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"JÚLIO DE MESQUITA FILHO"
FACULDADE DE MEDICINA

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Cafeína, Trigonelina e Ácido Clorogênico: Modulação da expressão de miRNAs na Hepatocarcinogênese associada à Fibrose

Tese apresentada à Faculdade de Medicina, Universidade Estadual Paulista "Júlio de Mesquita Filho", Câmpus de Botucatu, para obtenção do título de Doutor em Patologia.

Orientador: Prof. Dr. Luís Fernando Barbisan
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1. Fígado - Câncer. 2. Fígado - Cirrose. 3. Ácido fenólico. 4. Cafeína. 5. MicroRNAs.

Palavras-chave: Ácido clorogênico; Cafeína; Hepatocarcinogênese; MicroRNAs; Trigonelina.

Resumo

A expressão alterada de microRNAs contribui para o desenvolvimento do carcinoma hepatocelular (CHC). Em contraste, o consumo de café reduz em ~40% o risco de desenvolvimento de fibrose/cirrose hepática e CHC, enquanto o café descafeinado não. Esses efeitos são atribuídos à cafeína (CAF) ou à sua combinação com outros compostos comuns e altamente biodisponíveis no café, como a trigonelina (TRI) e o ácido clorogênico (ACG)? Assim, avaliou-se se a CAF individualmente ou combinada à TRI e/ou ao ACG atenuam a hepatocarcinogênese associada à fibrose, investigando a potencial modulação da expressão global de microRNAs. Camundongos C3H/HeJ foram submetidos a um modelo de hepatocarcinogênese associada à fibrose induzido por dietilnitrosamina/tetracloreto de carbono e tratados com CAF (50 mg/kg peso corpóreo (p.c.) 5x/semana, gavagem), CAF+TRI (50 e 25 mg/kg p.c.), CAF+ACG (50 e 25 mg/kg p.c.) ou CAF+TRI+ACG (50, 25 e 25 mg/kg p.c.) por 10 semanas. Somente o tratamento com CAF+TRI+CGA atenuou o desenvolvimento de focos pré-neoplásicos hepáticos. Somente a combinação de todos os compostos reduziu a proliferação celular (Ki-67) em focos pré-neoplásicos e aumentou a apoptose (caspase-3 clivada) em tecido adjacente. CAF+TRI+CGA reduziu o estresse oxidativo hepático, aumentando a atividade ou os níveis proteicos de membros do eixo antioxidante Nrf2/GSH-Px/SOD. O tratamento com CAF+TRI+CGA também atenuou o eixo pró-inflamatório IL-17/ NFκB, reduzindo a quantidade de macrófagos CD68+, a ativação de células estreladas (α-SMA) e a deposição de colágeno. Por fim, o tratamento com CAF+TRI+ACG aumentou a expressão hepática de miR-144-3p e miR-15b-5p, considerados supressores de tumor ou antifibróticos, reduzindo os níveis proteicos de EGFR (alvo de miR-144-3p) e de membros da família antiapoptótica Bcl-2 (Bcl-2, Mcl-1 e Bcl2l2, alvos de miR-15b-5p). Portanto, a combinação de compostos de café, ao invés de CAF isoladamente, atenuou a hepatocarcinogênese associada à fibrose, em parte, pela modulação da expressão de miRNAs.

Abstract

Aberrant microRNA expression implicates on hepatocellular carcinoma (HCC) development. Conversely, daily coffee consumption reduces by ~40% the risk for fibrosis/cirrhosis and HCC, while decaffeinated coffee does not. It is currently unknown whether these protective effects are solely related to caffeine (CAF), or to its combination with other common and/or highly bioavailable coffee compounds, such as trigonelline (TRI) and chlorogenic acid (CGA). We evaluated whether CAF individually or combined with TRI and/or CGA alleviates fibrosis-associated hepatocarcinogenesis, examining the involvement of miRNA profile modulation. Male C3H/HeJ mice were submitted to a diethylnitrosamine/carbon tetrachloride-induced model. Animals received CAF (50 mg/kg), CAF+TRI (50 and 25 mg/kg), CAF+CGA (50 and 25 mg/kg) or CAF+TRI+CGA (50, 25 and 25 mg/kg), intragastrically, 5x/week, for 10 weeks. Only CAF+TRI+CGA combination reduced the incidence, number and proliferation (Ki-67) of hepatocellular preneoplastic foci while enhanced apoptosis (cleaved caspase-3) in adjacent tissue. CAF+TRI+CGA treatment also decreased hepatic oxidative stress by enhancing the antioxidant Nrf2 axis. CAF+TRI+CGA had the most pronounced effects on decreasing hepatic pro-inflammatory IL-17/NFκB signaling, contributing to reduce CD68-positive macrophage number, stellate cell activation, and collagen deposition. The miRNAomic profile revealed that CAF+TRI+CGA upregulated tumor suppressors miR-144-3p and antifibrotic miR-15b-5p, frequently altered in human HCC. CAF+TRI+CGA reduced protein levels of pro-proliferative EGFR (miR-144-3p target) and antiapoptotic Bcl-2 family members (miR-15b-5p targets). Our results suggest that the combination of most common and highly bioavailable coffee compounds, rather than CAF individually, attenuates early fibrosis-associated hepatocarcinogenesis by modulating miRNA expression profile.

Sumário

Capítulo 1

Revisão de literatura

Observação: O capítulo 1 compreende as subsecções 1.1, 1.2 e 1.3. A subsecção 1.3, referente as evidências epidemiológicas e experimentais do café e seus compostos isolados sobre a carcinogênese do trato gastrointestinal e fígado, foi publicada na revista "*Food Research International*" (fator de impacto 3,579; JCR 2018).

1.1 Hepatocellular carcinoma: of murine and men

According to the World Health Organization (WHO), liver primary malignant neoplasias, mainly represented by Hepatocellular Carcinoma (HCC), are the sixth most incident while the fourth deadliest cancers worldwide, comprising around 800,000 new cases and deaths/year (WHO, 2018) (Figure 1). In addition, HCC-related incidence and mortality crude numbers are expected to increase by 60% from 2018 to 2040 (WHO, 2018) (Figure 2). Moreover, HCC is considered a poor prognosis disease, with a median survival time of 11 months after diagnosis, and 3-year survival rates ranging from 19 to 30% (Greten et al., 2005; op den Winkel et al., 2012). These epidemiological data evoke the importance of HCC as a global health problem and elicit the need for novel preventive and therapeutic strategies. Moreover, populational data on HCC display two important features: gender and geographical disparities (Figures 3 and 4). Standardized incidence rates (cases or deaths *per* 100,000 people) in Asian and African continents together are almost 2-fold higher than in Europe and North America (Figure 3). Noteworthy, HCC is not part of the top ten most incident and deadliest cancers in Brazil (INCA, 2018). Geographical disparity is clearly linked to the occurrence of the main risk factors for this malignancy. In addition, 70% of HCC cases and -related deaths occur in men (Figure 4). The mechanisms concerning gender disparity, involving the promoting activity of testosterone and protective effect of estrogens, were only elucidated with the use of preclinical rodent models, and will be further discussed.

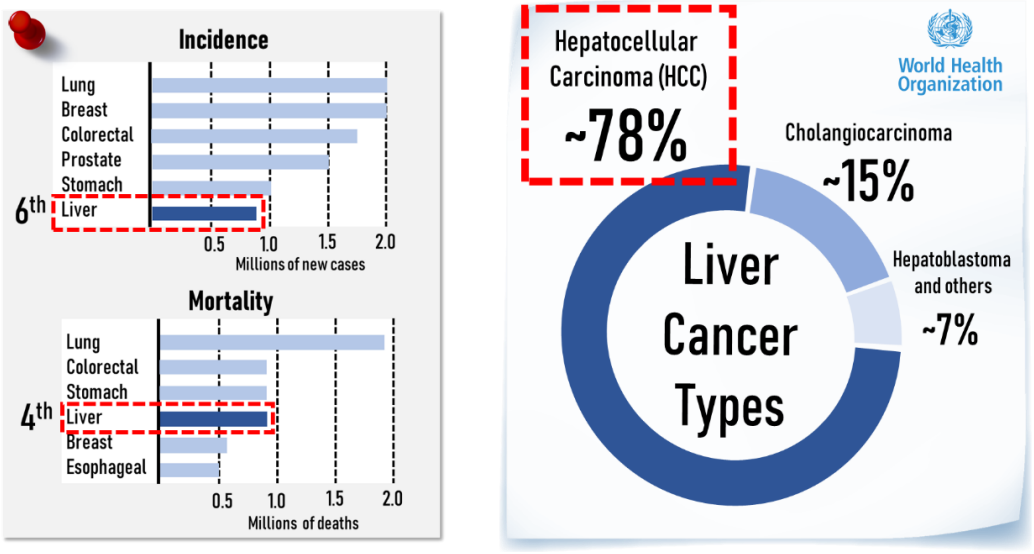

Figure 1. Infographics on HCC epidemiological global data in 2018. Primary liver cancers, mostly represented by HCC (responsible for 78% cases/deaths), are part of the top 10 ranked cancers in both incidence and mortality crude numbers (WHO, 2018).

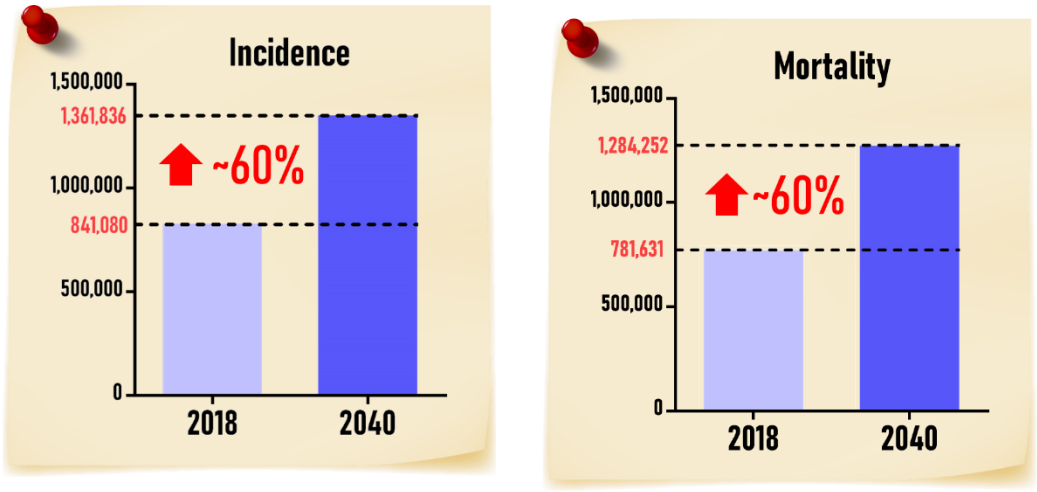

Figure 2. Estimated increase in both incidence and mortality crude numbers for HCC, considering 2018 to 2040 projection (WHO, 2018).

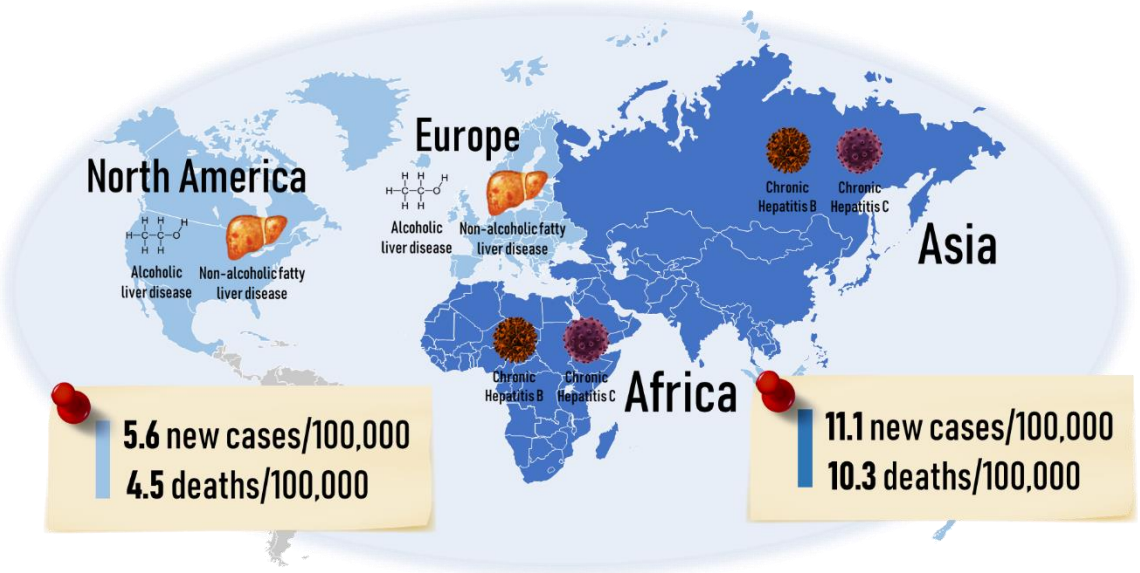

Figure 3. Geographical disparity feature of HCC-related standardized incidence and mortality rates between Africa/Asia and North America/Europe (WHO, 2018). The main risk factors are also presented according to the continent (Baecker et al., 2018)

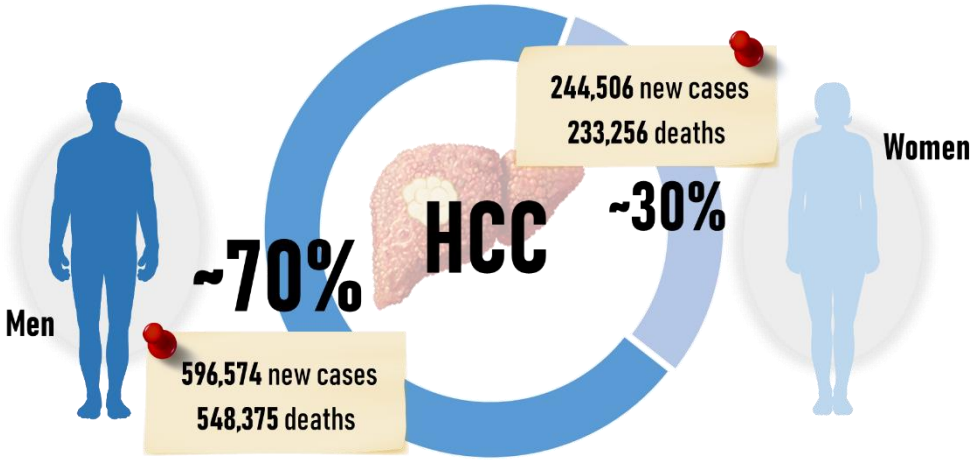

Figure 4. Gender disparity feature of HCC-related incidence and mortality crude number between men and women (WHO, 2018).

Around 70 - 90% of HCC cases develop in the background of liver fibrosis/cirrhosis caused by chronic hepatitis B (HBV) and C (HCV) virus infection, non-alcoholic fatty liver disease (NAFLD) and/or alcoholic liver disease (ALD) (Yang et al., 2011; Baecker et al., 2018). Globally, 44% and 21% of all cases are attributed to HBV and HCV chronic infections, respectively, whereas 26% and 16% are linked to ALD and NAFLD-related metabolic syndromes (mostly obesity and diabetes) (Baecker et al., 2018). Concerning Central Asia and Central Sub-Saharan Africa, HBV and HCV are indeed the most prominent risk factors, responsible for 57%-60% and 41%-50% of all cases, respectively (Baecker et al., 2018). On the other hand, in Central Europe and North America, ALD and NAFLD-related metabolic syndromes are the most protruding ones, linked to 30%-32% and 20%-24% of all cases, correspondingly (Makarova-Rusher et al. 2016; Baecker et al., 2018). Since some authors consider the chronic viral infections as the most important risk factors for HCC development, HBV/HCV-related HCC attributable fraction may in part explain geographical disparity among the cited continents (Figure 3). Nonetheless, recent studies have reported on the increasing prevalence of ALD and, specially, NAFLD in high income European countries and USA (Makarova-Rusher et al. 2016; Baecker et al., 2018). It is worthy of note that non-alcoholic steatohepatitis (NASH), the advanced stage of NAFLD, is the second leading etiology of HCC-related liver transplant in the USA (Wong et al., 2014; 2015). In general, HCC is considered a complex and multifactorial disease concerning its risk conditions since their co-existence of is often observed, as HBV and HCV co-infections and co-occurrence of ALD and NAFLD (Baecker et al., 2018).

The risk factors are proposed act through multiple different molecular mechanisms that predispose to chronic liver disease and culminate on the emergence of hepatocellular preneoplastic and neoplastic lesions. Overall, HCV and HBV chronic infections have both direct and indirect mechanisms (Levrero & Zucman-Rossi, 2016; Vescovo et al., 2016) (Figure 5). The direct ones are related to components of the viral particle (Figure 5). In order to induce viral replication, HBV genome integrates the host hepatocyte genome in a feature known as insertional mutagenesis (Minami et al., 2005). This integration may establish genomic instability and predispose to DNA damage and more severe genomic alterations, as mutations and chromosomal rearrangements in oncogenes or tumor suppressor genes (Minami et al., 2005). Moreover, the HBx protein, also responsible for orchestrating viral replication, is accounted for translational repression (as it promotes DNA methylation) or activation (as it promotes DNA acetylation) (Cougot et al., 2007; Zheng et al. 2009) (Figure 5). Regarding HCV, the core protein of the viral particle directly interacts with mitochondrial proteins, predisposing to mitochondrial stress and disfunction (Kim et al., 2013) (Figure 5). Core is implicated on post-translational modifications in important tumor suppressor proteins, as p53 protein, the “guardian of the genome” (Kao et al., 2004). The role of other

HCV proteins [nonstructural proteins (NS) NS3, N4B and NS5B] on intracellular signaling is also speculated, yet not fully understood. During chronic viral infections, HBV and HCV are able to evade the viral particle clearance mediated by both innate and adaptive immune systems through viral replication and mutations (Levrero & Zucman-Rossi, 2016; Vescovo et al., 2016). As long as viral particles are adapted to inflammatory microenvironment, the persistent inflammation may cause long-term hepatocyte damage and death, creating a necro inflammatory context that eventually progress to liver fibrosis and cirrhosis (Levrero & Zucman-Rossi, 2016; Vescovo et al., 2016). In this process, pro-inflammatory cytokine release, oxidative stress and cell death signals are the stimuli for hepatic stellate cell (HSC) activation (Tsuchida & Friedman, 2017) (Figure 6). Inactivated under physiological conditions, these cells progressively turn into proliferative, contractile and fibrogenic myofibroblast through epithelial-mesenchymal transition (EMT), being established as the central drivers of liver fibrosis and cirrhosis (Tsuchida & Friedman, 2017) (Figure 6). Chronic cycles of hepatocyte death and extracellular matrix (ECM) synthesis and accumulation, mainly collagen type I and III, lead to the progressive substitution of liver parenchyma by fibrous septa, characterizing fibrosis (Tsuchida & Friedman, 2017). The evolution of this condition, featuring profound liver architecture modification and functional and regenerative capacity loss, culminates on liver nodulation ("islands" of hepatocytes surrounded by ECM, Figure 6), is called cirrhosis (Tsuchida & Friedman, 2017). The interplay between macrophages and activated HSC through paracrine signaling mediated by growth factors [transforming growth factor-β (TGFβ), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF) and connective tissue growth factor (CTGF)] and cytokines (as interleukin 17) is also a key event on fibrosis establishment and progression to cirrhosis (Meng et al., 2012; Tsuchida & Friedman, 2017).

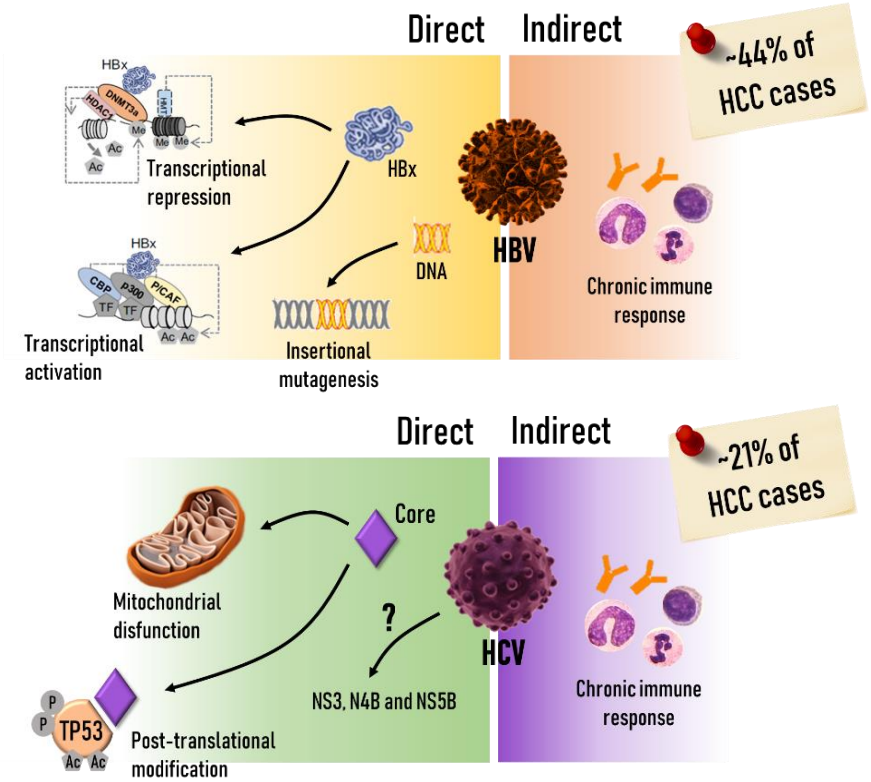

Figure 5. Molecular mechanism involved in HBV and HCV-related HCC, attributed to 44% and 21% HCC cases globally. Direct and direct mechanism are proposed (Kao et al., 2004; Cougot et al., 2007; Zheng et al. 2009; Kim et al., 2013; Levrero & Zucman-Rossi, 2016; Vescovo et al., 2016; Baecker et al., 2018).

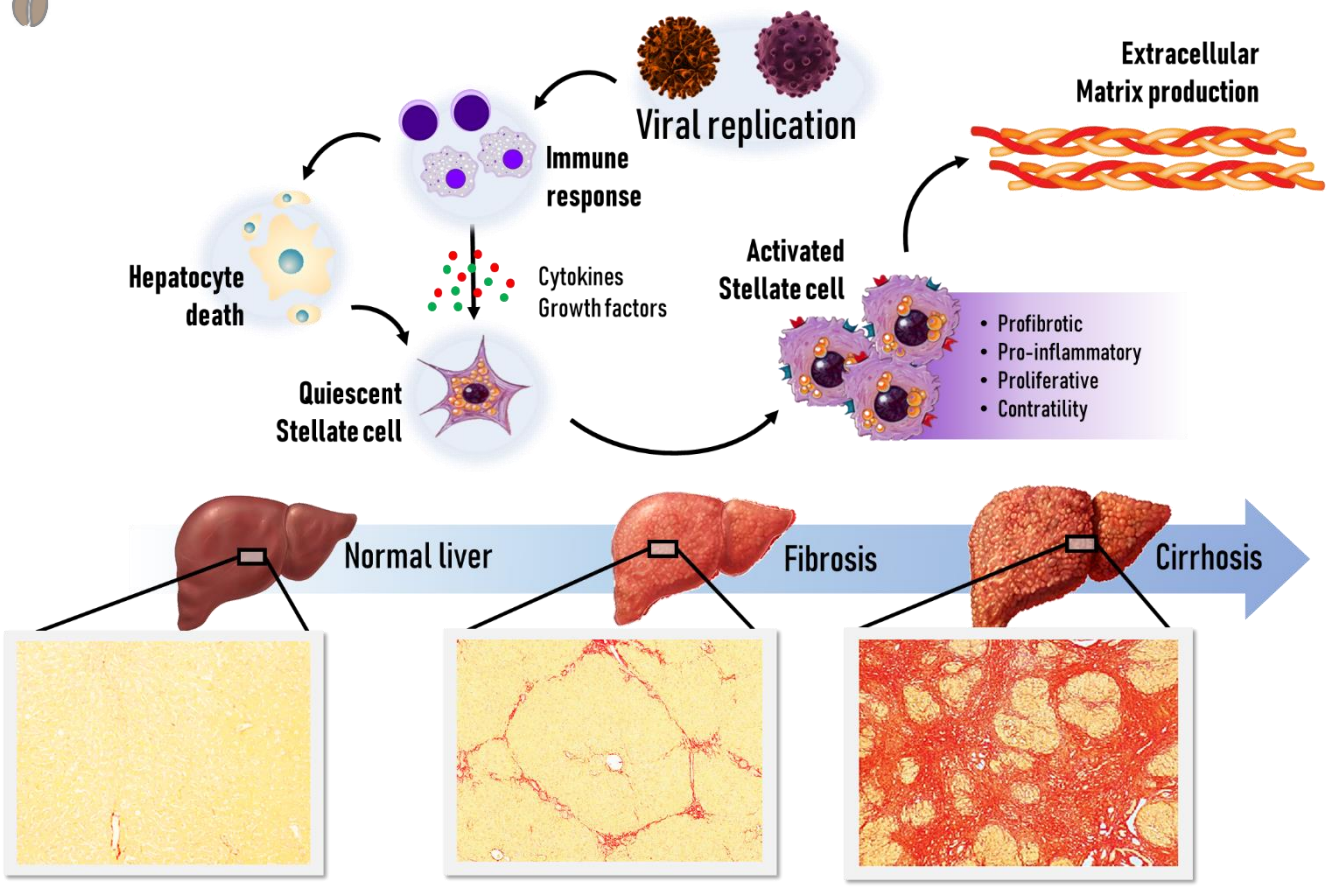

Figure 6. The main outcomes of HBV/HCV-related chronic liver disease. Chronic immune response and hepatocyte death are the stimuli for hepatic stellate cell activation, a key event on hepatic fibrogenesis. The progressive accumulation of collagen, as well as liver nodulation, are depicted in photomicrographs of Sirius red-stained sections of human liver. Adapted from Tsuchida & Friedman, 2017.

WHO estimates that ethanol (CH_3CH_2OH), the main component of alcoholic beverages, is linked to 12.6% of cancer-related deaths globally (WHO, 2018). Concerning excessive and chronic ethanol consumption, attributed to ~26% of HCC cases globally, epidemiological data point out to a linear positive correlation between alcohol intake and increased risk for HCC development (Chuang et al., 2015). It is estimated that a drink *per* day, corresponding to ~12 g of ethanol (half a pint of beer or one glass of wine), leads to 8% risk increase for HCC risk. Two (~24 g of ethanol) and four drinks (~48 g of ethanol) are associated with 19% and 54% risk increases, respectively (Chuang et al., 2015). Although some authors consider excessive and chronic ethanol intake as an independent risk factor for HCC, strong synergism is observed in the co-occurrence with viral infections and metabolic disorders (Yuan et al., 2004). After consumption (Figure 7), ethanol undergoes hepatic metabolism into acetaldehyde (CH_3CHO) by alcohol dehydrogenase (ADH), cytochrome P450 2E1 (CYP2E1) and/or, to a lesser extent, catalase (Cederbaum, 2012). Subsequently, acetaldehyde is oxidized into acetate (CH_3COOH) by aldehyde dehydrogenase (ALDH) (Cederbaum, 2012) (Figure 7). Acetaldehyde and acetate are highly reactive and prone to bind to biomolecules, specially DNA, forming stable DNA adducts (Figure 7). Ethanol metabolism

also leads to reactive oxygen species (ROS) release (Cederbaum, 2012). Both ROS and ethanol metabolites are accounted for genomic instability, a key hallmark for neoplastic transformation and progression. Oxidative damage may also cause hepatocellular death, activating immune response (Cederbaum, 2012). Oxidative stress, cell death and immune response are the stimuli for HSC activation and fibrosis, as previously highlighted (Tsuchida & Friedman, 2017). In addition, ethanol alters energy metabolism in the liver. ADH- and ALDH-mediated reactions lead to the accumulation of reduced nicotinamide adenine dinucleotide (NADH) (You & Arteel, 2019) (Figure 7). This high concentration of NADH inhibits fatty acid (FA) oxidation and promote FA synthesis. Progressively, triacylglycerols accumulate in the hepatocytes, leading to a condition known as “fatty liver”. The context of lipotoxicity in fatty liver also contributes to cell death and immune response that lead to HSC activation (You & Arteel, 2019). In addition to these mechanisms, ethanol causes enteric dysbiosis and subsequent intestinal barrier disruption (Stärkel et al., 2018) (Figure 7). Gut permeability enables the translocation of lipopolysaccharide (LPS), a bacterial endotoxin that reaches the liver *via* circulation and activates immune response, ultimately contributing to HSC activation and fibrosis (Tsuchida & Friedman, 2017; Stärkel et al., 2018).

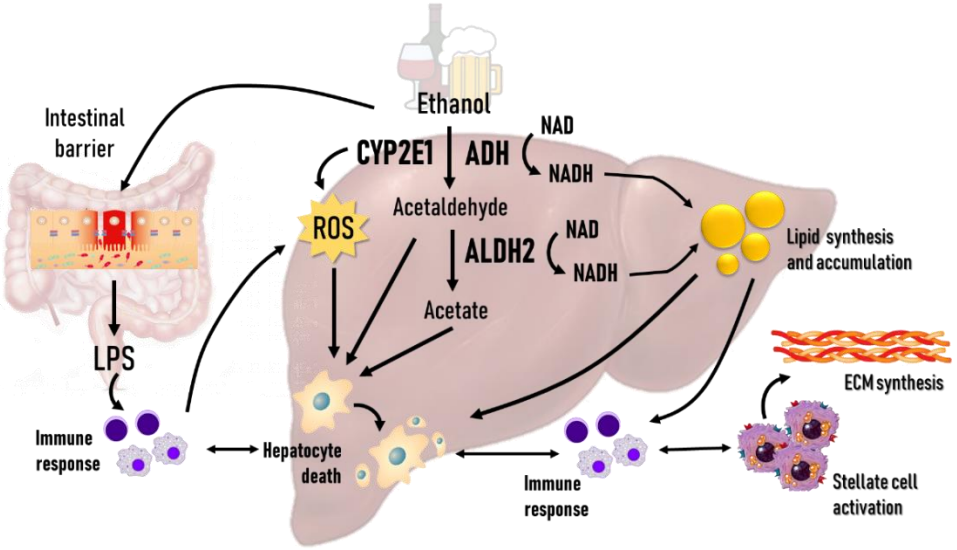

Figure 7. The main ethanol-related mechanisms on the induction on alcoholic liver disease (ALD), a growing risk condition for HCC in high income countries (Cederbaum, 2012; Tsuchida & Friedman, 2017; Stärkel et al., 2018; You & Arteel, 2019).

,Lastly, the mechanisms involved in NAFLD-related HCC are intrinsically related to the frequency of metabolic disorders, including type 2 diabetes mellitus (T2DM) and especially obesity, an imminent public health problem in high income countries (Makarova-Rusher et al., 2016). According to the Center of Disease Control (CDC) of the USA, the prevalence of obesity was 39.8% and affected about 93.3 million adults in 2015-2016 (CDC, 2018). Interestingly, ~34% of USA population presents NAFLD, indicating a potential causal relationship between obesity and this HCC risk condition

(Kim et al., 2013). The western fast food culture, established in 60's and globally widespread nowadays, contributes to the enhanced consumption of saturated fats and refined sugars (Fraser et al., 2010). A saturated fat-rich diet increases serum free fatty acids (FFA) levels, as well as FFA delivering to the organs, including the liver (Kutlu et al., 2018) (Figure 8). Increased FFA uptake drastically alters hepatic lipid metabolism, stimulating *de novo* lipogenesis while decreasing triglyceride disposal by fatty acid oxidation and by the secretion of triglyceride-rich lipoproteins (Kutlu et al., 2018). Some authors rely on the "two-hit" theory to explain NAFLD molecular pathogenesis (Kutlu et al., 2018). The first hit involves the triglyceride accumulation in more 5% of hepatocytes, characterizing simple steatosis (Kutlu et al., 2018). The progressive fat accumulation may establish a lipotoxic context that leads to hepatocyte death and triggers inflammatory response, which is considered the "second hit" (Kutlu et al., 2018) (Figure 8). Subsequently, chronic inflammation leads to HSC activation, fibrosis, and the coexistence of all these conditions is denominated NASH, which is the most advanced stage of NAFLD development (Kutlu et al., 2018). The consumption of high-sugar drinks, containing high fructose and glucose levels, also contribute to NAFLD (Jensen et al., 2018). Briefly, fructose and glucose metabolism increase acetyl-CoA bioavailability, stimulating *de novo* lipogenesis and triglyceride accumulation (Jensen et al., 2018) (Figure 8). A fat-rich diet consumption is also responsible for visceral body fat accumulation and gut permeability (Vonghia & Francque, 2015; Federico et al., 2016). Pro-inflammatory adipokines produced by increased visceral fat, as well as enhanced endotoxemia caused by gut leakiness also promote the "second-hit" events (Vonghia & Francque, 2015; Federico et al., 2016) (Figure 8). The chronic pro-inflammatory context in NASH contributes to the acquisition of "tumor-promoting inflammation" cancer hallmark.

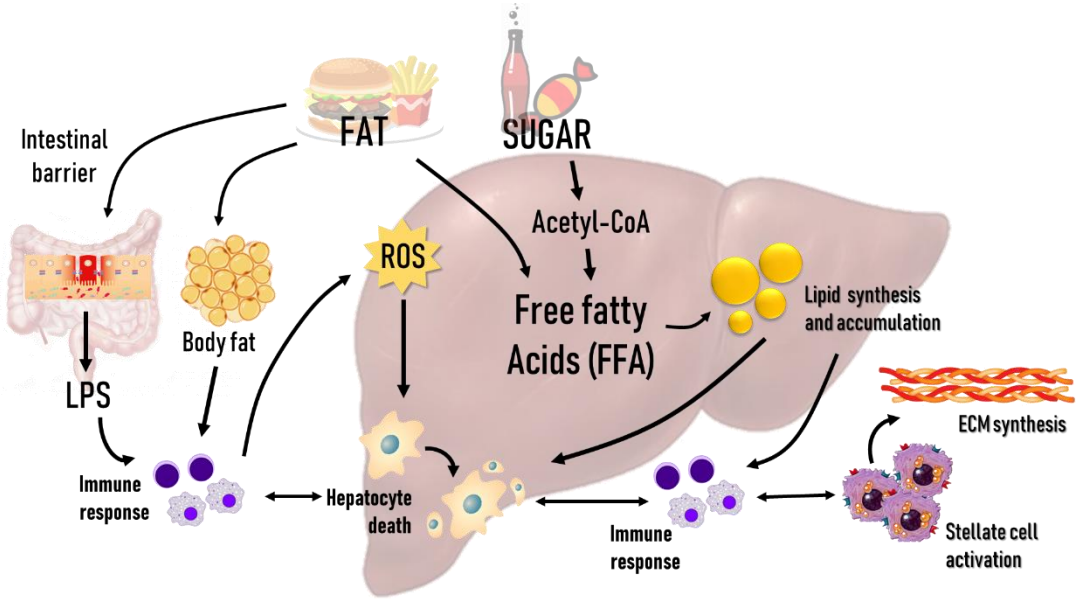

Figure 8. The main western diet-related mechanisms on the induction on alcoholic liver disease (NAFDL), another rising risk condition for HCC in high income countries. ROS = reactive oxygen species; LPS = lipopolysaccharide (Vonghia & Francque, 2015; Federico et al., 2016; Jensen et al., 2018; Kutlu et al., 2018).

The emergence of HCC, which is a complex and stepwise process, so called hepatocarcinogenesis, involves the progressive accumulation of molecular and morphological alterations under the mechanisms of the above cited risk factors. The cirrhotic background (up to 90% of HCC cases) and the metabolic alterations (as observed in ALD and NAFLD), confer "tumor-promoting inflammation" and "deregulated energetics" features to hepatic microenvironment, predisposing to molecular changes. The multistep sequence of (epi)genetic alterations disrupts core cellular functions, favoring the acquisition of sustained cell proliferation, immortality, genomic instability and mutation, migration and invasion, angiogenesis traits, known as hallmarks of cancer (Figure 9). Gradually, these features favor the development of benign hepatocellular lesions, as dysplastic nodules, HCC and metastatic tumors (Figure 9).

In humans, altered hepatocyte foci (AHF), microscopic alterations composed of 20-30 hepatocytes, are frequently observed in cirrhotic livers (around 68% of patients) (Su & Bannasch, 2003). These lesions display different phenotypes: small cell change (presenting decreased nuclear and cytoplasm ratio) or glycogen-storing foci (GSF) (Su & Bannasch, 2003; Libbrecht et al., 2007). Although similar alterations are frequently observed in experimental HCC mouse models, the relevance of AHF as early alterations in human hepatocarcinogenesis, as well as the importance of their screening, is yet to be elucidated (Su & Bannasch., 2003; Libbrecht et al., 2007). Generally, the earliest hepatocellular lesion with real clinical importance for human hepatocarcinogenesis develops in cirrhotic livers as macroscopic dysplastic nodules (DN). Further classified into low or high-grade DN, some of these lesions harbor Telomerase reverse-transcriptase (*TERT)* promoter mutations (~25%) (Nault et al., 2013). *TERT* gene encodes telomerase enzyme, responsible for telomere length maintenance. This activating mutation enables unlimited telomere stability, conferring the immortalization feature to DN (Nault et al., 2013; 2014). *TERT* mutations are also observed in HCC (comprising 44% of cases, considered the most common genetic alteration), whereas other DN-related mutations are not so frequent in this malignancy (Nault et al., 2014; Cancer Genome Atlas Research Network, 2017). For this reason, DNs are considered premalignant conditions and *TERT* mutations are predictive biomarkers of liver neoplastic transformation (Nault et al., 2014). Interestingly, HCC features not only mutations, but also *TERT* amplification is observed in 10% of HCC cases (Cancer Genome Atlas Research Network, 2017).

Among other important genetic alterations in HCC, there are inactivating mutations in tumor suppressor genes tumor protein (*TP53)* (31%), axin 1 (*AXIN1)* (8%), and RB transcriptional corepressor 1 (*RB1)* (4%) (Cancer Genome Atlas Research Network, 2017). Activating mutations on catenin beta 1 (*CTNNB1*) (27%) oncogene are also recurrent (Cancer

Genome Atlas Research Network, 2017). The widely-known *TP53* gene, that translates the most extensively studied protein in the solid tumors, has crucial roles on inducing cell cycle arrest (Cdkn1a and Gadd45a), DNA repair (Gadd45a) and apoptosis (BH3 domain-only proteins Puma, Noxa, Bad, Bax, and Bak)-related proteins (Chen, 2016). Thus, mutations on *TP53* may impair these cellular processes, contributing to the acquisition of "evasion of growth suppressors", "genomic instability and mutation" and "resistance to cell death" hallmarks, correspondingly. Besides *TP53* mutations, the hypermethylation of O6-methylguanine-DNA methyltransferase (*MGMT*) promoter (39% of cases) is also proposed to hinder DNA repair and predispose to genomic instability (Zhang et al., 2003; Cancer Genome Atlas Research Network, 2017).

AXIN1 and *APC* are negative regulators of the pro-proliferative Wnt/β-catenin pathway (Zhan et al., 2017). In the off-state of this pathway, β-catenin, which a transcriptional co-regulator, is phosphorylated, ubiquitinated and led to degradation by APC/Axin/GSK-3β-complex. In the presence of Wnt ligand (on-state), GSK-3β is displaced from APC/Axin complex, leading to β-catenin accumulation and translocation to the nucleus, where it binds to LEF/TCF transcription factors (Zhan et al., 2017). LEF/TCF/β-catenin promotes the expression pro-proliferative genes, including *Myc* and Cyclin D1 (*CCND1*) (Zhan et al., 2017). Deactivating *AXIN1* and activating *CTNNB1* mutations result on intrinsic activation of Wnt/β-catenin pathway in HCC, conferring "sustained cell proliferation" hallmark to this malignancy (Zhan et al., 2017). Aberrant methylation profile is also related to Wnt/β-catenin pathway, since increased hypermethylation of *APC* promoter is observed in 63.1% of HCC cases (Csepregi et al., 2008; Zhang et al., 2016). Despite of being quite heterogeneous, epidermal growth factor receptor (EGFR) overexpression is observed 32–66% of HCC tissues (Buckley et al., 2008; Panvichian et al., 2015). In addition, *EGFR* extra copy numbers are present in 45% of cases (Buckley et al., 2008). EGFR is transmembrane tyrosine kinase receptor activated by epidermal growth factor (EGF) and transforming growth factor α (TGF-α) ligands (Wee & Wang, 2017). Bindings results in EGFR dimerization and activation of downstream signaling pathways, including Ras-Raf-MAPK and phosphatidylinositol 3-kinase (P13K)/protein-serine/threonine kinase Akt, involved in cell growth, proliferation and survival (Wee & Wang, 2017). In HCC, increased EGFR signaling is proposed to contribute to "sustained cell proliferation hallmark", as well as Wnt/β-catenin pathway.

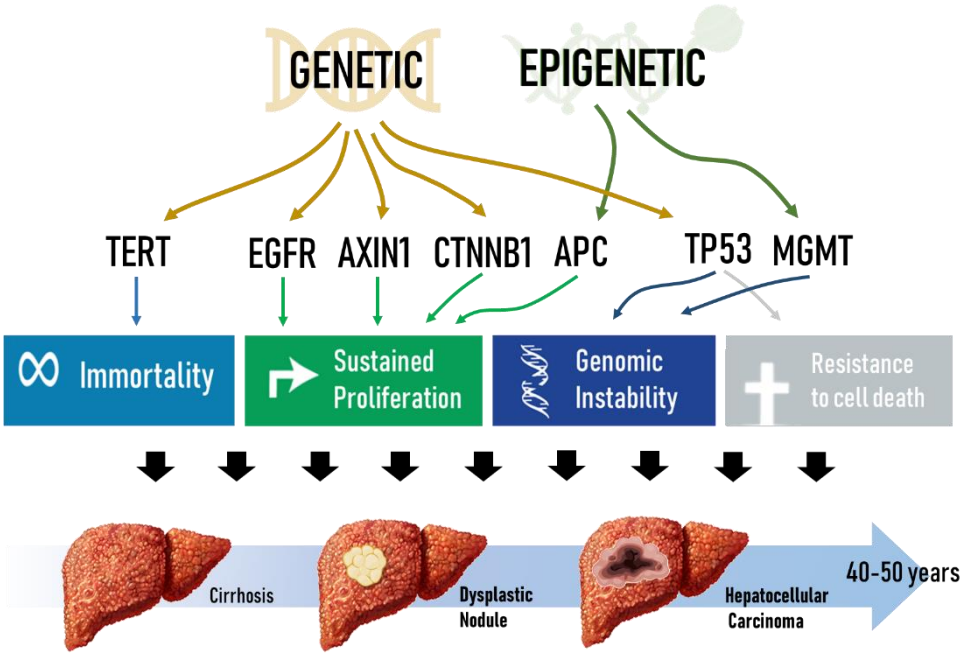

Figure 9. The main (epi)genetic alterations observed during human hepatocarcinogenesis. The multistep sequence of molecular alterations disrupts core cellular functions, favoring the acquisition of the hallmarks of cancer. Gradually, these traits favor the development of benign hepatocellular lesions and HCC (Zhang et al., 2003; Buckley et al., 2008; Nault et al., 2013; 2014; Panvichian et al., 2015; Cancer Genome Atlas Research Network, 2017).

In order to elucidate the molecular and morphological events involved in complex stepwise hepatocarcinogenesis, and to investigate preventive and therapeutic alternatives to HCC, a myriad of *in vitro* and *in vivo* models is applied in literature (as revised by Bakiri et al., 2013 and Hirschfield et al., 2018). The *in vitro* study of the multistage hepatocarcinogenesis process involves both primary cultures of hepatocytes and well-established HCC cell lines (Arellanes-Robledo et al., 2017; Hirschfield et al., 2018). By using primary hepatocyte cultures of human or animal origin, it is possible to understand the cellular and molecular events involved in malignant transformation, as genomic instability, cell proliferation and apoptosis (Arellanes-Robledo et al., 2017). On the other hand, a great number of human HCC cell lines have been established for the study of HCC progression stage (Arellanes-Robledo et al., 2017; Hirschfield et al., 2018). These cell lines have proved to be a useful tool in biomedical research, enabling the development of pharmacological therapies and screening of toxic compounds (Arellanes-Robledo et al., 2017; Hirschfield et al., 2018). Nonetheless, monolayer homotypical cell culture using HCC cell lines (Figure 10) lacks molecular and microenvironmental heterogeneity as observed in the corresponding human disease. Among all cell lines used, HepG2 (ATCC® HB-8065™),

which is derived from a 15-year-old American boy, has been the most used HCC *in vitro* model from 2000 to 2016, followed by Huh7, taken from a 57-year-old Japanese male (Arellanes-Robledo et al., 2017).

However, cell line heterogeneity is a limitation, since some of them harbor punctual resemblances with human HCC, while lacking many other molecular characteristics. The *CTNNB1* mutation, frequently observed in human HCC, was only observed in SNU-398 and C3A (Figure 10) cell lines (Hirschfield et al., 2018). SNU-398, Huh7 and HepG2 displayed *TERT* promoter mutations (Hirschfield et al., 2018). In addition, Huh7 also showed *TP53* mutation, while SNU-398, C3A and HepG2 did not. SNU-449 features *AXIN1* and *TP53* mutations while lacks *TERT* mutation (Hirschfield et al., 2018). Moreover, both SNU-398 and -449 present part of HBV genome, indicating insertional mutagenesis, which is not featured in Huh7, C3A and HepG2 (Hirschfield et al., 2018). Some cell lines, as HepG2 and its clonal derivative C3A, display HCC clinical characteristics such as increased serum α-fetoprotein (AFP) levels in the medium (Hirschfield et al., 2018). In order to mimic tumoral complexity and microenvironment, 3D heterotypical cultures using the above cited cell lines have been established (Figure 10) (Abu-Absi et al., 2004). Hepatocytes cultured on moderately adhesive surfaces or in suspension aggregate to form spheroids. In order to mimic a functional liver, tumoral cells are usually co-cultivated fibroblast-like cells, including the activated HSC (as human LX-2 and rat HSC-T6) and fibroblasts (as mouse NIH/3T3) (Abu-Absi et al., 2004; He et al., 2018; Khawar et al., 2018). Mixed cell 3D cultures systems sometimes contain three different cell types, as hepatocytes, HSC and macrophages (Prestigiacomo et al., 2017). In comparison to homotypical spheroids, mixed-cell spheroids display enhanced expression of collagen I and pro-fibrotic factors such as, TGF-β1 and CTGF (Khawar et al., 2018). In order to add microenvironmental complexity to spheroid model, some protocols are based on the co-activation of the fibroblast-like cells by treating the spheroids with pro-inflammatory LPS, IL-6, PDGF or TGFβ (Prestigiacomo et al., 2017; Ouchi et al., 2019; Pingitore et al., 2019). These examples of heterotypical 3D culture models, also called heterospheroids, are well-accepted models for the study of inflammation/fibrosis-associated-HCC (Figure 10). Recently, *in vitro* models of NASH-associated HCC have been established, using 3D heterotypical spheroid backbone, by adding FFA in the medium (Ouchi et al., 2019; Pingitore et al., 2019) (Figure 10). In comparison to non-exposed spheroids, FFA-treated ones accumulate of fat and exacerbate collagen synthesis (Ouchi et al., 2019; Pingitore et al., 2019).

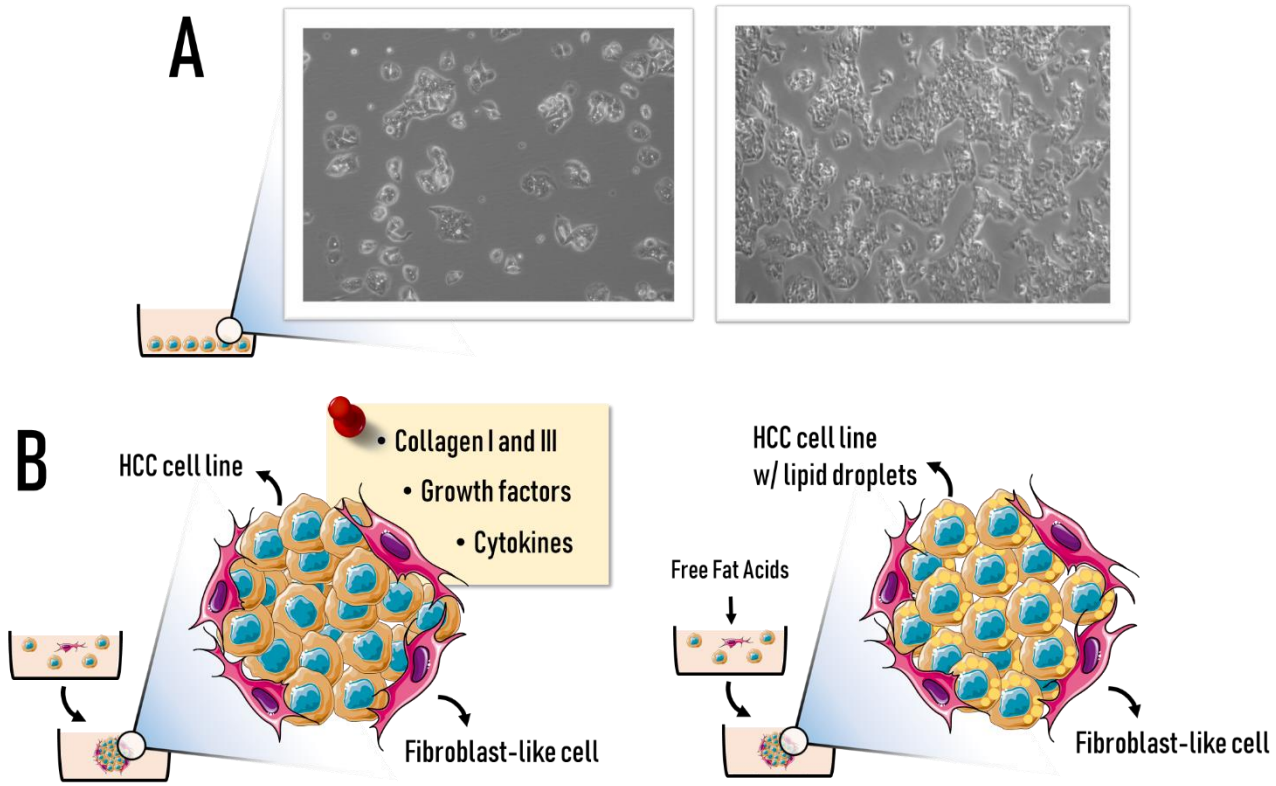

Figure 10. (A) Example of homotypical monolayer cell culture in low and high densities of C3A cell line (ATCC® CRL-10741™), a clonal derivative of HepG2, the most applied HCC cell line in research (Arellanes-Robledo et al., 2017). **(B)** Graphical examples of heterotypical 3D cell culture systems with HCC and fibroblast-like cells, applied as fibrosis-associated or NASH-associated HCC models (Khawar et al., 2018; Pingitore et al., 2019)

In addition to the *in vitro* bioassays, a relatively large number of rodent models to study hepatocarcinogenesis are presently available and can be organized as follows: (1) chemically-induced, (2) xenograft, (3) viral, and (4) genetically engineered mouse (GEM) models (Bakiri et al., 2013). Chemically-induced bioassays rely on the administration of genotoxic and non-genotoxic compounds that lead to the development of hepatocellular preneoplastic and malignant neoplastic lesions in mice (*Mus musculus*) and rats (*Rattus norvegicus*) (Heindryckx et al., 2009; Bakiri et al., 2013). Some of these preclinical bioassays present striking morphological and molecular similarity to the corresponding human disease, presenting potential translational application (Petrelli et al., 2014; Romualdo et al., 2017). Nevertheless, the greatest disadvantages on choosing these bioassays are the methodological inconsistency (*e.g.* different chemical compounds,

doses, frequencies of administration, etc.) and mouse inter-strain variability (less or more susceptible) (Heindryckx et al., 2009; Bakiri et al., 2013). Due to these reasons, literature lacks standard chemically-induced preclinical rodent models.

Among the most used chemicals in these models, diethylnitrosamine (DEN), also called N-nitrosodiethylamine (NDEA), is a N-nitrosamine naturally found in tobacco and cured meat (Scanlan, 1983) (Figure 11). The daily human ingestion of N-nitrosamines is around 0.5 to 1.0 µg (Scanlan, 1983). DEN holds the "Group 2A: probably carcinogenic to humans" classification according to the International Agency for Research on Cancer (IARC, 2019). DEN hepatocarcinogenic properties were first described in the 60's, when administered to rats and golden hamsters in drinking water (Dontenwill & Mhor, 1961; Thomas, 1961). In more recent protocols, DEN is usually administered intraperitoneally to rats or mice, in single or multiple doses (Romualdo et al., 2017; 2018). After DEN injection, its biotransformation happens in the liver by cytochrome P450 (CYP) 2E1, generating ROS and carbonium ions (Verna et al., 1996; Jin et al., 2007) (Figure 11). These highly reactive metabolites bind to biomolecules, including the DNA. DEN-induced DNA alkylation or oxidation predispose to DNA damage, genomic instability and mutation (Verna et al., 1996). Although the exact molecular alteration caused by DEN in hepatocytes is not known, DEN is considered a complete carcinogen that leads to the development of preneoplastic and malignant hepatocellular lesions (Verna et al., 1996) (Figure 12). DEN is frequently given to rodents in their neonatal (mice) or adult phases (rats and mice) (Vesselinovitch et al., 1984; Romualdo et al., 2017; 2018). The main advantage of using neonatal protocol in mice is the hepatic postnatal (PND) development context (Vesselinovitch & Mihailovich, 1983; Septer et al., 2011). Compared to adult liver, proliferation rates are increased in the liver of neonatal mice (Septer et al., 2011). When given to neonatal mice at the 15-20 PND, the pro-proliferative hepatic context promotes the clonal expansion of DEN-initiated hepatocytes, ultimately favoring hepatocellular (pre)neoplastic lesion development and shortening the time for HCC development (Vesselinovitch & Mihailovich, 1983).

Furthermore, inter-strain mouse susceptibility also interferes with the outcomes of chemically-induced hepatocarcinogenesis. In both adult or neonatal mice models, C3H/HeJ mice presents increased susceptibility to DEN-induced regimens compared to other commonly applied strains, as C3B6F1 (intermediate susceptibility), C57BL/6J and BALB/c (decreased susceptibility) (Lee et al., 1989; Weghorst et al., 1989). Although the molecular basis for liver tumor susceptibility have not been fully investigated, several loci that influence susceptibility and resistance have been mapped (Maronpot., 2009). The greater susceptibility of the C3H/HeJ strain is mostly attributed to hepatocarcinogenicity sensitivity 7 (*Hcs7*) locus, which promotes hepatocyte growth and proliferation, especially in preneoplastic lesions (Bilger et al., 2004; Maronpot, 2009). Indeed, due to this genetic feature, C3H/HeJ mice spontaneously develop HCC in the absence of

carcinogen in a long latency time (up to 76 weeks) (Connor et al., 2018). Nonetheless, even in neonatal mouse models using susceptible strains, a long latency time is necessary for these lesions to achieve high incidence and multiplicity (up to ~40 weeks, in a strain-, protocol-dependent manner) (Bakiri et al., 2013). Therefore, in order to reduce the experimental time, DEN is applied as genotoxic "initiator" and usually combined with another drug or surgical process (as 2/3 partial hepatectomy) as a "promoter" (Bakiri et al., 2013) (Figure 12). The promoter's main goal is to establish a context that favors the clonal expansion of DEN-initiated hepatocytes (Bakiri et al., 2013) (Figure 12).

One of the most applied promoters is carbon tetrachloride (CCl_4) (Uehara et al., 2013; Chappell et al., 2014; Romualdo et al., 2018) (Figure 11). Although DEN intraperitoneal administrations lead to the development of hepatocellular (pre)neoplastic lesions, these models lack the fibrosis/cirrhosis scenario observed in up to 90% HCC cases (Yang et al., 2011). Thus, the use combined DEN + CCl_4 regimen is suitable for the study of fibrosis- or cirrhosis-associated hepatocarcinogenesis process (Uehara et al., 2013; Chappell et al., 2014; Romualdo et al., 2018). When intraperitoneally administered to rats and mice, this haloalkane is majorly metabolized by CYP2E1 (CYP2B1, CYP2B2 and CYP3A also play minor roles) to form the highly reactive trichloromethyl ($*CCl_3$) and trichloromethyl peroxyl ($*OOCCl_3$) radicals (Weber et al., 2003) (Figure 11). This radical, as well as ROS generated by increased CYP2E1, can bind and damage to biomolecules, as lipids, proteins and nucleic acids (Weber et al, 2003). Lipid peroxidation reactions, initially mediated by $*OOCCl_3$, damage and increase the permeability of mitochondrial, endoplasmic reticulum, and plasma membranes (Weber et al, 2003). Protein oxidation impairs enzyme activity, while RNA damage may reduce protein synthesis (Weber et al, 2003). This context predisposes to hepatocyte death, triggering inflammatory response (Weber et al, 2003). Oxidative stress, cell death and inflammatory mediators are the stimuli for HSC activation and, ultimately, liver fibrosis (Weber et al, 2003) (Figure 11). Multiple intraperitoneal CCl_4 administrations, in unique or increasing doses, mimic cycles of viral replication and liver damage in the corresponding human disease (Uehara et al., 2013; Chappell et al., 2014; Romualdo et al., 2018). The establishment of a pro-inflammatory and pro-fibrotic background is thought to promote DEN-initiated hepatocytes (Uehara et al., 2013; Chappell et al., 2014; Romualdo et al., 2018). Growth factors and cytokines produced by immune cells during chronic inflammatory response may have paracrine mitogenic effects on (pre)neoplastic hepatocytes, inducing focal lesion growth (Uehara et al., 2013). Indeed, the combination of DEN initiation (single dose in neonatal mice) and CCl_4 promotion (twice a week for 14 weeks, starting at 8 weeks of age) regimens increased by 50% the incidence of HCC compared to DEN-initiated mice, suggesting an acceleration of HCC development (Uehara et al.,

2013). Even though CCl_4 is applied as a promoter, adduct formation between $*CCl_3$ and DNA may also function as initiator, leading to HCC development in a protocol-dependent manner (Weber et al., 2003; Uehara et al., 2013; Chen et al., 2015)

Other chemical promoters are recurrently used in models, as the non-fibrogenic phenobarbital (PB) and the fibrogenic thioacetamide (TAA). The mechanism regarding PB promotion is not fully elucidated, but its administration in low doses in drinking water or diet positively selects hepatocytes harboring the activating mutations of the *Ctnnb1* gene, which lead to activated β-catenin signaling (Aydinlik et al., 2001). For this reason, some authors denominate PB as a "tumor selector" rather than a promoter (Braeuning & Schwarz, 2016), since *Ctnnb1* mutations are not frequent in protocols using only DEN (Connor et al., 2018). Similar to CCl_4, TAA undergoes metabolic activation by CYP2E1, generating S-oxide (TASO) and S,S-dioxide (TASO(2)) compounds that sequentially exert biomolecule damage, cell death, inflammatory response, HSC activation, ECM synthesis and fibrosis/cirrhosis in a protocol-dependent manner (Hajovsky et al., 2012; Crespo et al., 2016; Romualdo et al., 2017). The use of fibrogenic promoters in chemically-induced models result on remarkable molecular and resemblances with human fibrosis/cirrhosis-associated HCC (Romualdo et al., 2017), usually caused by chronic viral infections. For ALD- and NAFLD- associated HCC bioassays, DEN initiation protocols are commonly combined with dietary interventions. For ALD models, Lieber-DeCarli (~5% of ethanol) liquid diet is usually given to rodents after DEN initiation, promoting β-catenin nuclear translocation and mRNA upregulation of its downstream targets (Mercer et al., 2014). Concerning NASH, recent models using "western diet" approach promote HCC in a context of progressive lipid accumulation, lipotoxicity, HSC activation and fibrosis (Kishida et al., 2016; Tsuchida et al., 2018). Mouse models frequently use high fat diet formulations (20-60% of fat) and fructose/glucose mix (55/45%) in drinking water (Kishida et al., 2016; Tsuchida et al., 2018). The combination of CCl_4 repeated applications and western diet was also applied for this purpose in order to establish a NASH context experimentally (Tsuchida et al., 2018)

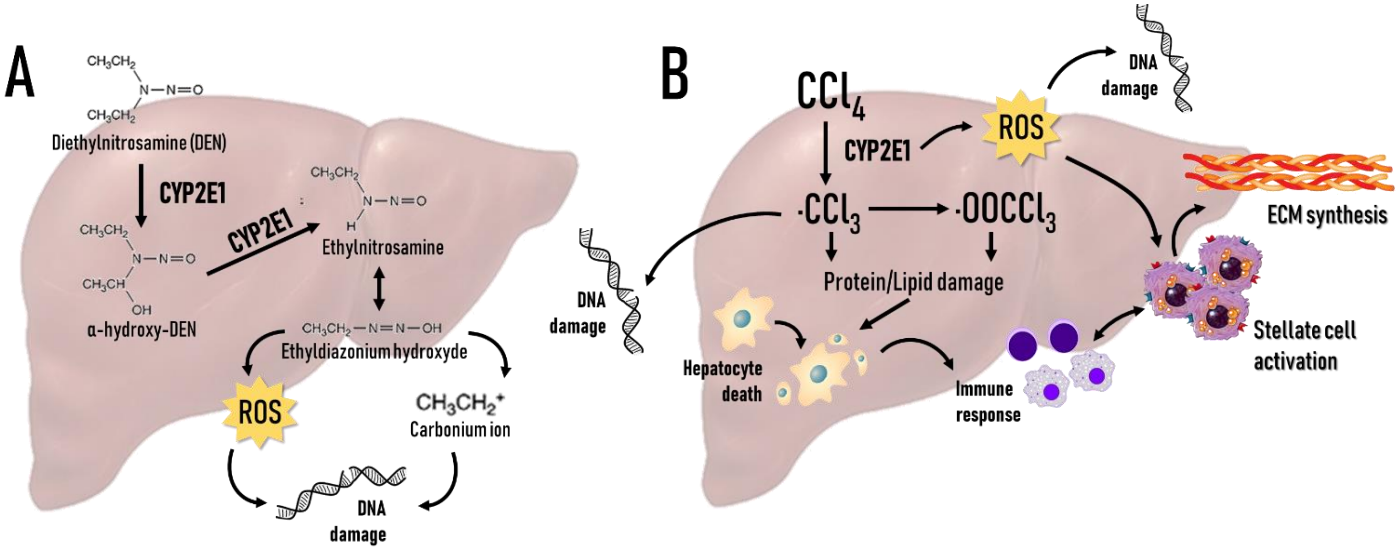

Figure 11. (A) Diethylnitrosamine (DEN) and **(B)** carbon tetrachloride (CCl_4) liver metabolism and their cellular outcomes (Verna et al., 1996; Weber et al., 2003). $*CCl_3$ = trichloromethyl radical; $*OOCCl_3$ = trichloromethyl peroxyl radical; ROS = reactive oxygen species.

The first and smallest morphologically recognizable lesion in chemically-induced models of hepatocarcinogenesis in mouse are AHF (up to ~20 weeks, in a strain- and protocol-dependent manner). The gold standard for AHF identification is the microscopic screening in Hematoxylin and Eosin (HE)-stained sections (Thoolen et al., 2010). Foci present clear phenotypical variations and are usually classified as basophilic, eosinophilic or clear cell foci according to the tinctorial characteristic of most hepatocytes (Thoolen et al., 2010) (Figure 13). Basophilic cell foci are composed of smaller cells than normal hepatocytes, and cytoplasm exhibits distinct basophilia due to free ribosomes or rough endoplasmic reticulum (Thoolen et al., 2010). Both clear and eosinophilic cell foci are composed of enlarged cells due to glycogen storage in excess (Thoolen et al., 2010). Strong eosinophilia may result from enhanced smooth endoplasmic reticulum, peroxisome, or mitochondria (Thoolen et al., 2010). These phenotypes seem not occur at random considering that these lesions are theorized to undergo a "metabolic turnover" (Figure 13). At first, DEN increases insulin growth factor II (IGF-II) levels, and IGF-II downstream signaling decreases glucose-6-phosphatase (G6Pase) activity, promoting the emergence of glycogen storage phenotypes (Lahm et al., 2002; Bannasch et al., 2003). IGF signaling also promotes ras-, raf-, mitogen activated signaling cascade, enhancing cell proliferation (Bannasch et al., 2003). Progressively, foci shift from anabolic to catabolic glucose metabolism in order to provide energy for the increasing cell proliferation, giving rise to basophilic phenotype (Bannasch et al., 2003) (Figure 13). Along with the "deregulated energetics" hallmark, some of AHF display *Hras* (15% of G6Pase-negative foci) and *Braf* (97.3% of basophilic foci) oncogene mutation (Buchmann et al., 1989; Yamamoto et al., 2017). These mutations may provide a proliferative and growth advantage to these lesions (Buchmann et al., 1989; Yamamoto et al., 2017). Although these molecular alterations are not common in human HCC, and considering that the importance of similar AHF in human hepatocarcinogenesis is not elucidated, mouse AHF are considered putative preneoplastic lesions in chemically-induced models and predisposed to neoplastic progression under adequate promoting stimuli (Figure 12) (Buchmann et al., 1989; Bannasch et al., 2003; Yamamoto et al., 2017). The screening of these lesions enables carcinogenicity testing during the early stages of hepatocarcinogenesis, avoiding the use of long latency chemically-induced protocols (Klaunig & Kamendulis, 1999; Bannasch et al., 2003, Ogawa, 2009). Indeed, AHF have been extensively used in research for the investigation of the early molecular events of hepatocarcinogenesis, as well as for the screening of preventive/promoting agents (Romualdo et al., 2016; 2017; Cast et al., 2018; Pedersen et al., 2019).

The subsequent molecular events that explain the malignant transformation of AHF are yet to be fully unraveled, but the initiation→ promotion→ progression stepwise theory is widely accepted in literature (Figure 12) (Klaunig & Kamendulis, 1999; Bannasch et al., 2003, Ogawa, 2009). Indeed, recent findings indicate that some hepatocytes of DEN-

induced AHF presenting oncogenic dephosphorylation of CCAAT/enhancer binding protein α (C/EBPα) acquire a "stemness" feature, being classified as potential tumor-initiating hepatocyte (PTIH) (Cast et al., 2018). Similar events were also described in early and late stages of aggressive human HCC, suggesting that the preneoplastic foci with PTIHs are the origin of mouse HCC (Cast et al., 2018). Other findings indicated that over 80% DEN-induced HCC displayed activating hotspot mutations in either *Hras* or *Braf* oncogenic drivers, what could also support that HCC may emerge from AHF (Connor et al., 2018). Activating *Egfr* oncogene mutations (~20%) were also observed, leading to Ras-Raf-MAPK pathway activation, similarly to the corresponding human disease (Connor et al. 2018). Truncating *Apc* mutations occurred in 21% of HCC, supporting a role for deregulation of Wnt/β-catenin signaling DEN-induced hepatocarcinogenesis, as also observed in human HCC (Connor et al., 2018). Chemically-induced models also reflect some of the epigenetic changes observed in humans. In a DEN-induced and CCl_4-promoted fibrosis-associated hepatocarcinogenesis model, the repair-related *Mgmt* gene was also hypermethylated in ~70% of HCC, leading to decreased expression of this repair-related gene (Chappell et al., 2014).

Finally, the establishment of reliable chemically-induced models enabled the translation investigation of the potential mechanisms involved in sexual disparity, since mice models reflect this feature of the corresponding human disease (Romualdo et al., 2018). Using neonatal DEN-induced mouse models, it was observed that androgen receptor (AR) knockout reduced the incidence and the number of tumors in male mice, suggesting the promoting effect of AR on hepatocarcinogenesis (Ma et al., 2008). AR activation may hinder p53-mediated DNA damage sensing/repair system and cell apoptosis, increasing cellular oxidative stress and DNA damage (Ma et al., 2008). In contrast, these models also provided a mechanism for a protective role of estrogen in female (Naugler et al., 2007). Estrogen may attenuate malignant transformation *via* downregulation of IL-6 release from Kupffer cells (Naugler et al., 2007). Other mechanisms are recently proposed, since the effects of sex hormones on lipid metabolism may interfere with HCC development (Greten, 2019).

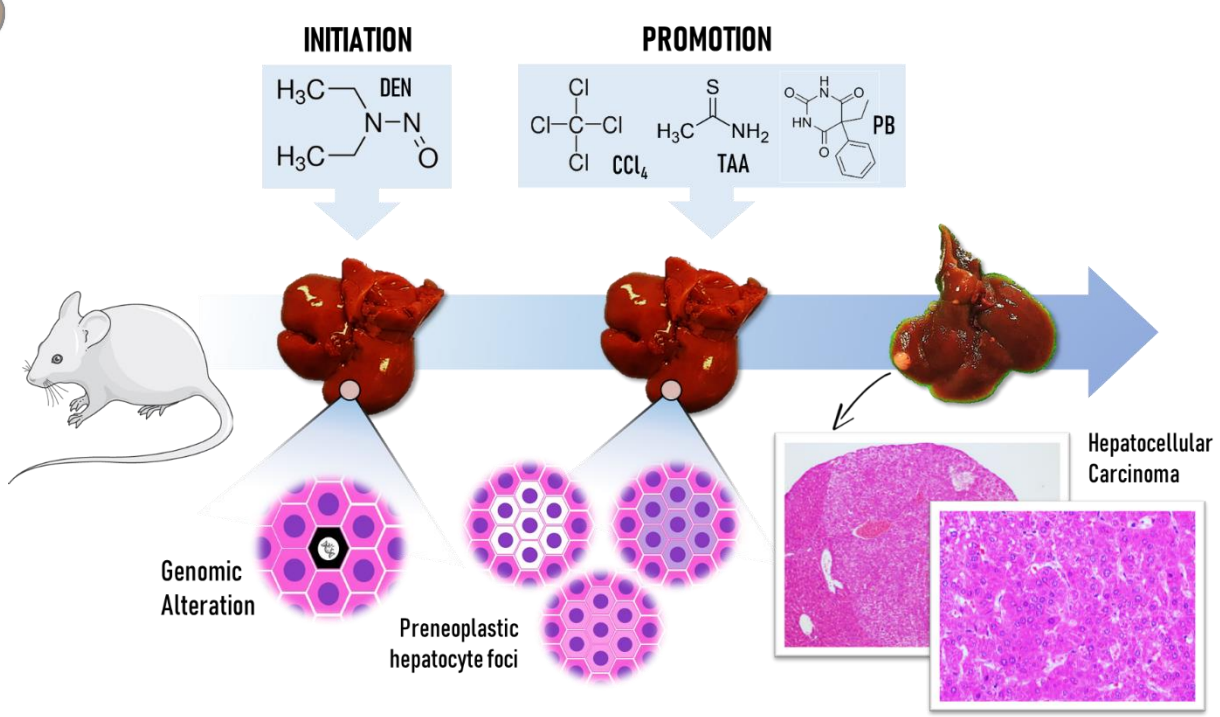

Figure 12. Multistep chemically-induced hepatocarcinogenesis in mice (Klaunig & Kamendulis, 1999; Bannasch et al., 2003, Ogawa, 2009). DEN = diethylitrosamine; CCl_4 = carbon tetrachloride; TAA = thioacetamide; PB = phenobarbital.

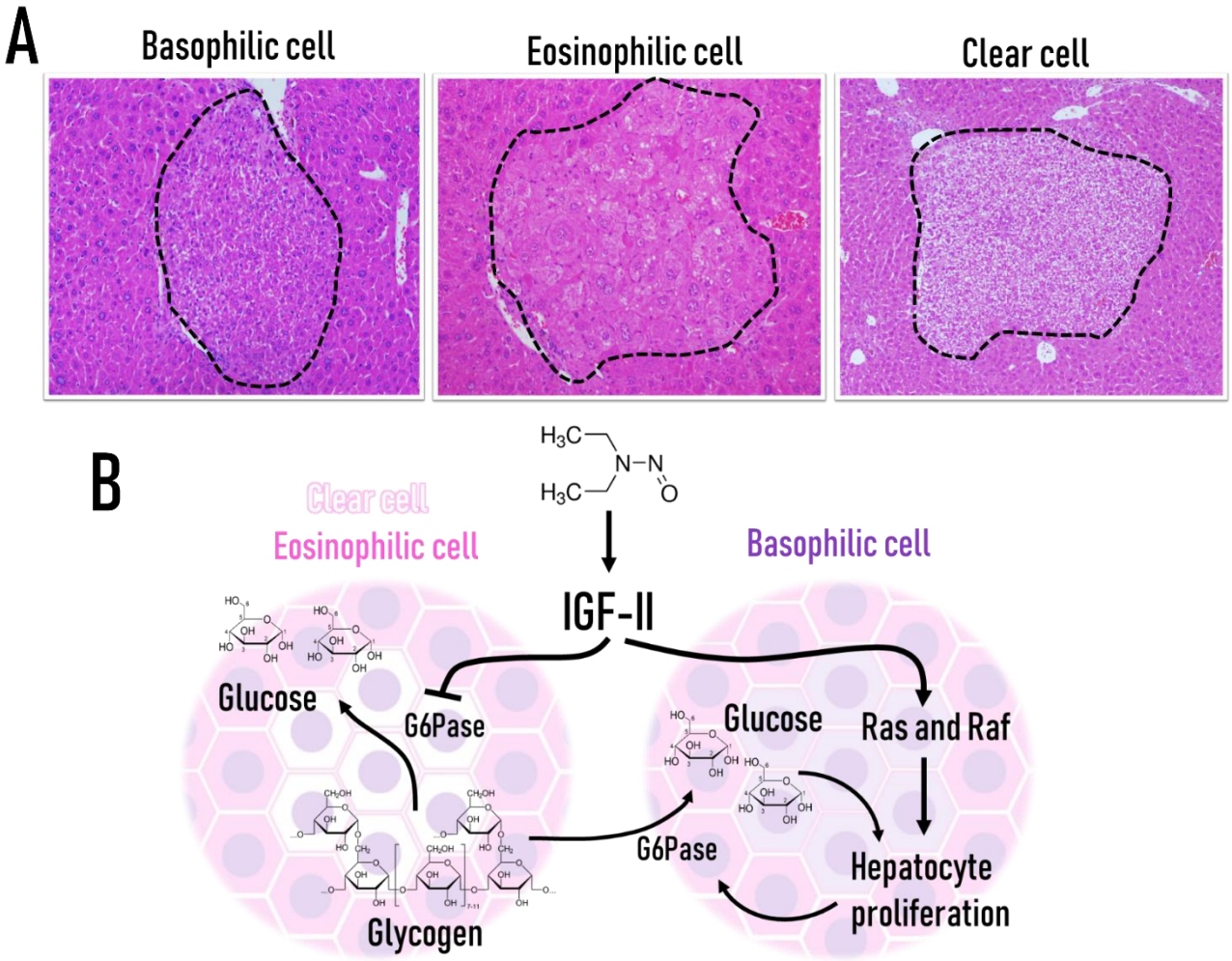

Figure 13. (A) Representative photomicrographs of HE-stained sections showing basophilic, eosinophilic and clear cell preneoplastic altered hepatocyte foci phenotypes found in DEN-treated mice. **(B)** Gradual metabolic shift from anabolic (clear cell and eosinophilic phenotypes) to catabolic (basophilic phenotype) glucose metabolism during DEN-induced hepatocarcinogenesis (Lahm et al., 2002; Bannasch et al., 2003). DEN = diethylnitrosamine; IGF-II =insulin growth factor II; G6Pase = glucose-6-phosphatase.

1.2 miRNAs: small molecules with great responsibilities

MicroRNAs (miRNAs) were first described in 1993 in *Caenorhabditis elegans* as small non-coding RNAs that display an average of 22 nucleotides in length (Lee et al., 1993). At the time of its discovery, lin-4, the first miRNA described, presented antisense complementarity to lin-14 gene, indicating that lin-4 would control lin-14 translation *via* an antisense RNA-RNA interaction (Lee et al., 1993). Three decades later, thousand of miRNAs have been identified (computationally and/or experimentally) in eukaryotes and even in viruses, and there is accumulating evidence on the post-transcriptional gene expression control by these small molecules, demonstrating strong interspecies sequence conservation (Li et al., 2010; De Rie et al., 2017). miRNA synthesis is classified into canonical and non-canonical pathways (O'Brien et al., 2018).

In the first step of canonical miRNA biogenesis (Figure 14), these non-coding RNAs are first transcribed by RNA polymerase II and III (Pol II) as 7-methylguanosine(m^7G)-capped structures with polyadenylated tails, so called primary miRNAs transcripts (pri-miRNAs) (Lee et al., 2004; Borchert et al., 2006). After transcription, pri-miRNAs fold into a double stranded molecule with an apical loop and basal stem, in a duplex hairpin-like structure (Figure 14). Most miRNAs are intragenic and processed within intronic regions of protein-coding genes, whereas others are intergenic, regulated by their own promoter (Kim & Kim, 2007) (Figure 14). Sometimes miRNAs are transcribed together as long polycistronic transcripts called clusters, which are further processed to the individual mature miRNAs (Tanzer & Stadler, 2004) (Figure 14). The clustered miRNAs transcripts harbor complex functional interactions, and around 25% of human and 31% of mouse miRNAs are transcribed from polycistronic precursors (Olive et al., 2015). The mature sequence of the functional miRNA is embedded in a hairpin-like sequence found on the pri-miRNA (Figure 14). Sequentially, still in the nucleus, pri-miRNAs are processed to single hairpin precursor (pre-miRNA) by protein complex called microprocessor (Denli et al., 2004). This protein complex mostly comprises DiGeorge Syndrome Critical Region 8 (DGCR8), which is a double-stranded RNA binding protein, and Drosha, a RNAse III enzyme. DGCR8 is responsible for recognizing the pri-miRNA, while Drosha cleaves the lower stem of the duplex hairpin structure, leaving a hydroxyl group (OH) at 3′ end, and overhangs of 2 nucleotides and one phosphate (P) at 5′ end (Denli et al., 2004; Alarcón et al., 2015) (Figure 14). Of note, 1917, 1.234 and 496 pre-miRNA hairpin sequences are currently annotated in miRBase V22 for human, mouse and rat genomes, respectively (Kozomara et al., 2019).

Then, pre-miRNAs are exported from the nucleus to the cytoplasm by protein complex mainly formed by exportin 5, that interacts with the 2 nucleotide 3' overhang through hydrogen bonds and ionic interactions (Okada et al. 2009)

(Figure 14). Then, pre-miRNAs are processed by RNase III endonuclease Dicer (Denli et al., 2004), that removes the terminal loop of the hair pin-like structure, resulting in a mature miRNA duplex (Denli et al., 2004) (Figure 4). In this doubled stranded (ds) RNA, the direction of the strand determines the name of the mature form: 5p strand comes from the 5' end, while the 3p strand originates from the 3' end. According to miRbase V22, 2654, 2013 and 769 mature sequence entries are found for human, mouse and rat genomes, respectively (Kozomara et al., 2019). Next, one of the strands is loaded on the RNA-induced silencing complex (RISC), which is key process in small RNA-mediated gene expression control (Khvorova et al., 2003). Both strands derived from the mature ds-miRNA can be loaded into the Argonaute (AGO) family of proteins (AGO1-4 in humans), which are core part of RISC (Khvorova et al., 2003). AGO interacts with the miRNA strand through three specific domains: the Piwi–Argonaute–Zwille (PAZ), the middle (MID) and the P-element induced wimpy testes (PIWI) (Nakanishi et al., 2012). The MID and PIWI hold the 5' end, while the PAZ domain binds to 3' end (Nakanishi et al., 2012). Although the process of strand selection by RISC is not fully understood, the proportion of AGO-loaded 5p or 3p strand fluctuates, greatly depending on the cell type or environment (Meijer et al., 2014). Some findings rely on a "strand bias" regarding AGO loading based on thermodynamic stability: the strand with lower 5' end stability or 5' uracil is preferentially loaded into AGO and is denominated the "guide" strand (Khvorova et al., 2003). However, many other structural preferences are proposed (Yoda et al., 2010). Upon AGO selection, the unloaded strand, which is called the "passenger" strand, will be unwound from the guide strand (Khvorova et al., 2003). In general, mature miRNAs are thought to be stable molecules that maintain long half-lives in the organs, including the liver (Gatfield et al., 2009).

The non-canonical miRNA biogenesis encompasses different pathways (Figure 15), majorly divided into Drosha/DGCR8-independent and Dicer-independent pathways (O'Brien et al., 2018). Of note, some of proteins involved in canonical synthesis are also participants of the non-canonical biogenesis. In Drosha/DGCR8-independent pathway, shorter pre-miRNA hairpins are generated from the same long primary transcript than mRNA *via* splicing machinery, that is proposed to substitute Drosha/DGCR8-mediated processing (Ruby et al., 2007) (Figure 15). Next, this pre-miRNA follows the canonical pathway, being exported to the cytoplasm by Exportin 5, cleaved by Dicer complex and loaded in RISC (Ruby et al., 2007). Another example of Drosha/DGCR8-independent biogenesis is the transcription of 7-methylguanosine (m7G)-capped pre-miRNA (Xie et al., 2013) (Figure 15). Since the transcript is already a pre-miRNA, it bypasses nuclear cleavage and is exported to cytoplasm by Exportin 1 (Xie et al., 2013) (Figure 15). After dicing, the 3' strand of the duplex has an advantage on AGO loading due to capped end of 5' strand (Xie et al., 2013). The miR-451 is an example of Dicer-independent biogenesis (Cheloufi et al., 2010). The Drosha processing of this miRNA generates a

short pre-miRNA hairpin that is not recognized by Dicer machinery (Cheloufi et al., 2010). Thus, the hairpin is directly loaded into AGO2 and 5p strand is cleaved by AGO2 catalytic region to generate an intermediate 3', while 3p strand is sliced (Cheloufi et al., 2010) (Figure 15).

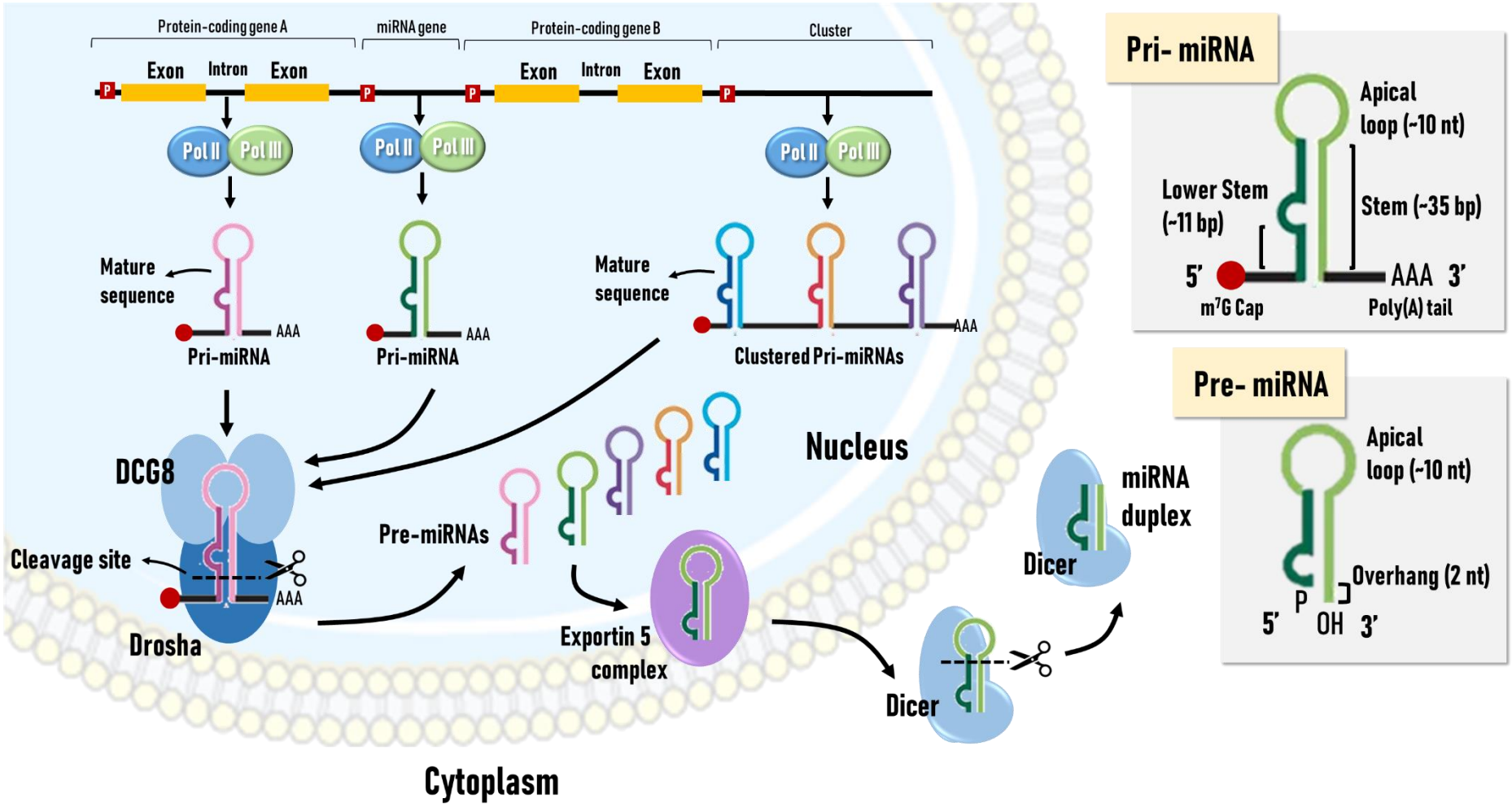

Figure 14. Canonical miRNA biogenesis (Denli et al., 2004; Lee et al., 2004; Borchert et al., 2006; Kim & Kim, 2007; Okada et al. 2009; Alarcón et al., 2015; Olive et al., 2015).

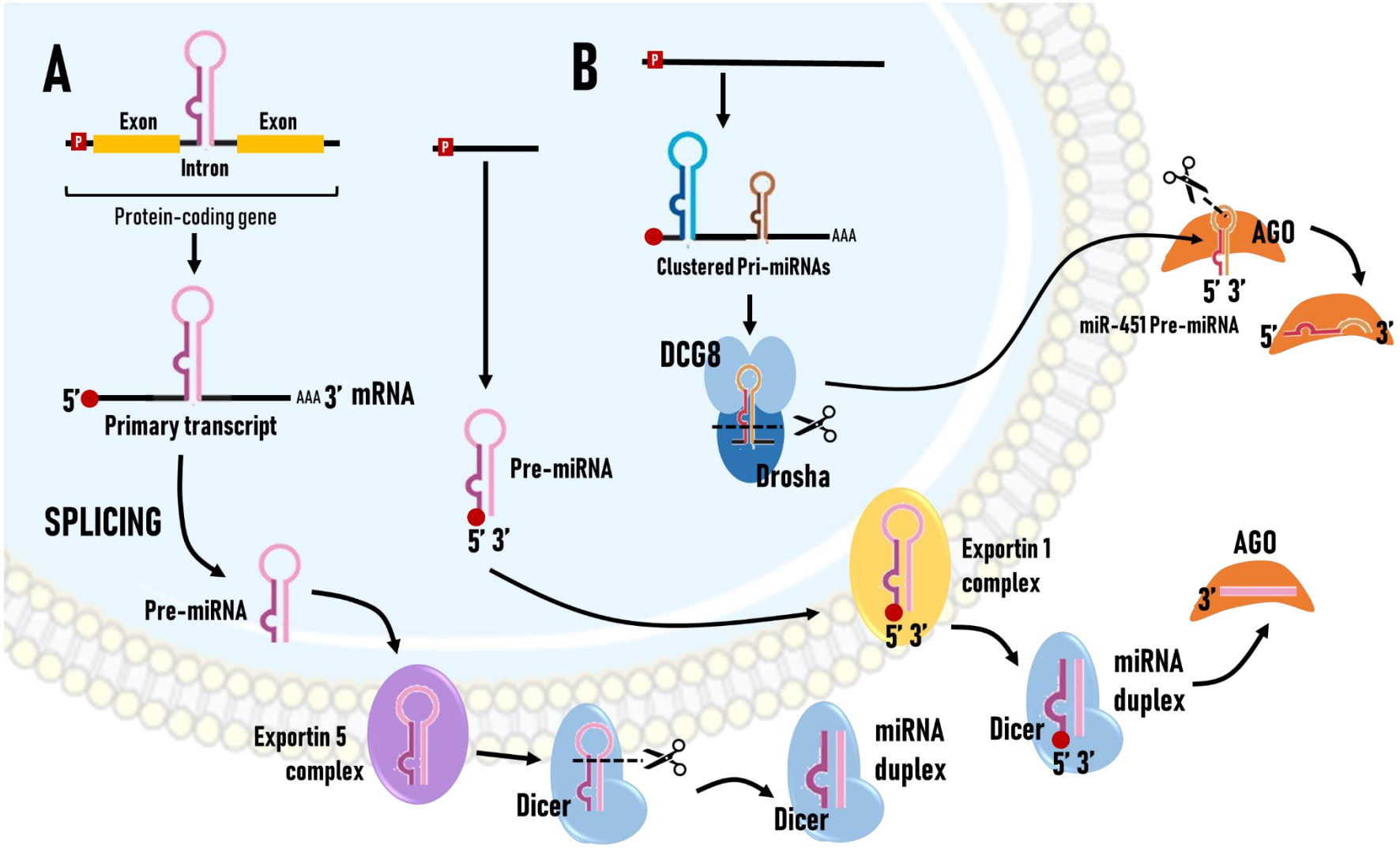

Figure 15. Non-canonical **(A)** Drosha/DCG8-independent and **(B)** Dicer-independent miRNA biogenesis (Ruby et al., 2007; Cheloufi et al., 2010; Xie et al., 2013)

Once loaded in the RISC complex, then denominated miRISC, the mature guide strand is proposed to interact with mRNAs, binding to specific sequences at the 3' untranslated region (UTR) of their target mRNAs (Figure 16). In bioinformatics, this interaction is the main criteria for target-site prediction algorithms. The nucleotides 2–8 from the 5' end of the miRNA are known as the seed, which is crucial for target mRNA recognition and matching (Bartel, 2009) (Figure 16). Once mRNA strand presents the complementarity for this nucleotide sequence (seed matching), it is predicted to be canonical target of the referred miRNA (Bartel, 2009). mRNA complementarity to 2–7 nt or 3–8 nt miRNA seed are much weaker but still considered canonical (Agarwal et al., 2015). This interaction is also supplemented with AGO binding (MID domain) with first nucleotide (nt1) of the target mRNA, preferentially an adenine (Schirle et al., 2015) (Figure 16). Other factors may influence a strong seed binding. These include additional base pairs towards the 3' end of the mature miRNA sequence, as ~13–16 of the miRNA, also called the "supplemental region" (Grimson et al., 2007) (Figure 16). It is predicted that more than 60% of all human protein-coding genes display at least one conserved miRNA-binding site (Friedman et al. 2009). To date, 3' UTR are the most accepted miRNAs seeds, but 5' UTR, coding sequence, and promoter regions are also proposed as potential interaction sites (Xu et al., 2014).

miRNAs encompass complex widespread networks of interactions with mRNA, as briefly depicted in Figure 16. One miRNA may interact with different mRNAs, and individually control entire cellular pathways (Selbach et al., 2008). miRNA clusters are also proposed to control the same cellular pathway (Mestdagh et al., 2010). Moreover, the same mRNA can be co-regulated by different miRNAs (Uhlmann et al., 2010). Some findings indicate cooperative repression of the same target by many miRNAs, what could explain the use of non-canonical interaction sites depending on the vacancy of the canonical ones (Flamand et al., 2017). The current model for post-transcriptional gene suppression occurs through (1) mRNA decay/cleavage and (2) translation repression (Figure 16). Upon seeding, miRISC recruits the glycine-tryptophan protein of 182 kDa (GW182) family of proteins [called trinucleotide repeat-containing gene 6 A/B/C (TNRC6A/B/C)] in humans, such as the poly(A)-deadenylase complex subunits 2 and 3 (PAN2 and PAN3) and carbon catabolite repressor protein 4 (CCR4-NOT), that promote the deadenylation the target mRNA (Behm-Ansmant et al., 2006). This process is initiated by PAN2/3 and completed by the CCR4-NOT complex (Behm-Ansmant et al., 2006). Subsequently, deadenylation stimulates the decapping by the mRNA-decapping enzyme subunit 1 (DCP1)–DCP2 complex (Behm-Ansmant et al., 2006). Next, an interaction between DCP1 and 5'–3' exoribonuclease 1 (XRN1) leads the mRNA to rapid 5'→3' degradation (Braun et al., 2012). In addition, miRISC inhibits the initial steps of translation by releasing initiation factor 4 A-I (eIF4A-I) and eIF4A-II hence inhibiting ribosome scanning and assembly, in a GW182-independent mechanism (Fukao et al., 2014). The mRNA

decay is generally responsible for most of miRNA:mRNA regulatory interactions (~84%), considered the predominant reason for gene silencing and hence reduced protein output (Guo et al., 2010). Some findings indicate that miRNA may exert a “fine tune” effect on gene expression, and the outcomes on reducing protein expression levels are about 20% (Baek et al., 2008). Nonetheless, the exact miRNA-mediated effect on protein levels are difficult to predict since miRNA function is increased on certain physiological and pathological contexts (Mendell & Olson, 2012).

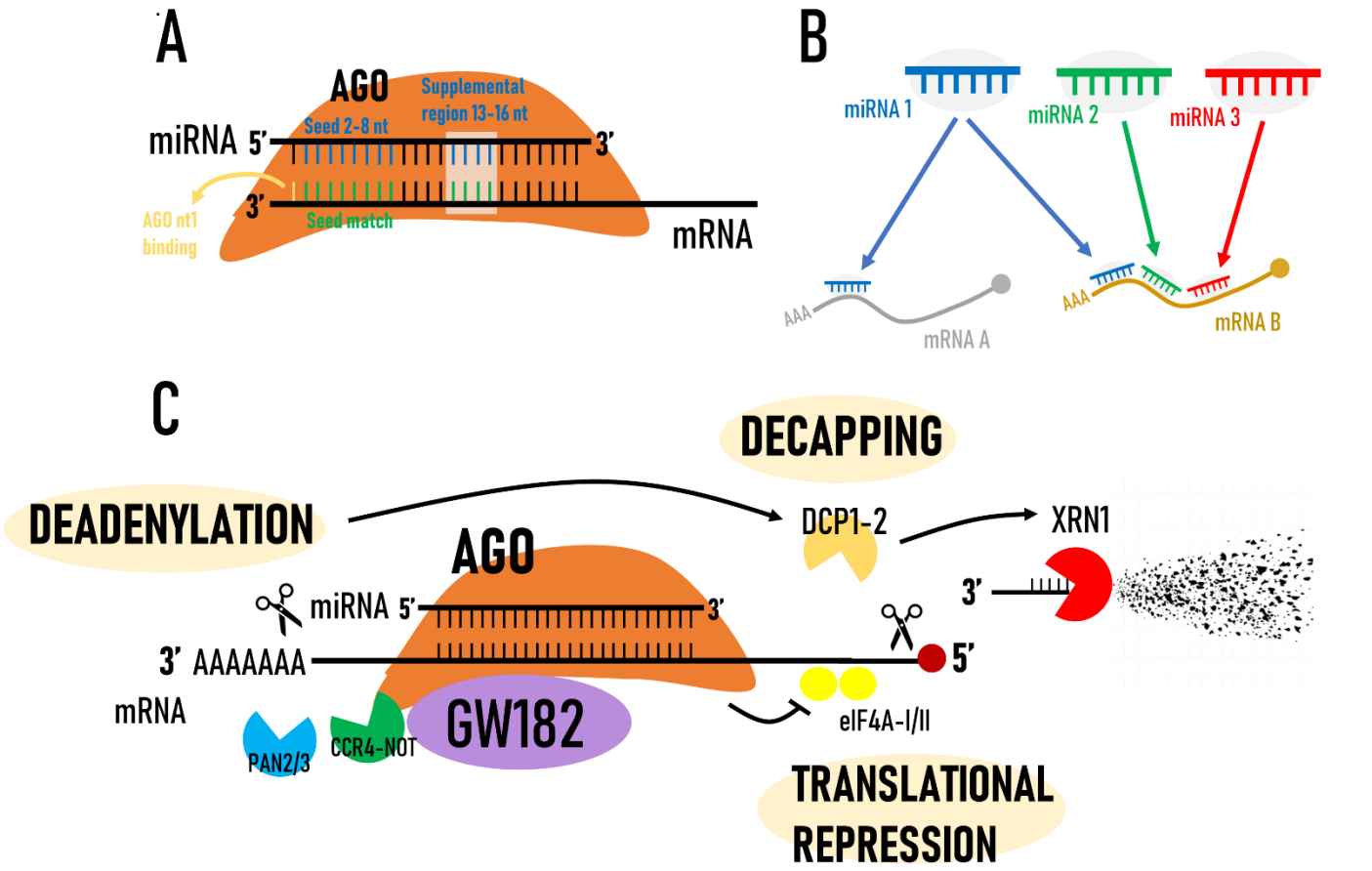

Figure 16. (A) miRNA and mRNA canonical interactions (Grimson et al., 2007; Bartel, 2009; Agarwal et al., 2015; Schirle et al., 2015); **(B)** Potential networks between miRNAs and mRNA (Selbach et al., 2008; Uhlmann et al., 2010); **(C)** miRNA-mediated mechanisms of posttranscriptional mRNA silencing (Behm-Ansmant et al., 2006; Braun et al., 2012; Fukao et al., 2014).

Due to miRNA-mediated gene silencing and outcome on protein expression, these small non-coding RNAs are proposed to participate on many key cellular pathways, contributing to a variety of physiological events (Bartel et al., 2009). On the other hand, deregulation of miRNA expression is associated with numerous diseases, particularly cancer (Xu et al., 2018). In this context, miRNAs can be considered oncogenes (called oncomirs) or tumor suppressors miRNAs, although

overall downregulation of miRNA expression is a hallmark of cancer (Xu et al., 2018). miRNAs may not only participate on tumoral biology, but also contribute with the molecular pathogenesis of preceding risk conditions, as liver fibrosis/cirrhosis (He et al., 2012). In this background, these non-coding RNAs are classified as antifibrotic or pro-fibrotic miRNAs (He et al., 2012) (Figure 17). The screening of deregulated miRNAs, as well as their molecular targets, have been encouraged in the last decades for their potential utility as biomarkers or novel therapeutic targets.

Both up or downregulation of some miRNAs may directly influence many key cellular events during liver fibrosis, mainly including HSC activation, and collagen synthesis and accumulation (He et al., 2012). The miR-29 family members (including miR-29a, miR-29b and miR-29c), so called 'master fibromiRNAs', are the most extensively studied in this HCC risk condition. All members of this family are found to be downregulated in human fibrotic livers and serum samples, being inversely corelated with fibrosis staging (Roderburg et al., 2012). Reduced levels of this miRNA family were also translationally confirmed by using both surgical (bile duct ligation) and chemically-induced (CCl_4-induced) mouse models of fibrosis (Roderburg et al., 2012). Cell isolation revealed miR-29 family is downregulated predominantly in HSC (Roderburg et al., 2012). miR-29 family targets collagen-related genes (*Col1a1*, *Col1a2*, *Col4a5*, and *Col5a3*), and its downregulation leads to collagen synthesis and accumulation, promoting liver fibrosis (Roderburg et al., 2012) (Figure 17). In contrast, the transfection of a miR-29b mimic, increasing miR-29b levels in a HSC cell line, led to decreased expression of these collagen-related genes, evidencing a potential antifibrotic role for miR-29 family upregulation in humans (Roderburg et al., 2012). Further roles of miR-29b on HSC activation were unveiled both *in vivo* (CCl_4-induced model in mice) and *in vitro* (human LX-1 and rat HSC-T6 cell lines), since the upregulation of this miRNA by ultrasound-mediated transfer or transfection decreased phosphoinositide-3-kinase regulatory subunit 1 (*PIK3R1*) and protein kinase B (*AKT3*) targets, responsible for signaling onset of HSCs activation (Wang et al., 2014) (Figure 17). With concern to collagen synthesis, miR-122 appears to negatively regulate this process *via* targeting prolyl4-hydroxylase subunit alpha-1 (*P4HA1*), a gene that encodes a component of prolyl 4-hydroxylase enzyme, responsible for collagen post-translational maturation (Li et al., 2013) (Figure 17). Indeed, miR-122 and *P4HA1* expressions are inversely correlated in human HSC LX-2 cell line and in the liver of CCl_4-treated mice (Li et al., 2013).

Recently, miR-34a-5p was also found to be downregulated in human liver fibrotic samples, human HSC cell lines, as well as in CCl_4-induced liver fibrosis in mice (Feili et al., 2018). This miRNA targets Smad4 mRNA in humans, which is a positive co-regulator of pro-inflammatory TGF-β/Smad2-3 pathway (Feili et al., 2018). In fact, when miR-34a-5p was upregulated by mimic transfection, it reduced the protein levels of Smad4 and, as a result, TGF-β/Smad2-3 (Feili et al.,

2018). Regarding TGF-β pathway, miR-17-5p targets the negative regulator Smad7, contributing to the activation of HSCs (Figure 17). miR-17-5p levels are increased in rat HSCs, human cirrhotic liver and serum samples, as well as in CCl_4-treated rat liver fibrotic tissues (Yu et al., 2015). In contrast, the use of a miR-17-5p inhibitor in rat HSC-T6 and in human serum restored Smad7 protein levels, increasing both mRNA and protein expression of α-SMA and collagen I in HSC-T6 cells (Yu et al., 2015). Another important upregulated miRNA in human HSC LX-2 cell line, human liver samples and liver samples from CCl_4-treated rats and mice is miR-214-3p (Ma et al., 2018). The increased miR-214 inhibits the expression of suppressor-of-fused homolog (Sufu), a negative regulator of the Hedgehog pathway, hence contributing to HSC activation and fibrosis (Ma et al., 2018) (Figure 17). In contrast, the use of an AntagomiR increases Sufu protein levels, ameliorating fibrosis *in vivo* (Ma et al., 2018). Altogether, these and other miRNAs are proposed orchestrate fibrosis-related cellular events thereby contributing to the establishment of a proinflammatory context that promote HCC development (He et al., 2012).

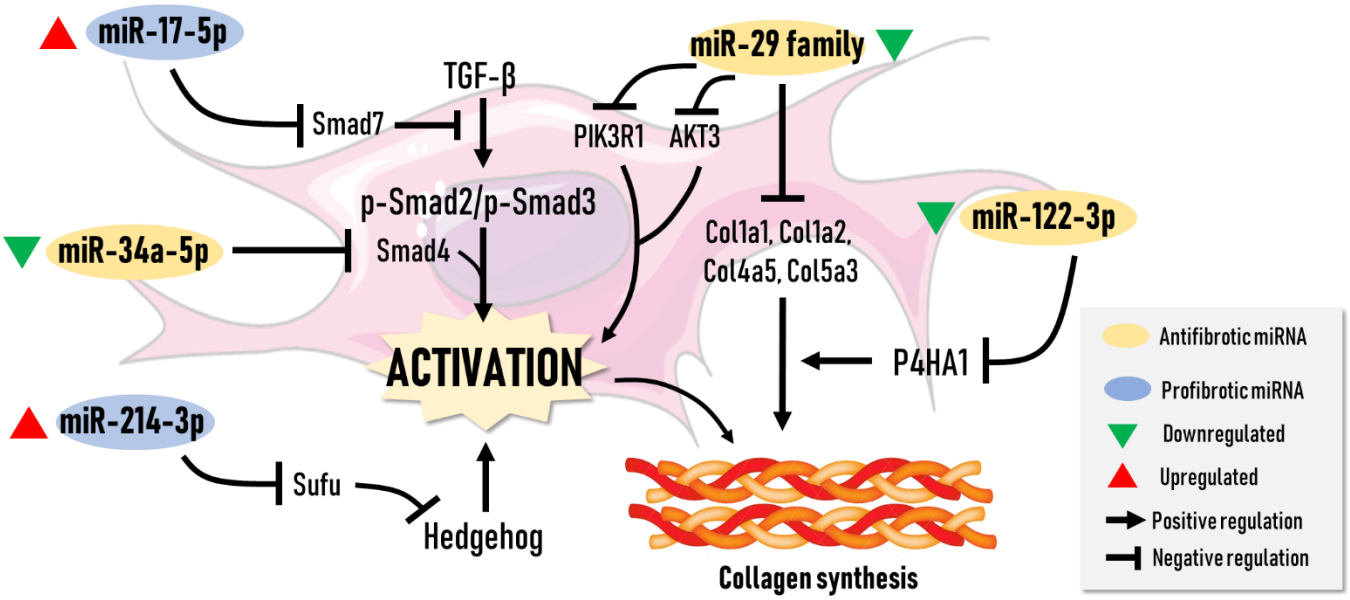

Figure 17. Some of the main miRNAs involved in liver fibrosis and their proposed targets and implications on fibrosis-related outcomes, focusing on hepatic stellate cell activation and collagen synthesis (Roderburg et al., 2012; Li et al., 2013; Wang et al., 2014; Yu et al., 2015; Feili et al., 2018; Ma et al., 2018).

Deregulated miRNA expression is also a common feature of HCC that contributes to the acquisition of key cancer hallmarks, as proliferation, apoptosis, invasion, metastasis and angiogenesis hallmarks (Xu et al., 2018). Some of these miRNAs and their biological properties are depicted in Figure 18. miR-144-3p is among the tumor-suppressor miRNAs with growing number of publications in the last years (Cao et al., 2014; Yu et al., 2016; Bao et al., 2017; Liang et al., 2017; Gu

et al., 2019). The downregulation of this miRNA is widely reported in many human HCC cell lines (HepG2, Huh7 and Hep3B) and samples, being inversely correlated with tumor staging (Cao et al., 2014; Yu et al., 2016; Bao et al., 2017; Liang et al., 2017; Gu et al., 2019). miR-144-3p is proposed to negatively control cell proliferation, migration and invasion hallmarks by directly targeting 3'UTR regions of Cyclin B1 (*CCNB1*) (Gu et al., 2019), *SMAD4* (Yu et al., 2016), E2F transcription factor 3 (*E2F3*) (Cao et al., 2014) and Zinc finger X-chromosomal protein (ZFX) (Bao et al., 2017) in humans (Figure 18). In general, the transfection of miR-144-3p mimics *in vitro* reduced the mRNA and protein of the targets, contributing to reduced cell proliferation, migration and invasion, as well as delaying tumor formation in xenograft models (Gu et al., 2019). The key role of miR-144-3p was also recently described in chemically-induced mouse models of HCC (He et al., 2017). In a neonatal DEN-induced model using susceptible C3H mouse strain, miR-144-3p levels were also decreased in HCC tissue. Intravenous administration of a miR-144-3p mimic decreased protein and gene expression of EGFR, a direct target of this miRNA in the liver, and its downstream Src/AKT signaling, resulting in decreased HCC size (He et al., 2017). Thus, upregulating miR-144 may be a therapeutic strategy to suppress tumor growth in HCC.

Other two key tumor suppressor miRNAs in HCC are miR-195-5p and miR-206 (Wu et al., 2017; Yu et al., 2017) (Figure 18). Both were downregulated in human HCC tissue, and miR-195-5p levels were inversely correlated to metastasis (Wu et al., 2017; Yu et al., 2017). Of note, miR-206 levels were also decreased in human HCC cell lines (HepG2, Hep3B and Huh7) and HCC from both cMyc and V-Akt murine thymoma viral oncogene homolog 1/neuroblastoma RAS viral oncogene homolog (AKT/Ras) transgenic mouse models (Wu et al., 2017). The yes-associated protein 1(YAP) gene expression is controlled by miR-195-5p, while cMET and cyclin-dependent kinase (CDK6) oncogenes are direct targets of miR-206 (Wu et al., 2017; Yu et al., 2017). *In vitro* treatments with miR-195-5p reduced migration and invasion of HCC cells by suppressing YAP (Yu et al., 2017), and *in vivo* miR-206 overexpression prevented HCC growth in cMyc mice by reducing *cMet* and *Cdk6* (Wu et al., 2017).

Although most of deregulated miRNAs in HCC are downregulated, some may function as oncogenes as they are upregulated in tumoral tissue (Xu et al., 2018). The oncomiR miR-155-5p and its direct target tumor suppressor PTEN were found to be upregulated and downregulated, respectively, in DEN-induced HCC rat tissue, human HCC tissue and cell line (Hep3B) (Fu et al., 2017). Besides, miR-155-5p upregulation and PTEN downregulation were significantly associated with HCC staging (Fu et al., 2017). Conversely, miR-155-5p inhibitors increased promoted proliferation, invasion and migration by decreasing PTEN and increasing PI3K/Akt pathway in xenograft models and Hep3B and HepG2 tumor cells (Fu et al., 2017). Similar findings were observed concerning oncomiR miR-454-3p (Yu et al., 2015), whose expression was increased

in human HCC cell lines (HepG2 and Huh7) and samples, displaying even higher expression in HCC-derived metastatic tumors (Yu et al., 2015). The expression of chromodomain–helicase–DNA–binding-5 (CHD5), a direct target of miR-454-3p, was inversely correlated with the expression of miR-454-3p in HCC tissues as well (Yu et al., 2015). In contrast, a miR-454-3p inhibitor decreased CHD5 mRNA and protein levels, reducing migration and proliferation *in vitro*, while decreasing tumor growth in a xenograft mouse model (Yu et al., 2015). Therefore, these studies have shed light on miRNAs as important biomarkers for cancer diagnosis, survival and treatment prediction, as well as novel strategies for cancer therapy. However, the number of mature miRNAs discovered in human and rodents keeps increasing, and their roles on both health and disease are yet to be unveiled.

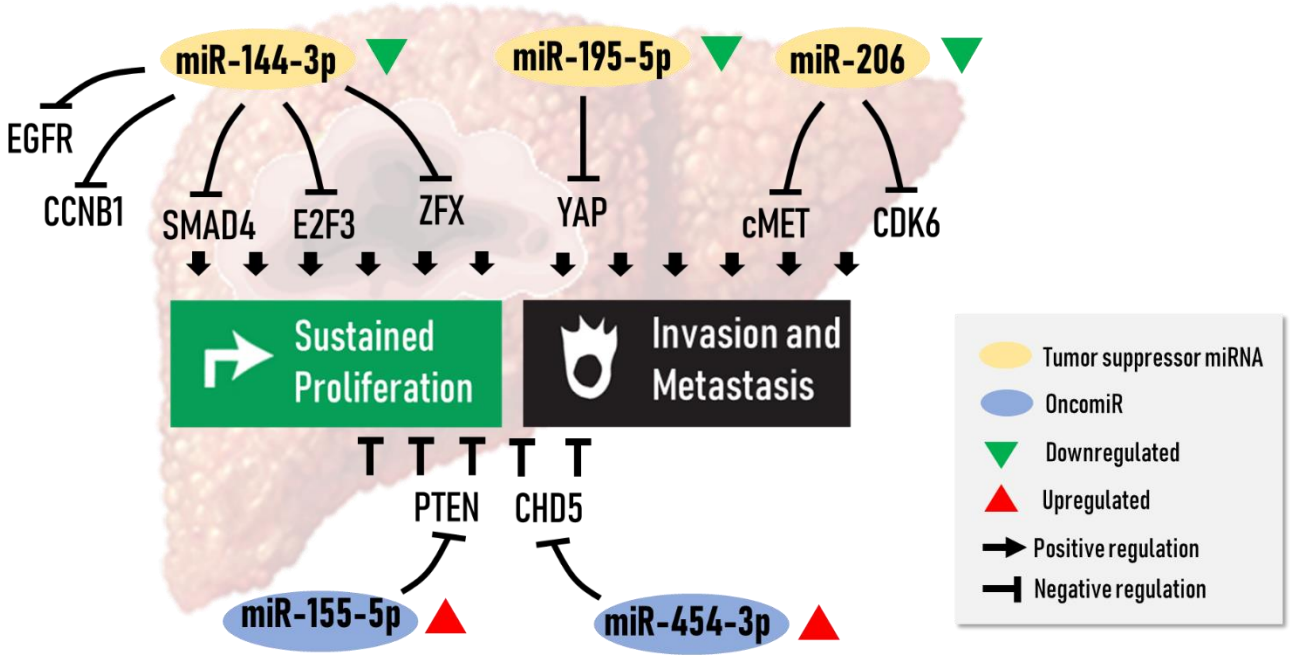

Figure 18. Some of the main miRNAs involved in HCC progression, focusing on sustained proliferation and invasion a migration features (Gu et al., 2019, Cao et al., 2014, Yu et al., 2015; Yu et al., 2016, Bao et al., 2017, Fu et al., 2017; He et al., 2017; Wu et al., 2017; Yu et al., 2017 Xu et al., 2018).

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