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Polyploidization of Hepatocytes: Insights into the Pathogenesis of Liver Diseases

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Abstract

Polyploidization is a process by which cells are induced to possess more than two sets of chromosomes. Although polyploidization is not frequent in mammals, it is closely associated with development and differentiation of specific tissues and organs. The liver is one of the mammalian organs that displays ploidy dynamics in physiological homeostasis during its development. The ratio of polyploid hepatocytes increases significantly in response to hepatic injury from aging, viral infection, iron overload, surgical resection, or metabolic overload, such as that from non-alcoholic fatty liver diseases (NAFLDs). One of the unique features of NAFLD is the marked heterogeneity of hepatocyte nuclear size, which is strongly associated with an adverse liver-related outcome, such as hepatocellular carcinoma, liver transplantation, and liver-related death. Thus, hepatic polyploidization has been suggested as a potential driver in the progression of NAFLDs that are involved in the control of the multiple pathogenicity of the diseases. However, the importance of polyploidy in diverse pathophysiological contexts remains elusive. Recently, several studies reported successful improvement of symptoms of NAFLDs by reducing pathological polyploidy or by controlling cell cycle progression in animal models, suggesting that better understanding the mechanisms of pathological hepatic polyploidy may provide insights into the treatment of hepatic disorders.

Key Words: Polyploidization, Hepatocytes, NAFLD, HCC

INTRODUCTION

Polyploidization is a process by which cells are induced to possess more than two complete sets of chromosomes or become polyploid. Whole-genome polyploidy is common among plants, insects, fish, and amphibia; however, in mammals, polyploidy is restricted in certain cell types including hepatocytes and mammary alveolar cells (Wertheim *et al*., 2013). Polyploidy of these cells results from both nuclear polyploidy, defined as an increase in the amount of DNA per nucleus, and cellular polyploidy, defined as an increase in the number of nuclei per cell (Donne *et al*., 2020).

Polyploidy was first recognized almost a century ago, and it has been generally considered as a consequence of adaptation during evolution (Comai, 2005). In mammals, the formation of polyploid cells is only observed in certain tissues, which are those that undergo development and differentiation, such as heart, placenta, bone marrow, pancreas, and liver (Pandit *et al*., 2013). Especially, polyploidy in the heart, kidney, and liver was found during regeneration and repair processes (Cao *et al*., 2017; Lazzeri *et al*., 2018; Donne *et al*., 2020). A high frequency of polyploidy has been detected as a consequence of aging in vascular smooth muscle cells, which is accompanied by symptoms of vascular smooth muscle hypertrophy and hypertension (Hixon and Gualberto, 2003). By contrast, unscheduled polyploidization has been observed in pathological conditions. In murine models of non-alcoholic fatty liver disease (NAFLD), the parenchyma of fatty livers displayed alterations of the polyploidization process. Biopsies from patients with non-alcoholic steatohepatitis (NASH) revealed the presence of alterations in hepatocyte ploidy compared with tissue from control individuals (Gentric *et al*., 2015). Polyploid and aneuploid cells are frequently seen in various solid tumors, probably because proliferating polyploid cells generate aneuploid daughter cells, leading to genomic instability, which is a risk factor for carcinogenesis (Davoli and Lange, 2011).

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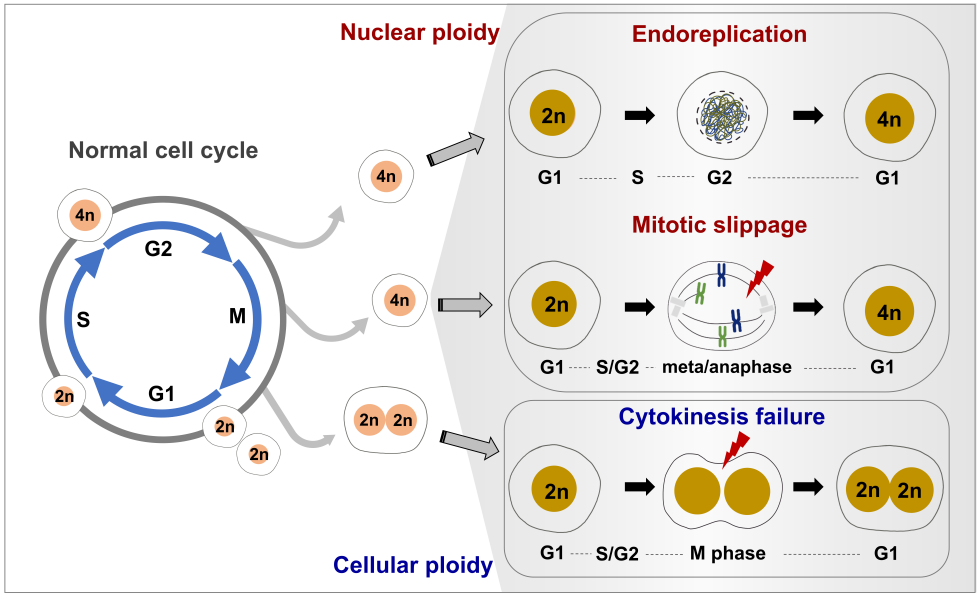

Fig. 1. Three alternative cell-cycle modes associated with polyploidy. Most polyploidization occurs during abnormal cell-cycle processes. To date, three different mechanisms have been proposed for cell-cycle-mediated polyploidization. Endoreplication is a cell cycle in which DNA is replicated in two successive S phases without continuing to the M phase, resulting in the formation of a mononucleated polyploid cell. Mitotic slippage is linked to the perturbed metaphase-anaphase transition. Cytokinesis is the final step in mitosis which divides the cytoplasm of mother cell into two daughter cells.

In this review, we summarize the current understanding of cellular and molecular mechanisms of polyploidization, and the importance of polyploidy in the normal physiology of the liver. In addition, we review the potential roles of polyploidy in pathological conditions of the liver including NAFLDs, and their implications for treatment of these hepatic disorders.

CELLULAR AND MOLECULAR MECHANISMS OF POLYPLOIDIZATION

Most polyploidization arises from abnormal cell-cycle processes, except for cell fusion, which is a cell-cycle-independent process. To date, three different mechanisms have been proposed for cell-cycle-mediated polyploidization: endoreplication, mitotic slippage, and cytokinesis failure (Gentric and Desdouets, 2014) (Fig. 1).

First, endoreplication, also referred to as endoreduplication or endocycling, is a cell cycle in which DNA is replicated through alternating gap and S phases, resulting in the formation of a mononucleated polyploid cells (Fox and Duronio, 2013). Trophoblast giant cells are the best-known example of endoreplication in mammals. The cells increase their DNA content to attain a large cell size to form a barrier between the maternal and embryonic tissues (Sher *et al*., 2013). Accumulation of the cyclin kinase inhibitors such as p57/Kip2 was proposed as a part of the mechanism of endoreplication-mediated cell growth and differentiation of trophoblast stem cells; however, the mechanism has not yet been fully elucidated (Ullah *et al*., 2008). DNA damage is also responsible for the endoreplication in specific species, cell types, and conditions. For example, UV irradiation causes double-strand breakage-triggered G2/M arrest, which is associated with endoreplication (Radziejwoski *et al*., 2011). Blockade of mitotic entry after G1/S progression was suggested as an important step leading to endoreplication. Degradation of key regulatory proteins controlling the S phase, such as CDK2, or inhibition of M phase progression factor CDK1, were suggested to cause blockade of mitotic entry (Fox and Duronio, 2013). Second, polyploid cells can be formed after a prolonged arrest in metaphase due to the activation of the spindle assembly checkpoint, which is called mitotic slippage. Subsequently, the perturbed metaphase–anaphase transition causes cell death following mitotic arrest, or mitotic slippage without undergoing anaphase and telophase, which progresses to form mononucleated polyploid cells (Gentric and Desdouets, 2014). Lastly, cytokinesis failure generates binucleated polyploid cells. Cytokinesis is the final step in cell division, leading to the physical separation of a mother cell into two daughter cells. Some cells are programmed to process cytokinesis failure during normal development. For example, hepatocytes exhibit destined cytokinesis failure, which generates multinucleated polyploid cells (Wang *et al*., 2017).

POLYPLOIDY ASSOCIATED WITH NORMAL PHYSIOLOGY IN THE LIVER

The liver is well-known to display ploidy dynamics in physiological homeostasis during its development (Gentric and Desdouets, 2014; Wang *et al*., 2017). All hepatocytes are diploid at birth, but the cells undergo polyploidization, leading to the gradual heterogeneity of hepatocyte ploidy thereafter. Postnatal binucleation seems to occur during suckling-to-weaning transition (about day 21), which is probably a result of insulin-mediated failure of cytokinesis (Celton-Morizur *et*

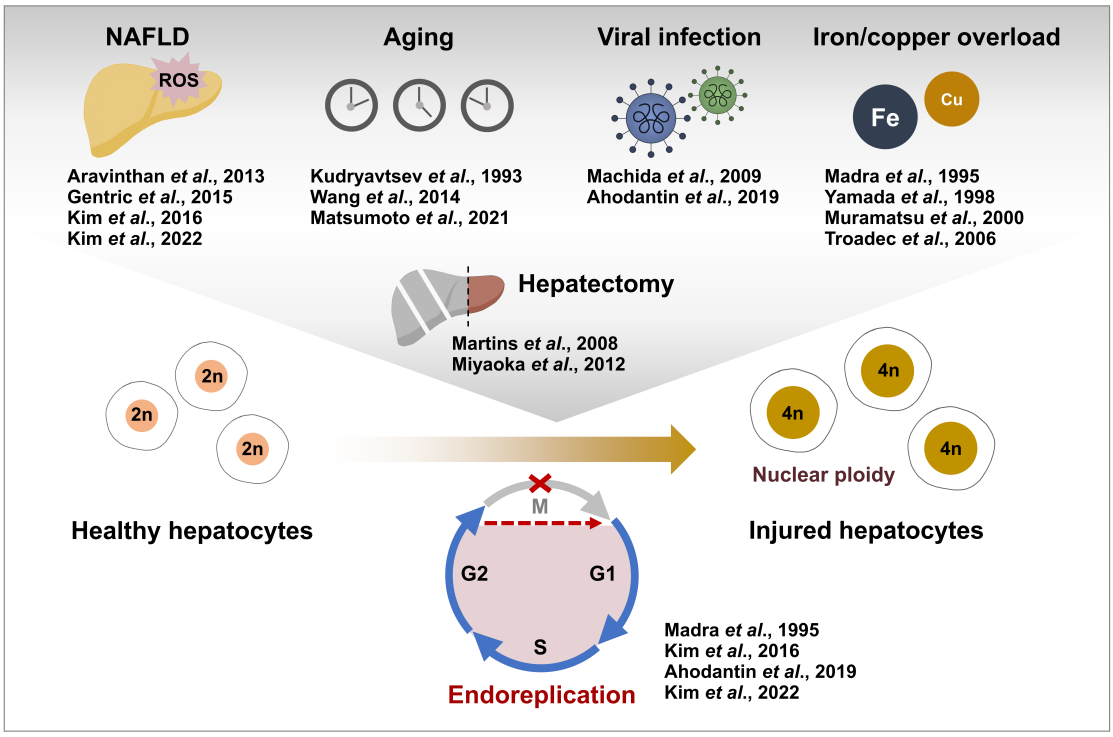

Fig. 2. Pathological polyploidization in the liver. Although hepatocytes in adult liver rarely enter cell cycle under normal conditions, hepatocytes possess potential for proliferation in response to hepatic injury from aging, viral infection, iron overload, surgical resection, and metabolic overload such as NAFLDs. Under these circumstances, compensatory proliferation actively takes place, resulting in marked heterogeneity in hepatic ploidy profile. Endoreplication, the specialized cell cycle in which mitosis is skipped, is one of the mechanisms producing nuclear ploidy in injured hepatocytes.

al., 2010). The resulting binucleated hepatocytes successively progress to form mononucleated polyploid hepatocytes (4n) or repeat the process generating multinucleated polyploid cells (2n+2n+2n+2n) to achieve postnatal liver growth. The ploidy level in hepatocytes effectively reaches a plateau at maturity and hepatocytes with octaploidy (8n) appearing in significant numbers of cells during the second and third months after birth (Celton-Morizur *et al*., 2010). Thereafter, aging-dependent polyploidization occurs during the lifecycle due to cellular senescence (Schwartz-Arad *et al*., 1989; Celton-Morizur *et al*., 2010).

The biological importance of polyploidy in hepatocytes has not been understood clearly. The physiological polyploidy of hepatocytes does not show a clear link to functional abnormality of the liver in that prevention of polyploidization by inactivating atypical E2Fs, important regulators of the mitotic and endoreplication cell cycle (vide infra) had no impact on differentiation, zonation, metabolism, or regeneration in the liver (Lammens *et al*., 2009; Pandit *et al*., 2012). Meanwhile, some research groups have addressed roles of polyploidy in the liver. Anatskaya and Vinogradov (2007, 2010) showed that gene expression patterns associated with fatty acid metabolism and aerobic respiration were altered in polyploid-associated hepatocytes and suggested a functional role of polyploidy in hepatic adaptation to energy storage, cell survival, and tissue regeneration under stressful conditions. Recently, Richter *et al*. (2021) analyzed the functional characteristics of 2n and 4n hepatocytes based on a single-nucleus RNA-seq2 method. Comparing 2n and 4n hepatocytes in the Gene Ontology database and informatics resource showed that 4n hepatocytes were more enriched in pathways involved in lipid, cholesterol, and xenobiotic metabolism, suggesting a potential role of polyploidy in different metabolic potential and position within the hepatic lobule of these cells.

POLYPLOIDY ASSOCIATED WITH PATHOPHYSIOLOGY IN THE LIVER

Although hepatocytes in adult liver rarely enter the cell cycle under normal conditions, hepatocytes possess the potential for proliferation in response to hepatic injury from aging, viral infection, iron overload, surgical resection, and metabolic overload, such as that from NAFLDs. Under these circumstances, compensatory proliferation actively takes place, resulting in marked heterogeneity in hepatic ploidy profile (Fig. 2).

During aging in humans, the rate of polyploid hepatocytes is significantly enhanced. The rate of accumulation of binucleate and polyploid cells is very slow until the age of 50 years, but after that, hepatocyte polyploidization is excessively activated, and cells with polyploid nuclei reach 27% by the age of 86-92 years (Kudryavtsev *et al*., 1993). Activation of p53-p21 and $p16^{ink4a}$-pRB pathways in hepatocyte senescence was suggested as a mechanism of polyploidization during aging in a mouse model (Wang *et al*., 2014). Recently, polyploid hepatocytes have been demonstrated as the source of cel-

lular turnover and been suggested as an important contributor to liver maintenance during aging (Matsumoto *et al*., 2021). However, the mechanisms linking polyploidy and aging-induced hepatic dysfunction are not clearly understood and warrant further investigation.

Chronic liver diseases caused by infection with hepatitis B virus (HBV) or hepatitis C virus (HCV) contributes to hepatic adaptation of polyploidy, probably due to cell-cycle defects. HBV X protein (HBx), a crucial viral factor for HBV replication, is assumed to participate in the development of hepatocellular carcinoma (HCC) (Kew, 2011). HBx led to aberrant polyploidization favoring DNA damage propagation and hepatocyte transformation through DNA-damage-mediated Polo-like kinase 1 (PLK1) activation in HBx transgenic mice (Ahodantin *et al*., 2019). Furthermore, early transition of G1/S phase and delay of G2/M progression observed in the HBx transgenic hepatocytes eventually led to an increase in the number of mononucleated polyploid cells and decrease in that of binucleated cells, which are representative phenomena of endoreplication (Ahodantin *et al*., 2019). In the case of HCV, infection with the virus or overexpression of its viral core protein induced defects in the mitotic checkpoint and aberrant chromatin segregation in hepatocytes, which showed frequent hepatic polyploidization accompanied by reduced Rb transcription and enhanced expression of E2F1 and MAD2 (Machida *et al*., 2009).

The liver is a major iron storage organ; however, it is also susceptible to injury from iron overload (Bonkovsky, 1991). Especially, iron overload is considered as a potential risk factor for hepatocarcinogenesis (Hino *et al*., 2021). It has been shown that the process of polyploidization during carcinogenesis was greatly accelerated in the liver of mice having received iron dextran (Madra *et al*., 1995). Iron overload induced cyclin D1, a protein involved in the G1 phase of the cell cycle, in a mouse model with carbonyl-iron supplementation or iron-dextran injection, leading to both hepatomegaly and hepatocyte polyploidization (Troadec *et al*., 2006). Wilson disease, a genetic liver disease accompanied by hepatic copper overload, has also been associated with increased hepatic polyploidy (Yamada *et al*., 1998; Muramatsu *et al*., 2000).

The most widely used model to study liver regeneration and hepatic polyploidy in rodents is partial hepatectomy (PHx) using a surgical resection procedure (Michalopoulos and DeFrances, 1997; Martins *et al*., 2008). A 70% PHx induced hypertrophy followed by hyperplasia, which caused a decrease in the number of diploid cells and binucleated polyploid cells, but an increase in the number of mononucleated polyploid cells. Mechanisms for pathological and regenerative polyploidization are quite distinct. According to the amount of resected liver, different patterns of cellular proliferation were observed during recovery of liver mass. Upon 70% PHx, all hepatocytes entered the cell cycle and rapidly continued through the S phase, but no hepatocytes entered mitosis, resulting in an increase in the number of polyploid hepatocytes. With a resection of >70% of the liver, severe liver injury occurred, and the regenerative capacity of preexisting hepatocytes was significantly impaired (Miyaoka *et al*., 2012). In this case, liver progenitor cells were activated and progenitor cell-mediated regeneration was the major process in the recovery of liver mass (So *et al*., 2020). However, after 30% PHx, remaining hepatocytes did not enter into the S phase; instead, hypertrophy of hepatocytes contributed to recovery of the original mass (Miyaoka *et al*., 2012).

HEPATIC POLYPLOIDIZATION IN NAFLDS

NAFLDs refer to a broad pathological spectrum of diseases ranging from simple steatosis to NASH, which can lead to cirrhosis and eventually to HCC (Hardy *et al*., 2016). The distinct heterogeneity of hepatocyte nuclear size is one of the unique features of NAFLD (Nakajima *et al*., 2010; Aravinthan *et al*., 2013). The degree of nuclear enlargement in patients with NAFLD is greater than that in normal individuals, and a larger hepatocyte nuclear area is strongly associated with an adverse liver-related outcome, including HCC, liver transplantation, and liver-related death (Nakajima *et al*., 2010; Aravinthan *et al*., 2013). The enlargement of nuclear size in hepatocytes is closely associated with polyploidization (Watanabe and Tanaka, 1982). In patients with NAFLDs, enrichment of mononucleated polyploid cells was observed throughout the liver parenchyma (Gentric *et al*., 2015).

In mouse models of NAFLD such as a high-fat diet-induced, methionine–choline-deficient diet-induced, and *ob*/*ob* mice, the proportion of mononucleated polyploid hepatocytes was increased, probably due to inefficient cell-cycle progression through the S/G2 phases. Oxidative stress promoted the appearance of highly polyploid cells, and antioxidant-treated NAFLD hepatocytes resumed normal cell division and returned the liver to a physiological state of polyploidy (Gentric *et al*., 2015). In mice, the liver-specific deletion of SSU72, a protein phosphatase involved in sister chromatin separation, led to polyploidization through endoreplication in chronic liver diseases such as steatohepatitis and fibrosis (Kim *et al*., 2016). A specific genetic loss of *Cdk1* in the liver yielded increased endoreplication in hepatocytes, which was accompanied by a hepatic phenotype of steatosis and fibrosis in mice (Diril *et al*., 2012; Dewhurst *et al*., 2020; Ow *et al*., 2020). Genetic deletion of *E2f7* and/or *E2f8* in hepatocytes led to decreased nuclear ploidy and increased G2/M transition, suggesting these genes as central transcription regulators that facilitate DNA endoreplication (Chen *et al*., 2012). Suppression of *E2f8* expression in the liver of zebrafish ameliorated diet-induced obesity and this observation may support the link between polyploidy and dysregulation of fat metabolism, although its relevance to human NAFLD was not well addressed (Shimada *et al*., 2015). Recently, a nuclear receptor RORα was demonstrated as a negative regulator of the *E2f7* and *E2f8* transcription. Deletion of hepatic RORα aggravated symptoms of NAFLD and enhanced hepatic polyploidy (Kim *et al*., 2022). These molecular factors that regulate hepatic polyploidy in diverse models are summarized in Table 1.

Polyploidization is closely linked to multiple pathogenic factors for NAFLDs, such as abnormal lipid metabolism and mitochondrial dysfunction. In murine hepatocytes, polyploid nuclei were negatively correlated with the expression of metabolic genes such as *Apoa5*, *Fabp1*, *Cyp4f15*, and *Pck1* (Kreutz *et al*., 2017). A modular biology approach and genome-scale cross-species comparison revealed that gene expression patterns of diploid cells and polyploid cells were different in that genes associated with lipid metabolism were downregulated in polyploid hepatocytes compared with diploid cells (Anatskaya and Vinogradov, 2010). It is noteworthy that expression of genes encoded in the mitochondrial genome has been negatively correlated with nuclear size to a high degree (Miettinen *et al*., 2014). Hepatocyte senescence resulting from irreversible cell-cycle arrest correlated closely with fibrosis stage and clinical outcome in patients with NAFLDs. The nuclear area of

Table 1. Molecular regulators of hepatic polyploidy associated with NAFLD/HCC in animal model

Animal model	Nuclear ploidy	Cellular ploidy	Molecular mechanism	NAFLD/HCC symptoms	References
E2f8 LKO mouse	Decreased	Decreased	Endocyclic gene regulation		Chen *et al*., 2012
Cdk1 LKO mouse	Increased		DNA re-replication due to an increase in Cdk2/cyclin A2 activity		Diril *et al*., 2012
Cdk1 LKO mouse	Increased	Decreased	DNA re-replication regulation	Blood ALT, bilirubin, and ALP levels increased Hepatic fibrosis area increased	Dewhurst *et al*., 2020
Ssu72 LKO mouse	Increased	Decreased	Rb-E2F signaling pathway	Serum ALT and AST levels increased Hepatic fat accumulation increased Hepatic fibrosis area increased	Kim *et al*., 2016
Mir-122 germline KO mouse	Decreased	Decreased	Cytokinesis regulation	Hepatic steatosis increased Hepatic fibrosis promoted	Hsu *et al*., 2016
Yap LKO mouse	Decreased		Akt-Skp2-p27/FoxO axis	Liver tumorigenesis enhanced	Zhang *et al*., 2017
Lis1 LKO mouse	Increased	Decreased		Hepatic steatosis induced and liver tumorigenesis accelerated	Li *et al*., 2018
RORα LKO mouse	Increased	No change	E2F7/E2F8 activated	Hepatic steatosis increased Hepatic collagen deposition increased	Kim *et al*., 2022

LKO, liver-specific knockout; ALT, alanine aminotransferase; ALP, alkaline phosphatase; AST, aspartate aminotransferase; Yap, yes-associated protein; Skp2, S-phase kinase-associated protein 2; LIS1, lissencephaly 1.

hepatocytes increased in patients with progressed fibrosis but was unchanged in patients with improved fibrosis (Aravinthan *et al*., 2013). Cellular senescence was also reported to act as a driver of age-dependent hepatic steatosis and nuclear enlargement was detected in the hepatocytes in senescence (Ogrodnik *et al*., 2017). Together, these observations suggest that hepatic polyploidization may drive progression of NAFLDs through control of multiple pathogenic factors for the diseases.

POLYPLOIDY AND HEPATOCELLULAR CARCINOMA

The contribution of polyploidization to development of HCC has been controversial to date. It was proposed that polyploidy provides a genetic buffer from mutation of tumor suppressor genes because polyploid hepatocytes contain extra chromosomes that probably contain an intact allele (Wang *et al*., 2021). Consistently, it was reported that mouse hepatocytes were more proliferative when in diploidy than in polyploidy (Wilkinson *et al*., 2019). The tumor-suppressive effect of polyploidy was demonstrated using genetically modified animal models. Diethylnitrosamine treatment of mice with deleted *Anln*, a gene encoding cytoskeletal scaffolding protein that regulates cytokinesis and might promote tumorigenesis (polyploid model) and *E2f7*/*E2f8* knockout mice (diploid model), showed that the polyploid mouse model was more resistant to the development of HCC after treatment with the hepatocarcinogen (Zhang *et al*., 2018; Wilkinson *et al*., 2019). Meanwhile, other investigators have presented evidence for an alternative concept, that is, the tumor-promoting function of polyploidy. Bou-Nader *et al*. (2020) showed that the percentage of mononucleated polyploid hepatocytes in patients was positively correlated with incidence of HCC and poor prognosis. In a murine model, treatment with diethylnitrosamine increased the generation of polyploidy hepatocytes that was accompanied by upregulation of Aurora kinase B (AURKB) (Lin *et al*., 2021). Further studies are required to address the circumstances and environment that determine the pro- or antitumor effects of polyploidy.

THERAPEUTIC IMPLICATIONS OF TARGETING PATHOLOGICAL POLYPLOIDY FOR HEPATIC DISORDERS

Due to various infections and metabolic abnormalities, there is a high frequency of developing diseases in the liver. Because of the growing epidemic of risk factors such as overnutrition, the incidence of NAFLD has risen sharply in the past three decades worldwide (Benedict and Zhang, 2017). Recently, the demand for drugs to treat NAFLD and the market size for them have been increasing; however, as yet there is no FDA-approved drug to treat NAFLD. The pathogenesis of NAFLDs is diverse and intertwined with lipotoxicity, inflammation, and fibrosis, in which various types of cells interact each other simultaneously and systematically (Kim and Lee, 2018; Peng *et al*., 2020; Loomba *et al*., 2021). However, the molecular mechanisms underlying the progression of NAFLDs remain ambiguous and heterogeneous, meaning that the identification of therapeutic targets has been challenging. Recently, many drug candidates targeting pathogenic drivers including lipogenesis, inflammatory response, mitochondrial stress, oxidative stress, and fibrogenesis have been developed (Loomba *et al*., 2021). Unfortunately, however, they have not achieved their ultimate goals due to insufficient efficacy or unexpected side effects (Neuschwander-Tetri *et al*., 2015; Ratziu *et al*., 2016; Mantovani and Dalbeni, 2021). Therefore, identification of new therapeutic targets is warranted to overcome the limits of classical strategies. Although whether polyploidy is a driver of NAFLDs or a consequence of progression of the diseases

is controversial, the mechanism of hepatic polyploidy may provide new insights into the development of therapeutic strategies for the treatment of the diseases.

Several recent studies in animal models found successful improvement of symptoms of NAFLDs by reducing pathological polyploidy or by controlling cell-cycle progression. Administration of JC1-40, an RORα activator that induced expression of E2F7/8, significantly reduced hepatic nuclear polyploidization and liver injury in a high-fat diet-induced model of NAFLD in mice (Kim *et al*., 2012, 2022). Hepatic steatosis was completely abrogated in *E2f1*-deficient mice with diet-induced NAFLD symptoms (Denechaud *et al*., 2016). In addition, genetic depletion of gankyrin, an oncogenic regulator of CDK4 and RB, significantly ameliorated liver fibrosis by inhibiting steatosis and blockade of hepatic proliferation in mice (Dawson *et al*., 2006; Cast *et al*., 2019). Moreover, administration of PD-0332991, a small compound that inhibits CDK4, prevented NAFLD development and reversed hepatic steatosis (Jin *et al*., 2016). Polyploidy of hepatocytes is associated with initiation of tumor formation (Lin *et al*., 2021; Matsumoto *et al*., 2021). Pharmacological inhibition of AURKB using AZD1152 reduced nuclear size and tumor foci (Lin *et al*., 2021). Clearly, further investigations are necessary to design novel strategies against pathological hepatic polyploidization to contribute to the development of therapeutics for diverse hepatic disorders including NAFLDs.

CONCLUSIONS

Polyploidy is closely related to both physiological and pathological contexts in the mammalian liver. Recent studies have now revealed not only the role of hepatic polyploidy in regeneration and development of the liver, but also its impact on liver metabolism and cancer. Although there are as yet unresolved controversies in the pathological role of polyploidy during progression of hepatic diseases such as NAFLDs and HCC, several studies have found beneficial effects of reducing pathological polyploidy or controlling cell-cycle progression to improve symptoms of NAFLDs in animal models. Thus, better understanding the mechanisms of pathological hepatic polyploidy and identification of relevant new targets may provide insights into the treatment of hepatic disorders.

CONFLICT OF INTEREST

The authors declare no competing interest.

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