Liver-specific microRNA-122
Biogenesis and function

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Abbreviations: miRNA, microRNA; miR-122, microRNA-122; HCV, hepatitis C virus; nt, nucleotide; UTR, untranslated region; Pol II, RNA polymerase II; HNF-4α, hepatocyte nuclear factor 4α; PPARβ/d, peroxisome proliferator-activated receptor β/d; CPEB, cytoplasmic polyadenylation element binding protein; CAT-1, cationic amino acid transporter 1; Aldo A, aldolase A; Ndur3, N-myc downstream regulated gene 3; Hfe, hemochromatosis; Hjv, hemojuvelin; HCC, hepatocellular carcinoma; IRES, internal ribosome entry site; miRISC, miRNA-induced silencing complex; LNA, locked nucleic acid

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MicroRNA-122 (miR-122) was one of the first examples of a tissue-specific miRNA. It is highly expressed in liver, where it constitutes 70% of the total miRNA pool. miR-122 expression is specific to the vertebrate lineage, where the sequence of the mature miRNA is completely conserved. miR-122 is a target for extensive study due to its association with cholesterol metabolism and hepatocellular carcinoma, and its important role in promoting hepatitis C virus (HCV) replication. This review will discuss the biogenesis and function of miR-122.

Identification of miR-122
MicroRNAs (miRNAs) are 21–23 nucleotide (nt) non-coding RNA molecules that post-transcriptionally repress gene expression; in animals, this is mediated by interaction with partially complementary target sites in the 3’ untranslated region (UTR) of target mRNAs. It became apparent that these RNA molecules are present in diverse eukaryotic systems when a number of different miRNA sequences were identified by cloning in HeLa cells, Drosophila and C. elegans. miR-122 was first identified in a subsequent study in which the cloning approach was applied to various mouse tissue samples. This uncovered a high level of tissue-specific miRNA expression and identified a number of novel miRNAs that are only detectable in certain tissues, including miR-122. miR-122 expression is developmentally regulated, increasing in the liver over the course of embryonic development.

Conservation and Genomic Location
miR-122 derives from a single genomic locus on chromosome 18 in humans.

This was confirmed by RNAseq protection analysis indicating that miR-122 is present at approximately 66,000 copies per cell in adult liver, making it one of the most highly expressed miRNAs in any tissue. miR-122 expression is developmentally regulated, increasing in the liver over the course of embryonic development.}

An early analysis of miR-122 demonstrated that it is conserved in different species, including human, frog and zebrafish. As of September 2011, miR-122 expression has been detected in 18 species, all of which are vertebrates (miRBase 17). Many miRNAs are members of families with closely related sequences and possibly some common targets. As the main determinant of miRNA target binding is complementarity to the seed (nt 2–8), and to a lesser extent to nt 13–16, miRNA sequence divergence outside these regions is common among paralogs. The sequence of mature miR-122 is completely conserved between all species in which it has been detected, and no paralogs have been identified, suggesting that the entire sequence is important for function (miRBase 17). In situ hybridization in zebrafish indicated that the highly liver-specific expression pattern of miR-122 is conserved across different species. No miR-122 orthologs have been detected in Drosophila or C. elegans, implying that miR-122 evolved with the vertebrate lineage, possibly in parallel with the emergence of the liver.
miRNAs are generally transcribed by RNA polymerase II (pol II) as long, nuclear transcripts known as pri-miRNAs, which undergo cleavage by Drosha to yield a ~70 nt pri-miRNA hairpin, nuclear export via Exportin 5, and cytoplasmic cleavage by Dicer to generate the mature miRNA.9 miRNA hairpins may be located in introns of protein-coding mRNAs, or in exons or introns of noncoding RNAs, and many are in clusters with other miRNA hairpins, which are subject to common transcriptional regulation.10-12 The human miR-122 locus is in a noncoding RNA exon and is not part of a cluster.13 The pri-miR-122 gene had previously been identified as a non-coding RNA known as hcr,6 and was recently characterized in detail.13 Identification of the transcription start site and 3' end indicated that pri-miR-122 is initially synthesized as a 7.5 kb transcript that is spliced to yield the 4.5 kb pri-miR-12213 (Fig. 1A). The pri-miR-122 sequence shows only short regions of

Figure 1. (A) The structure and transcriptional regulation of the human pri-miR-122 gene. The locations of the two exons, the pre-miR-122 sequence, and promoter elements are shown relative to the transcriptional start site as determined in reference 13. HNF-4α has been experimentally shown to activate pri-miR-122 transcription via the indicated binding site.13 Transcriptional repression during the circadian rhythm is driven by REV-ERBα. This repression is predicted to occur via the two ROREs shown in the promoter, one of which overlaps with the HNF-4α binding site. (B) The pre-miR-122 sequence is highly conserved between different species. The 23 nt form of mature miR-122 is shaded. (C) Relative levels of different miR-122 isoforms identified by deep sequencing of mouse liver.
Evolutionary conservation, of which the pro-miR-122 hairpin is the strongest (Fig. 1B). The promoter region was isolated, and showed liver-specific activity when tested in a reporter vector.19 The core promoter is highly conserved and possesses elements typical of a pol II promoter. It contains a conserved target site for the liver-enriched transcription factor HNF-4α for the liver-enriched transcription factor moter. It contains a conserved target site possesses elements typical of a pol II promoter, which stimulates miR-122 expression.18

**Regulated Transcription and Post-Transcriptional Modification**

pri-miR-122 transcription is regulated in a circadian fashion in mice.14 Many hepatic transcript functions are subject to circadian regulation, and analysis of liver miRNA expression during the murine circadian cycle demonstrates a diurnally changed in levels of pri- and pre-miR-122.14 Transcriptional repression of pri-miR-122 during the circadian cycle is driven by the orphan nuclear receptor REV-ERBs. Despite the clear circadian effects on pri-miR-122 transcription, the level of mature miR-122 does not change significantly, probably because of its high stability. However, circadian regulation of pri-miR-122 transcription does have functional consequences. Microarray analysis showed that miRNAs regulated by miR-122 were highly enriched for transcripts subject to circadian regulation, and the miR-122-dependent regulation of several important circadian miRNAs, including the Per2 gene, a circadian rhythm gene.20 This alteration in miR-122 target regulation in the absence of any change in mature miR-122 levels is not yet understood. It raises the intriguing possibility that newly synthesized miR-122 functions differently to the pre-existing pool.

Next-generation sequencing has revealed that post-transcriptional modification of miRNAs, usually by the addition of one or two 3' nucleotides, is a frequent event.15 In most cases the functional consequences, if any, of such modification are unclear, but for miR-122 3' adenylation is important in regulating miRNA stability. Mass spectrometry and deep sequencing identified several different isoforms of miR-122 in mouse liver (Fig. 1C), including a variant with a single 3' adenosine added by the non-canonical poly(A) polymerase GLD-2.16 In GLD-2−/− mice, the total level of miR-122 decreases and the proportion of the 21 nt isoform that is likely to be a precursor to degradation increases, suggesting that 3' adenylation stabilizes miR-122. The decrease in miR-122 levels was accompanied by an increase in the 7-methylguanosine-cap level of several known miR-122 targets.19 The degradation pathway of mammalian miRNAs is not yet established, but this role for 3' adenylation in maintaining miRNA stability provides an intriguing insight into this process. GLD-2 was recently shown to adenylate many other miRNAs and to reduce the effectiveness of targeting without significantly affecting stability,19 raising the possibility that adenylation has different effects on different miRNAs. Further research will be necessary to resolve these discrepancies.

A biological role for GLD-2 modification of miR-122 was observed in mouse embryonic fibroblasts, where stimulation of p53 polyadenylation/translation by the cytoplasmic polyadenylation element binding protein (CPEB) drives cellular senescence. miR-122 downregulates CPEB expression via two 3'UTR binding sites, and GLD-2 depletion stimulates CPEB and p53 expression by reducing the level of miR-122.17 The role for miR-122 in a non-liver cell line is interesting; it will be important to quantify miR-122 in these cells and to determine whether very low level miR-122 expression in tissues other than liver is sufficient for productive interaction with some targets. An unexplored possibility is that GLD-2 modification of other miRNAs that target p53 may be partially responsible for the observed effects.

**Endogenous Targets and Physiological Functions of miR-122**

The first miR-122 target to be identified was the Cationic amino acid transporter 1 (CAT-1), which provides an important example of regulated miRNA repression. In fed cells, miR-122 binds to several sites in the CAT-1 3'UTR and mediates translational repression, accompanied by a shift of the repressed mRNA to P bodies.18 However, under conditions of amino acid starvation of either cell stress, the RNA-binding protein HuR moves from the nucleus to the cytoplasm and interacts with the CAT-1 3'UTR adjacent to the miR-122 binding sites. This results in relief of miR-122 repression, release of the miRNA from P bodies and recruitment to polysomes.18 Although there are a few other examples of miRNA positively or negatively regulating miRNA activity by interaction with target 3'UTRs, this is currently the only example of regulated miR-122 repression. The possibility that such regulation is more widespread remains.

Several studies have used microarray technology following antisense-nucleotide-mediated inhibition of miR-122 in mouse liver to identify its targets.21,22 Target miRNAs, such as AldolaseA (Aldoa) and N-myc downstream regulated gene 3 (Ndrg3) were consistently identified in the different experiments, and confirmed using reporter assays. The overall effect of miR-122 sequestration in the liver of mice and primates is to reduce the level of plasma cholesterol, without inducing detectable liver toxicity.23-25 The pathways by which direct miR-122 targets regulate cholesterol biosynthesis may be important.19 miR-122 also regulates systemic iron homeostasis by repressing the target miRNAs hemochromatosis (Hfe) and hemojuvelin (Hjv). These miRNAs encode activators of the hormone hepcidin, which regulates iron availability, and mice with reduced miR-122 levels suffer iron deficiency.19 Many miRNAs have been implicated in various cancers either as oncogenes or tumor suppressor genes. miR-122 levels are frequently reduced in hepatocellular carcinoma (HCC) compared with normal liver,17 and lower miR-122 expression correlates with poor prognosis.21 Over-expression of miR-122 reduces tumorigenic properties of HCC cell lines, indicating that it functions as a tumor suppressor.22 miR-122 overexpression also sensitizes HCC cells to chemotherapeutic agents such as sorafenib and doxorubicin.23 Several miR-122 targets have been
implicated in tumorigenesis, including ADAM10, Igf1R, SRE, cyclin G1 and ADAM17. The factors governing reduced miR-122 expression in HCC have not been fully elucidated, but miR-122 levels in tumors correlate with those of several liver-specific transcription factors, including HNF-4α, suggesting a regulatory role for these proteins.39

miR-122 and HCV

A particularly intriguing function for miR-122 is its role in the hepatitis C virus (HCV) replication cycle. HCV is a positive-sense single-stranded RNA virus with a 9.6 kb genome that establishes persistent infections in the liver, eventually leading to cirrhosis and carcinoma.29 miR-122 is required for HCV replication in the cultured human hepatic cell line HuH7, and mediates this regulation by direct interactions with two adjacent binding sites in the 5'UTR of HCV RNA.30,31 Although the initial experiments were conducted using genotype 1a and 1b HCV RNA, the two miR-122 binding sites are highly conserved across all HCV genotypes. A requirement for miR-122 has also been observed in the live cycle of the genotype 2a JFH-1 strain, which establishes productive infections in HuH7 cells.32 Such positive regulation via 5'UTR sites is a very different process to the usual function of miRNAs in repressing gene expression via 3'UTRs, and suggests that miRNA function is potentially much more varied than is currently known.

The mechanism by which miR-122 regulates HCV has proved difficult to establish. HCV replication proceeds first through translation of the viral polyprotein from the genomic RNA, mediated by an internal ribosome entry site (IRES) immediately downstream of the miR-122 binding sites. The viral proteins then mediate replication of the viral RNA via a negative strand intermediate in membranous replication complexes.32 miR-122 stimulates translation via the HCV IRES to some extent,33,34 but this is not sufficient to explain its full effect on viral replication.35 A later stage of the viral replication cycle must therefore also be regulated, but this has proved difficult to identify. HCV RNA synthesis in either HuH7 cells or isolated replication complexes is insensitive to miR-122.36 It remains possible that the miRNA is involved in degradation of HCV RNA, in its localization to functional replication complexes, or in the translation to replication switch. miR-122 makes an unusual interaction with HCV RNA in which the miRNA 3' end overlaps the viral RNA 5' end, potentially protecting it from 5' to 3' exonuclease activity or cytoplasmic sensors of viral RNA.37 miR-122 regulation of HCV was shown to require the Argonaute proteins,38 although a different study indicated that this requirement could be overcome by overexpression of miR-122.39 The question of whether miR-122 regulates HCV in association with a normal miRNA-induced silencing complex (miRISC), or requires unusual components or accessory proteins, remains to be fully addressed. Examination of miR-122 activation of translation via the HCV 5'UTR suggests that the RNA requirements for this process are highly specialized,39 implying that similar mechanisms are unlikely to be used by other miRNAs and viruses, although this possibility has not yet been fully explored.

There is an urgent need for new therapies against HCV due to problems with inefficacy and poor tolerability of the existing regime of pegylated interferon-α plus ribavirin.40 As a conserved host factor that can be effectively inhibited without associated toxicity, and would not be expected to evolve resistance mutations, miR-122 presents a highly appealing antiviral target. Santaris Pharma has developed a locked nucleic acid (LNA)-based antisense oligonucleotide that is delivered to the liver and effectively inhibits miR-122 when injected intravenously. A pilot study in four chimpanzees chronically infected with HCV showed a substantial reduction in viral titer in the two animals given a higher dose of the inhibitor, and in one of the low dose animals.41 The reduced viral load was maintained over several weeks of therapy and was not accompanied by any acquisition of viral escape mutations or liver toxicity.41 The conservation of miR-122 between vertebrates and its high expression level in the liver suggest that it is likely to have important biological functions. The identification of miR-122 targets has allowed some of these roles to be determined, in particular its association with cholesterol metabolism. The relief of miR-122-mediated inhibition of CAT-1 by HuR, and the role for miR-122 in HCV replication, suggest that miRNA activity extends beyond straightforward repression and may take many different forms.

Important unanswered questions include the reasons behind the very high expression of miR-122: the tumor suppressive activity of miR-122 holds enormous promise as an antiviral therapy. The results of phase 2a clinical trials of the Santaris Pharma inhibitor, miravirsen, in human HCV patients were announced in November 2011. Excitingly, four of the nine patients receiving the highest miravirsen dose had no detectable HCV after four weeks of therapy.42

Conclusions

The conservation of miR-122 between vertebrates and its high expression level in the liver suggest that it is likely to have important biological functions. The identification of miR-122 targets has allowed some of these roles to be determined, in particular its association with cholesterol metabolism. The relief of miR-122-mediated inhibition of CAT-1 by HuR, and the role for miR-122 in HCV replication, suggest that miRNA activity extends beyond straightforward repression and may take many different forms.

Important unanswered questions include the reasons behind the very high expression of miR-122: the tumor suppressive activity of miR-122 in HCC suggests it is important in maintaining a healthy liver, but it is intriguing that it can be effectively depleted over the course of several weeks without any occurrence of liver toxicity.43 The
circuit regulation of miR-122 precursors and targets, despite unaltered mature miR-122 levels suggests that miRNA function may not be determined simply by expression level. The role of miR-122 in HCV replication is providing an existing avenue for development of a novel antiviral therapy; it will be very interesting to know whether it is effective in human patients. Finally, the mechanism of this regulation is an important question that has not yet been fully answered.

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