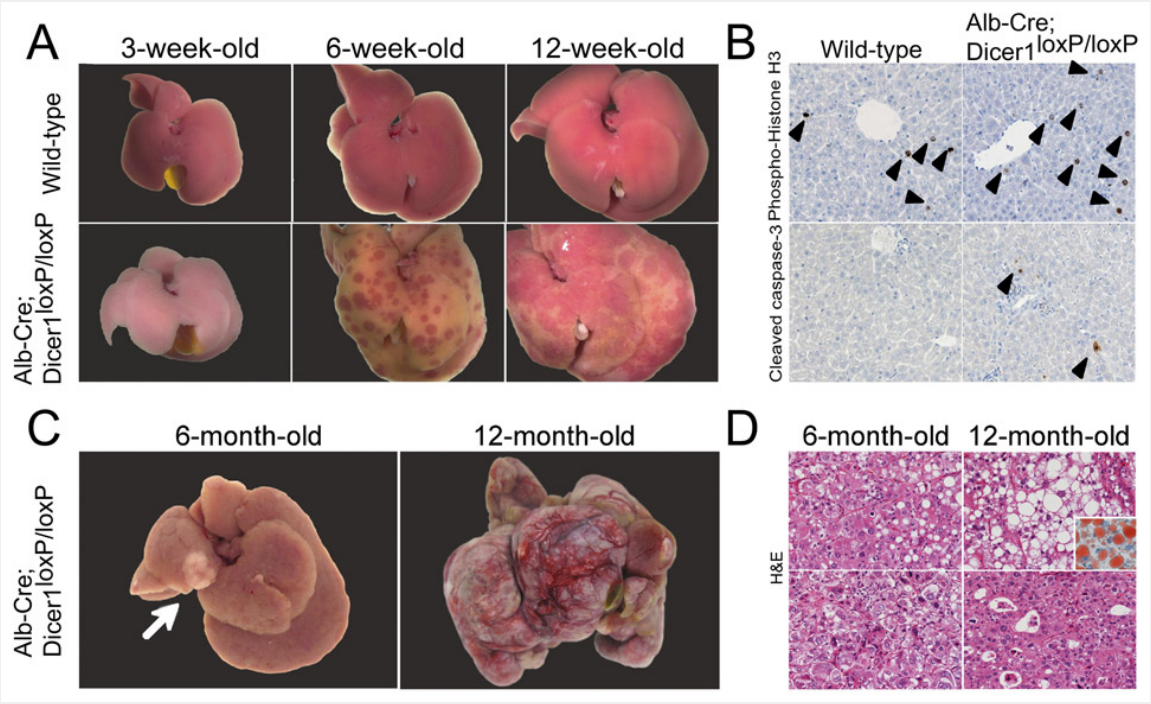

Figure 1

Disruption of Dicer1 induces dysregulated foetal gene expression and promotes hepatocarcinogenesis. (A) Macroscopy of wild-type and *Albumin-Cre;Dicer1*$^{loxP/loxP}$ livers at various time points postnatal (p.n.). At 3 weeks p.n. *Albumin-Cre;Dicer1*$^{loxP/loxP}$ mouse livers appeared pale when compared to wild-type livers. At 6 weeks, the *Albumin-Cre;Dicer1*$^{loxP/loxP}$ liver have developed a yellow colour with red areas. The normal-coloured areas had expanded at 12 weeks. (B) Immunohistochemical analysis for phospho-histone H3 and cleaved Caspase-3 in 3-week-old murine livers of *Albumin-Cre;Dicer1*$^{loxP/loxP}$ and wild-type mice. (C) HCC in *Albumin-Cre;Dicer1*$^{loxP/loxP}$ mice at as early as 6 and 12 months p.n.. One HCC nodule observed in a 6-month-old *Albumin-Cre;Dicer1*$^{loxP/loxP}$ liver (left panel, *white arrow*). At 12 months of age *Albumin-Cre;Dicer1*$^{loxP/loxP}$ livers carry multiple HCC (*right*). (D) On histological level HCC displayed mild steatosis, *top*, *left*), prominent steatosis (*top*, *right*), as indicated by oil red O staining (*inset*), poorly differentiated tumour cells with a solid growth pattern (*bottom*, *left*), and a pseudoglandular pattern (*bottom*, *right*). The figure was adapted from [83].

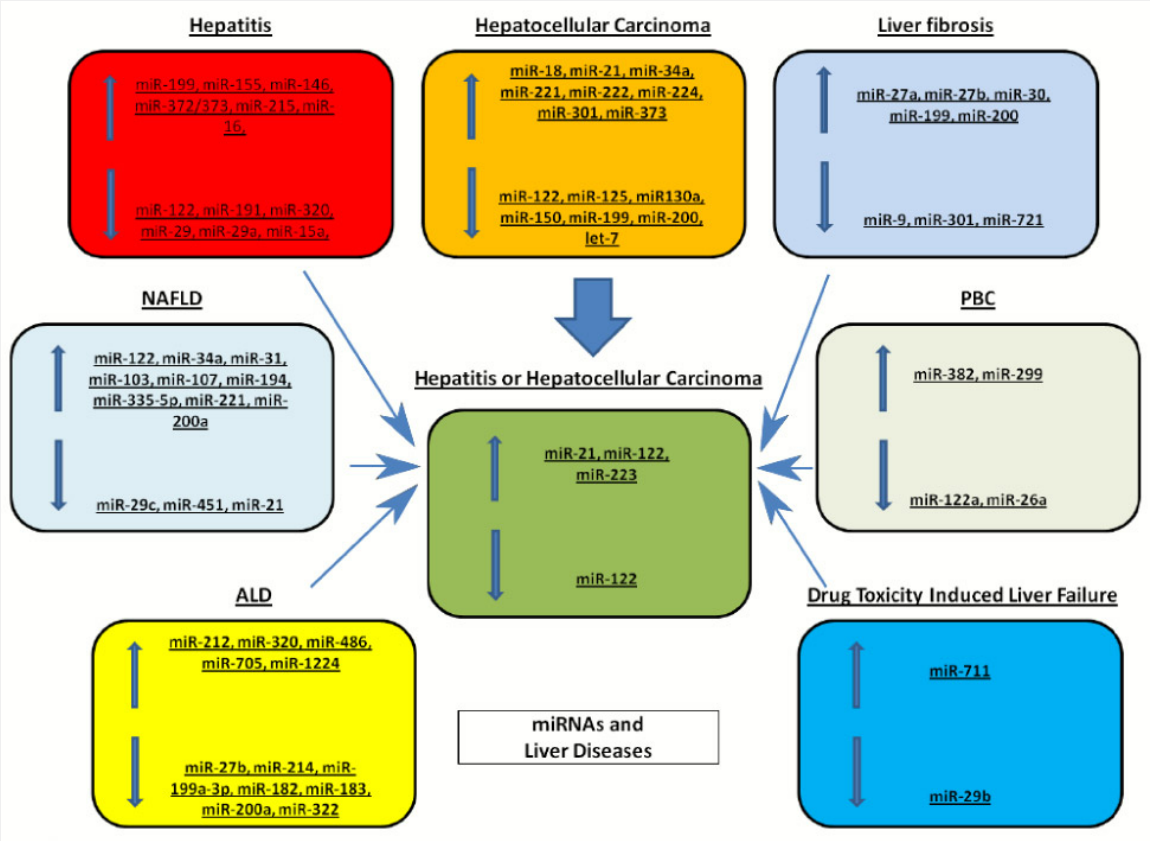

Figure 2

Schematic representation of miRNA expression and its deregulation in various liver diseases. This includes hepatitis, hepatocellular carcinoma (HCC), liver fibrosis, nonalcoholic fatty liver disease (NAFLD), hepatitis and HCC, primary bilary cirhossis (PBC), alcoholic liver disease (ALD), and drug toxicity induced liver injury. Arrows pointing upwards indicate upregulation. Arrows pointing downwards indicate downregulation.