INTRODUCTION

MOLECULAR BIOLOGY OF HEPATITIS C VIRUS

Hepatitis C virus (HCV) infection is one of the main causes of chronic liver disease worldwide. The long-term impact of HCV infection is highly variable, ranging from minimal histological changes to extensive fibrosis and cirrhosis with or without hepatocellular carcinoma (HCC).[1] The number of chronically-infected persons worldwide is estimated to be about 170 million.[2] However, the prevalence varies markedly from one geographic area to another and within the population assessed. In Western Europe, HCV prevalence ranges from 0.4% to 3%. It is higher in Eastern Europe and the Middle East, where the numbers are not precisely known.[3] Egypt has the highest worldwide prevalence, with 14.7% countrywide and up to 50% in certain rural areas, due to specific modes of infection.[4] The host immune response largely determines whether HCV is eradicated spontaneously or persists (as it does in the majority of patients).[5] Clinical care for patients with HCV-related liver disease has advanced considerably during the last two decades.[6] Until 2011, the combination of pegylated interferon (IFN)-α and ribavirin for 24 or 48 weeks was the approved treatment for chronic hepatitis C (CHC).[7] In 2011, telaprevir and boceprevir were licensed for use in addition to pegylated IFN-α and ribavirin in HCV genotype 1 infection. These two drugs are first-wave, first-generation direct-acting antivirals (DAAs) that target the HCV non-structural (NS)3/4A serine protease.[8,9] New HCV DAAs were licenced in the first half of 2014, for use as a part of a combination therapy. Sofosbuvir, a nucleotide analogue inhibitor of HCV RNA-dependent RNA polymerase and simeprevir, a second-wave, first generation NS3/4A protease inhibitor, has been approved.[10,11] Other drugs may be approved later in 2015.[6]

Hepatitis C virus genome:

Hepatitis C virus is a prototype member of the *Hepacivirus* genus of the *Flaviviridae* family.[12] It is a small enveloped virus with a positive-sense, single-stranded ribonucleic acid (RNA) genome that encodes a large polyprotein of 3010 amino acids.[13] The polyprotein is processed by host cell peptidases and viral proteases which cleave it into at least 10 different proteins: structural and NS proteins that are necessary for viral replication.[14] The HCV genome consists of a single-stranded positive-sense RNA of approximately 9600 nucleotides (nt) in length that contains a single open reading frame and is flanked by 5'-and 3'-untranslated RNA regions (UTRs) (Figure 1).[15,16]

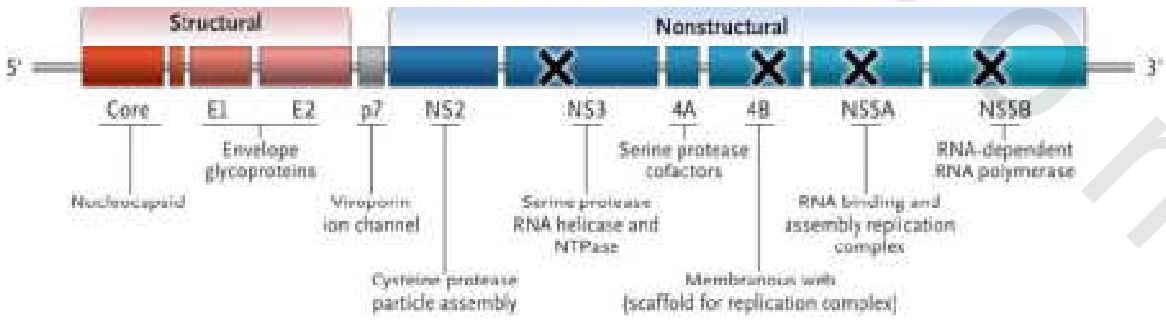


Figure 1: Hepatitis C virus polyprotein. [14]

HCV structural proteins:

The HCV structural proteins (core, envelop (E) and *p7*) are located at the N-terminus of the HCV polyprotein. They are released from the polyprotein by host signal peptidases that cleave signal peptides located between core/E1, E1/E2, E2/p7 and p7/NS2.[17] ***The HCV core*** is a highly conserved basic protein which makes up the viral nucleocapsid. It consists of HCV first 191 amino acids and can be divided into three domains on the basis of hydrophobicity.[18] The core protein is a cytosolic membrane-bound protein that can bind viral RNA via domain 1 (amino acids 1-74) [19] and associates with the endoplasmic reticulum (ER), lipid droplets, mitochondria and the nucleus.[20] It interacts with numerous cellular proteins and affects host cell functions such as gene transcription, lipid metabolism, apoptosis and various signaling pathways.[21] The HCV core protein is directly or indirectly involved in hepatocarcinogenesis and steatosis.[22,23] Also, it was identified as an immunomodulatory molecule suppressing T lymphocyte responsiveness through its interaction with complement receptor (gC1qR).[24] ***The HCV envelop proteins E1 and E2*** are 30-35 and 70 kDa glycoproteins forming a stable heterodimer. The E1 envelop glycoprotein of HCV contains 4 to 5 N-linked glycans and the E2 envelop glycoprotein has 11 N-glycosylation sites.[25] The E1 serves as the fusogenic subunit and the E2 acts as the receptor binding subunit of the HCV envelope.[26] The envelop proteins are thought to mediate cell entry by recognition of cellular membrane receptor protein.[27] The E2 contains two hypervariable regions (HVRs), HVR1 and HVR2, which are under constant selection for mutation probably because they are targets for neutralizing antibodies. The genetic heterogeneity of the HVR1 may enable the virus to evade the immune system and facilitate establishment of chronic infection.[28] ***The HCV p7*** is a 63-amino acid polypeptide located between HCV E2 and NS2 genes in the ER.[29] The HCV p7 has characteristics similar to those of a group of proteins called viroporins [30] and has been shown to be essential for virus particle assembly and release of infectious virions in a genotype specific manner.[31]

HCV non- structural proteins:

The HCV NS proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B) are situated at the C-terminus of the HCV polyprotein.[17] ***The HCV NS2*** is a 21-23 kDa transmembrane protein. It is essential for completion of the viral replication cycle in vitro and in vivo.[32] The NS2 contains highly hydrophobic N-terminal residues forming three or four transmembrane helices that insert into the ER membrane.[33] The C-terminal part of NS2 presumably resides in the cytoplasm and plays an important role in NS2/3 auto protease activity together with the N-terminal domain of NS3.[34] ***The HCV NS3*** is 67 kDa protein with multifunctional activity. NS3 N-terminal has serine protease activity and its C-terminal has NTPase/helicase activity.[35] The HCV NS3 protease last 185 amino acids at the N-terminus are involved in cleavage between NS3-4A, 4A-4B, 4B-5A and 5A-5B.[36] The NS3 serine protease recently turned out to influence the innate cellular host defense by inhibition of retinoic acid inducible gene-1 and Toll-like receptor (TLR) 3 signaling.[37] The enzymatic activity of the NS3 NTPase/helicase activity is indispensable for RNA replication. Putative functions during replication could be to unwind replicative double stranded RNA intermediates, to eliminate RNA secondary structures or to separate the genome from nucleic acid binding proteins.[38] ***The HCV NS4A*** is a 54 amino acids protein, which acts as a cofactor for NS3 protein. It has an N-terminus which is highly hydrophobic.[39] The last 20 amino acids of NS4A form a transmembrane helix, which

anchors the NS3/NS4A complex on the ER membrane.[40] The interaction between NS4A and NS3 allows activation of the NS3 active site and more efficient protease cleavage.[39] The NS4A is also required for the phosphorylation of NS5A and can directly interact with NS5A.[41] ***The HCV NS4B*** is a small hydrophobic 27 kDa protein, which plays an important role for recruitment of other viral proteins.[42] It interacts with NS4A and therefore indirectly with NS3 and NS5A.[43] Electron microscopy studies indicated that NS4B induced morphological changes to the ER forming a structure termed; the membranous web. All viral proteins were localized to this area suggesting a site for replication complex formation.[44] ***The HCV NS5A*** is a hydrophilic phosphoprotein which plays a role in viral replication, modulation of cell signaling pathways and IFN response.[45] It contains a region called the IFN-α sensitivity-determining region which confers resistance of the virus to IFN treatment. It has been proposed that the sequences in the IFN-α sensitivity-determining region could be used to predict sensitivity or resistance of HCV to IFN-α treatment.[46] It is also important in the assembly of infectious viral particles. Initial studies indicated the association of NS5A with other viral proteins which suggested its presence in replication complexes.[47] ***The HCV NS5B*** is a tail anchored protein of 65 kDa in size. NS5B acts as RNA dependent RNA polymerase (RdRp) and plays an important role in synthesis of new RNA genome.[48] The structural organization of NS5B is a typical 'right hand' polymerase shape with finger, palm, and thumb subdomains surrounding a completely encircled active site.[49] The HCV heterogeneity is primarily due to a high error rate of the RdRp encoded by the NS5B gene. As central component of the HCV replicase, the NS5B has emerged as a major target for antiviral intervention.[50] It lacks a ''proofreading'' function. Due to a high rate of error-prone replication, complex mutant swarms are generated.[48]

Hepatitis C virus life cycle:

Hepatitis C virus entry is the first step of interaction between virus and the target cell that is required for initiation of infection via attachment of the virus to the host cell surface.[51] Recent studies suggest that HCV entry is a slow and complex multistep process. Several host cell surface molecules including glycosaminoglycans, CD81, low-density lipoprotein receptor, scavenger receptor class B type 1, members of the claudin family (CLDN1, 6 and 9) and mannose-binding lectins DC-SIGN and L-SIGN have been identified as putative HCV receptors or co receptors.[52-54] The HCV E2 binds with high affinity to the large external loop of CD81 and CLDN1 and acts at a late stage of the entry process.[27] After the initial binding step, the virus linked to its receptor complex, internalize and then nucleocapsid is released into the cytoplasm. The virus is decapsidated, and the genomic HCV RNA is used both for polyprotein translation and replication.[55] Being a positive sense RNA, viral RNA acts as messenger RNA (mRNA) and is therefore directly translated. Translation of HCV RNA is initiated by binding the 5'-internal ribosome entry site (IRES) to ribosome and produces single polyprotein which is cleaved co- and post-translationally by cellular and viral proteases to produce structural and NS proteins.[56] Replication and post-translational processing appear to take place in a membranous web made of the NS proteins and host cell proteins called ''replication complex'', located in close contact with perinuclear membrane. Genome encapsidation appears to take place in the ER and nucleocapsids are enveloped and matured into the Golgi apparatus.[57] After the viral proteins, glycoproteins, and the genomic HCV RNA have been synthesized, these single components have to be arranged in order to produce infectious virions which will be released in the pericellular space by exocytosis (Figure 2).[58]

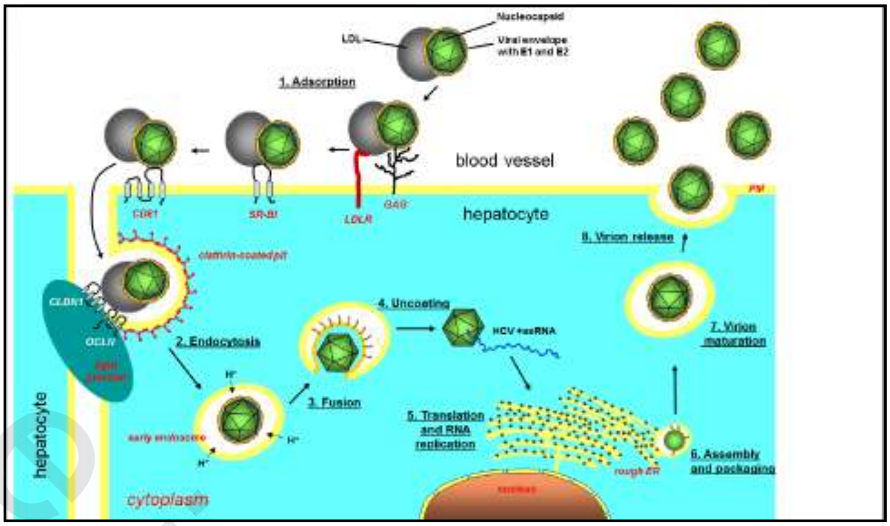


Figure 2: Hepatitis C virus internalization and release from hepatocyte.[58]

Hepatitis C virus genotypes and quasispecies:

Variation in the genome sequence of HCV isolates has enabled classification into six major types with a large number of subtypes within each genotype.[59] The predominant HCV genotype among Egyptians was found to be genotype 4, particularly subtype 4a.[60] All currently recognized HCV genotypes are hepatotropic and pathogenic. However, it has been suggested that different genotypes do vary in their infectivity and pathogenicity, thereby influencing the rate of progression to cirrhosis and the risk of HCC.[61] The duration of treatment should be based on the HCV genotype and the pretreatment viral load.[62] Moreover, it has been shown that HCV, like many other RNA viruses, circulates in infected individuals as a population of diverse but closely related variants referred to as quasispecies.[63] The mutation rate of HCV is estimated to be 1500-2000 base substitutions per genome site per year, due to the intrinsic lack of proof reading of the RNA dependent RNA polymerase.[64] Mutation rates vary in different regions. The E1 and E2 regions are the most variable, while the 5'-UTR and terminal segment of the 3'-UTR have the highest degree of sequence conservation.[28] This quasispecies model of mixed virus populations may contribute to viral persistence.[65]

OUTCOME OF HEPATITIS C VIRUS INFECTION

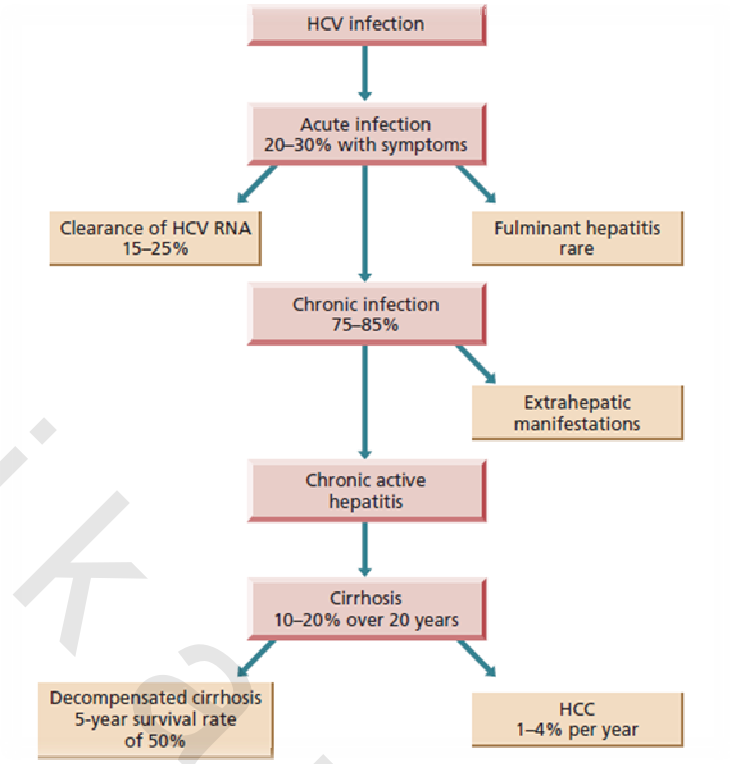


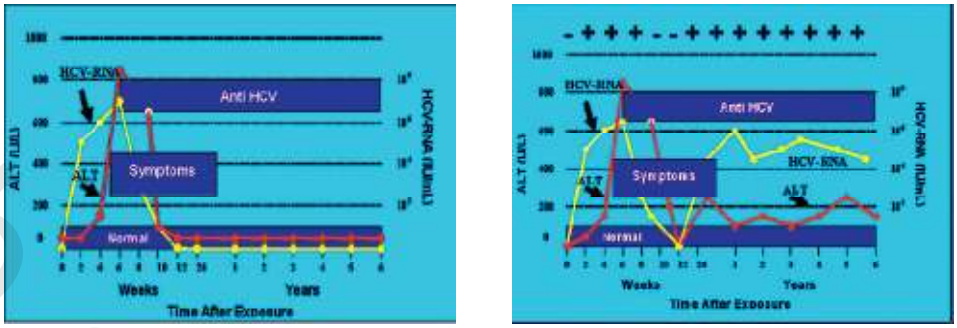
Figure 3: Natural history of HCV infection.[1]

The outcome of HCV infection includes acute and chronic hepatitis, which results in progressive fibrosis ultimately resulting in cirrhosis, liver failure and an increased risk of HCC.[1] The acute phase and progression to CHC is usually silent. Thus, the onset of CHC is often insidious and usually has gone unnoticed by the patient. Self-limited infections may be associated with a delayed clearance of HCV RNA, albeit this is relatively uncommon. The chronic disease is generally slowly progressive; cirrhosis develops within 20 years in about 10–20% of patients with chronic disease, but can remain indolent for a prolonged period of time (Figure 3).[1,66]

Acute hepatitis C:

Acute hepatitis C (detection of HCV RNA in the blood after initial exposure to the virus) is generally a benign disease. After initial exposure, HCV RNA can be detected in blood within 1 to 3 weeks and is present at the onset of symptoms. Antibodies to HCV are detected by enzyme immunoassay in only 50% to 70% of patients at the onset of symptoms, increasing to more than 90% after 3 months. Within an average of 4 to 12 weeks, liver cell injury is manifested by elevation of serum alanine aminotransferase (ALT) levels.[67] Symptoms are uncommon, but can include malaise, weakness, anorexia, and jaundice. Symptoms usually subside after several weeks as ALT levels decline. Acute infection can be severe but is rarely fulminant.[68] Acute infection evolves into chronic hepatitis in 50-84% cases – these variations being partially explained by the HCV pattern of transmission, viral factors and host capacity to initiate a strong cellular immune

response to eliminate the virus.[69] So, acute hepatitis C infection can evolve to spontaneous viral clearance or into chronic infection (Figure 4).[70]



(A) **(B)**

Figure 4: (A.) Course of acute resolving hepatitis C and (B.) Course of acute hepatitis C that evolves into chronic infection.(70)

Chronic hepatitis C and its sequelae:

Chronic hepatitis C means persistence of infection which is diagnosed by detection of HCV RNA in the blood for at least six months. In general, it has been shown that 60%-85% of HCV infected people develop chronic infection.[71] Most patients with CHC have few if any symptoms, the most common being fatigue, which is typically intermittent. Right upper quadrant pain (liver ache), nausea, and poor appetite occur in some patients.[66] Serum ALT levels are usually continuously or intermittently elevated, but the height of elevation correlates poorly with disease activity and at least a third of infected people have persistently normal ALT levels.[72] In these patients, the underlying disease is usually, but not always, mild and non-progressive.[73] Liver biopsy is still regarded as the reference method to assess the grade of inflammation and the stage of fibrosis.[74] Histological features (necroinflammation = grading; fibrosis = staging) should be reported using a structured, semi-quantitative method.[75] Various scoring systems have been validated for use in CHC. METAVIR and Scheuer's scores are more reproducible and less prone to observer variation.[76,77] A common histological feature of liver biopsies from HCV-infected individuals is hepatic steatosis, which is independently associated with fibrosis severity.[78]

Patients with CHC can present with extrahepatic manifestations or syndromes considered to be of immunological origin, such as rheumatoid symptoms, keratoconjunctivitis sicca, lichen planus, glomerulonephritis, lymphoma, and essential mixed cryoglobulinaemia.[79] Mixed cryoglobulinemia is the most known and studied syndrome associated with HCV Infection.[80] Also, thyroid disease (usually hypothyroidism) is more commonly seen in people with HCV than in the general population.[81] Other extrahepatic manifiestations for CHC infection include diabetes,[82] interstitial pulmonary fibrosis,[83] osteosclerosis,[84] dilated and hypertrophic cardiomyopathy.[85]

Many host factors increase the risk of liver disease progression due to CHC infection, including older age at time of infection, male sex [86] and an immunosuppressed state, such as that associated with human immunodeficiency virus (HIV) infection.[87]

Concurrent hepatitis B virus (HBV) infection also appears to increase the risk of progressive liver disease.[88] In addition, higher levels of alcohol use play an important role in promoting the development of progressive liver disease, with strong evidence for the detrimental effects of 30 g/day in men and 20 g/day in women.[89] Other factors includes iron overload,[90] non-alcoholic fatty liver disease (NAFLD), hyper-insulinemia/insulin resistance [78] and obesity.[91] Recently, genome wide association studies provide a powerful tool to identify genetic risk factors that predispose to disease progression in HCV infection. It is found that polymorphism within the interleukin (IL)28B region is more prevalent in patients with viral cirrhosis due to HCV in comparison to other etiologies.[92] Also, genetic polymorphisms have been evaluated for their influence on disease progression, with a focus on major histocompatibility class I and II alleles [93] as well as on profibrogenic cytokines.[94] Also, genetic polymorphisms contribute to individual variations in the characteristics of the body immune responses to HCV infection.[95]

Depending on the presence of co-factors, between 10% and 40% of patients with CHC will develop cirrhosis over a time period of 10–30 years.[96] Once cirrhosis has developed, complications including gastrointestinal bleeding, portasystemic encephalopathy, ascites and HCC are common. Liver failure or end-stage liver disease may become clinically apparent after decades in a minority of persons with CHC. The annual rate of decompensation among patients with cirrhosis is estimated to be about 4%.[97] Death related to the complications of cirrhosis may occur, at an incidence of approximately 4% per year, whereas HCC occurs in this population at an estimated incidence of 1–5% per year.[98] Patients diagnosed with HCC have a 33% probability of death during the first year.[99] Virtually all HCV-related HCC occurs among patients with cirrhosis. In a meta-analysis of 21 case-control studies, the risk for HCC was increased 17-fold in HCV-infected patients compared to HCV-negative controls.[100]

Immune responses in chronic HCV infection:

Immune responses against HCV play a crucial role in the pathogenesis of chronic HCV infection.[101] Vigorous HCV-specific immune responses against HCV multiple epitopes are necessary for spontaneous viral clearance during the acute phase, but the virus appears to have multiple strategies to evade the host immune response against the virus.[102] The resulting insufficient immune responses against HCV lead to persistent viral infection and disease chronicity.[103] Multiple lines of evidence suggest that HCV specifically interferes with the endogenous IFN induction and TLR response.[104] Several HCV-derived proteins are involved in the suppression on the signaling pathways inducing IFN regulatory factor-3, which is a key cellular antiviral signaling molecule.[105] Alternatively, HCV may directly suppress the function of natural killer (NK) cells and contribute to virus persistence. Envelope protein E2 cross links the HCV receptor CD81, thereby inhibiting cytotoxicity and IFN-γ production by NK cells.[106] Also, the expression of human leukocyte antigen (HLA) class I and inhibitory receptors on NK cells, has been reported in patients with CHC leading to inhibition of NK cell-mediated lysis.[107] Interestingly, HCV proteins inhibit dendritic cell (DC) maturation and impair DC functions.[108] A decrease in the number of plasmacytoid DC and impairment of IFN-α production by them have been reported, while the ability of myeloid DC to stimulate allogenic T-lymphocytes is reduced despite no reduction in overall frequency of these cells.[109] Also, DCs inhibit NK cell activation in HCV infection.[110] It is noted that the impairment of DCs is responsible for

the failure of HCV control in the early phase of primary HCV infection, where HCV continues to replicate in spite of high level of IFN-α expression in the liver.[111]

In addition to interference with the innate immunity, there is evidence that HCV escapes from adaptive immune response. The lymphocytic infiltrate characteristic of chronic HCV hepatitis contains a predominance of $CD8^+$ cytotoxic T lymphocytes (CTLs) over $CD4^+$ T helper (Th) cells. While $CD4^+$ cells are confined to the portal and periportal areas, $CD8^+$ cells contribute to the lobular infiltrate.[112] In CHC patients, HCV-specific $CD4^+$ T cells are functionally impaired and their responses are weak, narrowly selected and short-lived, which is in clear contrast with resolved cases.[113] The same as in $CD4^+$ T cells, the frequency of HCV-specific $CD8^+$ T cells decreases after HCV persistence develops and are stunned as demonstrated by an inability to produce IFN-γ and to proliferate in response to HCV antigens.[114] HCV escapes from T cell response through several different mechanisms such as mutational escape and functional anergy (failure) of virus specific T cells. HCV mutations affect virus specific T cell responses by decreasing T cell receptor recognition and impairing proteosomal processing of HCV antigens.[115,116] Another important possible mechanism of immune evasion is functional anergy of virus-specific T cells. HCV specific $CD8^+$ T cells may be impaired in their proliferative capacity, cytotoxicity, and ability to secrete tumor necrosis factor (TNF)-α and IFN-λ.[117] Moreover, the reduced DC numbers and activity might be responsible for the impaired Th1 polarization in chronic HCV infection.[118] A mutation of one amino acid within the epitope of the NS3 region recognized by Th1 cells results in a shift in cytokine secretion patterns from Th1 to Th2 cells leading to decreased antiviral responses.[119] In addition, HCV core-specific T regulatory cells (Treg) are induced in patients with CHC and IL-10 produced by these cells suppresses T-cell function directly leading to persistence of HCV infection.[120]

MICRORNAS

MicroRNAs (miRs) are a family of small non-coding RNAs, approximately 21-24 nt in length, that have become known as master regulators of gene expression.[121] Approximately 1–4% of the transcriptome (genome) in eukaryotic organisms consists of miRs.[122] MiRs have been identified in diverse animals, plants and even viruses.[123] Two decades ago, both the existence and the importance of miRs were completely unknown.[124] Until then, the scientific community focused on genes that codify for protein. Only in 1993 did the importance of miRs begin to be revealed.[125]

History of microRNA discovery:

The discovery of miRs began when Victor Ambros and colleagues performed a genetic screen to investigate defects in the temporal control of Caenorhabditis elegans development.[126] In an inspiring demonstration of perseverance and scientific proficiency, Ambros discovered that the gene lin-4, (a repressor of lin-14), did not code for a protein. Instead, they observed that lin-4 produces a pair of small RNAs of approximately 22 and 61 nucleotides in length. They also noticed that the lin-4 RNA had antisense complementarity to multiple sites in the 3′-UTR of the lin-14 gene, which suggested an antisense regulatory mechanism.[126,127] The shorter lin-4 RNA is now recognized as the founding member of this still growing family of small RNAs termed "microRNAs". The landmark discovery of lin-4 encoded RNAs hinted at a new mechanism of gene inhibition during development.[128] It was only 7 years later that the second miR was discovered. Indeed, before the year 2000, there was no evidence for an ortholog of lin-4 in any other species or for other similar non-coding RNAs within the nematodes. This all changed with the discovery of let-7, which encodes a 21 nucleotide small RNA that controls the transition from the L4 stage to the adult stage of C. elegans.[129] It was also shown that let-7 works similarly to lin-4 by binding to the 3′-UTR of its target (lin-41 and hbl-1) and by inhibiting its translation.[130] In 2008, a revolution occurred in the field when Lawrie et al, reported the detection of circulating miRs in blood.[131] Since then, hundreds of papers have been published revealing a specific signature of miRs for a wide range of diseases in blood, urine, saliva and many other biological fluids.[132-134] Then the links between miR dysregulation and human disease have been reported in almost all medicine fields. Currently, great efforts are being invested in understanding the involvement of miR deregulation in disease and unlocking the mechanisms by which they function. This new field of investigation has revealed the tremendous potential of miRs as diagnostic or therapeutic tools.[135,136]

MicroRNA biogenesis:

The *miR* genes can be located in the context of non-coding transcription units or in the introns of protein-coding genes (26-28). About 40–70% of miRs are encoded in introns of protein coding genes or introns and exons of non-coding RNAs, implying two or more possible mechanisms for their transcription.[137] Intronic *miR* genes are generally thought to be transcribed by RNA polymerase II; [138] while exonic *miR* genes are thought to utilize RNA polymerase III for transcription.[139] Regardless of the transcribing polymerase, miRs are initially synthesized as primary-miR (pri-miR) transcripts, which can extend over 1kb in length. These transcripts are processed by Drosha (a ribonuclease [RNase] III) and DGCR8, an RNA binding protein, which cleave the pri-miR to a ~70 nt precursor-miR

(pre-miR) within the nucleus. [140,141] The pre-miR is then exported out of the nucleus by exportin 5 in the presence of its cofactor Ran GTP, to be further processed in the cytoplasm.[142,143] Once in the cytoplasm, pre-miRNAs are cleaved by Dicer (an RNA endonuclease III) along with its partner protein TRBP (trans-activator RNA binding protein) to a ~21-22 nt double stranded RNA molecule.[144] This mature miR molecule is loaded onto the RNA-induced silencing complex (RISC), which contains Dicer, TRBP and an Argonaute protein that is present in multiple isoforms in mammalian cells. Within the RISC complex, the single stranded mature miR will guide RISC to the target mRNA, ultimately resulting in its degradation or translational repression (Figure 5).[145] The miR biogenesis is tightly regulated at transcription and each of the processing steps by complex regulatory mechanisms. Additionally, the half-life of individual miR species seems to be governed by a multitude of cis-acting modifications and trans-acting proteins.[146]

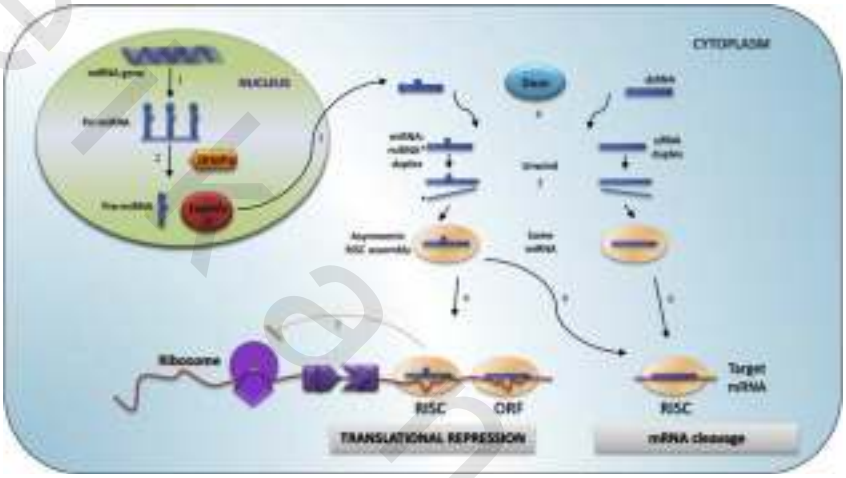


Figure 5: Schematic representation of microRNA biogenesis and target identification.[147]

MicroRNAs and polycistronic microRNA clusters:

To date, over 1500 miRs have been identified in the human genome so far, and their sequences are deposited in the miR database.[148] Most miRs are encoded individually as a single mature miR. However, many miRs are situated in "polycistronic" miR clusters, wherein multiple miRs are generated from the same primary transcript and are co-transcribed into a single precursor. In fact, at least one-third of human miR genes are clustered.[149] The majority of the miR polycistrons are regulated by the canonical miR biogenesis pathway, where microprocessor complexes and/or Dicer could confer differential processing on individual components.[144] The high conservation of miR clusters across species suggests evolutionary pressure to maintain such organization. Although the multiple miRs belonging to a particular cluster are often highly related to one another, having emerged via duplication events, the occurrence of miRs belonging to distinct “seed” families within the same cluster is also commonly observed.[150] Since each miR has the potential to regulate hundreds of target mRNAs, a miR polycistron cluster containing multiple components may possess a greater capacity for gene regulation, thus yielding pleiotropic biological effects through complex mechanisms of coordination. Increasing evidence has shown that members in a miR cluster tend to be coexpressed and play, as a whole, a role in various physiological and pathological processes.[151]

MicroRNA mode of action:

The miRs' main function is to inhibit protein synthesis by protein-coding genes at the post-transcriptional level.[152] MiRs bind to 3'-UTR coding sequences or 5'-UTR of target mRNAs, leading to mRNA degradation and/or translational repression and subsequently, gene silencing (Figure 5).[153] Although the entire sequence of a miR can bind to the target, evidence strongly indicates that the nucleotides at position 2-7, the so-called "seed" sequence, are the key determinants of target specificity for a miR. Thus, miRs with the same seed sequence are predicted to target highly overlapping sets of genes and are therefore grouped in the same "miR family".[154] In the mean time, recent data also suggest that under certain conditions, miR binding leads to a boost in translation.[155] Individual miRs may regulate several hundred genes and vice versa, that a single mRNA may be targeted by many different miRs, giving rise to intricate regulatory networks.[156] It is estimated that more than 30% of the human protein-coding genome is regulated by miRs.[157]

The actual mode of miR-mediated gene regulation depends on the amount of complementarity between miR and target mRNA.[158] Initial computational methods designed to identify the targets of miRs discovered that Watson–Crick base pairing between nucleotides 2 and 8 of miRs and their targets is an important determinant in target recognition.[159] Biochemical and biophysical evidences have shown that this region contributes to an increased binding affinity for target sequences relative to other parts of the miR.[160] Additionally, systematic reporter studies have shown that functional regulation by miRs is highly sensitive to base pair mismatches within nucleotides 2–8 of the miR, which have been defined as the seed region.[161] If the sequence match is perfect or near-perfect, the target mRNA can be cleaved or sliced and is degraded, if the complementarity is only partial then translational repression occurs. So, the mechanism of action is either one of two hypothesized mechanisms. First one is complete pairing in which the mRNA is degraded and it is predominant in plants, the second is imperfect pairing in which translation is repressed but mRNA remains intact and it is predominant in metazoans.[160] An individual miR can target numerous other mRNAs and can itself be targeted by multiple miRs, resulting in a vast regulatory potential.[162]

MicroRNAs and disease:

MicroRNAs are now recognized as major players in almost every biological process such as cell development, proliferation, apoptosis, differentiation, metabolism and senescence as well as angiogenesis, hematopoesis and organ development.[163] The emergence of miRs as modulators of gene expression identifies them as: (i) actors that underlie genetic predisposition toward several diseases,[164] (ii) novel candidate diagnostic biomarkers for various disorders [165] and (iii) promising targets of novel therapeutic strategies.[166]

It was found that the aberrant expression of miRs is closely associated with the initiation and progression of pathophysiologic processes in human diseases.[164] For example, miR deregulation has been consistently linked to malignant transformation.[167] The first human disease known to be associated with miR deregulation was chronic lymphocytic leukemia [168] and later many miRs have been found to have links with some types of cancer and are sometimes referred to as "oncomiRs".[169] Also, it has been found

that there is a relationship between miR deregulation and many different human diseases other than cancer including hereditary diseases,[170,171] skeletal and growth defects,[172] autoimmune diseases,[173] cardiovascular diseases,[174] diabetes mellitus [175] and neurodegenerative disorders [176] Moreover, miRs are linked to fibrosis in various organs and disease settings by regulating transforming growth factor (TGF)-β and participating in repairing and remodeling the matrix including collagens, matrix metalloproteinases (MMPs) and integrins.[177,178] In addition, miRs may be implicated in viral infections and can influence viral replication. Virally-encoded miRs can directly alter host physiology, including components of the immune system, while host cellular miRs can directly alter the virus life cycle.[179] MiR-122 was shown to function as an important host factor for HCV propagation by an unusual mechanism, in which two miR-122 molecules interact with the 5'-UTR of the HCV genome by binding to two miR-122 seed sites in association with Ago2 to protect the HCV 5'-UTR from nucleolytic degradation and thereby promotes viral RNA stability and propagation.[180]

Recently, it has been shown that miRs are present not only in tissues but also can be secreted from the cells and can gain access to body fluids (e.g. plasma, serum, urine, saliva and sputum).[181] Strikingly, miRs in the blood are incorporated into different types of vesicles, including apoptotic bodies, microvesicles and exosomes or protein complexes that prevent their degradation by endogenous ribonucleases in plasma (Figure 6).[182,183] In fact, serum miRs remain highly stable upon exposure to severe conditions that induce almost immediate degradation of free RNA, such as boiling, very low or high pH and extended storage. Evidence supports that any change in the cell free miRs profile is suggestive of a disease state.[184] Circulating miRs can be used as non-invasive, sensitive biomarkers for detecting disease.[185,186] Detection of miRs in blood is easy owing to highly sensitive real time polymerase chain reaction (RT-PCR) assay detection methods and the lack of post-processing modifications of miRs.[183]

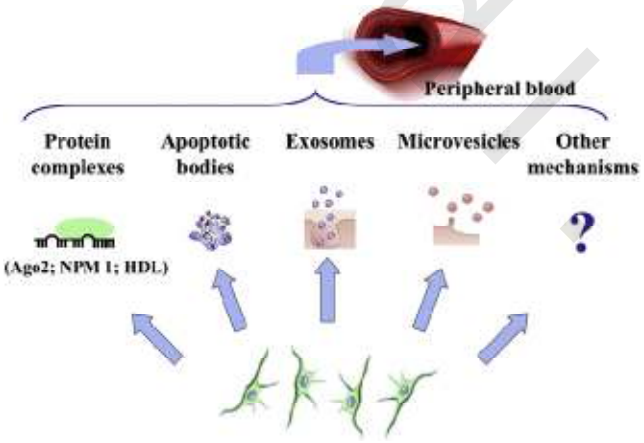


Figure 6: Mechanisms of microRNA release from cells in the peripheral blood. Ago2 = Argonaute2; NPM1 = nucleophosmin1; HDL= high density lipoproteins.[185]

Since miRs are aberrantly expressed in several diseases, it is not surprising that these small non-coding RNAs represent potential therapeutic targets for the diseases they are functionally associated with.[187] Two main strategies are employed for pharmacological modulation of miR activity in vivo: (i) inhibition of miRs that are upregulated in diseases either directly by chemically modified anti-miR oligonucleotides [188] or indirectly through downregulation of miR biogenesis pathway components and (ii) restoring the function of a miR using either synthetic miRmimics complex or viral expression constructs when miRs downregulation promotes disease.[189] However, the ubiquitous expression patterns reported for many miRs increases the risk of off-target effects by a miR modulator especially in indications that require chronic treatment. Thus, delivery of miR modulators to the cell type or tissue of interest is a key factor for successful development of miR-based therapeutics. This approach still needs to be evaluated in vivo because some challenges, such as stability and delivery strategies, need to be improved.[190]

MICRORNA 17~92 CLUSTER

The polycistronic miR-17~92 cluster is one of the earliest characterized miR clusters. It has initially been linked to tumorigenesis and was given the distinction of being the first 'oncomir' (known as oncomir-1).[191,192] The cluster is comprised of six miRs (miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1, and miR-92a-1). These miRs can be further grouped into four different seed families: (i) the miR-17 family with miR-17 and miR-20a, (ii) the miR-18 family with miR-18a, (iii) the miR-19 family with miR-19a and miR-19b-1 and (iv) the miR-92 family with miR-92a-1 (Figure 7).[191,193] The miR-17~92 cluster is strongly expressed in a wide array of tissues and the sequences of the mature miRs are highly conserved in all vertebrates.[194]

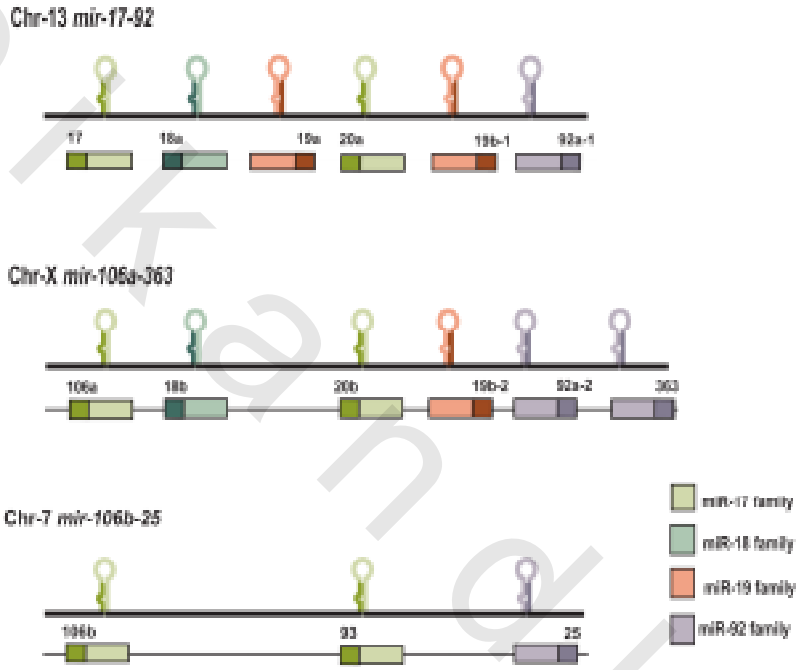


Figure 7: Gene structure of human microRNA-17~92 and its homologues.[191]

MicroRNA 17~92 cluster gene:

The study of the miR-17~92 cluster began with the identification of a novel gene which resides within the frequently amplified 13q31-q32 region in human B-cell lymphoma and designated as chromosome 13 open reading frame 25 (*C13orf25*) by Ota et al in 2004.[195] The *miR-17~92 cluster gene* is located in the third intron of *C13orf25* and is shown to be the actual effectors of the 13q31-q32 amplification. The cluster gene is known as the *miR-17 host gene* (*MIR17HG*) (formerly *C13orf25*, *MIRH1* and *MIRHG1*). The precursor transcript derived from the *MIR17HG* is a polycistronic miR transcript. It consists of two exons containing 5058-bp nucleotides and encodes miR precursors within about 1 kb on chromosome 13, which sequentially yields the six individual mature miRs (Figure 8).[193,195] It is worth noting that miR-18a exhibits a significant sequence homology with miR-17 and miR-20, despite one nucleotide difference within the "seed" regions. The distinct mature miR sequence of each miR-17~92 cluster component determines the specificity of target regulation, and ultimately the specificity of functional readout.[193]

Interestingly, although miRs are non-protein-coding, the primary transcript of *MIR17HG* contains also an open reading frame that encodes a polypeptide of 70 amino acids.[189] This polypeptide is produced by alternative splicing and has been designated as the "*MIR17HG* protein" (Figure 8).[195] Like miR components of the miR-17~92 cluster, the *MIR17HG* protein can gain access to body fluids (e.g. blood) and can be used as a simple, non-invasive biomarker for detecting disease.[181,186]

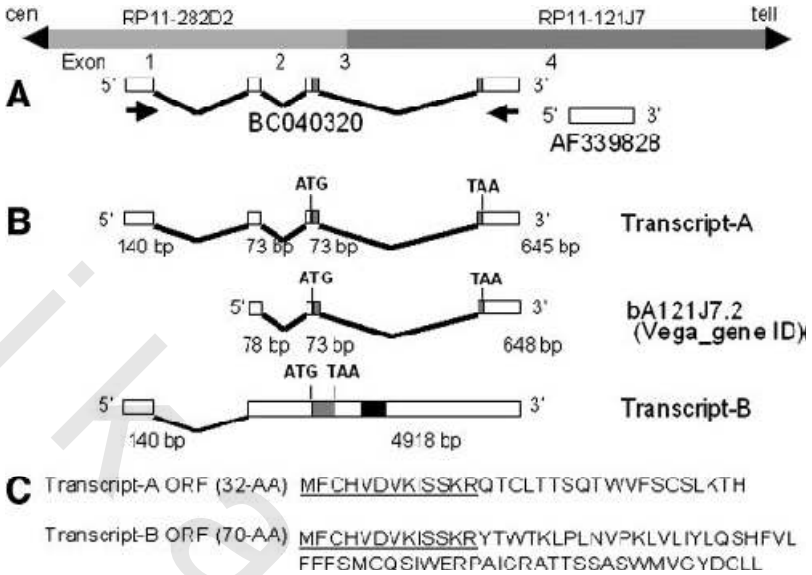


Figure 8: **Exon-intron structure of the C13orf25 gene.** (A.) the microRNA-17 host gene (MIR17HG) sequence is overexpressed with amplification at 13q31.3. (B.) The MIR17HG transcript obtained by reverse transcription-PCR (Transcript-B) consists of two exons containing 5058-bp nucleotides and encodes five precursor microRNAs and six mature microRNAs (miR-17, miR-18, miR-19a, miR-20, miR-19b, and miR-92) are obtained from the Transcript-B sequence, and are shown by the ■ in Transcript-B. Possible open reading frame is shown as ▭ (C.) the polypeptide sequence of 70 amino acids encoded by Transcript-B, designated as the "MIR17HG protein".[195]

The miR-17~92 cluster has two paralogs in the human genome; the miR-106b-25 cluster and the miR-106a-363 cluster. The miR-106b-25 cluster encodes for only three miRs; miR-106b, miR-93, and miR-25 and is embedded within the minichromosome maintenance protein 7 gene. The miR-106a-363 cluster encodes for miR-106a, miR-18b, miR-20b, miR-19b-2, miR92a-2 and miR-363. Reconstruction of the evolutionary history of the miR-17-92 cluster and its two paralogs revealed that the evolution of this cluster arose from tandem duplications of individual miRs, followed by duplications of entire clusters and subsequent loss of individual miRs. The miRs of the miR-17~92 cluster share a high degree of sequence homology with the miRs of the two paralogs, thus suggesting the possibility of overlapping cellular functions of these miR clusters (Figure 7).[191,193]

Regulation of microRNA 17~92 cluster:

A number of transcription factors have been shown to regulate the expression of the miR-17~92 cluster collaboratively (Figure 9).[196] The oncogene c-Myc (cellular avian myelocytoma virus gene) is the first identified regulator of the miR-17~92 cluster. The c-Myc directly activates transcription of the miR-17~92 cluster through binding to the (DNA) sequence CACGTG, known as an E box, located on 1484 nts upstream of the *MIR17HG* transcription start site and up-regulating the expression of all six miRs of the cluster.[197] Also, n-Myc has been shown to transcriptionally activate the miR-17~92 cluster.[198] Activation of the miR-17~92 cluster by c-Myc and n-Myc promotes cell proliferation, survival, angiogenesis, and metabolic reprogramming.[198,199] In addition, the E2 transcription factor (E2F) family (E2F1, E2F2 and E2F3), the direct downstream targets of c-Myc, are potent inducers of the miR-17~92 cluster. All E2Fs, especially E2F3, directly bind to the *MIR17HG* promoter and regulate its transcription.[200] The E2F family plays a central role in the regulation of G1 to S phase progression.[201] Also, a direct link has been found between the action of IL-6 and the expression of miR-18a and miR-20a mediated by signal transducer and activator of transcription (STAT)3.[202] Meanwhile, the miR-17~92 cluster is also subjected to transcription repression by key tumor suppressors. The miR-17~92 cluster is transcriptionally repressed by p53 under stress conditions such as hypoxia and DNA damage through binding to p53-binding site in the proximal region of the miR-17~92 promoter.[203] In addition, p53 decreases expression of the miR-17~92 cluster by an indirect mechanism through repression of E2F1.[204] It has been reported that p53 transactivates expression of the B-cell translocation gene 3 gene, which directly binds E2F1 and inhibits its activity.[205]

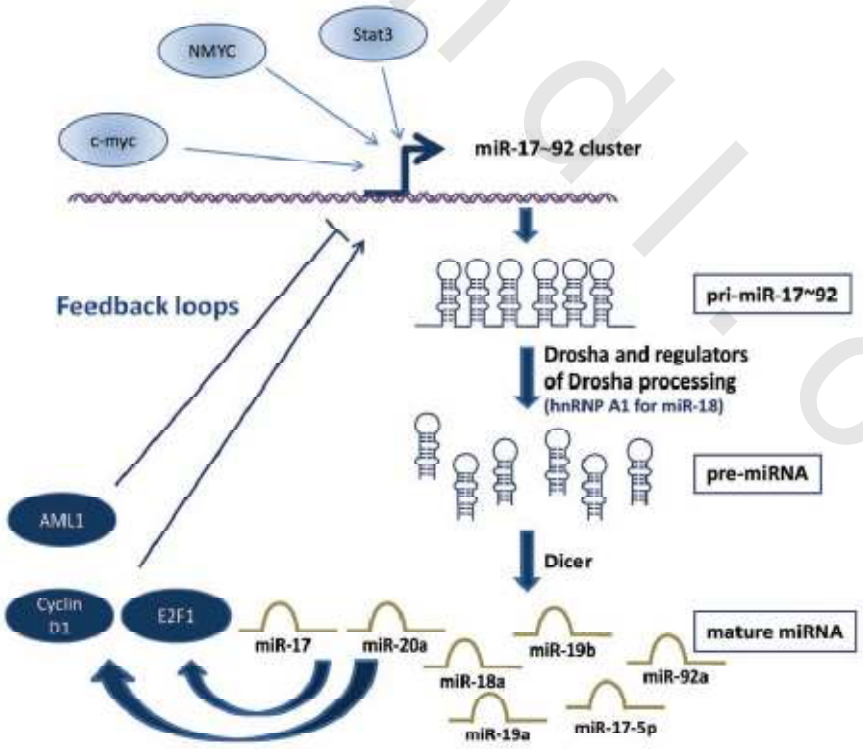


Figure 9: Proposed mechanisms transcriptionally and post-transcriptionally regulating the microRNA-17~92 cluster.[196]

Main targets of microRNA 17~92 cluster:

One of the least understood questions about the miR-17~92 cluster is the identity of its targets. Each miR component within the miR-17~92 cluster has the potential to repress several specific mRNAs. A number of miR-17~92 cluster targets has been identified, each of which is proposed to contribute to a specific functional readout of the miR-17~92 cluster (Figure 10).[206] Phosphatase and tensin homolog (PTEN) is one of the putative target genes of the miR-17~92 cluster mainly its miR-19a.[207] The miR-19 is the miR-17~92 component that is both necessary and sufficient to mediate PTEN repression through direct binding with PTEN 3'UTR.[208] Moreover, the transcription factor E2F1 is a target gene for the miR-17 seed family. Members of this family, miR-17 and miR-20a, can interact with the 3' UTR of the E2F1 transcripts and inhibit its translation, thus, establishing an auto-regulatory loop within the E2F transcriptional network. Also, miR-20a targets the 3'UTRs of both E2F2 and E2F3.[209]

In addition, the miR-17~92 cluster targets the pro-apoptotic protein Bcl2-interacting mediator (Bim) and the cyclin-dependent kinase inhibitor CDKN1A (p21), a negative regulator of the G1-S checkpoint cell cycle progression. This repression is mostly achieved by miR-17 and the related miR-20.[210] In particular, miR-17 and miR-20a directly target the TGF-β receptor II (TGF-βRII), whereas miR-18a targets Smad2 and Smad4, which are induced by TGF-β to negatively regulate the TGF-β signaling pathway.[211] Moreover, miR-92 negatively regulates an isoform of the cell cycle regulator p63, which functions as a molecular switch that initiates differentiation and regulates the proliferative potential of the cells.[212]

Furthermore, miR-18a and miR-19 directly repress the anti-angiogenic factors thrombospondin-1 and connective tissue growth factor (CTGF).[213] Also, hypoxia-inducible factor (HIF)-1α has been identified as a direct target for miR-17~92.[214] In addition, miR-17 and miR-20a participate in the regulation of the insulin gene enhancer protein and the T-box 1 protein.[215] Also, miR-18 and miR-19b target TNF-α-induced protein 3 (TNFAIP-3), which is a nuclear factor kappa B (NF-κB) pathway inhibitor and also inhibit other negative regulators of NF-κB signaling.[216,217] Another miR encoded by miR-17/92, miR-19a, augments STAT3 activity by targeting suppressor of cytokine signaling (SOCS)1, a known suppressor of STAT3 phosphorylation.[218]

Figure 10: Specific targets of microRNA-17~92 cluster.[191]

MicroRNA 17~92 cluster functions:

The miR-17~92 cluster has initially been linked to the initiation and progression of tumor development.[206,219] Meanwhile, recent functional dissection of the miR-17~92 cluster indicates that individual cluster components perform pleiotropic biological functions, which collectively regulate multiple related cellular processes such as cell proliferation, cell survival, apoptosis, cell cycle progression, the regulation of hematopoiesis and immune responses.[219] The structural complexity of miR-17~92 cluster along with the complex mode of interactions among its components, constitute the molecular basis for its unique functional complexity during normal development and disease.[220] Mutation or deregulation of the miR-17~92 cluster contributes to the pathogenesis of a variety of human diseases, including congenital developmental defects, immune diseases, viral infections, cardiovascular diseases, neurodegenerative diseases, aging and cancer (Figure 11).[196,221] However, it is unclear whether the six miRs encoded by the miR-17~92 cluster are functionally equivalent or control different molecular pathways, and the majority of their functionally relevant targets still remain to be identified. There is substantial evidence indicating that various members of the cluster can act synergistically, either by converging on the same targets or by targeting multiple nodes in the same pathway or biological output. [220]

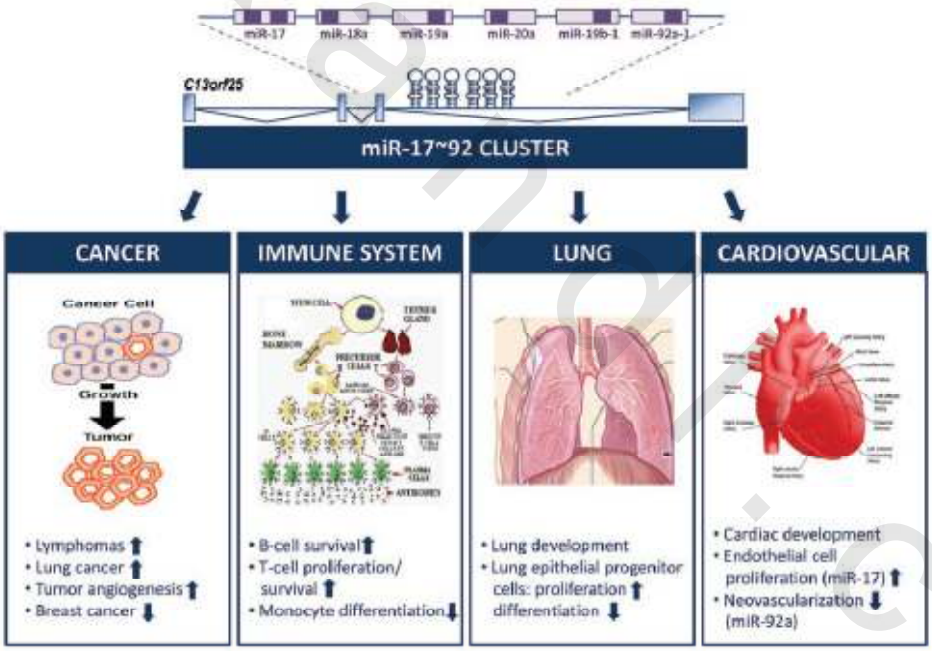


Figure 11: Summary of the proposed functions of the microRNA-17~92 cluster.[196]

***MicroRNA 17~92 cluster and normal development*:**

The miR-17~92 cluster is highly expressed in embryonic cells and has an important role in development.[194,222] The cluster was the first group of miRs to be implicated in a developmental syndrome in humans. Human patients with heterozygous microdeletions in the *MIR17HG* locus have autosomal dominant Feingold syndrome, characterized by multiple skeletal abnormalities in the fingers and toes, short stature and microcephaly. Some patients also show various degrees of learning and developmental disabilities.[223] Subsequent mouse studies showed that deletion of the miR-17~92 cluster results in postnatal lethality with multiple developmental defects, including lung hypoplasia and cardiomyopathy with cardiac defects as ventricular septal defect.[194,224] The miR-17~92 cluster is involved in normal lung morphogenesis, epithelial proliferation and branching through the targeting of STAT3 and mitogen-activated protein kinase (MAPK).[225] Also, the cluster plays an important role in heart development possibly through direct repression of PTEN, Connexin 43 and the Wnt/β-catenin signaling pathway in embryonic cardiomyocytes.[226,227]

MicroRNA 17~92 cluster, cell proliferation and apoptosis:

The miR-17~92 cluster particularly, miR-17 and miR-20a have been shown to control cellular proliferation and apoptosis by targeting the E2F family of transcription factors [197,209] and by down-regulating p21 and the pro-apoptotic protein Bim.[210] Also, the miR-17~92 cluster influences cell proliferation and survival by targeting the tumor-suppressor protein, PTEN thereby augmenting the phosphoinositol 3-kinase (PI3K)-AKT-mammalian target of rapamycin (mTOR) axis.[208] Moreover, gene expression analysis indicated that up-regulation of miR-17~92 cluster causes significant changes in the expression of several cell-cycle related genes, including CDK2 (cyclin-dependent kinase 2), cyclin-D2 and cAMP-response-element-binding protein.[228]

***MicroRNA 17~92 cluster and immune system*:**

The miR-17~92 cluster has a role in the innate and acquired immune response. Specifically, the miRs derived from the miR-17~92 cluster are selectively and/or highly expressed in immune cells and expression of the cluster is decreased upon cell differentiation.[229] The miR-17~92 cluster has been implicated in myeloid proliferation and differentiation.[230] Moreover, the cluster induces $CD34^+$ haematopoietic progenitor cells to differentiate into monocytes upon exposure to macrophage-colony stimulating factor.[231] In the adaptive immune system, the miR-17~92 cluster is crucial for promoting T-cell proliferation and protecting T cells from antigen-induced cell death, by down-regulation of the pro-apoptotic protein, Bim and the tumor suppressor, PTEN. Over-expression of this cluster resulted in autoimmunity that was characterized by enhanced T-cell proliferation and survival, particular effector $CD4^+$ T cells.[232] The miR-17 and miR-19b enhance Th1 cell responses and IFN-γ production, inhibit the differentiation of inducible Treg cells [233] and promote Th17 differentiation.[207] Mechanistically, PTEN is identified as the functionally important target of miR-19b, whereas the function of miR-17 is mediated by TGF-βRII.[233] and Ikaros Family Zinc Finger 4 (IKZF4).[207] Moreover, miR-19a promotes Th2 responses by direct targeting of the inositol phosphatase PTEN, the signaling inhibitor SOCS1 and the deubiquitinase A20.[234] In $CD8^+$ T cells, the miR-17~92 cluster exhibits a dynamic expression regulation during the acute anti-viral responses, being strongly

induced at the peak of effector responses, but greatly dampened during the formation of memory $CD8^+$ T cells. The miR-17-92 promotes cell-cycle progression and mTOR signaling in effector $CD8^+$ cells.[235] In addition, the miR-17~92 cluster is required for the pro-B to pre-B-cell transition during B-cell development where it inhibits cell death through the down-regulation of the proapoptotic protein Bim.[236]

MicroRNA 17~92 cluster and inflammation:

Accumulating evidences suggest that there is a link between the miR-17~92 cluster and inflammation and could be strongly proposed in diseases with inflammatory nature.[237] It was shown that the expression of miR-17~92 cluster could be triggered by proinflammatory cytokines such as TNF-α [216] and IL-6 [238] and NF-κB.[239,240] Also, the miR-17~92 cluster has been associated with important inflammatory processes such as the acute-phase response.[238] Moreover, the miR-17 and miR-19b are the two miRs in the miR-17~92 cluster that are responsible for enhancing Th17 responses,[207] and the miR-19a promotes Th2 cytokine production and amplified inflammatory signaling.[234] In addition, upregulation of the miR-17 and miR-20a promotes macrophage activation, phagocytosis and proinflammatory cytokine secretion through downregulating signal-regulatory protein α (SIRPα).[241] Meanwhile, the induction of the miR-17~92 cluster, specifically miR-18a, suppresses the induction of the anti-inflammatory Treg cells by targeting PTEN in T cells.[242] Furthermore, the miR-17~92 cluster has been linked to the process of inflammation via its ability to indirectly promote activation of NF-κB, a key player in the inflammatory responses, through targeting negative regulators of NF-κB signaling such as TNFAIP-3 [216,217] and other members of the A20/ TNFAIP3-ubiquitin editing complex (A20, Rnf11, Fbxl11/Kdm2a and Zbtb16) [217] and CYLD.[243] On the other hand, it has been recently proposed that miR-19a and b, belonging to the miR-17~92 cluster, have a repressive effect on inflammation through targeting TLR2.[244] Also, miR-20a is a negative regulator of inflammation by modulating expression of apoptosis signal-regulating kinase (ASK) 1, a key component of the TLR-4 pathway, upstream of p38 MAPK.[245]

MicroRNA 17~92 cluster and fibrosis:

Members of the miR-17~92 cluster are involved in the regulation of fibrosis although opposite effects have been reported. The miR-17~92 cluster plays a role in the progression of tissue fibrosis by targeting PTEN. Overexpression of PTEN inhibits the expression of α-smooth muscle actin (α-SMA) and the proliferation of fibroblasts.[246] Moreover, miR-17 and miR-20a inhibit senescence in fibroblasts by blunting the activation of p21WAF1.[247] On the other hand, the members of the miR-17~92 cluster were found to be down-regulated in idiopathic pulmonary fibrosis.[248] and in the context of liver and cardiac fibrosis.[249,250] The miR-17~92 cluster has been found to target profibrotic genes. The miR-18a and miR-19a/b inhibit CTGF and thrombospondin-1 (TSP-1).[249,250] Also, the miR-19b targets the TGF-β pathway. Overexpression of miR-19b inhibited the expression of TGF-βRII leading to decreased SMAD3 expression and reduced type-1 collagen production.[251,252]

***MicroRNA 17~92 cluster and cancer*:**

The polycistronic miR-17-92 cluster is the first miR cluster shown to play a role in tumorigenesis.[191,192] The miR-17~92 cluster was initially found to be amplified in diffuse cell lymphomas.[195] Also, the miR-17~92 cluster and its members were involved in the

pathogenesis of many hematological and solid organ malignancies.[253-260] The miR-20a was found to correlate with diagnosis to treatment time in B-cell chronic lymphocytic leukemia and thus can potentially serve as a blood biomarker.[253] Moreover, some members of the cluster can promote the growth of certain tumors as in case of miR-17 that was found to promote the growth of neuroblastoma cell lines.[254] In addition, expression level of miR-17, miR-18a, miR-19a and miR- 19b-1 was found to be increased in pancreatic cancer.[255,256] Also, miR-17-5p and miR-20a were overexpressed in lung cancer [257] and metastatic breast cancer.[258] Moreover, other studies have shown that miR-17, miR-18a and miR-20a were up-regulted in renal cancer.[259] All six members of the miR-17~92 cluster were found to be overexpressed in HCC.[260] The oncogenic activity of the miR-17~92 cluster is attributed to the downregulation of genes such as the well-established tumor suppressor retinoblastoma (pRB), the cell cycle inhibitor p21, as well as the pro-apoptotic protein Bim [210] and PTEN to promote cell survival and proliferation.[208] Moreover, the miR-17 inhibits the mitogen-activated kinase JNK2, which in turn leads to an increase in cyclin D1 expression, thereby promoting cell cycle progression.[209] In addition, the miR-17~92 cluster promotes the augmentation of tumor angiogenesis.[213] The c-myc-miR-17~92 axis blunts TGF-β signaling and production of multiple TGF-β-dependent antiangiogenic factors.[211]

PHOSPHATASE AND TENSIN HOMOLOG (PTEN)

Phosphatase and tensin homolog is a tumor suppressor gene located at the 10q23.31 locus. It was isolated and identified by three laboratories in 1997 and is also named as phosphatase and tensin homolog deleted on chromosome 10, mutated in multiple advanced cancers 1(MMAC1) and TGF-β-regulated and epithelial cell enriched phosphatase 1 (TEP1).[261-263] It is a non-redundant phosphatase that antagonizes the phosphatidylinositol 3-kinase (PI3K)/Akt (Protein Kinase B) signaling pathway, one of the most important and well-studied cancer promoting pathways.

PTEN Structure:

The PTEN gene comprises nine exons and encodes a protein of 403 amino acids.[264] The amino acid sequence of the PTEN tumor suppressor is considerably homologous to dual-specific protein phosphatases and tensin, a chicken cytoskeletal protein.[261] The crystal structure of PTEN revealed an expanded active site pocket for binding to its substrates and a C2 domain, which mediates membrane attachment of cell signaling proteins. Three other functional domains have also been identified: a short phosphatidylinositol-4,5-bisphosphate (PIP2) binding domain on the N terminus, PEST sequences and a PDZ interaction motif on the C-terminal tail that regulate protein stability and binding to PDZ domain-containing proteins respectively (Figure 12).[265] The binding of PIP2 to PTEN produces a conformational change in the enzyme leading to allosteric activation. The positive charge of PTEN's substrate binding pocket is also important for accommodating larger acidic substrates such as phosphoinositides (which are generated by many enzymes including a family of lipid kinases that are collectively known as the PI3Ks, which have important functions in the immune system).[266]

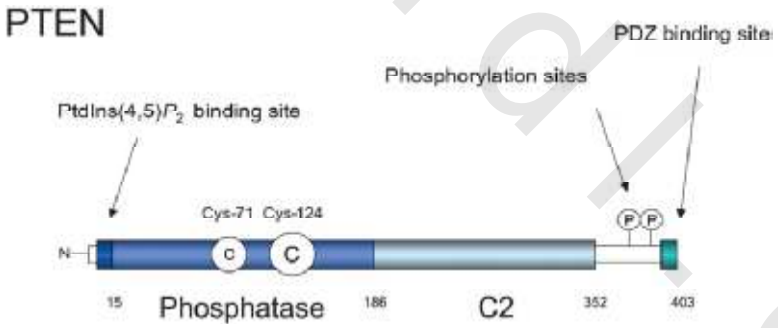


Figure 12: The structure of phosphatase and tensin homolog (PTEN).[265]

The PTEN phosphatase domain is evolutionarily conserved, and is the recipient of 40 % of its cancer-associated mutations, which occur most commonly through either a C124S mutation that abolishes both lipid and protein phosphatase activity or a G129E mutation that abrogates only its lipid phosphatase activity.[267,268] Although the phosphatase domain is responsible for PTEN's physiological activity, other PTEN mutations occur on the C-terminal C2 domain and tail sequence, highlighting an important role of the C terminus in maintaining PTEN protein stability. The fact that mutations occur in all PTEN functional domains indicates that each of these regions is biologically relevant to PTEN function.[269,270]

PTEN gene regulation:

PTEN expression and activity are tightly regulated at almost all possible levels: transcriptionally, translationally, and post-translationally.[271,272] Perhaps the most relevant mode of PTEN regulation, however, is through post-translational modifications, which may influence the phosphatase activity, the binding to the membrane, the localization to subcellular compartments, or the interaction with binding partners.[271,273] The recruitment of PTEN to membranes is further dictated through association with other proteins. In this respect, transmembrane, peripheral membrane, and membrane-associated scaffold proteins that interact with PTEN are particularly important in positioning PTEN within close proximity to the membrane. In effect, such protein-protein interactions prime PTEN function toward membranous substrates.[273] PTEN has been also shown to interact with plasma membrane receptors of the tyrosine kinase and G protein-coupled receptor families, although it is not always clear if these interactions are direct.[274] In addition, several PDZ domain-containing proteins interact with the C-terminal PTEN PDZ-binding motif and recruit PTEN to the plasma membrane.[275] PTEN-PDZ domain interactions may also serve in shifting PTEN into the open "active" conformation by sequestering the PTEN C-tail away from interacting with the C2-domain and unmasking the PTEN membrane-binding regions.[276]

Due to its principal function as a tumor suppressor, maintaining a stable level of PTEN expression is critical.[277] Positive regulators of PTEN gene expression include, EGR1 (early growth response protein 1), PPARγ (peroxisome proliferator- activated receptor γ) and p53; these were shown to directly bind to the PTEN promoter region.[278-280] Negative regulators include MAPK-4 (mitogen activated protein kinase kinase-4), TGF-β , NF-κB, transcriptional cofactor c-JUN and oncogene BMI1; these were shown to suppress PTEN expression in several cancer models.[277,281,282] Many miRs are also known to target PTEN expression and function in both normal and pathological conditions.[283] As mentioned before, PTEN is one of the main targets of the miR-17~92 cluster, which represses PTEN through direct binding with its 3'UTR.[208] Post-translational modifications such as active site oxidation, acetylation, phosphorylation, and ubiquitination can also regulate PTEN activity.[277,284] It has also been suggested that reactive oxygen species (ROS) inactivate PTEN, which subsequently leads to the insulin-mediated activation of protein kinase Akt.[285] Finally, genetic and epigenetic mechanisms such as point mutations, chromosomal deletions, and promoter methylation status, can also modulate the expression and activity of PTEN.[269]

PTEN and regulation of the PI3K/Akt Pathway:

PTEN is a unique lipid phosphatase that removes the phosphate from the D3 position of phosphatidylinositol-3,4,5-triphosphate (PIP3), a product of PI3K, thus directly antagonizing the action of PI3K.[286] The PIP3 accumulation at the plasma membrane through PI3K activity results in recruitment and activation of important kinases involved in cell growth and survival, including phosphoinositide-dependent kinase-1 and Akt family members, via their pleckstrin homology domains.[287,288] In this manner, PTEN acts as an endogenous negative regulator of the PI3K/Akt pathway, resulting in the suppression of Akt-mediated signal pathways, including NF-κB activation. Both PI3K and PTEN orchestrate cell responses to growth factors, cytokines, integrins and other intercellular mediators and contribute to the growth, motility, survival and metabolic responses of many

cell types.[287] The PI3K/Akt signaling plays a critical role in regulating growth responses, homeostasis, and longevity. At the cellular level, the PI3K/Akt pathway controls a broad range of cellular activities including cell growth, migration, differentiation, and survival. Numerous Akt substrates have been identified such as the Forkhead family of transcription factors, $p27^{KIP1}$, MDM2, GSK3, BAD, IKK-b, and mTOR.[289]

The precise intracellular localization of PTEN can greatly impact its stability and function. PTEN is largely located in the cytoplasm of human cells. In addition, significant nuclear staining of certain cell types has been reported, and in some cases nuclear staining predominates.[290] The level of functional PTEN in the nucleus is regulated through dynamic changes in its ubiquitylation.[291,292] So, unlike most tumor suppressor genes, PTEN protein can function in the nucleus as well as the cytoplasm. However, a small variable proportion of the protein is plasma membrane-associated, which is tightly regulated and functionally significant.[290]

PTEN functions:

Deregulation of PTEN has been associated with many diseases characterized by tissue destruction and remodeling. The most important function of PTEN is controlling cell survival, promoting proliferation and inhibiting apoptosis via its ability to negatively regulate the PI3K/Akt survival signaling pathway.[287,293] In addition to Akt, cytoplasmic PTEN also regulates epidermal growth factor signaling by inhibiting phosphorylation of SHC adaptor proteins, which in turn suppress activation of the Ras/MAPK pathway.[294] Also, PTEN can control cell migration, stretching and adhesion by regulating the activity of focal adhesion kinases (through dephosphorylation) and other membrane channels.[295] Moreover, nuclear PTEN can reduce the level of cyclin D1, up-regulate the negative cell cycle regulator p21 and induce G0-G1 cell cycle arrest and, through its non- enzymatic function, regulate the activity of the anaphase-promoting complex cyclosome. PTEN acts as part of a chemical pathway that signals cells to stop dividing and causes cells to undergo programmed cell death (apoptosis) when necessary.[293,296] There is also some evidence suggesting that PTEN helps the control of cell migration, adhesion of cells to surrounding tissues and angiogenesis, and plays a role in maintaining the stability of a cell's genetic information through the physical interaction with centromeres and control of DNA repair.[297]

Moreover, PTEN has direct or indirect role in immune system function and development. It has been documented that PTEN has the ability to suppress T-cell proliferation and survival through its antagonizing effect on the PI3K/Akt pathway.[298] In addition, the PI3K/Akt pathway has also been shown to be critical for facilitating Th1 differentiation and supporting IFN-γ production.[299,300] Also PTEN plays an important role in B-cell development and immunoglobulin production.[301] The PI3K/Akt signaling axis which is regulated by PTEN, is crucial for site-directed migration and diapedesis of immune effector cells, such as neutrophils and monocytes, to the site of inflammation and infection.[302]

Furthermore, PTEN plays a role in the regulation of tissue fibrosis.[304] Loss of PTEN expression in fibroblasts has been reported in idiopathic pulmonary fibrosis,[304] liver fibrosis,[305] myocardial fibrosis,[306] diffuse cutaneous systemic sclerosis, [307] and hypertrophic scar.[308] A decreased PTEN activity promotes proliferation, α-SMA

expression and collagen production of fibroblasts through activation of the PI3K/Akt signaling pathway.[246,309] Also, deletion of the PTEN gene has been shown to result in overexpression of CTGF, a proadhesive matricellular protein, in fibroblasts.[310] In addition, PTEN regulates cell interactions with the extracellular matrix (ECM) by inhibiting cell migration, spreading, and focal adhesions.[311] It becomes activated during collagen matrix contraction and is responsible for antagonizing PI3K activity and promoting a decline in phosphorylated Akt and fibroblast apoptosis in response to collagen contraction.[312] Moreover, PTEN expression decreases MMP-2 expression and activity by NF-κB-dependent pathway.[313,314]

In addition, PTEN plays a critical role in the regulation of lipid/cholesterol metabolism and contributes to steatosis. PTEN deletion stimulates the expression of genes related to lipogenesis through activation of the PI3K/Akt pathway.[315,316] It mediates the transactivation of sterol regulatory element binding proteins (SREBPs), major regulators of lipid metabolism that activate genes encoding the synthesis of cholesterol and fatty acids and cellular uptake of lipoproteins.[315] Also, PTEN inactivation has been reported to impair microsomal triglyceride transfer protein (MTP) activity.[317] In addition, PTEN directly dephosphorylates 3-hydroxy-3-methylglutaryl-coenzyme A (HMGCoA) reductase, the rate-limiting enzyme in cholesterol synthesis.[318] Moreover, PTEN deficiency induces peroxisome proliferator-activated receptor-γ (PPARγ), a key transcriptional activator for adipogenesis and lipogenesis, and its downstream target genes, such as the adipogenic genes (*adiponectin*, *adipsin*, and *aP2*) and β-oxidation-related genes (*acyl-CoA oxidase, peroxisomal enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase bifunctional protein, and peroxisomal 3- ketoacyl-CoA thiolase*) and increases the expression of fatty acid-modifying enzymes (*fatty acid synthetase, acetyl-coenzyme A carboxylase, and steroyl-CoA desaturase 1*).[319,320] Down-regulation of PTEN expression also affects import, esterification, and extracellular release of fatty acids.[321] In addition, PTEN down-regulation leads to constitutive activation of forkhead transcription factors, which mediate abnormal lipid accumulation in hepatocytes.[322] Meanwhile, PTEN is a regulator of insulin sensitivity in peripheral tissues.[323] PTEN inactivation leads to impaired insulin sensitivity by reducing insulin receptor substrate-1 (IRS-1) and inhibition of the insulin signaling pathway through negative regulation of PI3K/Akt signaling pathway.[321,324]