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MINIREVIEWS

Induced pluripotent stem cell-derived mesenchymal stem cells for modeling and treating metabolic associated fatty liver disease and metabolic associated steatohepatitis: Challenges and opportunities

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Abstract

The potential of induced pluripotent stem cells (iPSCs) for modeling and treating metabolic associated fatty liver disease (MAFLD) and metabolic associated steatohepatitis (MASH) is emerging. MAFLD is a growing global health concern, currently with limited treatment options. While primary mesenchymal stem cells hold promise, iPSCs offer a versatile alternative due to their ability to differentiate into various cell types, including iPSC-derived mesenchymal stem cells. However, challenges remain, including optimizing differentiation protocols, ensuring cell safety, and addressing potential tumorigenicity risks. In addition, iPSCs offer the possibility to generate complex cellular models, including three-dimensional organoid models, which are closer representations of the human disease than animal models. Those models would also be valuable for drug discovery and personalized medicine approaches. Overall, iPSCs and their derivatives offer new perspectives for advancing MAFLD/MASH research and developing novel therapeutic strategies. Further research is needed to overcome current limitations

and translate this potential into effective clinical applications.

Key Words: Metabolic associated fatty liver disease; Metabolic associated steatohepatitis; Nonalcoholic fatty liver disease; Nonalcoholic steatohepatitis; Mesenchymal stem cells; Induced pluripotent stem cells; *In vitro* liver models

Core Tip: Induced pluripotent stem cells (iPSCs) show promise for modeling and treating nonalcoholic fatty liver disease and nonalcoholic steatohepatitis. iPSCs can generate various cell types, including cells for therapy or disease modeling. Challenges remain, but iPSCs offer potential for drug discovery and personalized medicine approaches for nonalcoholic fatty liver disease/nonalcoholic steatohepatitis.

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INTRODUCTION

Metabolic associated fatty liver disease (MAFLD) encompasses a spectrum of liver conditions linked to metabolic dysfunction, with its more severe manifestation being metabolic associated steatohepatitis (MASH), characterized by inflammation and liver damage. Previously, these conditions were known as nonalcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH), respectively. Although MAFLD and MASH are the newly proposed nomenclature, this review will use the traditional terms NAFLD and NASH to maintain consistency with most of the existing literature and facilitate comparisons with previous studies.

NAFLD is the most common chronic liver disease worldwide, affecting approximately 30% of the global population and 70% of patients with type 2 diabetes mellitus. It is expected to increase to more than 400 million people in the United States, Europe, and Southeast Asia by 2030[1-3]. NAFLD typically stems from overnutrition, resulting in lipid accumulation in the liver. This leads to dysregulation of lipid metabolism, causing oxidative stress, endoplasmic reticulum (ER) stress, lipid peroxidation, inflammation, and tissue fibrosis[4].

NAFLD is diagnosed when more than 5% of the hepatocytes show fat accumulation. This can be detected through liver biopsy or imaging techniques in patients who consume little or no alcohol and do not have factors that contribute to fatty liver disease[5]. Commonly, these patients also have coexisting metabolic disorders, such as obesity, diabetes mellitus, and dyslipidemia. Additional factors, such as genetic and epigenetic factors or changes in gut microbiome, can contribute to NAFLD[3,6].

NAFLD is divided into two main categories. The first is nonalcoholic fatty liver, with hepatocytes exhibiting fat accumulation without liver injury, affecting approximately 75% of patients. The second category is NASH, which is considered the advanced form of NAFLD. In NASH, hepatocytes show steatosis and inflammation, and fibrosis is frequently present in the liver[3,6]. NAFLD can progress to NASH, which can consequently lead to cirrhosis and hepatocellular carcinoma. The precipitating factors for this progression include a high-fat diet, genetic factors, oxidative stress, and inflammation caused by high levels of cytokines or changes in the gut microbiome[7,8].

Several therapies are being studied for the treatment of NAFLD, including the regulation of metabolism through peroxisome proliferator-activated receptor (PPAR) agonists, thyroid hormone receptor beta-agonists, and fibroblast growth factor analogues. Other research areas focus on the gut microbiome, targeting oxidative stress, microRNA, and apoptosis inhibitors[7]. However, there is currently no approved therapy for NAFLD, despite multiple drugs being tested in clinical trials[9], and the most common therapeutic approach involves lifestyle changes, such as adjustments of diet and physical activity.

The use of stem cells, particularly of primary tissue-derived mesenchymal stem cells (MSCs), and their secreted factors has been widely studied as a promising alternative therapeutic for treating NAFLD/NASH patients. Jiang *et al*[10] provided a comprehensive overview of the current situation of the utility of tissue-derived MSCs to address NAFLD/NASH in a recent volume of the *World Journal of Stem Cells*. However, significant progress has been made with induced pluripotent stem cells (iPSCs), particularly in understanding the reprogramming process and differentiation methodologies. These advancements position iPSC as a potential source of MSCs and MSC-derived factors for disease treatment and as a means to model the disease with human cells. In this review, we focused on the potential of iPSC and iPSC-derived MSCs (iMSCs) for modeling and as treatment alternatives of NAFLD/NASH.

MSCS AND NAFLD/NASH

MSCs are multipotent cells readily available in bone marrow (BM-MSC), adipose tissue (AD-MSC), and perinatal tissues, such as umbilical cord tissue, among other tissues/organs. These cells have the ability to adhere to plastic *in vitro* and must be able to differentiate into adipocytes, chondrocytes, and osteoblasts. MSCs are characterized by the expression surface markers such as CD73 and CD90 and the absence of CD11b, CD14, CD19, CD34, CD45, CD79, and HLA-HR expression[11]. However, depending on the donors, MSCs may exhibit some heterogeneity, and some AD-MSCs have been shown to display CD34[12].

MSCs are, nonetheless, a versatile type of stem cells with multiple promising autologous or allogeneic clinical and tissue engineering applications that are explored. Those cells present several advantages including their potential to differentiate into various cell types under appropriate conditions, such as adipocytes, chondrocytes, osteoblasts, and others. In addition, MSCs have a high proliferative ability and low immunogenicity and display immunomodulatory properties that are valuable for the treatment of inflammatory and autoimmune diseases. Therefore, MSCs are a promising therapeutic alternative for the treatment of NAFLD/NASH[13]. Indeed, MSCs may exert several beneficial effects at different stages of NAFLD/NASH by: (1) Secretion of trophic factors and extracellular vesicles (EVs), which induce proproliferative and antiapoptotic effects; (2) Secretion of proteins responsible for triglyceride synthesis and reduction of lipid concentrations, which limit metabolism dysregulation and consequent inflammation; (3) Reduction of fibrosis through paracrine effects and expression of metalloproteinases, which degrade fibrotic matrix; and (4) Regulation of the immune response by inducing a shift in macrophage polarity and reducing T cell activation[13-18].

Despite the properties of MSCs, no registered clinical trials (research performed on November 20, 2024) specifically mentioned "mesenchymal stem cells," "NASH," or "NAFLD" in the Clinicaltrials.gov data base. However, there is a multi-institutional clinical trial registered in the Japanese UMIN Clinical Trial Registry (UMIN000022601) that investigated the potential to treat liver cirrhosis in patients with NASH or fatty liver disease with autologous AD-MSCs. In this pioneering trial, 7 patients injected with AD-MSC *via* intrahepatic arterial infusion showed an improvement in serum albumin concentration and thrombin activity, with no adverse effect detected, demonstrating the efficacy and safety of this approach. These promising results emphasize the need for further studies with extended observational periods to fully elucidate the ability of AD-MSCs to treat NASH or fatty liver disease and determine long-term efficiency and safety of this strategy to treat these conditions[19].

IPSCS AND IMSCS

The generation of human iPSCs has unveiled new possibilities for disease modeling, drug discovery, and regenerative medicine[20]. Indeed, iPSCs are a category of pluripotent stem cells generally derived from somatic cells *via* the exogenous coexpression of the transcription factors octamer-binding transcription factor 4 (OCT4), sex-determining region Y-box 2, Kruppel-like factor 4, and c-MYC (also known as Yamanaka factors), which display properties similar to those of embryonic stem cells with less ethical concerns[21]. Human iPSCs can also be generated from patient-specific cells, enabling the establishment of cellular model systems to study diseases and test drugs or therapies personalized to the patient. Human iPSCs have some drawbacks, such as interclonal and intraclonal heterogeneity in gene expression acquired during the reprogramming process, (epi)genetic variability and genomic instability, and the potential to develop teratomas, among others[20]. Of interest, iPSCs can be used to alter the genome through CRISPR-based methodologies to evaluate the impact of genes or genetic variants in the disease of interest.

iMSCs are a promising alternative to primary MSCs, displaying high proliferation rate and low susceptibility for senescence and showing robust characteristics of standard MSCs[22]. Like primary MSCs, iMSCs express standard MSC superficial markers, have the ability of self-renewal and differentiation, and may even have a propensity for a better proliferation capacity than primary MSCs, as demonstrated by Wei *et al*[22]. These features make iMSCs excellent cellular models for studying new drugs that can even be adapted for personalized treatments if iMSCs are derived from the patient's iPSCs (Figure 1). In addition, iMSCs may be used for cell-based therapies and are suited to generate a large quantity of cells often necessary for therapeutic approaches[23].

However, iPSC-derived cells have some drawbacks. Indeed, iPSCs themselves acquire the potential for teratoma formation during the reprogramming process, which raises significant safety concerns in the use of iPSC-derived cells that may not be properly depleted of undifferentiated iPSC[24]. Teratomas are benign tumors composed of multiple germ layer cell types. Thus, robust safety profiles of produced iMSCs must be carefully established to ensure their safe clinical application. Thus, iMSC usage faces limitations, necessitating the optimization of differentiation protocols and the assessment of cell quality and safety before any clinical applications.

To ensure that iPSCs differentiate properly with no undifferentiated iPSCs remaining among them, cell sorting methodologies (flow cytometry and fluorescence-activated cell sorting, magnetic bead separation based on antibodies targeting pluripotent specific markers) may be used either for removal of undifferentiated iPSCs or to isolate properly differentiated iMSCs harboring specific MSCs markers. Complementary methods, such as immunohistochemistry and quantitative PCR, may also be used to confirm the elimination of cells that do not present proper markers of iMSCs to improve the safety and purity of iPSC-derived therapies[23,25-27].

Although, genomic alterations are not desirable in cells destined for clinical applications, some strategies are developed to introduce "suicide genes" into iPSCs allowing for their selective elimination of undifferentiated cells by administering a specific drug or agent[28]. The establishment of standard, efficient, and reliable differentiation protocols is essential for consistent production of functional iMSCs and is paramount for their clinical application. Indeed, any variability in iMSC

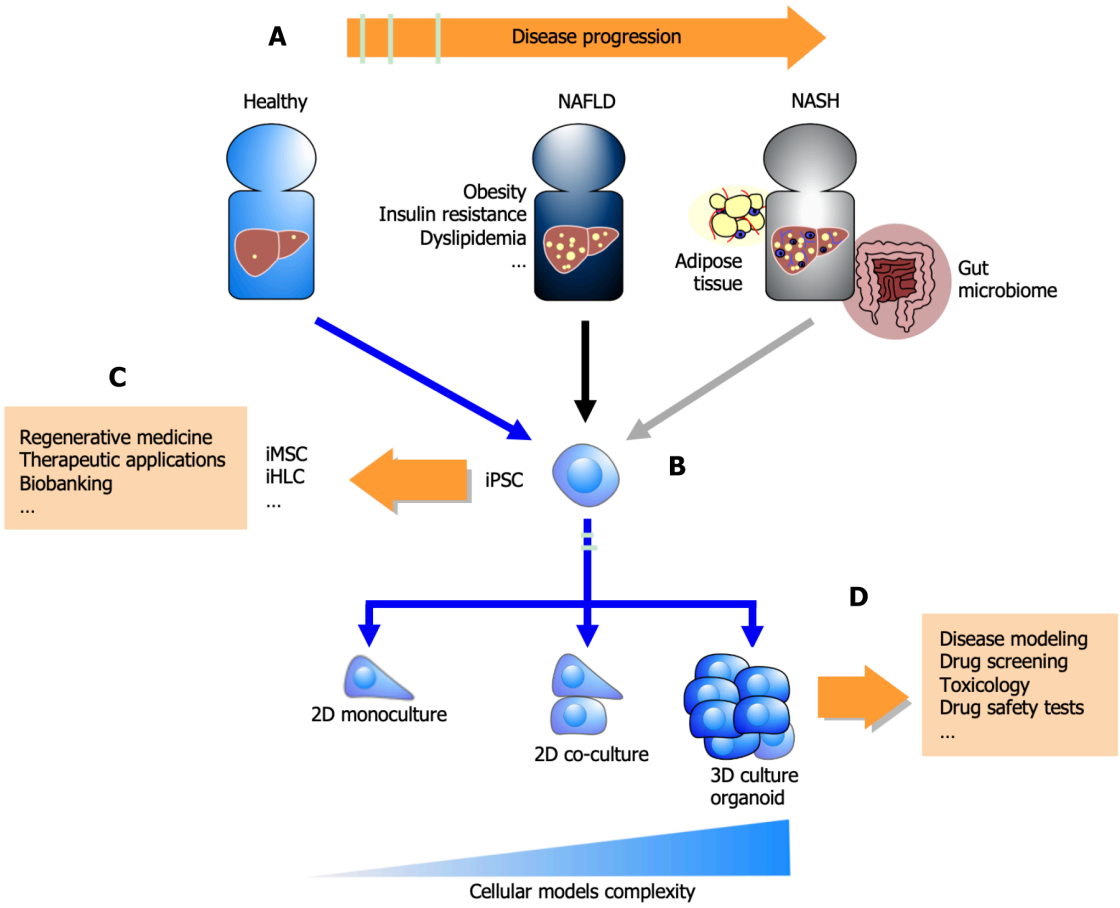

Figure 1 Applications of human induced pluripotent stem cells for nonalcoholic fatty liver/non-alcoholic steatohepatitis disease modeling and therapy. A: Nonalcoholic fatty liver (NAFLD) arises from abnormal lipid accumulation in hepatocytes unrelated to excessive alcohol consumption and is associated with dyslipidemia, insulin resistance, obesity, metabolic syndrome, and type 2 diabetes. The progression of NAFLD to nonalcoholic steatohepatitis (NASH) can be triggered by lipid peroxidation, mitochondrial dysfunction, oxidative stress, hepatocyte apoptosis, the production of proinflammatory cytokines, and alterations in the microbiome. NASH may further deteriorate into cirrhosis due to the development of liver scarring and advanced hepatocellular damage; B: Induced pluripotent stem cells (iPSCs) can be reprogrammed from somatic cells of healthy individuals or patients with NAFLD/NASH; C: Functional hepatocytes, mesenchymal stem cells, and other cell types can be derived from iPSCs for use in regenerative medicine and other therapeutic approaches. Additionally, iPSCs and their derived cells can be characterized and preserved in biobanks worldwide for allogenic cell-based therapies in human leukocyte antigen-matched patients; D: Cells differentiated from iPSCs can be used to create two-dimensional (2D) monocultures, 2D co-cultures, and complex 3D cellular systems, such as liver organoids, to model normal or diseased hepatic tissue. These human *in vitro* liver models are valuable for studying NAFLD/NASH disease progression as well as for testing drugs to assess their efficacy, safety, and toxicity. If the iPSCs are derived from a patient, these models can help identify drugs with better efficacy for treating that specific patient, enabling personalized medicine. iMSC: Induced pluripotent stem cell-derived mesenchymal stem cell; iHLC: Induced pluripotent stem cell-derived hepatocyte-like cell.

properties may result in unpredictable therapeutic outcomes. Moreover, inconsistent differentiation processes may result in heterogeneous iMSC populations impairing their clinical effectiveness. Additionally, the maintenance of protocol reproducibility and quality of iMSCs have to be achieved in large-scale production systems to provide the necessary cell numbers for clinical usage. Of importance, these processes will have to preserve the immunomodulatory function of iMSCs that are crucial for NAFLD/NASH treatment. Therefore, optimizing and standardizing differentiation protocols are vital to ensure that iMSCs are produced consistently with the necessary functional characteristics, thereby supporting their successful clinical application.

Despite iMSCs and primary MSCs exhibiting similar functional characteristics, there are differences in their overall gene expression profiles. For instance, Frobel *et al*[29] reported that genes associated with T cell activation and immune response were expressed at higher levels in primary BM-MSCs compared with iMSCs. Consequently, iMSCs demonstrated a reduced immunomodulatory effect attributed to their decreased ability to suppress T cell proliferation [29]. Diederichs and Tuan[30] found significant differences in gene expression related to differentiation between iMSCs and BM-MSCs from the same donor, with iMSCs showing inferior trilineage differentiation potential compared with BM-MSCs, highlighting that they are not identical entities.

Building on these studies, Xu *et al*[31] compared various iMSC and BM-MSC lines to further investigate their differences. They showed that both cell types were able to efficiently differentiate into osteoblasts, but iMSCs displayed a reduced capacity to differentiate into adipocytes and chondrocytes. iMSCs exhibited a gene expression profile similar to vascular progenitor cells derived from mesodermal origins, possibly explaining their limited capacity to differentiate into the three pilar cell types, namely adipocytes, chondrocytes, and osteoblasts, in comparison with primary MSCs. This underscores the authors' call for broader assessments beyond the International Society for Cellular Therapy,

recommending the inclusion of diverse mesodermal progenitor markers in future evaluations[31].

The immunomodulatory effects of MSCs are mainly attributed to paracrine signals, which include EVs secreted by those cells. Thus, MSC-derived EVs are viewed as therapeutic replacement to MSCs for the treatment of several diseases. Of particular interest for liver-related diseases, MSC-derived EVs are small and accumulate in the liver when delivered by systemic injection. Making the liver a privileged target of EVs could be used to minimize the homeostatic hepatic imbalance during disease[32,33]. For efficient EV-based therapies, a large quantity of EVs would be required, and primary MSCs do not produce large amounts of EVs. Therefore, iMSCs are considered a better source for EV production, as their numbers or iPSCs before differentiation can be expanded in culture for long periods without undergoing senescence.

Moreover, Kim *et al*[34] demonstrated that EVs derived from pan PPAR agonist-stimulated iMSCs were able to treat NASH in both *in vitro* (primary hepatocytes) and *in vivo* (mouse) models by promoting tissue repair, reducing ER stress, stimulating mitochondrial biogenesis, and decreasing reactive oxygen species generation. These results indicate that priming of iMSCs may result in EVs with enhanced capacity to treat NAFLD, and more research has to be performed in this field. Despite some drawbacks, iMSCs appear to be promising cells for drug testing, personalized therapies, and large-scale cell and EV production, which have great potential for treating liver diseases by targeting hepatic repair mechanisms. Nevertheless, optimizing differentiation protocols and ensuring cell safety are crucial before clinical applications can be realized.

MODELLING NAFLD/NASH

To understand the etiology and pathophysiology of NAFLD/NASF and test potential therapies, animal models have been very valuable. Those models are numerous and to some extent reflect the genetic, metabolic, and behavioral abnormalities that contribute to NAFLD/NASH. They provide valuable insights into the specific pathways and mechanisms underlying the pathology. However, these models do not completely reproduce the full spectrum of heterogeneity and complexity observed in human NAFLD/NASH pathology. The mouse model that most closely mimics human NAFLD is called "Diet-induced Animal Model of Nonalcoholic Fatty Liver Disease". Mice are fed a high-fat and fructose diet, leading to progressive stages of NAFLD and eventually liver cancer. However, this model is genetically constrained, and the phenotype takes a few months to develop[35].

Furthermore, ethical and public pressure is increasing to minimize or even abolish the use of animals in scientific, medical, and other procedures to preserve animal welfare and reduce pain and suffering. This is also scientifically justified by the genetic, physiological, and other differences between animals and humans, which can lead to inaccurate modeling of human diseases. Additionally, novel human two-dimensional (2D) and three-dimensional (3D) cell-based *in vitro* models are now available and are alternatives offering more relevant and humane research approaches for disease studies and drug development.

2D CULTURE CELL MODELS

NAFLD is a disease centered on hepatocyte dysfunction. Thus human primary hepatocytes, iPSC-derived hepatocytes, and hepatoma cell lines are interesting cellular systems for studying NAFLD (Figure 1). Primary human hepatocytes (PHHs) are the most abundant cell type in the liver and are responsible for liver-specific functions[35]. Thus, PHHs are commonly used as an *in vitro* model to study liver diseases due to their ease of isolation from liver resection or tissue. However, PHHs have some limitations, such as interdonor variability that makes reproducibility difficult and their ephemeral capacity to proliferate and maintain their liver-specialized functions[36,37]. Despite those limitations, PHHs are physiologically relevant for NAFLD investigation, and their exposure to free fatty acids (FFAs) (oleic or palmitic acid) replicates liver steatosis *in vitro*. Those cells were successfully used to unravel pathways contributing to liver injury, such as transforming growth factor β[38] and to identify protector molecules of hepatocytes, such as GLP-1 analogues that promote autophagy and reduce hepatocyte ER stress-induced apoptosis[39]. GLP-1 is an incretin involved in insulin secretion, satiety, and the suppression of glucagon release when nutrients are digested[40,41]. In the presence of an agonist, a decrease in the fatty acid load within hepatocytes is observed, making it a potential therapeutic target for NAFLD/NASH[39].

Human hepatoma cell lines, such as HepG2 and Huh-7, were derived from cancers and adapted to *in vitro* culture and conserved some of properties of primary hepatocytes. However, they are genomically instable and fail to be fully representative of a primary hepatocyte since they present an altered metabolism and lack the expression of some drug-metabolizing enzymes[36,42]. Nevertheless, these models have been utilized to elucidate some mechanisms underlying the pathology of NAFLD and define some drugs with potential for treatment of the disease. Indeed, exposing HepG2 cells to oleic acid results in lipid accumulation, elevated production of tumor necrosis factor-α, decreased expression of PPARα, increased lipid peroxidation and apoptosis, and reduced cellular proliferation, which are key events contributing to the pathogenesis and progression of NAFLD[43]. The Huh-7 cell line, which shows an expression profile of metabolic enzymes similar to those of PHHs, has been used to test drugs to improve steatosis, such as toyocamycin and an inhibitor of the transcription factor X-box binding protein 1[37,44]. Overall, hepatoma cell lines are not the best cells to model NAFLD/NASH.

The pluripotent nature of human iPSC and embryonic stem cells enables their differentiation virtually into any cell of the adult organism, and protocols have been established to generate hepatocyte-like cells (iHLCs) from those cells. iHLCs express hepatocyte-specific proteins and display biochemical functions specific to liver. Of interest, the exposition of iHLCs to oleic acid led to the accumulation of FFA and metabolic changes recapitulating the characteristics of NAFLD, with an upregulation of genes associated to lipid or glucose metabolism and to a PPAR-related pathway. Interestingly, the treatment of iHLCs with compounds modulating PPAR activity has demonstrated the beneficial effects of PPARα activation seen in patients by reducing the expression of enzymes involved in lipid and cholesterol synthesis[36]. Thus, iHLCs are promising models for NAFLD *in vitro* despite their limitations, such as an immature state compared with primary hepatocytes. Several strategies have been proposed to promote the maturation of iHLCs: (1) Adjusting amino acid levels in the culture media to increase CYP3A4 activity[45]; (2) Using small molecules such as activin A, LY294002 or FH1[46,47]; and (3) Co-culture with other cell types, such as fibroblasts[48].

Another group used undifferentiated iPSCs to study NAFLD by directly placing these cells in contact with oleate to verify their capacity to accumulate lipids. Compared with iHLCs, iPSCs did not present different levels of oleate. They demonstrated the potential of patient-derived iPSCs for initial research on NAFLD and other diseases characterized by lipid accumulation, particularly genetic factors. This allows the identification of high-risk individuals and the understanding of variations in treatment response[49].

To increase the complexity and attractiveness of 2D models, several research groups have developed co-culture systems. Barbero-Becerra *et al*[50] tested a co-culture of Huh-7 hepatocytes and LX-2 human hepatic stellate cells (HSCs) using cell inserts that allow the cells to grow independently while sharing the same culture medium. HSCs are specialized liver cells storing vitamin A, supporting liver regeneration by secreting growth factors, and producing and remodeling the extracellular matrix (ECM) to maintain liver structure. Upon liver injury, HSCs become activated and transform into myofibroblast-like cells, producing excessive ECM, especially type I collagen, leading to liver fibrosis and potentially cirrhosis.

In NAFLD, HSC activation is a key event in the progression from simple steatosis to NASH and fibrosis. HSCs also play a role in immunomodulation by interacting with immune cells and regulating inflammation[51]. The study concluded that the response of HSCs to FFA exposure and its effect on Huh-7 hepatocytes is independent of FFA exposure but requires cell-to-cell or proximity contact with hepatocytes. This is mediated by genes involved in ECM remodeling, such as *α-COL1* and matrix metalloproteinase-2, which exert regulatory effects *via* paracrine mediators[50]. A significant challenge of these 2D models is optimizing cell culture conditions for the various cell types involved[37].

For a better replication of the (patho)physiological characteristics and context through 2D models, several research groups have developed co-culture models. For instance, Barbero-Becerra *et al*[50] tested a co-culture of Huh-7 hepatoma cells and LX-2 human HSCs, where the two cell types grow separated by inserts allowing only the exchange of diffusible molecules and proximity contacts. Supplementation of FFA to this co-culture 2D cell complex led to the indirect activation of HSCs through cell-cell or proximal molecular interactions with Huh-7. This activation was attributed to the expression of genes involved in ECM remodeling, such as α-COL1 and matrix metalloproteinase-2, and paracrine mediators[50].

3D MODELS FOR NAFLD/NASH

Unlike 2D cellular models, 3D co-culture models recreate a more realistic 3D environment of the hepatic organ/tissue with spatial organization of cells and physiology that are a more accurate representation of cell-cell interactions occurring in NAFLD/NASH disease processes. Methods to produce 3D models may include hydrogels, synthetic scaffolds, and spheroid cellular formation. Other groups used microfluidic devices using PHHs or HepG2 hepatoma cells, where cells were exposed to FFA during a defined time point and metabolic and phenotypic changes were observed[52,53]. The standard method involves a 2D sandwich culture, in which hepatocytes are placed between two layers of collagen[54]. 3D spheroid models originated from PHHs of healthy and pathological donors have been used to study steatosis and insulin resistance. Despite variations among donors, such as age and phenotypes, spheroids exposed to FFA for 21 days mimicked pathological effects such as hepatic steatosis and triglyceride accumulation. Notably, the induced steatosis was reversible, suggesting that this model is ideal for studying NAFLD[55]. In 3D spheroids, PHHs were viable for more than 21 days allowing potential high throughput screening experiments. However, the generation of these models remains time-consuming and requires exhaustive validation.

ORGANOIDS TO INVESTIGATE NAFLD/ NASH

In 2009, Sato *et al*[56] successfully generated the first intestinal organoids (“mini-guts”) from intestinal stem cells, demonstrating the feasibility of producing complex. 3D structures that replicate the architecture and functions of specific organs. This groundbreaking achievement spurred significant interest across various research fields, inspiring the generation of organoids from numerous tissues and organs. Consequently, organoid models have become invaluable tools for studying diseases, drug testing, and applications in regenerative medicine.

Organoids are essentially 3D miniaturized and simplified versions of organs produced *in vitro* from stem cells. They arise from stem cells cultured under conditions that support spatial self-organization and differentiation, enabling them to mimic the cell-type composition, structural characteristics, and key functions of the original organ. Compared to traditional 2D cell cultures, organoids are more physiologically relevant, offering a more accurate architectural and

functional complexity representation of organs *in vivo*[57-59]. Moreover, organoids can be maintained long-term in culture, cryopreserved, biobanked without genetic alterations, and derived from stem cells or iPSCs to create personalized models[60].

Hepatic organoids, for instance, replicate the *in vivo* characteristics of liver tissue, including 3D cellular interactions and interactions with the ECM. Moreover, as multicellular structures, the organoids display functional units such as bile duct-like structures and express metabolic and detoxifying enzymes, transporters, and proteins involved in secretion of bile and other metabolites. Importantly, these organoids can be generated from patient-specific liver tissues or iPSCs and preserve genetic traits unique to each individual donor, which are critical for personalized medicine as they may influence drug metabolism and response. Personalized organoids may be instrumental to test and identify the medication with the more effective effects and least toxicity for the donor individual. Thus, organoids not only provide a powerful platform to advance our understanding of NAFLD/NASH mechanisms pathophysiology, but they may facilitate the development of improved therapeutic strategies[61].

Generating hepatic organoids from iPSCs involves protocols designed to guide the transition from 2D cells into self-organized 3D structures through different stages of differentiation. Once formed, these organoids are cultured under specific conditions to support either their expansion or differentiation into various hepatic lineages, including unicellular or multicellular hepatic organoids[61].

In vitro modeling of NAFLD using organoids can follow similar methods to traditional 2D approaches. Typically, a high concentration of FFAs is used to transition from simple steatosis and inflammation to fibrosis. Ramli *et al*[62] analyzed the gene expression profiles of organoids, comparing those exposed to FFAs with those not exposed, using samples from healthy individuals and those from patients with NASH. They found that organoids incubated with FFA exhibited gene expression patterns akin to those in liver tissues from patients with NASH[62].

By using iPSCs from healthy and diseased patients, Ouchi *et al*[63] established multicellular organoids composed of hepatocytes, stellate-like cells, and Kupffer-like cells, recapitulating progressive inflammation, steatosis, and fibrosis, in response to high doses of FFAs. This method proposed a novel approach to personalized treatments for inflammation and fibrosis in humans, enabling the identification of new therapies[63].

Hendriks *et al*[64] utilized expandable human fetal liver organoids to reproduce steatosis either by loading high doses of FFAs, expression of a patatin-like phospholipase domain-containing 3 variant (I148M) associated with a high risk of NAFLD, or by knockout of APOB or MTTP (mutations in these genes are linked to lipid disorders). Exposure of wild-type organoids to low concentrations of FFAs had no effect since the hepatocytes present within the organoids were able to metabolize those lipids, while at higher concentrations, hepatocytes progressively accumulated lipids. Organoids harboring patatin-like phospholipase domain-containing 3 variants or null for either APOB or MTTP showed high intracellular lipid accumulation[64]. Thus, liver organoids derived from stem cells or patient-specific iPSCs, hold potential for understanding NAFLD/NASH progression due to their ability to mimic disease hallmarks like steatosis and fibrosis.

DISCUSSION

NAFLD is an increasing health problem worldwide, which carries the risk of progression to more severe conditions, such as NASH, and even cirrhosis and hepatocellular carcinoma[3,6]. Despite many therapeutic approaches that are under investigation, including the search for metabolic regulators and novel strategies targeting oxidative stress and inflammation, no approved pharmacological therapy is currently available for NAFLD. MSCs have emerged as attractive cells for the treatment of NAFLD since these cells with immunomodulation and tissue regenerative properties are easily harvested from many tissues[13]. An ongoing clinical trial exploring autologous AD-MSCs for liver cirrhosis associated with NASH or fatty liver disease provides encouraging preliminary results, demonstrating improvements in liver function without adverse effects[19]. Despite the positive results of this clinical trial, further research is needed to understand the long-term safety and efficacy of AD-MSCs. Compared to current treatment options for NAFLD/NASH, which primarily focus on lifestyle modifications and managing symptoms, stem cell therapies offer the potential for a more targeted and regenerative approach.

A drawback of employing MSCs in therapies is cell heterogeneity where they can significantly impact the efficacy and consistency of treatments and impair the implementation of standardized MSC preparation[65,66]. A critical point in the production of an MSC-based advanced therapy medicinal product is its quality control, which includes identity, purity, safety, and potency tests because it enhances its safety and efficacy for administration to patients[66]. Therefore, it is essential to identify additional key control points like the assessment of heterogeneity through transcriptome sequencing, along with the monitoring of chromosomal instability and tumorigenicity[66]. *In vitro* culture of MSCs can result in the accumulation of genetic and epigenetic changes, exhibiting genetic instability that could account for their tumorigenic potential.

A significant challenge in developing MSC-based products is the considerable heterogeneity among cell sources, which display varying marker profiles, gene expressions, differentiation potentials, and immunomodulatory and paracrine characteristics. Additionally, factors such as the donor's age and health, along with the manufacturing process (encompassing culture medium composition, substrate properties, and oxygen concentration) are likely to influence the features of MSCs in various mechanisms[66].

To date, numerous single-cell transcriptome studies have been performed to explore MSCs heterogeneity. A study demonstrated that BM-MSCs presented higher transcriptomic heterogeneity than AD-MSCs, suggesting that adipose-tissue derived cells are more suitable for certain cell transplantation treatments[67]. Another study used 361 single MSCs

derived from two umbilical cord-MSC donors and demonstrated that umbilical cord-MSCs expanded *in vitro* present limited heterogeneity and are associated with cell cycle status[68]. Nonetheless, these two studies have limitations, including a small donor pool and the focus on only one or two tissue types. In response, Wang *et al*[69] conducted large-scale multiplexing single-cell RNA sequencing to create an atlas comprising 130000 single MSC transcriptomes from various tissues, including bone marrow, umbilical cord, adipose tissue, and dermis, sourced from 11 donors. This study revealed significant global variability among tissues, highlighting that ECM-related inflammation, aging, and antigen processing and presentation differ greatly within tissue-specific subpopulations, yet these variations are not linked to cell cycle status[69].

To date, the characterization of iMSCs and their heterogeneity have not been the primary focus of MSC transcriptome studies. Indeed, a unique transcriptome analysis has compared pluripotent stem cells, intermediate cells involved in iMSC derivation, mature iMSCs, BM-MSCs, and fibroblast gene expression. This study showed that iPSC-derived MSCs share a similar immunophenotype with BM-MSCs. While pluripotency markers are silenced, the epigenetic signature linked to the iPSC origin remains intact, which does not impede the differentiation potential of iPSCs. Additionally, as somatic cells were reprogrammed into iMSCs, genes associated with mesenchymal identity and lineage commitment were activated. A group of 52 genes with a higher expression profile distinguished MSC types from other cell types[70].

Another important consideration is the tumorigenic potential of MSCs. Stucky *et al*[71] used single-cell RNA sequencing to analyze MSCs during osteogenic differentiation and identified a subpopulation of differentiation-resistant MSCs. These cells exhibited upregulated expression of the *YAP1* gene network, which is involved in stemness, proliferation, and epithelial-mesenchymal transition, a key processes in cancer initiation and progression. They performed a meta-analysis of clinical cancer data, demonstrating a high degree of coexpression among CDH6, YAP1, and OCT4 across various solid tumors. These findings suggested that the *YAP1* gene network plays a key role in the tumorigenicity of MSC subclones that are resistant to differentiation[71]. Conversely, Malvicini *et al*[72] demonstrated that OCT4 is fundamental to MSC to differentiate into adipocytes and osteoblasts. Collectively, these studies underscore the necessity of a balanced expression of these factors in MSCs to maintain their multipotency and prevent tumorigenicity and the necessity to understand MSCs gene expression dynamics to develop safe and reliable an advanced therapy medicinal products.

The ability to generate patient-specific iPSCs has opened avenues for tailoring treatments and setting up complex *in vitro* models to unravel disease mechanisms at a molecular level. It is crucial for advancing precision medicine approaches in NAFLD/NASH. Indeed, functional and well-characterized iMSCs and iHLCs represent a promising opportunity for (personalized) therapies[20]. Functional iHLCs could be reintroduced in the diseased liver to substitute lost functional hepatocytes. On the other hand, iMSCs could be substitutes to tissue MSCs, which are still unstable and difficult to culture for reaching large numbers of cells required for therapies. However, ethical considerations surrounding the use of iPSCs and potential side effects of MSC therapy need to be carefully addressed. Indeed, the use of iPSC-derived cells in therapy generally lacks long-term safety data. Addressing these concerns is crucial, as both iPSCs and iMSCs may present risks of tumorigenicity if not carefully selected. Additionally, it is essential to provide clear and accurate information to patients participating in early-phase clinical trials, as they may have unrealistic expectations or misunderstandings regarding the efficacy of these pioneering therapies.

Both iMSC and iHLCs are useful for disease modeling, drug screening, and personalized therapies[20,23]. Of interest, EVs and proteins secreted by MSCs display the cellular properties to modulate lipid metabolism, reduce inflammation, and promote tissue repair. Therefore, EVs could be substituted for MSCs for NAFLD/NASH treatments in a safer manner than cells since they do not bear genomic DNA material[32,33]. Further research into priming strategies, such as enhancing EV production from iMSCs through targeted molecular interventions, holds promise for enhancing therapeutic efficacy in NAFLD/NASH. Overall, while challenges persist, the potential of stem cells, particularly iMSCs and their secreted EVs, in reshaping the landscape of NAFLD treatment underscores their importance as a focal point for future research and clinical translation efforts. Importantly, optimizing differentiation protocols of desired cells and production/isolation of EVs, ensuring safety, and addressing regulatory concerns are required before stem cell-based therapies can be widely implemented clinically[31]. Beyond priming strategies for EVs, exploring broader future research directions in the field, such as optimizing cell delivery methods or combination therapies with existing treatments, would provide a more comprehensive picture.

Hepatic, intestinal, and many organoids, which are *in vitro* structural and functional representations of the organs, have originated from stem cells including iPSC to represent dysfunctional organs or healthy organs[60]. Hepatic organoids are promising cellular structures for retracing the etiology and progression of NAFLD/NASH[61,63]. However, improvements are needed to optimize their use. Reproducibility in size and cellular organization, especially in multicellular organoids, must be enhanced, as structural variations can affect cell interactions and responses to stimuli. Organoid models created from iPSCs exhibit low hepatic maturity, affecting disease modeling and drug testing. A key limitation is their insufficient vascularization, which limits nutrient and stimulus distribution, restricts organoid size, and obstructs the formation of complex structures for *in vivo* pathology modeling[61]. Those different organoids could be coupled through microfluidic devices to provide a more controlled environment and simulate blood flow for the exchange of metabolites and proteins.

Overall, iPSC and iPSC-derived cells are promising for modeling NAFLD/NASH and the discovery of novel therapeutic strategies. However, while challenges persist, the potential of stem cells, particularly iMSCs and their secreted EVs, in reshaping the landscape of NAFLD treatment underscores their importance as a focal point for future research and clinical translation efforts.

CONCLUSION

Stem cell-based therapies, particularly those utilizing MSCs and HLCs derived from iPSCs, hold significant promise for the treatment of NAFLD/NASH. Unlike current therapies that primarily focus on symptom management, iPSC-derived therapies offer the potential to replace damaged cells and tissues in the liver, promoting the restoration of liver functionality. However, for these therapies to become clinically viable, several critical challenges must be addressed. A key challenge lies in the optimization of cell differentiation protocols to generate high-quality, functional iPSC-derived HLCs and iMSCs that closely replicate the characteristics of their *in vivo* counterparts. Furthermore, scaling up the production of these cell populations, essential for clinical applications, without compromising their functionality may constitute a significant obstacle. Safety concerns also play a central role in the clinical translation of iPSC-derived therapies. A primary concern is the risk of tumorigenicity associated with the use of iPSC-derived cells, such as iMSCs. Rigorous screening and quality control measures are essential at every stage of cell generation and clinical application. Protocols must include mechanisms for detecting and eliminating undifferentiated iPSCs in the final therapeutic cell population to mitigate the risk of teratoma formation. Additionally, the detection of chromosomal abnormalities in these cells should be incorporated into quality control protocols to ensure the safety and stability of the cells used in therapy.

In parallel, the utilization of advanced models such as organoids and single-cell profiling techniques will significantly enhance the understanding of the underlying mechanisms of NAFLD and NASH. These innovative models allow for detailed disease modeling and drug screening, facilitating the development of more effective and personalized therapeutic strategies. The translational potential of iPSC-derived therapies is particularly noteworthy in the context of personalized medicine. By generating patient-specific iPSCs and liver models, such as liver organoids, researchers can better investigate the unique genetic and environmental factors that contribute to NAFLD/NASH. These models offer a robust platform for drug screening, efficacy assessment, and prediction of treatment outcomes on an individualized basis. Moreover, iMSCs demonstrate the capability to modulate fibrosis and inflammation in NASH, providing a targeted therapeutic approach that may surpass the limited efficacy of conventional pharmacological treatments in reversing disease progression.

The integration of iPSC-derived cells into clinical practice holds the potential to transform the treatment landscape for liver diseases by offering regenerative and personalized solutions that traditional therapies cannot achieve. Crucial to the success of these therapies is the development of robust strategies to ensure the engraftment, long-term survival, and proper integration of transplanted cells within the host liver tissue. Addressing these factors is vital for achieving sustained therapeutic outcomes and enhancing patient responses to treatment.

Ethical considerations surrounding iPSC-based therapies must also be addressed. This includes ensuring that informed patient consent provides accurate and comprehensive information, particularly for individuals participating in early-phase clinical trials. Transparent communication regarding the potential adverse effects of these therapies, as well as contingency plans in case of unexpected outcomes, is essential. Furthermore, the accessibility of such cutting-edge therapies must be equitable, ensuring that all patients, irrespective of their socioeconomic status or geographic location, have the opportunity to benefit from these advancements.

While significant challenges remain, the potential of iPSC-derived therapies to address the unmet needs in NAFLD/NASH treatment is substantial. With continued optimization of differentiation protocols, safety measures, and scalability, along with the integration of personalized medicine strategies, these therapies could bring about a transformative shift toward more effective, individualized interventions for liver disease.

FOOTNOTES

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