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Contents

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REVIEW

Induced pluripotent stem cells for therapy personalization in pediatric patients: Focus on drug-induced adverse events

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Abstract

Adverse drug reactions (ADRs) are major clinical problems, particularly in special populations such as pediatric patients. Indeed, ADRs may be caused by a plethora of different drugs leading, in some cases, to hospitalization, disability or even death. In addition, pediatric patients may respond differently to drugs with respect to adults and may be prone to developing different kinds of ADRs, leading, in some cases, to more severe consequences. To improve the comprehension, and thus the prevention, of ADRs, the set-up of sensitive and personalized assays is urgently needed. Important progress is represented by the possibility of setting up groundbreaking patient-specific assays. This goal has been powerfully achieved using induced pluripotent stem cells (iPSCs). Due to their genetic and physiological species-specific differences and their ability to be differentiated ideally into all tissues of the human body, this model may be accurate in predicting drug toxicity, especially when this toxicity is related to individual genetic differences. This review is an up-to-date summary of the employment of iPSCs as a model to study ADRs, with particular attention to drugs used in the pediatric field. We especially focused on the intestinal, hepatic, pancreatic, renal, cardiac, and neuronal levels, also discussing progress in organoids creation. The latter are three-dimensional *in vitro* culture systems derived from pluripotent or adult stem cells simulating the architecture and functionality of native organs such as the intestine, liver, pancreas, kidney, heart, and brain. Based on the existing knowledge, these models are powerful and promising tools in multiple clinical applications including toxicity screening,

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Core tip: Adverse drug reactions (ADRs) are major clinical problems, especially in pediatric patients, who may respond differently to drugs with respect to adults. This up-to-date review focuses on the employment of patient-derived induced pluripotent stem cells and related systems (*i.e*. stem cell-derived organoids) to study ADRs in adults, and wherever available, in the pediatric field. We especially focused on the intestinal, hepatic, pancreatic, renal, cardiac, and neuronal levels, in which the major ADRs are usually observed. Due to their genetic and physiological species-specific differences, these models may be accurate in predicting drug toxicity.

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ADVERSE DRUG REACTIONS

Adverse drug reactions (ADRs) are major clinical problems[1], especially for special populations such as pediatric patients[2,3]. Indeed, a significant number of drugs can cause adverse effects leading, in some cases, to patients' hospitalization, permanent disability, or even death. ADRs are costly for pharmaceutical industries in terms of drug withdrawal from the market[4], but also in terms of clinical and economical efforts needed to resolve side effects[1]. In order to avoid these problems that affect patients' health and greatly increase costs related to treatment, it is necessary to improve clinical trial strategies. In addition, both *in vivo* and *in vitro* models are needed that better assess and evaluate drug safety. Due to newly discovered technologies and progress made in this area, this goal seems achievable[5-7]. Indeed, to date, it is possible to establish more sensitive and personalized assays leading to a better comprehension, and thus prevention, of ADRs. One of the most important advances in this field is the possibility to set up groundbreaking patient-specific assays. Indeed, ADRs are related to individual genetic patients' background, leading to a wide range of toxicities of different severities[8]. Another important point is that pediatric patients may respond differently to drugs than adults and are susceptible to developing different patterns of ADRs, leading, in some cases, to more severe consequences[1-3]. One of the most powerful tools that can be used to individually model drug response and ADR development is represented by induced pluripotent stem cell (iPSC) technology, which was discovered by Takahashi *et al*[5] more than 10 years ago.

iPSCs can be used to evaluate safety during drug preclinical screening, possibly replacing the use of animal models or immortalized human cell lines. Due to genetic and physiological species-specific differences, these models may be accurate in predicting drug toxicity, especially when this toxicity is related to individual genetic differences[9]. Indeed, many factors may influence drug pharmacokinetics and pharmacodynamics as well as the development of adverse effects, for instance: (1) Polymorphisms in genes encoding for drug-metabolizing enzymes and transporters, ion channels and receptors, possibly affecting their expression and/or activity[10]; (2) Epigenetic alterations such as DNA methylation, histone modification, microRNAs, mRNA instability, and nucleosome positioning[11]; and (3) Environmental and nongenetic factors including body mass index and behavioral patterns, concomitant diseases, in particular liver and kidney diseases, and developmental factors, especially those in early life[12].

In this review, we explored the state of the art of iPSCs as a model to study ADRs in adults, and wherever available, in the pediatric field. In particular, we focused on

intestinal, hepatic, pancreatic, renal, cardiac and neuronal levels, also discussing the progress in the creation of organoids (Figure 1).

ADVERSE DRUG REACTIONS IN PEDIATRIC PATIENTS

Children can respond differently to many drugs and can present different ADRs than adults[2,3]. The reason is mainly the variation in the pharmacokinetic and pharmacodynamic profiles between pediatric and adult patients[13,14]. In the era of personalized medicine, it is important to screen and identify genetic variations related to the predisposition to ADR development for certain drugs[15,16]. However, in pediatric patients, ADRs have not been studied as thoroughly as in adults. The clinical and research experience on drug safety for children is limited and more works are needed[17]. One of the reasons for the lack of these data is that, for ethical reasons, the effects of most drugs have been analyzed in clinical trials only in adults, resulting in limited knowledge on children's responses[17]. To date, there is strong interest into therapy personalization for children to further explore, study, and prevent possible new and severe ADRs. In order to avoid the well-known ethical limitation of pediatric trials, to increase the comprehension of drug response in children, and establish safer and personalized treatment, it is possible to use innovative technologies such as iPSCs[5,18]. Indeed, several specific pediatric problems could be addressed using iPSCs; for example, this technology is ideal to develop innovative patient-specific models of rare or genetic diseases that often occur in pediatric populations[13]. Moreover, several drugs used in pediatric patients lead to adverse effects in organs that are not easily accessible, such as pancreatitis for asparaginases[18] for the treatment of acute lymphoblastic leukemia.

INDUCED PLURIPOTENT STEM CELLS, THERAPY PERSONALIZATION, AND ADRs

Patients' somatic cells can be reprogrammed into iPSCs using the four Yamanaka's factors[5]. iPSCs preserve the donor's genetic heritage and are useful for creating patient-specific models[19]. For a decade, scientists have been using iPSCs to study complex diseases and to obtain tissues or cells otherwise not simply accessible from patients[20]. Moreover, these cells have allowed scientists to set up innovative research to study ADRs in a more personalized way[18,21-24]. The focal point of this technology is the possibility to differentiate iPSCs into almost any cell of the human body[20]. Progress made in this last decade has allowed the development of groundbreaking tailored models for single individuals, contributing to the modern era of therapy personalization, finding the appropriate treatment for the right patient, and avoiding the development of ADRs[25]. To date, a number of studies based on iPSCs for therapy personalization and to study ADRs, especially in the adult field, are available[18,21-24]. However, more efforts are needed in the pediatric field, even though some works are already available[26-28]. iPSCs may be a great tool to model sensitivity to new, but also old drugs in children, shedding light on the mechanisms of toxicity, resolving in part the problem of the lack of data. Indeed, with a simple blood sample, it is possible to reprogram peripheral blood mononuclear cells into iPSCs that can be subsequently differentiated into somatic cells of interest.

ORGANOIDS AS GROUNDBREAKING PATIENT-SPECIFIC MODELS FOR PHARMACOLOGICAL STUDIES

Organoids are three-dimensional (3D) *in vitro* culture systems derived from pluripotent stem cells (embryonic stem cells or iPSCs) or adult stem cells, and can simulate the architecture and functionality of native organs[29]. Organoids are generated from multiple organs including the intestine[30,31], stomach[32], kidney[33], liver[34], pancreas[35], brain[36], and lung[37] and can be used in multiple clinical applications including drug screening, disease modeling, and regenerative medicine. Moreover, the development of human patient-derived organoids may enable personalized medicine. Considering that organoids cultures based on a specific disease and from a specific individual can be expanded in culture maintaining a stable genetic and epigenetic signature[38], they are suitable for biobanking and high-throughput screening. Therefore, organoids represent a powerful tool for drug efficacy and drug toxicity screening. Future analyses may be performed using these

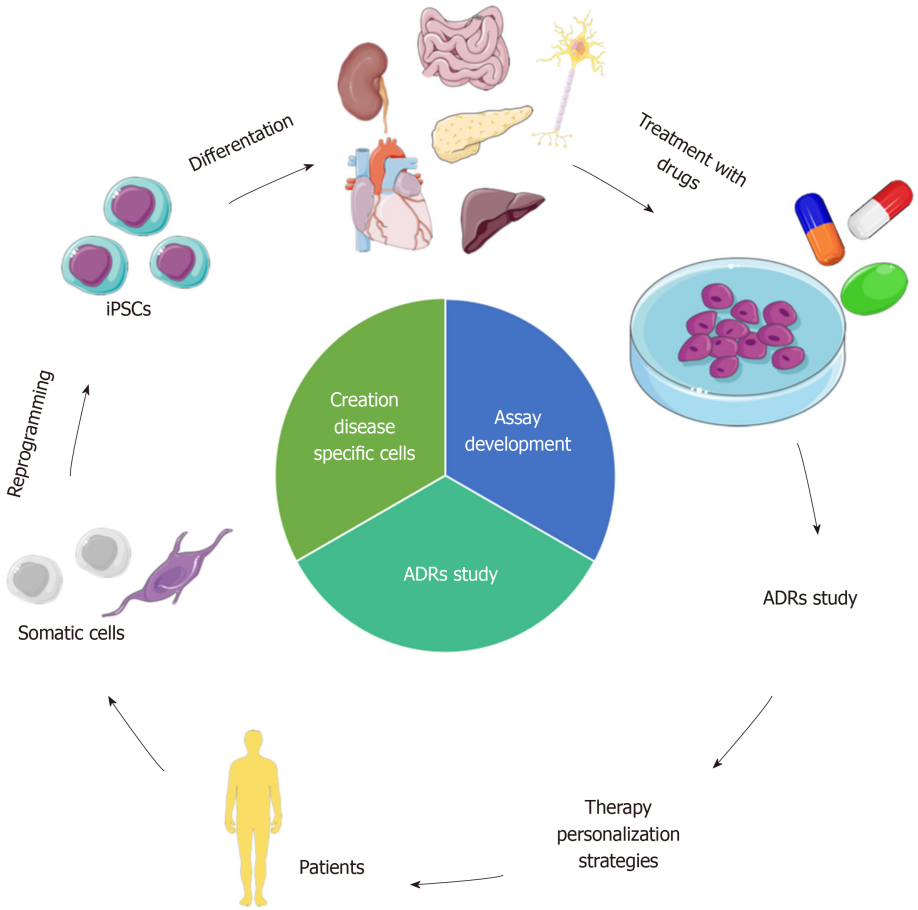

Figure 1 iPSCs to model ADRs for therapy personalization. iPSCs: Induced pluripotent stem cells; ADRs: Adverse drug reactions. Image adapted from: https://smart.servier.com/.

biobanks to identify new drugs, but also to know in advance which patients may benefit from an existing drug treatment. In toxicology screenings, 3D organoids could replace the use of cell lines and animal models. Renal and hepatic toxicities are common in drug administration and hepato-biliary organoids generated together with kidney organoids enable the accurate study of drug metabolism and toxicity. Interestingly, 3D organ-on-a-chip is an experimental system in which tissue architecture and cellular composition are assembled on a fabricated synthetic matrix[39]. This technology represents a novel approach to overcome the limitations of conventional model systems, especially to model complex disorders affecting several tissues, and for future pharmacological target identification, safety, and efficacy testing as well as personalized medicine.

GASTROINTESTINAL TOXICITY

Drug-induced gastrointestinal toxicity is very common and may result in different consequences that range from nausea and dyspepsia to life-threatening events such as the development of complicated gastroduodenal ulcers[40,41]. To study and prevent these ADRs, researchers have started to create patient-specific *in vitro* models using iPSCs. These models are based on the differentiation of iPSCs into enterocytes, using innovative protocols, and involve the creation of groundbreaking models based on iPSC-derived human organoids.

Intestinal ADRs

The small and large intestines are one of the most frequent sites of ADRs. The severity of symptoms can vary from very easy-to-manage to life-threating illnesses. Well known examples of intestinal ADRs are mucosal damage, hemorrhage, colitis, changes in motility, and malabsorption[42]; an important example of frequent gastrointestinal ADR are those caused by nonsteroidal anti-inflammatory drugs

(NSAIDs). To manage and prevent the development of intestinal ADRs, scientists tried to model enterocytes, the most abundant intestinal cells with fundamental functions in nutrient/drug absorption and metabolism. In this regard, iPSCs could be a great tool, as demonstrated by some recent works. In 2014, Iwao *et al*[43] generated for the first time pharmacokinetically functional enterocytes starting from iPSCs, with the aim of building an intestinal pharmacokinetic evaluation system to better study drug absorption and biotransformation. The protocol developed by this group allowed the authors to obtain mature enterocyte-like cells (ELCs) after about 30 days of stimulation using activin A and fibroblast growth factor (FGF)-2 in order to obtain definitive endoderm cells. Then these immature cells were differentiated into ELCs using epidermal growth factor in 2% fetal bovine serum. ELCs displayed increased expression of the specific intestinal markers sucrase-isomaltase, villin 1, intestine-specific homeobox, CDX2, dipeptidyl peptidase 4, and SLC46A1/proton-coupled folate transporter. The authors also noticed that a longer period of differentiation made the process more efficient in terms of the number of mature ELCs obtained.

In a subsequent study, the same group tried to ameliorate the efficiency of the already established protocol using different small molecules[44]. In particular, the authors analyzed the effect of GSK-3 inhibitor XV (a highly potent inhibitor of GSK-3), dorsomorphin (a selective bone morphogenetic protein [BMP] inhibitor), PD98059 (a mitogen-activated protein inhibitor), 5-aza-20-deoxycytidine (an inhibitor of DNA methylation), and A-83-01 (a potent and selective transforming growth factor beta [TGF-β] pathway inhibitor). Overall, the differentiated cells expressed intestinal markers, drug transporters and metabolizing enzymes, such as cytochrome P450 1A1/2 (CYP1A1/2), CYP2C9, CYP2C19, CYP2D6, CYP3A4/5, UDP-glucuronosyltransferase (UGT), and sulfotransferase. To analyze the intestinal differentiation of iPSCs after small molecule treatment, the authors considered mRNA expression of sucrase-isomaltase and CYP3A4, which were both markedly increased, while those of intestinal stem cell markers decreased. In particular, CYP3A4 mRNA expression level was mainly induced by the addition of 1α,25-dihydroxyvitamin D3. Intestinal drug transporter peptide transporter 1 was also increased by PD98059, 5-aza-29-deoxycytidine, and A-83-01. With this work, the authors created a model of enterocytes that is definitely more useful than primary cells given their poor viability, short life span, limitation of passage number, and difficulty in obtaining human intestinal tissue samples. Moreover, ELCs seem to be a better model with respect to the widely used Caco-2 cells, since the drug transporter expression levels and carrier-mediated drug permeability in Caco-2 are different from those of the human duodenum, and the expression level of CYP3A4 is quite low.

A similar work was performed by Ozawa *et al*[45], who generated an ELC monolayer characterized by drug absorption and metabolism functions. The authors[45] analyzed the barrier formation capacity of the obtained monolayer, measuring the transendothelial electrical resistance (TEER). By this test, the authors found that the ELC monolayers have a weaker barrier function than Caco-2 cell monolayers. Importantly, it is known that TEER values in Caco-2 cell monolayers are higher than those in the small intestine. Therefore, the authors concluded that the ELC monolayers might be a more suitable *in vitro* model for evaluating the absorption of hydrophilic drugs than Caco-2 cell monolayers. Overall, these studies provide a basis for exploiting ECL models to study drug absorption, metabolism, and ADRs.

Regarding our current knowledge, only a very recent work[46] applied a 2D ELC model to study ADRs. In particular, Matsunaga's group[46] used ELCs, obtained using the protocol already set up and described above[43,44], to study human drug-induced intestinal mucosal damage. In this work[46], mucin 2 (MUC2) mRNA expression as a marker of mucosal damage was analyzed. MUC2 is the main component of intestinal mucus and its expression in the intestine is decreased by several drugs that induce mucosal damage, such as NSAIDs, and is increased by other protective agents including rebamipide. First, the authors compared MUC2 expression in the human intestine, ELCs, and Caco2 cells to validate the ELC model. The results showed that after 26 days of differentiation, ELCs presented MUC2 levels comparable to those of the human intestine, while levels in the Caco2 line were 100 times lower. Authors also examined the effects of NSAIDs and of the mucosal protective agent rebamipide on MUC2 expression in the ELC model, confirming that NSAID exposure reduces MUC2 levels while rebamipide increases it. Given that the fluctuation of MUC2 seemed to be influenced by different cytokines, they also analyzed the mRNA expression levels of cyclooxygenase-2, interleukin (IL)-1β, nuclear factor (NF)-κB, and tumor necrosis factor (TNF)-α after drug treatment. In particular, as reported in other studies[47-51], they found that IL-1β, NF-κB, and TNF-α mRNA expression levels were decreased by indomethacin, but increased by rebamipide. Therefore, taken together, the authors concluded that it seems reasonable that these factors may be involved in MUC2 expression changes in enterocytes.

Regarding the advances for obtaining intestinal models used in drug screening, intestinal organoids also represent a valuable tool[52]. Self-renewal of the intestinal epithelium is driven by the proliferation of stem cells and their progenitors located in crypt regions[30]. Wingless/integrated 3A (WNT3A), R-spondin, and noggin are factors needed in organoid protocols to promote *in vitro* self-proliferation of intestinal stem cells; moreover, these organoids are composed by different cell types such as enterocytes, Paneth cells, goblet cells, and enteroendocrine cells and manifest many enteric characteristics[53].

Recently, Lu and Rosenbaum[54] described the application of crypt organoid cultures from genetically modified mice as a model to evaluate drug metabolism. Irinotecan metabolism and toxicity were studied using crypt organoids generated from both $Ugt1^{F/F}$ (control) and $Ugt1^{\Delta IEC}$ (deletion of the Ugt1 locus) mice. These 3D cultures metabolize the drug to the active topoisomerase inhibitor metabolite SN-38, which is further metabolized by UGT1A1-dependent glucuronidation to form an SN-38 glucuronide. In the absence of Ugt1 gene expression, $Ugt1^{\Delta IEC}$ crypt cultures exhibit very limited production of SN-38 glucuronide, concordant with increased apoptosis in comparison with $Ugt1^{F/F}$ crypt cultures. Glucuronidation is an important phase II pathway responsible for the metabolism of many drugs used also in the pediatric age, such as opioids or acetaminophen[55]. These results demonstrate that intestinal organoid cultures can be employed to study drug metabolism, under conditions of altered pharmacogenetics.

Colonic organoids derived from iPSCs have been used to evaluate the toxicity of rapamycin and geneticin; an impairment of cell proliferation was only observed after treatment with rapamycin indicating that this compound can harm the healthy colon[56].

Hepatic ADRs

Drug-induced liver injury (DILI) is a severe ADR characteristic of more than 1000 drugs[57]. The estimation of DILI worldwide is between 1 in 10000 and 1 in 100000 inhabitants, even though some recent studies have reported a higher occurrence[58]. In the last several decades, the study and prevention of this ADR have gained increasing interest in the scientific community, given its increasing incidence caused mainly by the number of new drugs on the market[57]. Moreover, DILI is the most common cause of drug withdrawal from the market with consequent high costs for industries[59]. DILI is divided into acute or chronic conditions including hepatitis and acute liver failure, leading, in the most severe cases, to liver transplant and aggressive treatments[57-60]. The mechanism of DILI development can be dose-related or idiosyncratic.

Further efforts are needed to manage and prevent this ADR, given the limitations of current *in vivo* and *in vitro* models[61,62]. iPSCs are a great tool to overcome these limitations and to obtain more predictive results. Indeed, several studies have already shown how to differentiate human iPSCs into hepatocyte-like cells (HLCs) to create a source of cells for different purposes such as *in vitro* drug studies. One example is the study performed by Kondo *et al*[63], where iPSCs were differentiated into HLCs using a three-step protocol, using three growth factors (activin A, hepatocyte growth factor, oncostatin M) and two small molecules (dimethyl sulfoxide, dexamethasone). With this work, the authors established a reproducible and relatively inexpensive method to obtain a greater number of hepatic cells, with respect to other protocols, to perform, for example, pharmacological studies. The differentiated cells expressed the hepatocyte markers hepatocyte nuclear factor 4 alpha (HNF4-α), albumin (ALB), and alpha-fetoprotein (AFP) at similar or higher levels in comparison to primary human hepatocytes (PHH) and HepG2 cells. HLCs were also characterized by the expression of drug metabolizing enzymes such as CYP3A4 and UGT1A1. Authors demonstrated that the mRNA expression levels of CYP3A4 and UGT1A1 were increased by the CYP inducers dexamethasone, rifampicin, and omeprazole. However, the expression levels of drug-metabolizing enzymes were very low compared to those in PHH and mature liver. Two years after Kondo's work, Kang *et al*[64] confirmed the greater similarity of HLCs and PHH, with respect to HepG2 cells, analyzing acetaminophen hepatotoxic effects on all three cell types. In particular, HLCs were more similar to PHH in comparison to HepG2 cells both in terms of cell viability after acetaminophen exposure and CYP450 levels, with similar downregulation of CYP1A2 and CYP3A4 genes by cytotoxic concentration of both agents. In contrast, HepG2 cells showed an increment in CYP levels.

Takayama *et al*[21] investigated whether HLCs, obtained differentiating iPSCs of different donors, could reproduce the interindividual difference in hepatic biotransformation and drug response. In this regard, HLCs were generated from human iPSCs, established by reprogramming donor PHH. iPSCs were generated using a non-integrative method, based on Sendai Virus vectors, a process essential to avoid insertional mutagenesis. After this, they compared the drug metabolism and

drug responsiveness of HLCs to those of their parental PHH. The main purpose of this work was to establish a panel of HLCs that represents the diversity of genetic polymorphisms in humans, in order to use these cells to determine the appropriate drug dosage for the single individual. In particular, they focused on CYP activity levels in the HLCs with respect to the parental PHH. Results showed that CYP activity and drug responsiveness of individual HLCs reflected those of parental cells, suggesting that it might be possible to predict individual CYP activity using HLCs and to perform personalized drug treatment analyzing HLCs of the single patient. Moreover, the presence of a single nucleotide polymorphism (SNP) in genes encoding CYP2D6, related to a different metabolism and drug responsiveness, was successfully reproduced in HLCs. Also, Liu *et al*[65] successfully differentiated iPSCs into HLCs with a relatively simple three-step protocol, using the commercial hepatocyte maturation medium HepatoZYME (Life Technologies, Frederick, MD, United States). These authors reprogrammed peripheral blood mononuclear cells (PBMCs) instead of PHH, with obvious advantages, given the greater ease of access of blood with respect to liver biopsies. Similarly, Wilson *et al*[66] generated iPSCs and then HLCs from a cohort of individuals affected by alpha-1 anti-trypsin deficiency (AATD), a genetic disorder related to liver cirrhosis and pulmonary emphysema and characterized by low levels of AAT, the main protease inhibitor (PI) in human serum. In particular, the most common deficient allele involved in the development of AATD is the PI homozygous for the Z allele (termed PiZZ by authors). The most common disease variant is caused by an inherited single base pair mutation of the serpin family A member 1 gene, which results in a glutamate to lysine substitution and production of a mutant version of the PI AAT, known as Z AAT. Interestingly, the authors found that the global transcriptomes of iPSCs, carrying PiZZ mutations, diverge from that of the healthy controls (three control individuals without any known disease) only after differentiation to HLCs, when the AAT gene is expressed. Moreover, the obtained HLCs successfully model key features of AAT-associated liver disease, including intracellular accumulation and reduced secretion of AAT protein as well as increased autophagic flux. The authors confirmed an increase in autophagic flux upon treatment with the drug carbamazepine as previously described in mice carrying the mutation. Subsequently, authors tested if the PiZZ mutation can increase the toxicity of different drugs with respect to healthy PHH, and exposed both HLCs and controls to acetaminophen and other drugs known to cause hepatotoxicity. In each case, HLCs carrying the PiZZ mutations were more sensitive to drugs with respect to PHH. However, further studies need to be done to evaluate the cytotoxic mechanisms of drugs in HLCs carrying the PiZZ mutations with respect to healthy HLC controls. The authors concluded that these findings support the utility of iPSCs as tools for drug development or prediction of toxicity. More recently, Kvist *et al*[67] deeply analyzed the critical differences in drug metabolic properties of different human hepatic cellular models including PHH, HLCs, and the hepatoma cell lines HepG2 and HepaRG. Surprisingly, these authors showed that HLCs, obtained differentiating iPSCs, should not be used as a model to study drug metabolism, and thus ADRs, since critical differences were detected with respect to human PHH. This conclusion, in contrast with other works[21,63,64], arises from a different analysis performed by Kvist's group analyzing the expression and function of key hepatic proteins important for the metabolic fate of drugs such as CYP enzymes. A principal component analysis to study and compare gene expression of HLCs, PHH, and the hepatoma cell lines HepG2 and HepaRG showed a distance between the two iPSC-derived hepatocytes, as well as HepG2 and HepaRG cells, and the three PHH donors and PHH pool, which were clustered more closely together. This finding was confirmed by another analysis, which clustered HepG2 close to HLCs in terms of gene expression of 91 genes related to the liver function or CYP450. Moreover, HLCs were found to have low activity of several CYPs such as CYP3A and CYP2C9, barely detectable activity of CYP1A2, 2B6, 2C8, 2C9, 2C19, 2D6, and a high expression of several extrahepatic P450s such as CYP1A1 and 1B1 that may have significant effects on biotransformation profiles. On the other hand, HepaRG cells showed a CYP profile very similar to PHH, suggesting that this cell line can be a good model in drug metabolism studies and ADRs. The authors concluded that, to date, HLCs derived from patients' iPSCs should not be used as a substitute for PHH in drug toxicity studies. To improve the performance of the HLC model, the authors suggested to culture cells in a 3D rather than the current 2D monolayer, because the 3D model has been shown to improve the performance of PHH[68]. In 2018, Smutný *et al*[69] used HLCs to study the toxicity of phytochemicals saikosaponin D, triptolide, deoxycalyciphylline B and monocrotaline known to cause DILI, in comparison with hepatoblastoma-derived HepG2 cells and long-term culture of primary human hepatocytes (LTHHs). In order to compare the cytotoxic effects of the tested phytochemicals, the authors analyzed hepatocyte key markers in the HLCs compared to the HepG2 and LTHHs controls. First, they analyzed ALB level, a

specific protein produced only by hepatocytes and hepatoblasts by immunofluorescence staining and real time PCR. Both HLC and HepG2 cells exhibited intense staining of ALB. The mRNA level of ALB in the HLC cells was similar to that of HepG2 cells but was lower than that in reference LTHHs of the two donors. Then, transcription of HNF4α, a liver-enriched transcription factor associated with the regulation of many liver-specific genes, was confirmed in the HLC model, as well as the expression of CYP3A4, which however was lower with respect to LTHHs. Interestingly, HepG2 cells were negative for the important CYP3A4 drug-metabolizing enzymes. Additionally, the authors analyzed the maturation of HLCs studying three markers: AFP, a typical liver marker expressed in hepatoblasts and fetal hepatocytes, but not in adult hepatocytes; cytokeratin 19 (CK19), a marker of cholangiocytes and hepatic progenitors; and CYP3A7, a CYP450 enzyme expressed mainly in fetal hepatocytes and at a very low level in adult hepatocytes. Overall, the results showed that HLCs resemble to be closer to an immature hepatic phenotype expressing both AFP and CK19 markers. After characterizing HLCs, they analyzed the potential of HLCs, HepG2, and LTHHs to predict DILI using hepatotoxic compounds. Overall, HLCs appeared more sensitive to triptolide and saikosaponin D in comparison to both HepG2 cells and LTHHs. Interestingly, the authors noticed an atypical response of HepG2 cells with less toxicity at higher concentrations of triptolide. This atypical effect could be related to particular resistance mechanisms characteristic of the HepG2 line, such as induction of metabolizing enzymes and/or efflux transporters induced by high doses of this phytochemical. Also, saikosaponin D treatment produced higher cytotoxic effects in HLCs, although LTHHs also showed high sensitivity. However, the HepG2 cells resulted less sensitive to saikosaponin D with effects observed at the highest concentration tested. Regarding monocrotaline, no cytotoxic effect was reported in all lines tested. The authors commented that this observation may be related to targeting by monocrotaline principally of hepatic sinusoidal endothelial cells. However, it would be interesting to further explore the causes of this resistance. Finally, they analyzed deoxycalyciphylline B effects, finding a mild decrease in mitochondrial activity at the maximal tested concentration only in LTHHs cells, while neither HLCs nor HepG2 exhibited any toxic effect. The authors concluded that this study provides a basis for further in-depth studies to confirm HLCs as a competent *in vitro* liver cell model for toxicological assessment; however, further efforts are needed to develop HLCs with a more mature phenotype, expressing typical adult hepatocyte markers such as CYP3A4, HNF4α, and ALB despite the expression of immature markers typical of fetal hepatocytes and hepatic progenitors such as AFP and CK19. Another recent and interesting work was performed by Yamazaki and Murayama[70], which analyzed CYP450 expression levels of commercial HLCs at different culture times. Authors found a significant increase of CYP450 activities after 3-4 weeks with respect to HLCs cultured for 1 week. After 4 weeks, HLCs reached CYP450 levels similar to those in HepaRG cells. The increase in activity was associated with increasing CYP450 2C9 and 2C19 mRNA levels. This finding can help researchers perform more precise and repeatable studies on HLCs and drugs and is in contrast with the manufacturer's instructions that suggest the use HLCs after 1 week of culture.

In this context, the recent development of liver organoid culture systems derived from iPSCs provides another promising strategy to study drug-induced hepatotoxicity[71,72]. Liver organoids closely resemble *in vivo* human liver, preserving their genetic and epigenetic integrity over months in culture[73].

The most recently published method to generate functional hepatobiliary organoids from iPSCs cultured on Matrigel was developed by Wu *et al*[74]. The protocol is based on inclusion at differentiation stages I and II (days 1–15) of 25% mTeSR culture medium into hepatic differentiation medium to induce endodermal and mesodermal commitment; subsequently, at stage III (days 15-45), 10% cholesterol and other small molecules (a Chinese patent pending product called cholesterol$^+$MIX) were added to the maturation medium to promote the formation and maturation of hepatobiliary organoids by activating the NOTCH2 and TGF-β signaling pathways. Concerning drug metabolic functions, the expression of several P450 enzymes was measured: the organoids displayed significantly higher levels of CYP3A4 and CYP2E1 than fetal liver, with comparable expression of CYP2A6, CYP2B6 and CYP2D6. On the contrary, the expression of most P450 enzymes (except for CYP3A4) in liver organoids was significantly lower than that in the adult liver, indicating that these organoids present intermediate maturity between the fetal and adult liver.

Liver organoid culture systems allow hepatotoxicity testing. Recently, Leite *et al*[75] established a method to detect hepatocyte-mediated and drug-induced liver fibrosis based on this platform. After a single dose or repeated exposure for 14 days to the pro-fibrotic compounds allyl alcohol and methotrexate, hepatic organoids displayed fibrotic features such as activation of hepatic stellate cells (HSCs), the major collagen-

producing cells during conditions of sustained hepatic injury. The tested drugs caused significant upregulation of HSC activation-associated mRNAs collagen, type I, alpha 1, collagen, type I, alpha 3, and lysyl oxidase homolog 2 in the organoids. Acetaminophen is another compound identified by these organoids as an inducer of hepatotoxic-mediated HSC activation, which was also confirmed in an *in vivo* model.

Pancreatic ADRs

Drug induced-pancreatitis is a serious problem for both the patient and the health system. About 0.1%-2% of drugs are related to the development of this ADR and cases can be mainly divided into mild and severe. Severe cases may lead to death, while mild ones lead to patient hospitalization. Recently, our group reviewed the possible application of iPSCs to study and prevent this ADR[18,76]. Here, we provide an up-to-date revision of iPSC technology to model patient-specific human pancreatic cells with the purpose to obtain a platform for drug-induced pancreatitis studies. iPSCs can be differentiated into both endocrine and exocrine pancreatic cells applying protocols that provide the addition of specific stimuli to the culture medium. Regarding endocrine differentiation, many protocols are available; however, the main current limitation is the complete maturation of differentiated cells due to its lower hormone release profiles with respect to human islets, thereby resembling more immature fetal endocrine cells. However, to overcome this limitation, some progress has been made in the last several years. The work published by Pagliuca *et al*[77] is an example of the progress made in this field. IPSCs were differentiated into hormone-secreting cells using a six-step protocol and cells were cultured in clusters into spinner flasks. Cells obtained were implanted in mice, resulting, after 3-4 months, in functional β-cells and polyhormonal cells, a particular type of early endocrine cells that appears during pancreas development, able to secrete insulin, glucagon, somatostatin, and pancreatic polypeptide and localized in the walls of pancreatic ducts. These cells can be a great tool for diabetes studies and identification of new therapeutic approaches.

To the best of our knowledge, protocols regarding exocrine differentiation are limited. The most efficient, already described in our previous works[14,53], was developed by Takizawa-Shirasawa *et al*[78].

Even if the progress made to obtain patient-specific pancreatic cells is increasing, the experience on the application of differentiated cells to create a platform for drug-induced pancreatitis studies is still limited and only two works are available. Indeed, to date, only Hohwieler *et al*[79] developed a model of 3D pancreatic organoids generated from iPSCs of patients with cystic fibrosis. Their work, already reviewed by our group[18], has provided a basis for the development of new research based on this topic. Also, Huang *et al*[80] developed an efficient protocol to obtain exocrine pancreatic organoids starting from human embryonic stem cells. On the other hand, even if works based on iPSCs and the generation of exocrine pancreatic cells are limited, there are several solid works concerning pancreatic organoids created from tumor cells. Therefore, considering these two facts, it is reasonable to believe that more progress can be made in this field to fill the existing lack of pancreatic ADR studies.

NEPHROTOXICITY

Drug-induced renal injury (DIRI) is a frequent side effect, especially in critical patients undergoing complex pharmacological treatments. DIRI is common in the pediatric field with an incidence of about 25% pediatric patients taking intensive pharmacotherapy[81,82]. This high incidence is principally due to the excretory function of the kidney, which is exposed to high concentration of drugs or metabolites. DIRI can lead to severe acute renal failure, which contributes to prolonged hospitalization, and increased costs for healthcare and morbidity. To prevent devastating consequences for patients, it is important to identify markers of this adverse event, taking measures to avoid it. To date, the main clinical indicators of DIRI are serum creatinine levels; however, novel markers are needed to more efficiently prevent its development[83]. It is known that this adverse effect can be caused by drugs through different mechanisms that are divided on the basis of the affected kidney component[84]. A major problem of DIRI management is the lack of *in vitro* models to test nephrotoxicity of drugs or to find predictive biomarkers of renal drug toxicity[85,86]. iPSCs from patients represent a promising model to develop more precise therapies, better studying the mechanisms related to drug-induced nephrotoxicity and creating the possibility to prevent DIRI development.

The differentiation of iPSC to renal cells involves different steps: starting from mesendoderm formation, the intermediate mesoderm can be obtained, from which it is possible to obtain the ureteric bud or metanephric mesenchyme. From the latter, the

differentiation continues with the renal vesicle, from which the podocyte, proximal tubule, or distal tubule can be obtained[87]. Taguchi *et al*[88] analyzed a differentiation protocol allowing metanephric nephron progenitors starting from iPSC to be obtained in 14 days. The protocol involved different steps of differentiation: starting from the formation of embryoid bodies (EBs), epiblast, nascent mesoderm, posterior nascent mesoderm, posterior intermediate mesoderm, and finally metanephric mesenchyme. The different steps were obtained with appropriate concentrations and exposure times to stimuli added in the medium: activin-A, and bone morphogenetic protein 4 (BMP-4), CHIR99021, retinoic acid, fibroblast growth factor 2 (FGF-2), FGF-9. Immunohistochemical analysis confirmed the differentiation[89]. Xia *et al*[89] derived ureteric bud progenitor-like cells from iPSC in 4 d. The culture medium was supplemented with BMP-4 and FGF-2 for 2 d and then with retinoic acid, activin-A, and BMP-2. The differentiation was evaluated using real-time PCR and immunostaining[89]. Musah *et al*[90] developed a differentiation method of iPSC in kidney glomerular podocytes with a feeder-free and serum-free protocol in 21 days. The differentiation procedure was established in three commercial iPSC lines: PGP1, IISH3i-CB6, and IMR-90-1. During differentiation, the cells were cultured on tissue plates coated with lamin-511 E8 fragment and the mesoderm was obtained adding Rho-associated kinase inhibitor Y27632, CHIR99021, and activin-A; the intermediate mesoderm was obtained with CHIR99021 and BMP-7; and finally podocytes were obtained by stimulating the cells with BMP-7, retinoic acid, activin-A, vascular endothelial growth factor (VEGF), and CHIR99021. The differentiation markers analyzed were goosecoid, HAND1, and brachyury in the case of the mesoderm; Pax2 for the nephron progenitor cell markers; WT1 and OSR1 for intermediate mesoderm; and finally, for podocytes WT1, podocin, and nephrin proteins and specific genes such as *MAF, PODXL, SYNPO*, and *EFNB2*, together with a decrease of progenitor marker genes (*e.g*., *SALL1* and *PAX2*) and pluripotency genes (such as *SOX2, MYC, NANOG, POU5F1*)[90,91]. The authors also created an organ-on-a-chip microfluidic model of glomerular function. Organ-on-a-chip culture models can better reproduce the structure, function, and environment of human organs. The chip was formed by two parallel micro-channels separated by a poly(dimethylsiloxane) membrane: the intermediate mesoderm, subsequently differentiated into podocytes was cultured in the upper part of the channel and in the opposite part the primary human glomerular microvascular endothelial cells were seeded. In this way, it was possible to recreate the podocyte-endothelium interface. Furthermore, two hollow chambers were added on the sides of the central channels and a cyclic suction was applied to mimic the cyclical pulses of the renal blood flow that cause relaxation or motion and dynamic mechanical stretching *in vitro*. The podocytes were obtained by differentiation in the presence or absence of fluid flow or with a combination of fluid flow and mechanical strain; nephrin expression analyses indicated that differentiation may be influenced by mechanical forces. Further analysis showed that with cultures in the presence of flow or the flow-mechanical combination, there was an increase in the number of processes of the podocytes and greater production of VEGF-A (necessary for development of the glomerulus *in vivo*). They analyzed the percentages of retained ALB and inulin filtration in the presence of cyclin mechanical strain and noted that 99% of ALB is retained while 5% of inulin is filtered, suggesting that this represents a good *in vitro* model for glomerular filtration barrier. They also analyzed the production of collagen IV (mainly produced by glomerular podocytes in the mature glomerular basement membrane) and noted that this type of collagen is produced by both cell types present in the chip, even though the greatest production occurs in differentiated podocytes under mechanical strain, demonstrating the greater differentiation efficiency in the presence of mechanical strain. Finally, they analyzed the damage induced by a continuous flow of the adriamycin anti-tumor drug on this organ-on-a-chip. The results revealed an interruption of the podocyte layer and cell detachment in a dose-dependent manner, together with decreased viability and non-selective loss of ALB from vascular channel. This indicated that the tested drug produced lesions and that this model is useful for analyzing glomerular function, therapeutic development, and drug-induced toxicity[91].

Kandasamy *et al*[92] reported an example of proximal tubular-like cell differentiation from iPSCs used to predict drug-induced nephrotoxicity. They used commercial iPS (foreskin)-4, from WiCell Research Institute and differentiated these cells in human proximal tubular-like cells (HPTC-like) in 8 days, using commercial renal epithelial growth medium supplemented with BMP-2 and BMP-7. They analyzed the expression of markers to evaluate the different steps of differentiation in HPTC-like cells. In summary, the expression of iPSC characteristic genes such as *SOX2, NANOG, DNMT3D*, and *OCT3/4* decreased starting from day 1 of differentiation, while the characteristic genes of proximal tubular cells (*AQP1, GGT*, and *KSP-CAD*) are expressed in HPTC-like cells. The authors also analyzed the expression of 31 different

genes in HPTC-like cells, including genes coding for transporters, epithelial markers and kidney injury markers. Then they analyzed the nephrotoxic effect of two compounds, rifampicin and citrinin, in terms of IL-6 and IL-8 expression and noted an increase in the two ILs after drug treatment. They used the IL-6/IL-8-based assay to test 30 compounds that included substances for which a nephrotoxic effect was known and substances that did not produce such toxicity and treated HPTC-like cells derived from iPSC differentiation and a commercial line of HPTC (American Type Culture Collection, Manassas, VA, United States). To classify the compounds as toxic and non-toxic, an automated classifier was used and the system was trained to recognize the two types of compounds. Finally, they tested how predictive the developed system was and concluded that cells differentiated from iPSCs have higher test accuracy than HPTC. Therefore, HPTC-like cells can be used to predict toxicity using this automatic system. In addition, performance predictions were also analyzed using HPTC derived from nephrectomy samples from two tumor patients and an increased variability in performance was observed. The authors concluded that the use of HPTC-like cells differentiated from iPSCs in this prediction system can avoid inter-donor variability problems and thus could be useful to predict nephrotoxicity of drugs. Finally, authors tested the anti-cancer drug cisplatin using different biomarkers. The treatment produced HPTC-like DNA double-strand breaks and reactive oxygen species (ROS) production. The authors reported that the results obtained are in line with clinical data and animal experiments, and concluded that iPSC-differentiated HPTC-like cells are an effective model for the *in vitro* study of cisplatin-induced toxicity[92].

Although the number of studies on drug-induced nephrotoxicity based on iPSCs is currently limited, such a model represents a good tool to investigate the pathophysiology of many renal diseases and may allow the investigation of more effective therapies. In fact, for many kidney diseases, specific *in vitro* models are not yet available. The use of pluripotent stem cells (including iPSC and embryonic stem cells) have allowed the study of renal diseases due to genetic mutations such as renal cysts, diabetes syndrome, Wolfram syndrome, focal segmental glomerulosclerosis, systemic lupus erythematosus, Wilms tumor, and Alport syndrome[86].

With respect to iPSC-derived proximal tubular cells, 3D kidney organoids are characterized by distinct cell types such as endothelial cells, nephron progenitors, and podocyte-like cells and are therefore promising systems for nephrotoxicity testing[93].

Recent data have shown that treatment of kidney organoids generated from human iPSCs (CRL1502) with nephrotoxic cisplatin induced specific acute apoptosis in mature proximal tubular cells, whereas immature cells did not respond to the drug[93]. These results were further confirmed on patient-derived organoids obtained from renal normal tissue of neoplastic patients: a consistent activation of caspase 3 (CASP3), an indicator of apoptosis, after 72h of incubation with cisplatin was detected by different techniques and only tubule cells suffered after drug exposure without affecting organoid architecture[94].

Furthermore, interesting results have been obtained when organoids derived from cultured murine nephron progenitor cells were treated for 24 h with gentamicin at different concentrations; the percentage of CASP3+ cells co-stained with *Lotus tetragonolobus* lectin, a proximal tubule marker, selectively increased up to 80%. In contrast, immunofluorescence analyses of cells stained for podocalyxin, a glomerulus marker, treated with gentamicin showed no cells positive for CASP3, suggesting that the drug caused proximal tubule injury without affecting glomerular structures[95].

The most recently published paper about kidney organoids described the use of glomeruli isolated from iPSC-derived kidney organoids for toxicity screening[96]. The method for isolation of intact glomeruli from kidney organoids is based on enzymatic dissociation of mature organoids that generates 3D aggregates of podocytes representing forming glomeruli. In particular, the cultured organoid glomeruli were exposed to increasing concentrations of doxorubicin, and after 48 hours, activation of the pro-apoptotic pathway was evident at the lower doses; reduction in glomerular size following doxorubicin treatment was also detectable.

These preliminary results are encouraging; however, more drugs must be evaluated before kidney organoids can be used as a promising drug testing platform.

CARDIOTOXICITY

Drug-induced cardiotoxicity may be triggered by several mechanisms of action. In general, drugs can cause different effects at the cardiac level: heart failure due to abrupt decrease of contractile performance, decrease in left ventricular ejection fraction, arrhythmias, and prolonged cardiac repolarization. The latter, associated

with a prolonged QT interval, may increase the risk of serious cardiac arrhythmias. In most cases, the prolonged repolarization phase is caused by drug effects at the ionic channels or pump levels[97]. In general, variations in the electrical currents that stimulate the contractions of cardiomyocytes (CMs) are due to alterations in the fluxes of ions such as Ca^{2+}, K^+, and Na^+ through ionic channels in cardiac cells[98]. An example of a potentially lethal arrhythmia is the Torsade de Pointes, often determined by the prolongation of the action potential that affects rapid K^+ current by inhibition of the ether-a-go-go-related gene channel (hERG) in cardiac muscle and is related to prolongation of the QT interval[99]. Furthermore, alterations of the ions flux not only have consequences on CM contraction, but suggest oxidative stress condition, a common mechanism of toxicity that may lead to apoptosis[100].

One of the major problems during new drug development in preclinical trials is represented by the potential cardiotoxicity of the therapeutic agent[9]. Indeed, during the registration phase of a new drug, 23% of candidates fail for this reason[101]. In this view, prolongation of the QT interval has been suggested as the major problem related to the potential cardiotoxicity of drugs by the U.S. Food and Drug Administration (FDA)[102]. Hence, in 2005, the International Conference On Harmonisation Of Technical Requirements For Registration Of Pharmaceuticals For Human Use created guidelines for cardiotoxicity screening in preclinical[103] and clinical studies[104].

Cardiotoxicity is not only a frequent cause of failure of preclinical studies for new drugs, but can also be the cause of drug withdrawal from the pharmaceutical market. Between 1994 and 2006, 45% of all drugs removal from the market were due to cardiotoxicity[105]. For instance, some of the most recent withdrawals from the market of drugs due to cardiotoxic effects involve sibutramine and rosiglitazone. The anorectic sibutramine was recalled in 2010 because of increased risk of nonfatal myocardial infarction and nonfatal stroke in patient with pre-existing cardiovascular disease[106]. The anti-diabetic rosiglitazone was also recalled in 2010. Although an increase in mortality risk has not been identified, this drug was associated with an increased risk of myocardial infarction and heart failure[107].

Also in this case, a promising approach is the use of iPSCs. iPSCs allow patient-specific studies, since these cells can be easily differentiated into CMs (iPSC-CMs). In addition, iPSC-CMs have the advantage of being cryopreserved to facilitate their use as well as being grown in large numbers and high purity[108]. Di Baldassarre *et al*[109] reported three main approaches for the generation of iPSC-CMs: differentiation of the monolayer culture system for which various protocols have been described, co-culture of iPSCs with visceral-endoderm–like cells, and formation of embryonic bodies (3D culture)[109]. In general, the protocols provide, as a first step, differentiation towards the mesoderm layer by activation of WNT, activin/NODAL, and/or BMP pathways. For example, the combination of CHIR99021 with differentiation factors such as activin-A and BMP4 are used to activate the WNT pathway[97]. The activation set of these pathways, together with the Matrigel sandwich method, has been proven effective[110]. However, the obtained iPSC-CMs are qualitatively and quantitatively immature compared to adult CMs. This can significantly impact the validity of *in vitro* studies using these models, in terms of modeling the disease and/or prediction of the drug effects[110]. The differences between iPSC-CMs and adult CMs are related to: structural gene expression[111,112], structural features[113], metabolism[112], and contractile function[113]. Very recently, Machiraju and Greenway reported successful methods for the maturation of iPSC-CMs, including: biochemical approaches (manipulation of growth conditions through the addition of small molecules or changes in culture medium), environmental manipulation (through various mechanical and electrical forces), and 3D approaches (creation of 3D cultures of CMs, called organoids). According to the authors, the optimal conditions of maturation can be achieved by combining these different approaches[110].

A good example of the use of iPSC derived from patients to perform patient-specific studies to evaluate drug toxicity was reported by Liang *et al*[114]. In this study, iPSC-CMs were obtained from healthy donors and patients with inherited long QT syndrome (LQT), familial hypertrophic cardiomyopathy (HCM) or familial dilated cardiomyopathy. The authors initially characterized iPSC-CMs by immuno-fluorescence staining (noting that there were phenotypic differences in patients' iPSC-CMs compared to healthy iPSC-CMs due to the associated pathology) and ion channel expression (noting that they are present in all types of cells and therefore potentially able to modulate the electro-physiological responses to drugs). Then, they analyzed divergent aspects such as the morphology of action potentials and the action potential duration in nodal, atrial, and ventricular waveforms in all iPSC-CMs. On these cell models, the authors showed that the iPSC-CMs of patients affected by LQT and HCM treated with cisapride (a gastroprokinetic agent capable of blocking hERG channels) showed a higher susceptibility to arrhythmias, suggesting that the greater sensitivity

to cardiotoxicity induced by this drug could be associated with LQT or HCM mutations. Thus, it is conceivable that iPSC-CMs can be used as good models for evaluation of the patient-specific cardiotoxicity of drugs[114].

On iPSC-CMs, different physiological parameters can be monitored as a measure of possible toxic outcomes induced by drugs at the cardiac level. For instance, continuous real-time monitoring of the beating frequency of CMs is possible thanks to the xCELLigence Real Time Cell Analysis Cardio Instrument, a technique useful for the dynamic monitoring of CM contraction and beating, measured by an electric field differentially modulated by the number of cells covering the electrodes, their morphology and the strength of cell attachment. Nguemo *et al*[115] showed that this technique is a useful tool for characterization of the potential cardiotoxicity of drugs. The most recent study using this tool to analyze drug-induced cardiotoxicity on iPSC-CMs was carried out evaluating the cardiotoxicity of etoposide (ETP), a broad-spectrum anti-neoplastic drug. The iPSC-CMs were treated with ETP for 48 hours followed by 2 days of drug washing. An irreversible increase in the beating rate of iPSC-CMs was observed with 30 and 15 μmol of ETP with alterations in the beating profile and arrhythmic beating (measured with XCELLigence). Furthermore, treatment with 10 μmol ETP resulted in initial changes of the beating profile, that however, returned to baseline level after drug washing. A dose-dependent increase in the extracellular level of lactate dehydrogenase after treatment with ETP was observed, indicating membrane damage in iPSC-CMs. The authors also performed gene expression analyses (deregulation of 58 genes and upregulation of 5 miRNAs were found), intracellular calcium handling and mitochondrial membrane potential analyses, immunostaining, and transmission electron microscopy (to confirm the cytoskeletal and mitochondrial damage). Finally, they showed that the apoptosis inhibitor, pifithrin-α, could protect iPSC-CMs from ETP-induced cardiotoxicity[116].

Another useful technique is represented by microelectrode array (MEA), a method that allows measurement of the electric field potential (homogeneous and electrically coupled populations) of cardiac cells[117]. The most recent study using this tool to analyze drug-induced cardiotoxicity was carried out on a commercially available iPSC-CM cell line (iCell CMs, Cellular Dynamics International, Madison, WI, United States) testing 25 drugs, with or without serum in the medium, using a MEA system to analyze the field potential duration (FPD) and arrhythmic events. In the case of serum-free medium, only some drugs induced significant changes in FPD, nine with an extension and four with a reduction. In the case of medium with serum, on the other hand, the number of drugs with prolonged FPD was 11. The authors concluded that the presence or absence of serum can affect the results; indeed, components present in the serum such as serum ALB can create optical artifacts during this electrophysiology assays, and therefore, this should be considered during the analysis[118].

Cardiotoxicity is a relevant problem for anti-tumor drugs[101]. For example, anthracyclines, especially doxorubicin and its derivative epirubicin, are widely used as anti-cancer drugs in hematological malignancies (*e.g.*, lymphoblastic or aggressive myeloblastic leukemia) and solid tumors (such as breast, endometrial and stomach tumors). The cardiotoxicity induced by doxorubicin can cause congestive heart failure, tachycardia and arrhythmias, asymptomatic reduction of left ventricle ejection fraction, cardiomyopathy, and myocardial infarction[119]. The cardiotoxicity induced by this drug acts on several levels: activation of the apoptosis pathway by alteration of mitochondrial functions, generation of ROS, and alteration of gene transcription due to inactivation of topoisomerase II and double-strand breaks[120]. One of the most recent studies of cardiotoxicity induced by doxorubicin on patient-specific iPSC-CMs was reported by Burridge *et al*[119]. The study was carried out in iPSC-CMs derived by three groups of female patients: healthy controls, breast cancer patients treated with doxorubicin or equivalent who did not experience clinical cardiotoxicity (DOX), and breast cancer patients treated with doxorubicin or equivalent who did experience clinical cardiotoxicity (DOXTOX). Once derived from each of these patients, iPSCs were differentiated into iPSC-CMs that were subsequently exposed to doxorubicin to evaluate the drug–induced cardiotoxicity. iPSC-CMs have been tested for: toxicity of doxorubicin by immunofluorescent imaging to assess the concentration of the sarcomeric disarray, detecting a concentration-dependent increase in DOXTOX cells, but not in DOX cells at 0.1 μmol, as well as an increase in beating rates, more severe in DOXTOX cells; (2) cell viability that was reduced in DOXTOX cells; (3) level of double–stranded DNA damage (by staining for phosphorylated H2A histone family member X), which was higher in DOXTOX patients; and (4) oxidative stress, demonstrating that ROS and H_2O_2 production was higher while antioxidant glutathione levels decreased in the DOXTOX patients. Moreover, the effect of doxorubicin on patient-specific gene expression was evaluated by RNA sequencing analysis. An association between homozygous non-synonymous variants in BRCA1

and cardiotoxicity in DOXTOX patients was found. Because of these results, the authors concluded that the study of drug-induced cardiotoxicity using iPSC-CMs allows the evaluation of its molecular mechanisms and genetic bases[119].

Another example of iPSC-CMs used to study the cardiotoxic effects induced by anti-cancer drugs was reported by Sharma *et al*[121], who investigated the tyrosine kinase inhibitor (TKI)-induced cardiotoxicity. TKIs are anti-cancer agents that act on the tyrosine kinase receptor by inhibiting its phosphorylation. TKIs act in terms of proliferation, migration and survival of the cells. However, reduced left ventricular ejection fraction, heart failure, myocardial infarction, or arrhythmias are common cardiac adverse effects caused by TKI treatment. In this study, patient-specific iPSC-CMs were obtained from the somatic tissues of 11 healthy individuals and 2 cancer patients (patients with kidney cancer and treatment with sunitinib as first-line and axitinib as second-line without significant clinical cardiotoxicity) to evaluate the cardiotoxicity of 21 FDA-approved TKIs. Different endpoints were evaluated such as cytotoxicity, contractility, and the effects on QT intervals. The authors noted the cytotoxic effects of some TKIs (sorafenib, regorafenib, and ponatinib induced the greatest effects) in iPSC-CMs obtained by healthy patients and there were no significant differences when compared to iPSC-CMs obtained by patients treated with sunitinib or axitinib. Regarding the effects on cell contractility, healthy iPSC-CMs were exposed to doses lower than the drugs' median lethal dose, and nilotinib and vandetanib altered the beating rate. The authors concluded that before the death of CMs, they manifested an effect on the beat profile. Finally, regarding the effects on QT interval, prolongation of the contraction time of CMs was observed in healthy iPSC-CMs treated with nilotinib or vandetanib, in addition to a decrease in the beating rate and prolongation of the transitional duration of calcium. In this article, it was also hypothesized that insulin/IGF signaling (that was upregulated after treatment with VEGFR2/PDGFR-inhibiting TKIs) may protect iPSC-CMs from TKI toxicity. The authors concluded that iPSC-CMs can be used to evaluate TKI-induced cardiotoxicity[121].

The response to some drugs is different between adults and children and/or during childhood; for example, in the case of warfarin, in pediatric patient age has a more important effect on pharmacokinetics than polymorphisms (for example in *VKORC1* or *CYP2C9* genes)[122]. Hence, of crucial importance is the establishment of patient-specific studies to evaluate the cardiotoxic potential of drugs in pediatric patients. Also in this case, patient-specific iPSCs are a useful tool. For example, Visscher *et al*[123] reported the relationship between the SNP in a panel of genes and anthracycline-induced cardiotoxicity (ACT) in children (independent cohort of 218 patients who included patients treated with anthracyclines who had or not developed cardiotoxicity). Genomic DNA was extracted from blood, saliva, or buccal swabs and 23 SNPs were selected for which an association with ACT was already known. The results showed an association between rs17863783 in *UGT1A6* and ACT, while the association with two SNPs in *SLC28A3* (rs7853758 and rs885004) and one in *SULT2B1* (rs10426377) was close to being significant. They also analyzed the influence of sex and age at the start of treatment: The variant *SULT2B1* rs10426377 was associated with an increased risk of ACT only in males, while the two variants of *ABCB4*, rs4148808, and rs1149222 were only associated with an increased risk in females. Regarding age, an association with ACT was found in the case of the *HNMT* variant rs17583889 in the case of younger children (< 5.3 years)[123]. This study highlights the ability of a specific patient study to improve the risk assessment of ACT in children, potentially helping to improve the safety of anti-cancer therapy.

With respect to the traditional 2D cell culture, 3D-engineered human cardiac organoids generated from stem cell-derived CMs provide another functional *in vitro* model for disease modeling and drug screening. Biochemical-inducing factors can be combined with several tissue engineering approaches based on 3D printing and bioscaffold technologies to direct spatial organization of 3D tissue to build human cardiac organoids[124,125]. Despite the progress in this area, protocols for the maturation of CMs toward an adult phenotype in defined conditions still need to be further elucidated.

The study published by Voges *et al*[126] demonstrated that human cardiac organoids primarily contain CMs and stromal cells at a ratio that is comparable with the fetal/neonatal heart, and form functional sarcomere units. Moreover, these cellular models are able to completely recover cardiac function following injury, showing many features of regenerative neonatal heart tissue.

Fluid-ejecting 3D human ventricular-like cardiac organoid chambers (hvCOC) can mimic physiologically complex behaviors, such as pressure-volume relationships, and have been used for detecting contractile responses to different pharmacological compounds[127,128]. The hvCOC system can accurately identify inotropic effects of pharmacological compounds such as isoproterenol and levosimendan, with increased

sensitivity with respect to human ventricular-like cardiac tissues strips.

The utility of these structures has been additionally demonstrated for environmental contaminants screening, for example heavy metal and pesticide, confirming the accurate responses to external stimuli[129].

These preliminary results demonstrate that in the future, these platforms could provide patient-specific models for personalized drug screening to achieve optimal therapeutic applications.

NEUROTOXICITY

Neurotoxicity can be caused by physical, chemical, or biological agents exhibiting adverse effects at the central or peripheral nervous system level, altering its function or structure. Neurotoxicity can affect attention, executive functions, decision making, and memory, based on the severity and location of the injury, compromising the quality of life. Brain functions can be altered by drug activation of different neurotransmitter systems, including dopamine and glutamate[130].

Drug-induced neurotoxicity is divided, according to the damaged region, in: myelinopathy, induced by drugs such as amiodarone, which causes damage to Schwann cells[131]; assonopathy, induced by drugs such as vinca alkaloids (*i.e.* vincristine and paclitaxel), inducing microtubule-dependent axons damage[132]; and neuronopathy, induced by platinum-based compounds (cisplatin and oxaliplatin) due to oxidative or mitochondrial stress resulting in death of the dorsal root of ganglion neurons[132].

An example of ADR is represented by seizure, a serious neurological complication that is commonly associated with treatment with antibiotics[133]. Seizures involve an abnormal and transient discharge of neurons at the brain level. To test drug-induced seizure-liability many models are currently available, for example acute slide assay carried out on surgical slides obtained from any part of the brain (especially hippocampus), organotypic slide cultures, primary central nervous system cultures, iPSC-derived cultures. The latter are useful to study drug-induced neurotoxicity using different *in vitro* techniques, such as calcium imaging and MEA. This method allows the measurement of the electrophysiological activities of the neural networks in a non-invasive way[134]. For instance, Odawara *et al*[135] used this tool to evaluate the efficiency of response to two convulsant agents (pentilentetrazole, a GABA blocker, and 4-aminopyridine, a K^+-channel blocker) in an *in vitro* system constituted by co-cultures of commercial neurons and astrocytes derived from iPSCs. The results obtained indicated that the synchronized bursts firings, indicative of functional maturation in synaptic transmission, and the analysis of the peaks in the synchronized bursts firings allows the neurotoxic effects of these two drugs to be distinguished. Moreover, the co-culture improves the spontaneous activity of neuronal networks[135]. The latter result was obtained by Ishii *et al*[136], who analyzed the response to drugs (gabazine and kaliotoxin) on synchronized burst and spontaneous firing, demonstrating that the co-culture system is more efficient as an *in vitro* model than the individual cultures of commercial astrocytes or iPSC-derived neurons[136].

One of the protocols reported in the literature for the differentiation of human iPSCs into a mix of neurons and glia cells takes about 28 days. The first step is the generation of embryonic bodies, followed by the generation of neuroepithelial aggregates (rosettes) that requires 5 days, and then the dissociation of the rosettes and the neuronal differentiation (20 days) in a mix of neurons and glial cells. This protocol allows heterogeneous cultures of glutamatergic, dopaminergic, and GABAergic neuronal cells to be obtained, together with glial cells[137]. Mukherjee *et al*[138] reported that the main method of differentiation of iPSCs involves the mimics of development signals using culture medium added with morphogens, small molecules, and/or growth factors. Differentiation can occur basically using two different methods: culture in suspension in a single cell or in adhesion with the subsequent formation of aggregates of embryonic bodies and the cultivation in medium, allowing the formation of the definitive neuroectoderm and neural rosettes; and inhibition of one important protein of the small mothers against decapentaplegic family in iPSCs cultured as a monolayer and the formation of rosettes by inhibition of BMP. The main differences between the two methods are the efficiency of differentiation and the duration of culture, while the involved pathways are similar (BMP/TGF-β/Wnt). The cortical GABAergic neurons are obtained by adding, before the phase of terminal differentiation, inhibitors of sonic hedgehog (SHH) and/or Wnt, for the dopaminergic neurons of the caudal midbrain (mesencephalon) with the FGF-8, SHH, and Wnt agonist, while using FGF-2 and insulin it is possible to obtain Purkinje cells[138].

Also, for drug-induced neurotoxicity, as for other forms of toxicity, there is a

correlation between patient-specific gene variability and the development of adverse drug-induced effects. In line with this observation, also in this case, iPSCs offer the advantage of specific patient studies to better understand the correlation between gene expression and drug toxicity.

An example was described by Ohara *et al*[139]. In this study, iPSCs were obtained from PBMCs or T-lymphocytes of two patients with Charcot-Marie-Tooth disease (CMT), with a mutation of a gene encoding a mitochondrial protein (mitofusin-2), and two healthy individuals. iPSCs were differentiated to motor neurons, noting that the mutation also remains in the differentiated neurons, and analysis at the mitochondria level indicated that the neurons derived from patients affected by CMT present mitochondrial dysfunctions. The results showed that two drugs (vincristine and paclitaxel) cause mitochondrial aggregation (a parameter of mitochondrial abnormalities to evaluate the neurotoxic effects) in healthy and CMT-derived neurons, but the greatest effect was observed in patients. The authors concluded that the effect of the tested drugs is different between patients and healthy donors and that the analysis of mitochondria is a good parameter to study neurotoxicity[139].

Permanent peripheral neuropathy is the most common non-hematologic toxicity of anti-cancer chemotherapy, with an incidence of around 20%-40%[140]. It has been hypothesized that calcium signaling, oxidative stress, or mitochondrial changes are effects caused by anti-cancer drugs and that these effects may induce neuropathy[132]. Several studies are available on commercial cells to study neurotoxicity. Wheeler *et al*[140] studied the neurotoxic effects of three anti-cancer drugs (paclitaxel, vincristine, and cisplatin) on neurons derived from commercial human iPSCs (from Cellular Dynamics International). They analyzed the neurite outgrowth phenotype and observed a dose-dependent decrease in neurite processes in the case of treatment with the drugs (the greatest effect was observed for vincristine). Using the Caspase-Glo 3/7 assay, drug-induced apoptosis was evaluated, noting that the greatest effect of increased caspase activity was present in the case of treatment with cisplatin, followed by treatment with vincristine, while paclitaxel had no effect[140]. The effect of paclitaxel on neurite outgrowth without affecting caspase 3/7 activation was also observed by Wing *et al*[141]. In this study, neurotoxicity of other anti-cancer drugs was evaluated on two commercial human iPSC-derived neuronal cell lines: iCell® Neurons (Cellular Dynamics International) and Peri.4U (Axiogenesis, Cologne, Germany), demonstrating a differential sensitivity of neurons to different chemotherapeutics[141]. Another study carried out on Peri.4U neurons (from Axiogenesis) is reported by Rana *et al*[142], who studied the effect of 16 chemotherapeutic agents. One of the most important findings was that some drugs such as epothilone, taxane, and vinca alkaloid chemotherapeutics did not produce cytotoxicity even though a reduction in the length of the neurite was observed[142].

Snyder *et al*[143] evaluated the neurotoxic effects of different classes of chemotherapeutics on various commercial neurons derived from iPSC: Peripheral iPSC-neurons (Axiogenesis), iCell® Neurons (Cellular Dynamics International), ReproNeuro glutamatergic neurons (ReproCell, Glasgow, United Kingdom), and human cerebral cortical neurons (Axol Bioscience, Cambridge, United Kingdom). Initially, they evaluated the expression of the characteristic markers of each type of iPSC neurons by gene expression and protein quantification, while the neurotoxic effect of the compounds was evaluated as neurite dynamics and apoptosis[143].

Another example was reported by Yamada *et al*[144], who evaluated the neurotoxicity and the influence on neuronal development of neuronal cells derived from commercial iPSCs of 5-fluorouracil. These cells have been used as an *in vitro* model of human fetal stage. Real-time PCR results showed that this drug has inhibitory effects on the early neural differentiation of iPSCs. All of these studies have shown that commercial neurons derived from iPSC may be useful models for the study of drug-induced neurotoxicity, especially chemotherapeutics.

Considering the different physiological characteristics between children and adults, particular care should be taken in the investigation of ADRs in children, also in the case of drug-induced neurotoxicity. An example is represented by anesthetics, as children seem to be more vulnerable to the adverse effects of these drugs. The problem arises by the fact that many of the anesthetic protocols used in children were developed because of those used in adults[145]. Numerous studies have indicated that neuronal damage due to anesthetic depends on many factors such as duration of exposure, developmental phase at the time of exposure, activated receptor subtype, and dose and route of administration. These agents can damage immature neurons, inducing morphological changes and functional impairment of mitochondria, releasing ROS that can further damage the tissue[146].

Neurotoxicity induced by chemotherapy is another problem in children as it may compromise the quality of life and longevity. For example, vincristine is used for ALL, which is the most common childhood cancer. However, this drug causes motor and

sensory dysfunction and neuropathic pain. Diouf *et al*[147] performed genotyping analysis on two cohorts of pediatric patients with ALL treated with vincristine and noted that the rs924607 polymorphism (*CEP72* gene) could be associated with the development of vincristine-induced neuropathy. Analysis of the neurite development such as outgrowth, number of processes and number of branches on commercial iCell neurons derived from iPSC (Cellular Dynamics International) showed a relationship between *CEP7* gene expression and vincristine-induced neurotoxicity; a reduced expression of this gene was associated with a greater sensitivity to the drug. This may suggest that patient-specific therapy would be required for a safer use of vincristine[147].

Considering the extreme complexity of the 3D structure of the brain, the study of its development and modeling of disease processes in 2D cultures could have several limitations. Therefore, the use of human iPSCs for the generation of 3D *in vitro* organoids has revolutionized the research in this field by recapitulating the organization of different regions of the nervous system such as retina, cerebral cortex, and choroid plexus[148-150]. The protocol described by Lancaster and Knoblich[148] to develop cerebral organoids begins with the generation of embryoid bodies from iPSCs. Embryoid bodies are subjected to neural induction in a culture medium that favors only the neuroectoderm to expand and are then transferred into droplet of Matrigel, which allows outgrowth of neuroepithelial buds. In the end, the tissues are moved to a spinning bioreactor, or in an orbital shaking plate, which contributes to increase oxygen and nutrient interchange and further development into defined brain regions.

Human brain organoids provide insights into the pathogenesis of neurological disorders and may offer new approaches for investigating the mechanism of the neurotoxicity of drugs. For instance, Liu and collaborators[151] used this *in vitro* model to study the neurotoxic effects of vincristine treatment. In particular, human iPSC (SC101A-1) were cultured to generate cerebral organoids and subsequently were treated with different concentrations of vincristine for 48 hours; the treatment resulted in a dose-dependent growth inhibition evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide test and caused the enhanced expression of cleaved caspase 3, indicating the induction of apoptosis[151]. In the same paper, several cellular markers were analyzed in cerebral organoids exposed to the drug, highlighting the inhibition of the microtubule-associated protein tubulin, fibronectin, and the downregulation of matrix metalloproteinases 10.

Another recent report published by Huang *et al*[152] describes the cerebral organoids as an innovative tool to investigate the toxicity of drugs. The authors showed that tranylcypromine, which is used to treat refractory depression, caused neurotoxicity on brain organoids generated from human iPSCs (SC101A-1), leading to a decreased proliferation activity and apoptosis induction[152]. These data were confirmed by measuring the expressions of the neuron-specific markers TUJ1 and GFAP, which stained for neurons and astrocytes respectively: tranylcypromine treatment impaired cell density and arrangement affecting both cell types. Moreover, tranylcypromine treatment abolished the transcriptional activity of BHC110/LSD1-targeted genes and increased the expression of histone di-methylated K4.

Limits of iPSCs

One of the first problems that can be highlighted regarding iPSCs technology is the low efficiency in reprogramming. The most important problem is related to the use of viral vectors such as retrovirus and lentivirus, which deliver the reprogramming transcription factors by integration in the host cell genome, leading to chromosomal instability and tumorigenesis from insertional mutagenesis.

However, during the past several years, new safer and non-integrative reprogramming methods have been developed, reaching better efficiency and avoiding the already mentioned issues. Another interesting point to discuss regards epigenetic memory and clonal variability. iPSCs can present an epigenetic memory of the parent somatic cells that can influence the differentiation propensity and therefore the outcomes of studies applied to this technology. This peculiarity of iPSCs, not characteristic of ESCs, may predispose them to differentiate more readily into their parental cells than others and can be useful for example in cell replacement therapy.

Kim *et al*[153] observed in iPSCs obtained by transcription factor-based reprogramming, a residual DNA methylation pattern characteristic of the parent cells, which increase their propensity to differentiate along lineages related to the donor tissue, while restricting alternative cell fates. The authors tried to reset the epigenome of the parent tissue by differentiation and serial reprogramming, or by treatment of iPSCs with chromatin-modifying drugs. These approaches could help researchers in increasing the differentiation efficiency of iPSCs, limiting the problem related to epigenetic memory. However, other aspects could affect epigenetic memory of iPSCs,

such as the number of passages performed. For example, Polo *et al*[154] demonstrated that increasing the number of passages there is a decrement in differentially methylated regions with respect to the parent cells. One of the reasons that can explain the spontaneous loss of epigenetic memory was postulated by the authors; probably, the loss of parent cells pattern is based on a slow replication-dependent process. However, further studies must be performed to consolidate this hypothesis as indicated by authors and, to date, the origins of epigenetic loss remain to be determined.

Another important point related to iPSCs limits is the intra-variability differences of clones of the same patient. Thatava *et al*[155] addressed this problem analyzing the variation in terms of pancreatic beta-cells differentiation of three iPSCs clone lines of three patients suffering from type 1 diabetes. Interestingly, a notable intra-patient variation, comparable to interpatient one, was identified by authors leading to conclude the necessity for a comprehensive fingerprinting of multiple patient-specific clones to obtain a representative pool of cells useable for biomedical applications such as ADRs studies. In addition to Thatava study, also Yokobayashi *et al*[156] found that the efficiency in iPSCs clones differentiation capability into primordial germ-cell was different; in particular, they observed that the XX clones were less efficient in germ-cell differentiation. However, interindividual variability is more important to consider than intraindividual variation for complex genetic disorders[157]. Deneault *et al*[158] analyzed both types of variabilities in iPSCs-derived neuron cells of patients with autism spectrum disorder, a heterogeneous and complex genetic condition, finding significant intra-variability only on few lines, corroborating what has just been said. Concluding, given the contrasts still present on this aspect, the intra-variability between different iPSCs clones should be investigated and it is advisable to analyze the genetic and epigenetic features of the clones generated. To study ADRs, the clonal variability is a key point to keep in mind in order to set up useful standardized tools. Therefore, before developing a model useful to predict patients' sensitivity, different clones should be first genetically checked to exclude for instance chromosomic aberrations, alterations in differentiation efficiency and variability in DNA methylation profiles. Also, the sensitivity to the drugs of interest should be analyzed in the different clones from the same patients, to exclude a variability in the response.

Besides iPSCs, the direct differentiation of somatic cells could be used to obtain the cells of interest. This approach has both advantages and limitations with respect to iPSCs technology regarding the study of ADRs (Table 1). A great advantage of direct differentiation is the short period of time necessary to obtain the cells of interest, avoiding the iPSCs step. Moreover, using this direct method, all DNA instability problems in generating and expanding iPSCs, such as point mutations and karyotypic abnormalities, can be avoided. In addition, to study ADRs affecting aged populations, it is desirable to obtain cells reflecting the disease-related DNA mutations together with the age of the patient. However, there is still an open debate if the best method to recapitulate the age of the patient is the iPSCs technique or the somatic direct differentiation[159]. The direct method has also several limitations; iPSCs have a higher proliferation capacity and they can self-replicate in culture differently from parental primary cell lines used in the direct method that can be cultured for a very short time with fast accumulation of epigenetic changes. Regarding ADR studies, it is important to generate a great number of differentiated cells of interest and this purpose can be more easily achieved with iPSCs rather than with the direct method based on the great expandability of iPSCs, as discussed above. Heterogenicity of the differentiated cells is another critical point for both techniques. This point is particularly critical for cell therapy and there are several works trying to ameliorate this aspect. Some limitations have to be underlined also for organoid technology: the complex culture medium contains a cocktail of growth factors and molecular inhibitors which might affect the drug responses of organoids; the extracellular matrix Matrigel, used to grow the organoids, might hamper drug penetration and limit their potential in drug screening; genetic and epigenetic aberrations might occur during long-term culture making organoids unsuitable for transplantation/regenerative medicine. Therefore, further efforts are needed to overcome these limits and improve this promising technology for clinical applications.

CONCLUSION

ADRs are one of the major clinical problems, especially in populations at risk, such as pediatric patients. One of the most important tools to improve the comprehension, and thus the prevention, of ADRs, is represented by the possibility to set up groundbreaking patient-specific assays. This goal has been powerfully achieved using

Table 1 Main differences of differentiation techniques based on iPSCs and direct somatic differentiation technologies

	iPSCs	Direct somatic differentiation
Reprogramming time	Long	Not necessary
Differentiation time	Long	Brief
DNA instability	Occasional	Rare
Proliferation of pre-differentiated cells	High (iPSCs)	Low (*e.g.*, fibroblasts)
Self-renewal of pre-differentiated cells	Yes (iPSCs)	No (*e.g.*, fibroblasts)
Heterogenicity of the differentiated population	Critical	Critical

iPSCs: Induced pluripotent stem cells.

iPSCs, being characterized by genetic and physiological patient-specific differences and being able to be differentiated ideally into all the tissues of human body. The knowledge gained so far has demonstrated that iPSCs can be fruitfully used in multiple clinical applications, including not only toxicity screening in the frame of ADRs characterization, but also disease modelling, personalized and regenerative medicines. In addition, the recent development of related models, such as 3D organoids derived from pluripotent or adult stem cells, resembling the 3D architecture of different organs in a more physiological way, has paved the way to better characterize ADRs in a personalized way.

It should be considered that the number of studies on this topic is still limited, especially concerning the pediatric field. In this case, particular care should be taken, considering that children are a population at risk for ADRs and because of the difficulties to obtain biological materials necessary to establish iPSCs models or stem cells-based organoids. However, based also on studies carried out on these models derived from adult patients or by commercial suppliers, a major advancement has been made to develop and establish differentiation procedures to obtain more efficient differentiation protocols and toxicity screening methods. It is therefore conceivable that in the near future, thanks to this progress, also the number of pediatric studies will increase, enabling the possibility to efficiently study and predict ADRs also in pediatric patients.

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REVIEW

Influence of olive oil and its components on mesenchymal stem cell biology

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Abstract

Extra virgin olive oil is characterized by its high content of unsaturated fatty acid residues in triglycerides, mainly oleic acid, and the presence of bioactive and antioxidant compounds. Its consumption is associated with lower risk of suffering chronic diseases and unwanted processes linked to aging, due to the antioxidant capacity and capability of its components to modulate cellular signaling pathways. Consumption of olive oil can alter the physiology of mesenchymal stem cells (MSCs). This may explain part of the healthy effects of olive oil consumption, such as prevention of unwanted aging processes. To date, there are no specific studies on the action of olive oil on MSCs, but effects of many components of such food on cell viability and differentiation have been evaluated. The objective of this article is to review existing literature on how different compounds of extra virgin olive oil, including residues of fatty acids, vitamins, squalene, triterpenes, pigments and phenols, affect MSC maintenance and differentiation, in order to provide a better understanding of the healthy effects of this food. Interestingly, most studies have shown a positive effect of these compounds on MSCs. The collective findings support the hypothesis that at least part of the beneficial effects of extra virgin olive oil consumption on health may be mediated by its effects on MSCs.

Key words: Olive oil; Mesenchymal stem cells; Cellular differentiation; Aging; Cellular

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niche; Mediterranean diet

Core tip: Extra virgin olive oil consumption is healthy and prevents unwanted effects of aging. Mesenchymal stem cell (MSC) populations may be positively affected by olive oil components, through their antioxidant capacity and interactions with cell signaling pathways, accounting for at least part of olive oil's beneficial health effects. In the absence of specific studies on olive oil and MSCs, the objective of this work is to provide a critical review of the current information on how compounds that are part of extra virgin olive oil, including residues of fatty acids, vitamins, squalene, triterpenes, pigments and phenols, affect MSC maintenance and differentiation.

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INTRODUCTION

Olive oil is obtained from olives, which are the fruits of the olive tree. Depending on the extraction method and fruit quality, different oils can be produced: (1) Extra virgin olive oil, resulting from the first pressing of olives, harvested the same day, to avoid spoilage. Extraction is mechanical only, at temperatures below 28 °C, with acidity (content of free fatty acids) below 0.8%. Such oil retains the highest amount of hydrophilic compounds, including both simple and complex phenols, as well as lignans. The total content of polyphenols is about 55 mg/100 g for this oil; (2) Virgin olive oil is similar to the previous one but with slightly higher acidity, below 2%. It also contains a high amount of hydrophilic phenolic compounds, albeit less than half of the previous one (about 21 mg/100 g); (3) Refined olive oil is obtained from olive pulp (generated after first pressing), using chemicals (acids and bases) and higher temperatures than the former ones. It has lower phenolic content, without significant antioxidant capacity; and finally; and (4) Olive pomace oil, produced from olive pulp and skin remains from the previous processes. Hexane is used as solvent, being further refined. It has the lowest antioxidant activity and is of less quality than the previous three.

Olive oil is a fundamental component of the Mediterranean diet and is associated with human health benefits[1,2]. This diet was first described by Ancel Keys, and it is based on preferential consumption of unprocessed food from plant origin (*i.e.*, fruits, vegetables and cereals), fish and poultry meat as the main protein sources, with low consumption of meat, dairy products, eggs and animal grease, and with extra virgin olive oil being the main lipid source[3]. The effects of olive oil on prevention of several diseases have been revealed in cohort studies[4-6] and randomized controlled trials[7]. As an example, the Spanish cohort of the European Prospective Investigation into Cancer and nutrition (commonly referred to as EPIC-Spain) has provided some of the most clear evidence to date that olive oil consumption, independent of the Mediterranean diet pattern, is related to reduction in global mortality, with a strong inverse association to other death causes (*i.e.*, respiratory, digestive, and nervous system diseases)[8].

Beneficial effects of extra virgin olive oil on health are due to its particular profile of healthy residues of fatty acids, which are mainly monounsaturated (one double bond between carbons of the hydrocarbon chain), rendering olive oil more resistant to oxidation, in comparison to oils that are rich in polyunsaturated fatty acid residues. Besides, extra virgin olive has a large group of bioactive compounds. They may reach 1% to 2% of total content, including unsaponifiable (squalene, sitosterols, triterpenes, *etc*.) and soluble (α-tocopherol and other phenols) fractions (Table 1)[9]. They have antiinflammatory activities, promoting cholesterol metabolism, reducing oxidative stress, having antiaggregating effect, and improving mitochondrial function[1]. Because of that, olive oil can be considered a nutraceutical functional food.

Olive oil has cardioprotective, antitumoral, neuroprotective, antidiabetic and antiaging effects. Beneficial cardioprotective effects of this food have been partially associated with reduction of low-density lipoprotein oxidation. This is mostly due to

Table 1 Principal components of extra virgin olive oil

Saponifiable fraction	98%-99% of total
Fatty acid residues	Percentage of total
Oleic acid (C18:1)	55-83
Linoleic acid (C18:2)	3.5-21
Linolenic acid (C18:3)	0.1-1.5
Palmitoleic acid (C16:1)	0.3-3.5
Palmitic acid (C16:0)	7.5-20
Stearic acid (C18:0)	0.5-5
Unsaponifiable fraction	1%-2% of total
Vitamins	
Alpha tocopherol	> 100 mg/kg
Vitamin K	Approximately 500 μg/kg
Beta carotene	2-4 mg/kg
Squalene	
Squalene	200-7500 mg/kg
Sterols	1000-2000 mg/kg
Common sterols	750-1800 mg/kg
4-Methyl sterols	50-360 mg/kg
Triterpene alcohols	350-1500 mg/kg
Triterpene dialcohols	< 4.5% of total sterol
Triterpene acids	40-185 mg/kg
Oleanolic acid	Approximately 50% of total triterpene acids
Maslinic acid	Approximately 50% of total triterpene acids
Pigments	
Lutein	3.5 mg/kg
Pheophytins	0-30 mg/kg
Phenols	100-300 mg/kg
Oleuropein derivatives	1.2-120 mg/kg
Hydroxytyrosol	1.1-75 mg/kg
Luteolin	0-19 mg/kg
Apigenin	0-19 mg/kg
Vanillic acid	11-18 μg/mL
Syringic acid	32-65 μg/mL
4-Hydroxybenzoic acid	5-25 μg/mL
p-Coumaric acid	2-8 μg/mL
Caffeic acid	1.9-4.5 μg/mL
Tyrosol	1.1-45 μg/mL
Ligostride derivatives	0-78 μg/mL
Pinoresinol	2-55 μg/mL
Other compounds (fatty alcohols, diterpene alcohols, volatile compounds, *etc*)	

the protective effects from its phenolic compounds[10]. Additionally, olive oil improves functionality of high-density lipoprotein particles[11]. That represents a protective factor, preventing atherosclerosis. Also, its antiinflammatory effects and ability to reduce platelet aggregation have been associated with reduction of cardiovascular risks[9,12]. Olive oil consumption has also been associated with reduced cancer risks, including for prostate, colorectal, and breast[13,14]. Such beneficial effects are mainly attributed to its minor components[15].

The Mediterranean diet, including olive oil, has been associated with risk reduction of mild cognitive impairment and Alzheimer's disease[16]. For instance, mice with diet including extra virgin olive oil showed reduction in neuropathologies[17]. Risk of type 2 diabetes was also reduced with a diet having high olive oil intake. That is mainly due to its high content of monounsaturated fatty acid residues, as well as to the presence of polyphenols[18]. Indeed, replacement of a diet rich in saturated fatty acid residues by extra virgin olive reduced glycosylated hemoglobin between 0.3% and

2.0%[9].

Aging is characterized by oxidative stress, telomer shortage, genomic instability, epigenetic alterations, proteostasis (protein homeostasis) reduction, nutrition deregulation, mitochondrial dysfunction, cellular senescence, dysfunction of stem cell populations, and altered cellular communications. Interestingly, beneficial effects of olive oil and its components on health include attenuation of the negative alterations of aging[19]. For example, polyphenols of olive oil prevented DNA breakage induced by oxidizing agents by scavenging free radicals, as well as modulating DNA repair mechanisms[20]. Indeed, a Mediterranean diet rich in extra virgin olive both reduced adiposity and increased telomer length[21]. On the other hand, oleuropein, an abundant polyphenol in extra virgin olive oil, stabilized the proteasome, during induction of replicative senescence, and extended life span[22]. Additionally, olive oil and its components positively modulated different stem cell populations, which can promote tissue regeneration and reduce negative effects of aging[19,23].

Adult mesenchymal stromal cells, also called mesenchymal stem cells (MSCs), comprise heterogeneous populations of undifferentiated cells, exhibiting high capacity for proliferation and differentiation into other cell types. MSCs can be found distributed in different tissues of the body, and they function as a cellular reservoir for tissue homeostasis maintenance and regeneration of damaged tissues[24]. They can be isolated from different tissues, like bone marrow, muscle, adipose tissue, hair follicles, dental roots, placenta, dermis, perichondrium, articular cartilage, umbilical cord, lungs, and liver[25]. Such cells were originally identified in bone marrow, derived from nonhematopoietic cells, and exhibiting osteogenic potential and ability to adhere to plastic surfaces[26].

Yet, this cell population has not been completely characterized, due to lack of specific biomarkers. The minimum criteria to define MSCs by the International Society for Cellular Therapy include: Adherence to plastic; capacity to differentiate into osteoblasts, adipocytes and chondrocytes *in vitro*; presence of CD73 (ecto-50-nucleotidase), CD90 (Thy1) and CD105 (endoglin, SH2) markers, as well as the absence of CD45, CD19, CD19 or CD79, CD14 or CD11b and human leukocyte antigen –DR isotype (HLA-DR) hematopoietic markers[27].

More recently, different "-omic" sciences have been used for a better characterization of the MSCs. Indeed, several genomic and proteomic studies have proposed that MSCs are characterized by specific expression of a set of genes and protein biosynthesis[28,29]. Another characteristic of MSCs is that they are metabolically very active, producing extracellular matrix components, as well as an important variety of cytokines. Among other actions, they can modulate the adaptive and innate immune system[30]. The possibility to isolate, and *in vitro* culture and differentiate MSCs has opened the door to their usage as therapeutic agents, especially for cellular therapy and regenerative medicine[31]. The putative therapeutic usage of their paracrine and antiinflammatory activity is also being evaluated currently[32,33].

Aging produces changes in cellular niches and MSCs themselves, which may affect their functionality and differentiation capacity. One of the most illustrative examples of that is the increase of bone marrow adiposity with aging, partially due to MSC differentiation into adipocytes instead of osteoblasts[34,35]. Such a phenomenon also happens in obese people[35]. MSC aging depends on intrinsic factors, such as reactive oxygen species (referred to as ROS), and accumulation of DNA damage and damaged proteins[36]. But extrinsic factors are also involved, acting through the cellular niche that is made of medium and other cellular types interacting with the MSCs. Niches are essential not only to maintain the pool of stem cells but also their functionalities. Because of that, it has been suggested that some changes associated with aging could be reversed, through niche manipulation-for instance, adding antiaging factors from serum of young people, or reducing medium factors related to aging[37]. Among those factors that promote aging are the increase in oxidative stress as well as the accumulation of proinflammatory cytokines produced by senescent cells, the population of which increases with age[38].

Interestingly, some recent studies have shown that diet may affect the proliferative and differentiation capacities of stem cells in adult organisms[39]. Curiously, a caloric restriction or fasting diet usually improves DNA repair, stem cell functionality, and longevity. On the contrary, diets rich in fats may have negative effects[39]. The diet may produce changes in serum composition and, therefore, affect stem cell functionality. Indeed, it has been shown that *in vitro* proliferation of rat hippocampal neural progenitor cells and MSCs increased when culture medium was supplemented with serum from old rats, which were fed with NT-020 food supplement (made by blueberry, green tea, vitamin D3, and carnosine) for 28 d, in relation to serum from rats that did not take such supplement[40]. This suggests that diet may alter the stem cell niche.

Fortunately, the knowledge of how diet modulates stem cells may allow for the

design and use of specific diets to reduce, and even prevent, degenerative processes associated with some pathologies and aging. In this scenario, extensive data support the healthy effect of extra virgin olive oil consumption. As indicated above, its consumption produces changes in serum lipid composition, decreases oxidative stress, and provides bioactive compounds that can modulate cellular processes. These effects may affect the physiology of MSCs. The state of the MSC population in an individual will directly affect its regenerative capacity and health. Thus, a new approach to studying the healthy effects of olive oil is through its putative influence on MSC functionality (Figure 1). Therefore, the main objective of this critical review is to synthesize the current knowledge on how this food and its components may modulate MSC biology, in general, and differentiation capacity, in particular. That should allow a better understanding of the physiological and metabolic mechanisms by which olive oil positively modulates health, preventing diseases and the degenerative effects of aging.

SAPONIFIABLE FRACTION OF OLIVE OIL AND MSCs

The saponifiable fraction of olive oil is composed of saturated and unsaturated fatty acids, almost entirely esterified with glycerol to form triglycerides (triacylglycerols). Extra virgin olive oil is characterized by being rich in unsaturated fatty acid residues (~ 85%), in relation to saturated ones (~ 14.5%). It should be taken into account that unless the word "free" is used, we are dealing here with fatty acids residues, forming part of the triacylglycerol molecule, because free fatty acids represent acidity, which is an unwanted characteristic of olive oil. Such lipidic molecules account for one of the healthiest properties of olive oil. The most abundant unsaturated fatty acids are oleic acid (C18:1; monounsaturated omega-9), linoleic acid (C18:2; diunsaturated omega-6), palmitoleic acid (C16:1; monounsaturated omega-7), and α-linolenic acid (C18:3; triunsaturated omega-3). Saturated fatty acid residues include the ones from palmitic acid (C16:0) and stearic acid (C18:0) (Table 1).

The fatty acid content in the medium may modulate MSC physiology, including its differentiation potency. In fact, it has been found recently that high content of fatty acids in bone marrow fluid is positively correlated to bone marrow adiposity in patients with osteonecrosis in the femoral head[41]. Oleic acid is found in the plasmatic membrane lipids. Concentration of this compound in MSCs seems to be related to the cellular differentiation stage. Indeed, nonosteoporotic individuals showed an increased concentration of oleic acid residues. Yet, osteoporotic ones showed an increase in palmitic acid residues, with cells remaining at a mostly undifferentiated stage[42]. Such results suggest that supplementation with lipids containing different fatty acids may modulate MSC differentiation.

In fact, it has been described that neuronal differentiation of human endometrial-derived stem cells was enhanced *in vitro* when culture medium was supplemented with oleic acid[43]. This fatty acid also modulates cellular adhesion and migration. For instance, it increased the migration and capacity to accelerate skin wound healing of MSCs, derived from human umbilical cord, in an *in vivo* model[44]. Also, as related to its capacity to increase the regenerative capacity of the MSCs, another study showed that supplementing MSC culture medium with oleic acid for 7 d reduced cellular proliferation, increasing biosynthesis of interleukin (IL)-6 and vascular endothelial growth factor (commonly known as VEGF), as well as nitrite concentration. Since the latter factors are angiogenic, it has been suggested that MSC preconditioning with oleic acid may activate its therapeutic capacity for wound treatment and tissue regeneration[45].

Other relevant unsaturated fatty acid residues in olive oil are the essential linoleic and linolenic acids, previously described. As with oleic acid, they are found in plasma membranes. Interestingly, its concentration increased during osteoblastogenesis in MSCs from osteoporotic persons[42]. Additionally, its presence in MSC culture medium inhibited cellular proliferation and activated synthesis of IL-6, IL-8 and VEGF inflammatory and angiogenic factors, as well as nitrite production[45]. On the other hand, linolenic acid is a substrate for biosynthesis of unsaturated n-3 fatty acids (omega-3), like eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3), through a process of desaturation/elongation[46]. Consumption of olive oil increases concentration of omega-3 fatty acid residues in blood plasma. That has been associated with a risk reduction for cardiovascular and inflammatory diseases[47]. In relation to the effect on MSCs, we have previously demonstrated that: (1) Arachidonic acid (omega-6 fatty acid derived from linoleic acid) favored adipogenic differentiation and inhibited osteoblastogenesis *in vitro* for MSCs derived from human bone marrow; and (2) EPA and DHA did not affect adipocyte differentiation, having little effect on

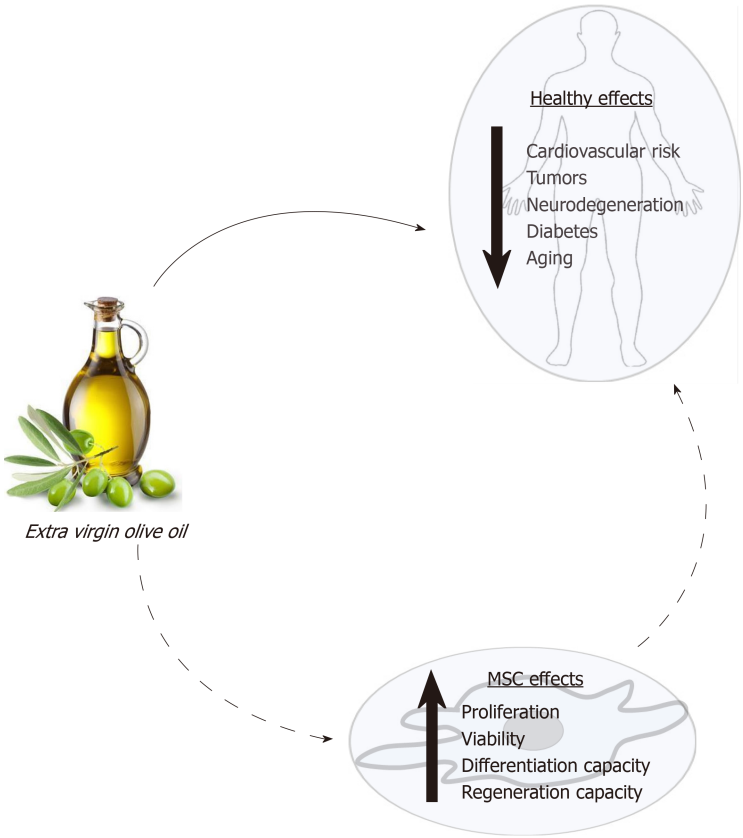

Figure 1 Effects of extra virgin olive oil on health and mesenchymal stem cells. Consumption of extra virgin olive oil has relevant health benefits, preventing cardiovascular and neurological pathologies, diabetes, and some types of cancer, and attenuating degenerative processes associated with aging, like osteoporosis, among others. Some of these healthy properties may be due to the modulation of biochemical pathways and positive effects of the constituents of this food on MSC populations. All that may have positive consequences for other cells, tissues, organs, and the whole organism. MSCs: Mesenchymal stem cells.

osteoblastogenesis. These results suggest that increasing the omega-3/omega-6 ratio may prevent the loss of mineral bone mass with aging[48]. Besides, it has been recently observed that MSCs derived from placenta, treated with EPA or DHA, increased their angiogenic capacity and, therefore, their regenerative potential[49].

Palmitoleic acid is another unsaturated lipid, generally being scarce in food. Yet, it is abundant in fatty tissue. Therefore, it has been suggested that it is mainly produced through lipogenesis[50]. Curiously, this fatty acid residue, besides others like oleic acid and linoleic acid, have capacity to induce adipogenic transdifferentiation of different tumor cell lines[51]. These results support the idea that such fatty acid residues change the cellular niche, modulating MSC differentiation.

On the other hand, olive oil palmitic and stearic (saturated) fatty acids are also abundant in the human body. In particular, palmitic acid represents between 20% and 30% of the total fatty acid of phospholipidic membranes[52]. The organism has capacity for regulating palmitic acid homeostasis though lipogenesis, independent of the diet. Nevertheless, a diet unbalanced for it and polyunsaturated fatty acid, as well as having excess of carbohydrates, may increase the concentration of palmitic acid in tissues, producing dyslipidemia, hyperglycemia, accumulation of ectopic fat, and enhancement of the inflammatory condition[52]. MSC cultures treated with palmitic acid had reduced angiogenic capacity[53]. Additionally, excess of palmitic acid in the bone marrow niche may induce apoptosis in MSCs. For example, concentrations of 0.125 mmol/L to 0.5 mmol/L of palmitate induced apoptosis of MSCs *in vitro*[54]. Additionally, palmitic acid upregulated expression of adipogenic genes, produced lipotoxicity, and increased synthesis of proinflammatory IL-6 and IL-8 in MSC cultures. That is in contrast to oleic acid effects, which also included the capacity to counteract the negative influence of palmitic acid[41,55]. Finally, the effect of stearic acid on MSCs is unknown. Nevertheless, studies carried out with animals other than humans have shown that a diet rich in it increases adiposity, reducing insulin sensitivity[56]. Such data suggest that it could affect MSC differentiation.

In summary, the amount and type of fatty acids in the medium may modulate MSC

functionality and their capacity to differentiate. Therefore, the modification of the plasmatic lipidic profile after olive oil intake may affect MSCs and partly explain the beneficial effects of such food in health (Figure 2).

EFFECTS OF UNSAPONIFIABLE FRACTION OF OLIVE OIL IN MSCS

The unsaponifiable fraction of olive oil corresponds to 1% to 2% of this food. It is composed of a large variety of compounds (Table 1). They are largely responsible for the taste and aroma (flavor) of olive oil, besides contributing to relevant biological activities. Interestingly, they can modulate the redox system and different cellular signaling pathways. Therefore, olive oil consumption may have effects on metabolism, in general, and physiological processes in MSCs, in particular, as described below and summarized in Figure 3.

Vitamins

Olive oil contains different vitamins, including E, K and A precursor (beta-carotene). Vitamin E accounts for 2% to 3% of the extra virgin olive oil unsaponifiable fraction, with 90% of alpha-tocopherol. Its main biological activity is that of antioxidant, preventing ROS[57]. Extracts of by-products of olive oil production are rich in that vitamin. We have found that intake of such extracts by postmenopausal women changed their blood serum composition. Interestingly, supplementation of culture medium with such serum promoted MSC differentiation *in vitro*, enhancing osteoblastogenesis, and inhibiting adipogenesis[58]. Additionally, vitamin E prevented oxidative stress induced by H_2O_2, reducing apoptosis and increasing cellular viability, in MSC cultures of bone marrow from rat and fatty tissue from pig[59,60]. Vitamin E pretreatment of MSCs from rat bone marrow increased chondrogenesis and proteoglycan production, after being implanted in a surgically-generated experimental osteoarthritis model of rat[59]. Also, using an experimental gentamicin-induced acute renal failure rat model, less kidney injury was observed when such animals were treated with a combination of MSCs from bone marrow administered together with vitamin E (80 mg/kg)[61]. These results show that vitamin E not only protects MSC populations but also improves their regenerative capacities.

On the other hand, vitamin K is found in extra virgin olive oil at about 500 μg/kg, which is considered a modest amount for this vitamin. It has several biological activities, including a relevant role in blood clotting. Other physiological activity of vitamin K involves modulating osteocalcin levels in bone. Low levels of carbonylated osteocalcin have been associated with loss of bone mass and higher fracture risk[62]. In this regard, treatment of human MSC cultures with vitamin K together with vitamin D favored osteoblastic differentiation and higher carboxylated osteocalcin levels[63]. All those findings highlight the relevance of this vitamin for maintenance of bone health.

Finally, beta-carotene, together with other carotenoids found in extra virgin olive oil, are partially responsible for the color of this food, besides the chlorophylls and pheophytins described below. Concentration of beta-carotene in extra virgin olive oil ranges from 2-4 mg/kg[64]. Vegetables like carrots contain much higher amounts of this provitamin, but its presence prevents olive oil photo-oxidation, adding further health benefits to this food. Beta-carotene is metabolized into retinol and retinoic acid. The latter is the active metabolite of vitamin A. It influences the physiology of MSCs and, in particular, their differentiation capacity, favoring osteoblastogenesis and inhibiting adipogenesis[65-67]. Interestingly, bead-on-string mats based on poly(lactic-*co*-glycolic acid) released beta-carotene, favoring osteoblastogenic differentiation of MSCs seeded on scaffolds, without supplementation with other osteogenic inducers[68]. These studies suggest that vitamin A may improve bone health. Retinoic acid also favored differentiation of MSCs into neurons and smooth muscle cells[69,70]. Therefore, these studies show that beta-carotene can modulate MSC differentiation. On the other hand, vitamin A repressed expression of proinflammatory factors in MSCs *in vitro*, after being stimulated by lipopolysaccharides[71]. This indicates that it also regulates the inflammatory response of MSCs.

Squalene

Squalene is an aliphatic triterpene, being the most abundant hydrocarbon in olive oil, reaching 0.7% of the total content and between 30% to 50% of the unsaponifiable fraction. That sets it apart from other oils, which have a much lower total content of this triterpene (between 0.002% and 0.03%)[72]. Squalene has a high antioxidant capacity, being associated with cardioprotective and antiaging effects[73,74].

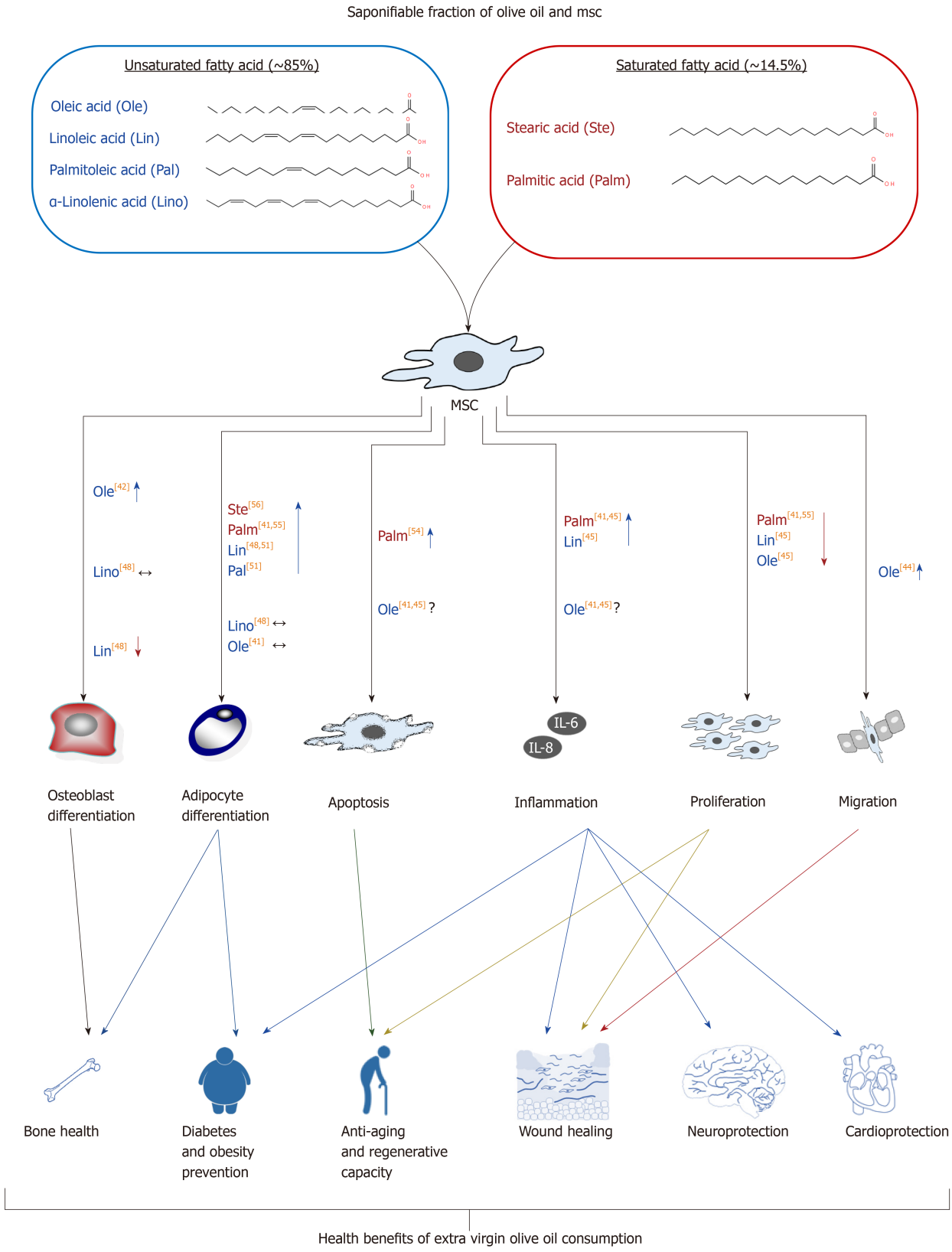

Figure 2 Effects of saponifiable fraction of olive oil on mesenchymal stem cells. Unsaturated fatty acid residues present in olive oil have effects on several biological activities of mesenchymal stem cells (MSCs), including differentiation, proliferation, inflammation, migration, and apoptosis, among others. Such effects can be related to different aspects of human health, being modulated by olive oil consumption. They include better bone health, less incidence of diabetes and obesity, and better regenerative, neurological and cardiovascular capacities, among others. Several residues of fatty acids studied (other than oleic acid) may increase adipogenesis and inflammation, inhibiting MSC proliferation. Fortunately, oleic acid accounts for the highest percentage (55% to 83%) of total fatty acid residues of olive oil. Therefore, its positive effects prevail after olive oil intake. Among others, they include increase of osteoblastogenesis and MSC migration, not affecting adipogenesis, apoptosis or inflammatory status. Symbols: ↑ and ↓ represent increase or reduction of effect, respectively, due to involved fatty acid residues; The ↔ indicates that the studies carried out so far have not found significant effects on the evaluated parameter; The ? indicates contradictory data. The numbers in square brackets indicate the bibliographical references. MSCs: Mesenchymal stem cells.

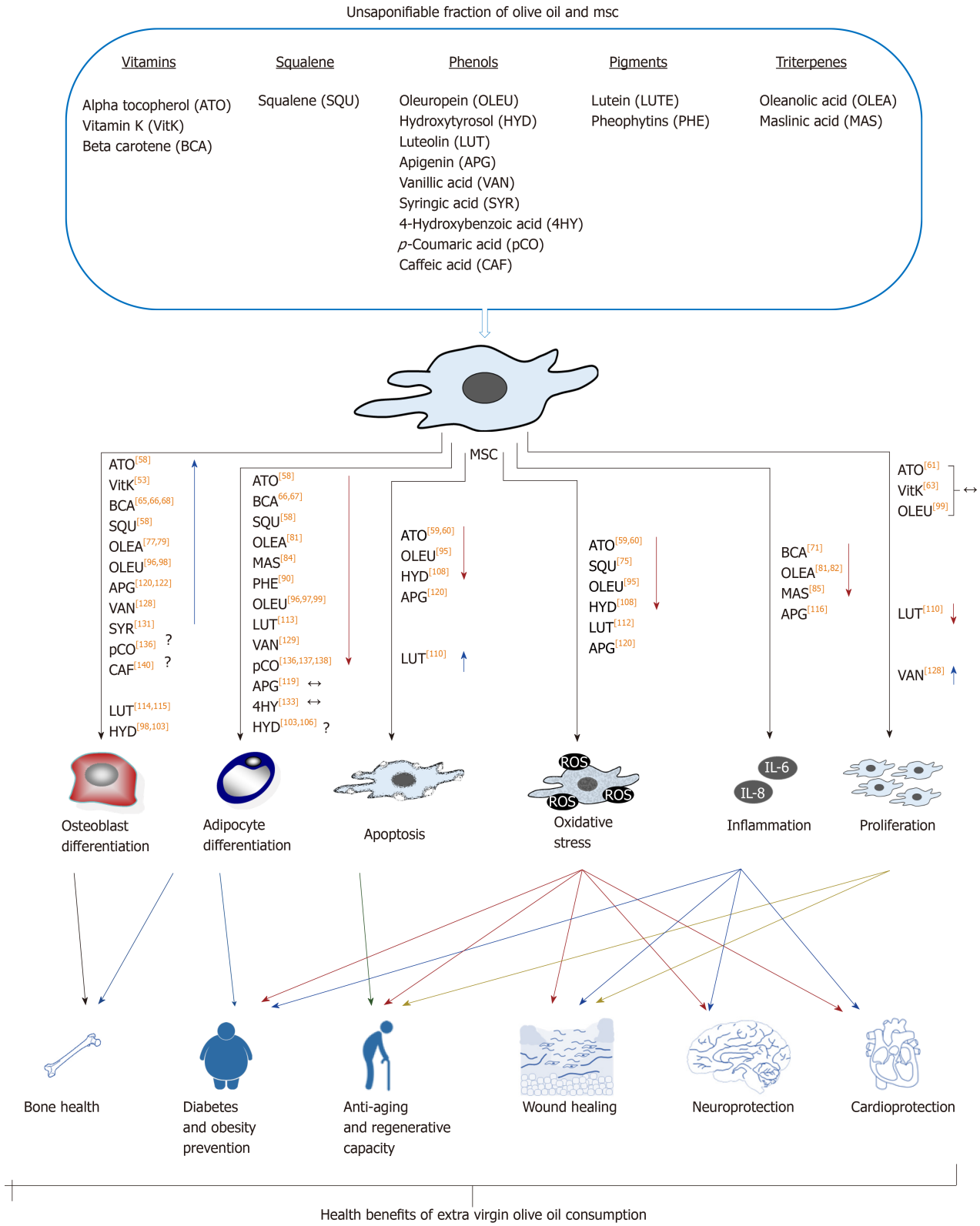

Figure 3 Effects of unsaponifiable fraction of olive oil on mesenchymal stem cells. The different groups of compounds included in the unsaponifiable fraction of olive oil have positive effects on mesenchymal stem cells (MSCs). Among others, they include differentiation, proliferation and inflammation capacities, reducing oxidative stress and apoptosis. These effects may be related to different aspects of human health, which are positively influenced by consumption of this food. Among others, they involve better bone health, lower incidence of diabetes and obesity, and better regenerative, neurological and cardiovascular capacities. In general, available data show that the studied compounds present in olive oil enhance bone *vs* fat formation. They also reduce apoptosis, oxidative stress, and inflammatory status of MSCs. All that may have a positive impact on health, explaining at least partially the beneficial effects of olive oil consumption. Symbols: ↑ and ↓ represent increase or reduction of effect, respectively, due to involved fatty acid residues; The ↔ indicates that the studies carried out so far have not found significant effects on the evaluated parameter; The ? indicates contradictory data. The numbers in square brackets indicate the bibliographical references. MSCs: Mesenchymal stem cells; ATO: Alpha tocopherol; VitK: Vitamin K; BCA: Beta carotene; SQU: Squalene; OLEU: Oleuropein; HYD: Hydroxytyrosol; LUT: Luteolin; APG: Apigenin; VAN: Vanillic acid; SYR: Syringic acid; 4HY: 4-Hydroxybenzoic acid; pCO: p-Coumaric acid; CAF: Caffeic acid; LUTE: Lutein; PHE: Pheophytins; OLEA: Oleanolic acid; MAS: Maslinic acid.

Interestingly, we have recently found that serum from postmenopausal women (previously treated with an extract of by-product of the olive oil extraction process, containing more than 7% squalene) enhanced osteoblastogenesis and inhibited adipogenesis of MSC cultures[58]. Indeed, squalene may have an important role in the MSC niche in stroma of bone marrow, as suggested by its protection of MSCs in the presence of chemotherapeutic and anticancerogenic agents, like cisplatin and carboplatin[75]. These studies suggest that, unlike other oils, the consumption of squalene-rich olive oil may positively influence MSC populations.

Triterpenes

Triterpene concentration in olive oil ranges from 8.90-112.36 mg/kg, mainly including oleanolic acid, maslinic acid, uvaol, and erythrodiol[76]. Triterpenes of olive oil exhibited interesting biological activities, including antitumoral, antioxidant, antiinflammatory, antimicrobial, and hepatoprotective and cardioprotective effects, which may have positive impact on health[76]. Effects of oleanolic and maslinic acids on MSCs have been reported; for instance, some authors have reported that the former reduced MSC viability[77], although others have not found such an effect[78]. Such triterpene modulates bone formation. Indeed, some authors have found that this triterpene prevented bone mass loss in ovariectomized rats. They also observed that 1 μmol/L and 10 μmol/L of oleanolic acid induced rat MSC differentiation into osteoblasts *in vitro*, and suggested that this effect was mediated by the Notch signaling pathway[79]. These results have been reproduced in human MSCs. Such experiments showed that this triterpene inhibited Notch signaling and induced the expression of osteoblastic genes[77]. It has been reported that Notch signaling maintained the MSCs undifferentiated stage, whereas the inhibition of this pathway promoted differentiation into osteoblasts[80]. Besides, oleanolic acid enhanced the effect of bone morphogenetic protein 2 (commonly known as BMP2) as an osteoblastic inducer[77].

Since the factors that induce MSC differentiation into osteoblasts usually inhibit adipogenic differentiation, a negative effect of oleanolic acid on adipogenesis is expected. Indeed, treatment of 3T3-L1 mouse preadipocytes with such triterpene downregulated peroxisome proliferator-activated receptor gamma (*PPARγ*), which is the master regulator of adipocyte differentiation. In addition, it decreased accumulation of fat droplets in these cells during adipocyte differentiation[81]. Also, oleanolic acid reduced inflammatory factor production, like that of visfatin and resistin, in 3T3-L1 preadipocyte cells[81,82]. Therefore, consumption of this triterpene may have beneficial effects on pathologies associated with overproduction of inflammatory adipokines.

On the other hand, maslinic acid (as oleanolic acid) showed osteoprotective effects in ovariectomized mice. But, in this case, such an effect has been associated with its capacity to inhibit bone resorption, by suppressing osteoclastic differentiation promoted by the receptor activator of nuclear factor-kappa B (NF-κB) ligand (commonly known as RANKL)[83]. Yet, the putative positive effects of this triterpene on MSC osteoblastogenesis have not been found so far. Nevertheless, it inhibited 3T3-L1 preadipocyte differentiation *in vitro*, reducing fat droplets and increasing glucose uptake[84]. Such results, besides the antiinflammatory activity of maslinic acid[85], suggest that its consumption, together with other triterpenes of olive oil, may affect MSC populations.

Pigments

Pigments are responsible for the coloration of olive oil, with the carotenoids and pheophytins (derived from chlorophylls) being the most important. Among the carotenoids are beta-carotene (also known as provitamin A, as previously described) and lutein. The latter is found at a concentration of 3.5 mg/kg in extra virgin olive oil[86]. Relevance of food rich in lutein for eye health is well established[87]. Yet, knowledge of the putative effects on MSCs is scarce. Interestingly, it has been recently found *in vivo* that a diet rich in lutein reduces bone resorption in mice. That is accomplished through inhibition of osteoclastogenesis, additionally increasing bone formation[88]. These results suggest that lutein may promote osteoblastogenesis or differentiation of osteoblasts into osteocytes. Nevertheless, more research is needed to properly ascertain its putative role in MSC differentiation.

On the other hand, pheophytins are responsible for the attractive greenish color of olive oil. Their concentrations range from traces to 30 mg/kg, depending on olive tree cultivar, ripening stage of the olive fruit, and technology used for the olive oil extraction, among other factors. Their biological activities include antiinflammatory and antidiabetic effects[89,90]. Although the putative influence of pheophytins on MSCs are unknown, some authors have found that they inhibited adipogenic differentiation of 3T3-L1 murine cells, reducing fat droplets when induced to differentiate into

adipocytes[90]. These results suggest that pheophytins probably inhibit adipogenic differentiation of MSCs. Again, further research is required to shed more light on this exciting topic.

Phenolic compounds

Phenolic compounds of extra virgin olive oil represent 18% to 37% (100-300 mg/kg) of the unsaponifiable fraction. They are partially responsible for the high oxidative stability of olive oil. Their composition and concentration depend on the olive tree cultivar, weather conditions, fruit ripeness, and technology used for olive oil extraction[91]. The main types of phenolic compounds of extra virgin olive oil are flavonoids, lignans, phenolic acids, phenolic alcohols and secoiridoids, the last two being the most abundant. Indeed, secoiridoids represent between 85% and 99% of the total phenolic compounds of olive oil[92]. Their intake is healthy, being antioxidants (preventing oxidative stress) and interacting with several signaling pathways. Actually, they have antiinflammatory, anticarcinogenic, antiatherogenic and antithrombotic effects. Besides, they regulate lipidic metabolism, among other beneficial activities[15]. Interestingly, the biological activity of phenolic compounds also modulates MSC physiology, including their proliferation, viability, regenerative capacity and differentiation, as demonstrated by numerous studies in the last two decades.

Oleuropein is a relevant secoiridoid, ranging from 1.2-120 mg/kg in extra virgin olive oil[93]. It has numerous beneficial health properties, including antioxidant, antiinflammatory, antiatherogenic, anticancerogenic, antimicrobial, antiviral and antiaging effects, among others[94]. Interestingly, oleuropein prevents both oxidative stress and apoptosis induced by H_2O_2 in MSCs. That is accomplished by inhibiting the B-cell lymphoma 2 (referred to as Bcl-2)-like protein 4 (referred to as BAX) proapoptotic protein, activating the antiapoptotic Bcl-2 and myeloid leukemia cell differentiation protein (referred to as Mcl-1), and modulating autophagy-related death signals[95].

Additionally, oleuropein also affects MSC differentiation. We have described that this phenolic compound upregulates osteoblastic marker genes, increasing alkaline phosphatase activity, mineralization and osteoprotegerin (*OPG*)/*RANKL* expression ratio in human MSCs derived from bone marrow[96]. These results suggest that oleuropein favors osteogenesis, inhibiting bone resorption. The latter is accomplished by activating expression of *OPG*, which is a decoy receptor for the receptor activator of RANKL, which is an osteoclastogenesis activator. Therefore, an increase in *OPG* expression reduces the ability of RANKL to activate osteoclastogenesis.

We have reported that oleuropein inhibits adipogenic differentiation of MSCs by downregulating *PPARγ* and other adipogenic genes, reducing fat droplet formation in treated cultures[96]. We have also carried out transcriptomic analyses of MSCs induced to differentiate into adipocytes in the presence of oleuropein. Interestingly, this compound restored expression of 60% of the genes repressed during adipogenesis, activating some signaling pathways, like Rho [family of guanosine triphosphate (GTP) ases] and beta-catenin, and inhibiting others related to mitochondrial activity, which are induced during adipogenesis. This indicates that the presence of oleuropein keeps MSCs that have been induced to adipocytes in a more undifferentiated state[97]. Therefore, since this compound favors osteoblastogenesis *versus* adipogenesis, it can have osteoprotective properties and its consumption may prevent some diseases, like osteoporosis. It can also be beneficial in physiological processes affecting bone health, like aging. That rationale is supported by the fact that treatment with 10 mg of oleuropein/kg every 3 d prevented loss of trabecular bone in the femurs of ovariectomized mice. Besides, the authors also showed in *in vitro* studies that this compound favored mineralization of MC3T3-E1 preosteoblastic cells from mice and inhibited osteoclastogenesis[98].

Additionally, oleuropein can prevent formation of ectopic fat, preventing obesity. Indeed, it has been reported that this phenolic compound prevented formation of visceral fat in obese mice and inhibited adipogenesis in 3T3-L1 preadipocytes[99]. Oleuropein may also have antiaging effects on MSCs, maintaining a better regenerative capacity of the organism with advanced age. This is because it can inhibit the phosphatidylinositol 3kinase/Akt/mammalian target of rapamycin (commonly known as mTOR) signaling pathway on MSCs[100]. The inhibition of phosphoinositide 3-kinases (commonly known as PI3Ks)/Akt/mTOR pathway maintained high proliferative and differentiation capacities in MSCs[101]. Akt stands for Ras-related C3 botulinum toxin substrate 1, (RAC)-alpha serine/threonine-protein kinase, also known as protein kinase B (or its more common abbreviation of PKB).

Hydroxytyrosol is another of the most abundant phenolic compounds present in extra virgin olive oil. Its concentration ranges from 1.1 mg/kg to 75 mg/kg[93]. This phenolic alcohol has many healthy effects, including antiinflammatory, antimicrobial,

cardioprotective, neuroprotective and antitumoral activities, among others. Thus, it has also been considered as a nutraceutical[102]. Regarding its effect on MSCs, we have evaluated the effects of 1 μmol/L and 100 μmol/L hydroxytyrosol on osteogenic and adipogenic differentiation of MSCs from human bone marrow. The highest concentration of this compound (but not the lower one) reduced the number of cells, repressed expression of osteoblastic gene collagen type-I alpha-1 (*COLIA1*) and reduced mineralization in cultures induced to differentiate into osteoblasts[103]. On the other hand, mostly the highest concentration increased both expression of the *PPARγ* gene as well as of generation of fat droplets, indicating induction of adipogenesis[103].

Yet, other authors have described that such high concentration of hydroxytyrosol inhibited adipogenesis in 3T3-L1 preadipocytes from mice[104,105]. Discrepancies were probably due to different cellular types and different methodologies used to induce differentiation. Interestingly, mitotic clonal expansion is an important event during adipogenic differentiation of 3T3-L1, but does not take place in human MSCs. This compound inhibited mitotic clonal expansion of 3T3-L1[104,105]. Therefore, its effects on mice *versus* human adipogenesis may be different[103]. Additionally, it has been recently reported that 30 μg of hydroxytyrosol/mL (~ 200 μmol/L) inhibited adipogenesis on human preadipocytes (not affecting mature adipocytes), further increasing apoptosis[106].

Nevertheless, it should be taken into account that plasma levels of hydroxytyrosol have been found to be about 4.5 ng/mL (~ 0.029 μmol/L) in 30 min after consumption[107]. Therefore, the effect of high concentrations of hydroxytyrosol, reported by others and ourselves, on osteoblastic and adipogenic differentiation of human MSCs *in vitro* would require a pharmacological *in vivo* intake. Interestingly, studies with animals have shown that both oleuropein and hydroxytyrosol protected 6-wk-old ovariectomized mice of trabecular (albeit not cortical) bone loss in femur. Yet, it is not clear if that was due to increased osteoblastogenesis, inhibition of osteoclastogenesis, or both[98].

Additionally, hydroxytyrosol has positive effects on chondrocytes, which are other cells also derived from MSCs. For instance, in an osteoarthritis model in which chondrocytes have been stimulated with growth-related oncogene alpha (commonly known as GROα) to promote hypertrophy and terminal differentiation, the presence of hydroxytyrosol reduced oxidative stress and apoptosis, which are induced in such pathology[108]. These results suggest that this compound might have a relevant role in MSC physiology in bone marrow, modulating bone metabolism. Nevertheless, further research is required to properly ascertain its putative effects *in vivo*.

Extra virgin olive oil also contains other phenols, known as flavonoids, mainly luteolin and apigenin. Their concentrations range from 0-19 mg/kg[93], therefore representing a minority. Despite that, their intake has been associated with reduced risk to suffer cardiovascular disease, cancer, and neurodegenerative disorders[109]. Both flavonoids upregulate the octamer-binding protein 4 (*OCT4*) and sex-determining region Y box-containing gene 2 (*SOX2*) genes in MSCs[110]. Such genes are mainly expressed in embryonic stem cells, encoding transcription factors that regulate cell cycle and maintenance of totipotency or pluripotency. They are considered additional MSC molecular markers in adult tissues, being repressed after cell differentiation[111]. Therefore, luteolin and apigenin may delay the loss of regenerative capacity of MSCs with aging. In addition, also related to aging, luteolin prevented oxidative stress *in vitro*, induced with $FeCl_2$ and H_2O_2 in MSCs[112].

Luteolin can also affect MSC differentiation. Indeed, it inhibited adipogenic differentiation of 3T3-L1 murine cells, reducing triglyceride accumulation, as well as downregulating genes encoding the PPARγ and CCAAT-enhancer-binding proteins (*C/EBPa*) adipogenic transcription-factors[113]. Regarding osteogenic differentiation, some authors have reported that luteolin reduced alkaline phosphatase activity and viability of MC3T3-E1 preosteoblastic cells[114]. Yet, other authors using the same cellular type but much lower concentrations of this flavonoid have found that it prevented the osteoblastogenesis inhibition induced by glucocorticoids. Indeed, they showed that luteolin prevented bone mass loss, by downregulating apoptotic genes, increasing *OPG/RANKL* gene expression ratio, and activating the beta-catenin pathway in an animal model of osteoporosis induced by glucocorticoids[115]. Therefore, these results suggest that this compound has an osteoprotective role in bone marrow.

On the other hand, apigenin is a phytoestrogen that has antiinflammatory effect on MSCs. Interestingly, apoptosis produced after the inflammatory response, triggered by lipopolysaccharide, was enhanced by the presence of this flavonoid[116]. MSC differentiation is also influenced by apigenin. Different studies have suggested that it inhibits adipogenic differentiation of MSCs. Indeed, it has been found that apigenin downregulated *PPARγ* and inhibited fat droplet formation in 3T3-L1 cells that had been induced to differentiate into adipocytes[117]. A negative effect of apigenin on adipogenesis *via* inhibition of the early differentiation processes, including mitotic

clonal expansion, has also been reported. Yet, apigenin supplementation did not have effect in the advanced stages of adipogenic differentiation[118]. Nevertheless, contrary to results obtained with mouse preadipocytes, this flavonoid did not inhibit adipogenesis in human MSCs derived from fatty tissue[119]. Further studies are required to shed more light on putative roles of apigenin on human MSC differentiation into adipocytes.

The effect of apigenin on osteoblastogenesis has also been studied. This compound reduced apoptosis and ROS production, maintaining mitochondrial membrane potential, under oxidative stress conditions induced by H_2O_2 in MC3T3-E1 mouse preosteoblasts[120]. Besides, its antioxidant activity maintained expression of osteoblastic genes in such cells and conditions[120]. However, other authors have reported that this compound reduced viability and osteoblastogenesis in such cells, in a dose-dependent manner. Nevertheless, they reported that 10 mg apigenin/kg intraperitoneally administered to ovariectomized mice prevented loss of trabecular bone[121].

These *in vivo* results are in agreement with the ones obtained with human MSCs *in vitro*. For example, apigenin supplementation to osteogenic medium increased expression of osteoblastic markers and mineralization of extracellular matrix in human MSC cultures. Such effects activating osteoblastogenesis were mediated by increased phosphorylation of c-Jun N-terminal kinases (commonly known as JNKs) and p38 mitogen-activated protein kinases (commonly known as MAPKs)[122]. The role of p38 in MSC osteoblastogenesis has also been described[123]. Interestingly, apigenin effects on MSCs may modulate bone metabolism, preventing bone mass loss in diseases like osteoporosis[122] and physiological processes like aging.

The last important group of phenolics in extra virgin olive oil is the one of phenolic acids. These acids are simple phenols, usually found at very low concentrations in such food. Nevertheless, sometimes they are found at higher concentrations, like in some Tunisian olive oils, in which they can reach up to 38.39% of the total phenolic content[124]. The most relevant phenols in this group include vanillic acid, syringic acid, 4-hydroxybenzoic acid, *p*-coumaric acid, and caffeic acid. Vanillic acid has hepatoprotective and cardioprotective effects[125,126]. Indeed, it has been observed that it reduced bone mass loss in ovariectomized mice[127]. That may be related to its effects on cells derived from MSCs. For instance, this phenolic compound favored proliferation and differentiation of rat osteoblast-like UMR 106 cells, increasing expression of osteogenic markers and ratio of *OPG*/*RANKL* expression. These effects were mediated by its activity as phytoestrogen[128]. It has also been suggested that vanillic acid may modulate adipogenesis because it reduced triglyceride content, without affecting cellular viability, in 3T3-L1 cells induced to differentiate into adipocytes[129].

On the other hand, syringic acid also has relevant healthy properties. Indeed, it is mainly a powerful antioxidant, being also antidiabetic, cardioprotective, antiinflammatory, *etc*[130]. Yet, only its effect on MSC differentiation into osteoblasts have been studied. For example, it has been found that the presence of this compound in culture medium of mouse MSCs increased expression of osteogenic genes, alkaline phosphatase activity, and mineralization. This phenolic acid promoted osteoblastogenesis by inducing expression of the miR-21 microRNA, thus reducing expression of the so-called "mothers against decapentaplegic homolog 7" (*SMAD7*) gene, since it is the target of such microRNA[131]. SMAD7 protein inhibits osteoblastogenesis. Therefore, its repression in MSCs promoted differentiation into osteoblasts[131,132]. In relation to 4-hydroxybenzoic acid, little is known about its putative effects on MSCs. Just one *in vitro* study has been carried out in relation to adipogenic differentiation of mouse 3T3-L1 and human MSCs from adipose tissue. Yet, in neither of these cell types did it produce significant changes during adipogenesis[133].

With respect to *p*-coumaric acid, it is usually found in extra virgin olive oil at concentrations lower than 1 mg/kg[134]. Its intake is healthy, due to several biological activities, including antioxidant, antimicrobial, antiviral, antitumoral, antidiabetic, *etc*[135]. Interestingly, extracts from the fragrant eupatorium plant (*Eupatorium japonicum*) favored osteoblastogenesis and inhibited adipogenesis in both multipotent C3H10T1/2 and primary bone marrow cells from mouse and rat, respectively. Besides, it prevented body weight increase and bone mineral density decrease in ovariectomized rats. Those effects were partially due to the biological activities of this phenolic compound[136]. Similar results showed that *p*-coumaric acid inhibited adipogenic differentiation of 3T3-L1 preadipocytes[137,138]. Interestingly, this compound also inhibited myogenic differentiation of C2C12 cells from mouse, which may negatively affect development of skeletal muscle[138].

Finally, caffeic acid has antidiabetic and antioxidant activities[139]. Yet, its putative biological activity on MSCs is unknown. Possibly, it can induce osteoblastic

differentiation, since this compound increased alkaline phosphatase activity and changed the phenotype through a process involving antigen expression in human MG-63 osteosarcoma cells[140]. Nevertheless, its mechanism of action and putative effects on undifferentiated cells remains unknown, requiring further research.

In summary, most studies about phenolic components of olive oil on MSCs have focused on how these compounds affect MSC differentiation into osteoblasts and adipocytes. In general, results have shown that such chemicals may modulate proliferation and differentiation of osteoblast precursor cells, suggesting that their consumption may influence bone health. The fact that phenolic extracts of extra virgin olive oil from different olive tree cultivars (Picual, Hojiblanca, Arbequina and Picudo) increased proliferation of MG-63 preosteoblastic cells supports that hypothesis[141]. Indeed, olive oil consumption is associated with better bone health. That is partially due to the effects of its phenolic components on osteoblastic differentiation of MSCs. Therefore, it has been proposed that intake of this food, as well as other products derived from the olive tree, may help to prevent bone mass loss, due to diseases like osteoporosis[142,143] or physiological aging.

CONCLUSION

Numerous studies and clinical assays have confirmed the health benefits of extra virgin olive oil consumption, due to its nutraceutical properties[144]. Its molecular mechanisms of action have been investigated in different cells, tissues, organs, and organisms. Results have shown prominent biological activities, including antioxidant, antiinflammatory and chemoprotective, modulating different cellular signaling pathways[145,146]. The putative effects of extra virgin olive oil intake on maintenance and functionality of MSC populations have not been investigated to date. Such knowledge should shed light on this subject and help to better explain the health benefits of olive oil. Fortunately, the effects of specific compounds present in this food have been evaluated in relation to viability and differentiation of MSCs.

A summary of research results is shown in Figures 2 and 3. It is evident that further investigations are required to gain more knowledge on putative effects on MSC biology of some of these compounds, after *in vivo* intake. They should be studied alone and combined, taking into account putative synergistic interactions between them. Interestingly, available data indicate that the profile of fatty acid residues (saponifiable fraction) of extra virgin olive oil can change the composition of the MSC niche and therefore its physiological behavior. For instance, the high content of oleic acid in extra virgin olive oil can favor migration and differentiation of MSCs. Besides, the increased ratio of omega-3/omega-6 fatty acid residues enhanced by consumption of extra virgin olive oil favors both viability and differentiation into osteoblasts (instead of adipocytes) of MSCs from bone marrow.

On the other hand, although the unsaponifiable fraction of extra virgin olive oil represents just 1% to 2%, it is comprised of a large variety of compounds with relevant biological activities, which also influence MSC biology. For instance, phenolic compounds, vitamins, squalene and other compounds of this fraction modulate signaling pathways. That, in turn, can impact MSC viability and differentiation. In general, most of these compounds protect MSCs from oxidative stress and aging, favoring osteogenic *versus* adipogenic differentiation. Indeed, they have healthy properties, including regenerative, osteoprotective, antidiabetic and antiobesity effects, partly because of their activity on MSCs.

In summary, the currently available data show positive effects of different components of extra virgin olive oil, related to maintenance and functionality of MSC populations. That is in agreement with the fact that consumption of this food prevents bone loss due to pathologies like osteoporosis or physiological processes like aging[19]. Yet, it should be taken into account that most studies have been carried out with purified compounds *in vitro*, usually at concentrations higher than the physiological ones. Therefore, further *in vivo* research is needed to ascertain the putative effects of extra virgin olive oil consumption on MSC viability and regenerative capacity. That should shed light on the molecular mechanisms underlying such effects, which is required to increase our current knowledge on this topic. Studies should focus on analyses of specific MSC populations-for instance, including the ones in bone marrow or fatty tissue. The rationale is that niche compositions may be different for different tissues, changing with differential patterns as well, in response to extra virgin olive oil consumption.

Additionally, it should be considered that different components of this food may work synergistically on MSCs. Therefore, the effects due to combination of even small quantities of different compounds might be equal or even superior to the ones of

higher amounts of such individual chemicals. Therefore, it would be interesting to carry out both *in vitro* and *in vivo* studies with different combinations of these compounds or extracts of extra virgin olive oil. That should allow a better understanding of the biological effects of MSCs. Additionally, such knowledge should allow a better description of the composition and desirable characteristics of extra virgin olive oil. The goal is that its consumption reaches the highest possible positive impact on the MSC populations and, thus, the organism's overall health.

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REVIEW

Three-dimensional cell culture systems as an *in vitro* platform for cancer and stem cell modeling

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Abstract

Three-dimensional (3D) culture systems are becoming increasingly popular due to their ability to mimic tissue-like structures more effectively than the monolayer cultures. In cancer and stem cell research, the natural cell characteristics and architectures are closely mimicked by the 3D cell models. Thus, the 3D cell cultures are promising and suitable systems for various proposes, ranging from disease modeling to drug target identification as well as potential therapeutic substances that may transform our lives. This review provides a comprehensive compendium of recent advancements in culturing cells, in particular cancer and stem cells, using 3D culture techniques. The major approaches highlighted here include cell spheroids, hydrogel embedding, bioreactors, scaffolds, and bioprinting. In addition, the progress of employing 3D cell culture systems as a platform for cancer and stem cell research was addressed, and the prominent studies of 3D cell culture systems were discussed.

Key words: Three-dimensional cultures; Cancer; Stem cells; Disease modeling; *In vitro* screening platform

Core tip: Three-dimensional cell culture systems are considered an *in vitro* platform for cancer and stem cell research, which hold a great potential as a tool for drug discovery and disease modeling. With such systems, the success rate in disease modeling, drug target identification, and anticancer screening could be accelerated and result in an emergence of a novel and effective therapeutic means as well as the development of tissue replacement substances that may transform our lives.

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INTRODUCTION

Cancer is one of the most serious diseases causing almost one in six deaths globally, which is estimated to equal 9.6 million deaths in 2018[1]. Considerable efforts have been intended to develop effective approaches to cure cancer. Among them, drug discovery could be one of the most important approaches aiming to identify and verify new and potent anticancer agents for both daily medication and chemotherapy. For testing the capability of novel anticancer drugs, the experiments are performed on cell-based assays, which offer information about cellular responses to drugs in cost/time effective and high throughput manners.

Currently, two-dimensional (2D) platforms in which flat monolayer cells are cultured is still the most commonly used for the research of cell-based assays. The 2D cell culture systems are easy, convenient, cost-effective, and widely used. However, various drawbacks and limitations are still of concern. The first drawback of a 2D cell culture systems is that an actual three-dimensional (3D) environment in which cancer cells reside *in vivo* is not accurately mimicked[2]. The irrelevant 2D environment may provide misleading results regarding the predicted responses of cancer cells to anticancer drugs[3]. Generally, standard preclinical screening procedures for therapeutic agents involve identification of compounds from the 2D cell culture system tests and animal model tests and then to the introduction of clinical trials[4]. Along with each phase, the percentage of efficient agents dramatically decreases. Less than 5% of anticancer agents and small molecule oncology therapeutics passed the clinical trials and were finally approved for marketing by the regulatory agencies[3]. One possible cause of the failure is that drug responses of 2D cell cultures systems did not consistently predict the outcome of clinical studies[5-7].

The key limitation of traditional 2D culture is the failure to imitate the *in vivo* architecture and microenvironments. As a consequence, there are many different features that 2Dcultured cells possess compared with *in vivo* cells such as morphological characteristics, proliferation and differentiation potentials, interactions of cell-cell and cell-surrounding matrix, and signal transduction[8,9]. Such concerns inspired the emergence of 3D cell cultures systems, a promising approach to overcome the inconsistency between cell-based assays and clinical trials. The 3D cell culture systems provided the novel cell-based assays with more physiological relevance, especially the behavioral similarity to the *in vivo* cells. Over the last decade, a variety of *in vitro* platforms was developed to achieve the 3D culture systems for cancer and stem cell applications such as novel drug development, cancer and stem cell biological research, tissue engineering for *in vivo* implantation, and other experimental cell analyses[10-12]. Thus, the study of cellular phenomena in a conditions that closely imitates *in vivo* scenery could be elaborately constructed *in vitro*[11,13,14].

Here, we aim to demonstrate the necessity of novel 3D cell culture systems and describe, compare, and contrast the 3D cell cultures techniques that has been developed to date. In addition, we also present the possibility to be applied in cancer and stem cell aspects.

CELL CULTURE AS A RESEARCH MODEL

In 1907, Harrison *et al*[15] implemented the cell culture technique to his research, exploring the origin and the development of nerve fibers. Since then, the technique has been continuously improved, and cell-based experiments can be effortlessly conducted based on such cell culture technique due to cell banking[16]. The selection of cell culture procedures for cancer research is the key for the better understanding of tumor biology, resulting in the optimal and effective conditions for radio/chemotherapy as well as the discovery of new cancer treatment strategies[17]. At the very beginning of the cell culture era, the cultures were mostly carried out under an adherent condition, which is called the 2D monolayer cell culture model[18]. However, the *in vivo* environment provides cell-cell and cell-extracellular matrix

(ECM) interactions in a 3D structure[19], and the 2D monolayer cells might not accurately mimic the actual 3D environment of the *in vivo* cells. The clear evidence was the experiments using the immortalized tumor cell lines grown in the 2D culture systems resulted a 95% drug response failure rate in human subjects. It indicated that the 2D cell culture model could be an inaccurate model for drug development[20]. Therefore, the drug discovery and validation processes should integrate both 2D cell culture screening and animal study, complying with the standard procedure prior to clinical trials. Nonetheless, the data collected from the 2D cell system are often misleading for *in vivo* responses as previously mentioned, and the animal models are expensive, time consuming, controversial with ethical dilemmas, and inconsistent due to species differences[21]. The development of novel models is needed to resolve the inconsistency between the 2D cell culture systems, animal models, and clinical trials. Therefore, the 3D *in vitro* cell culture platforms could be the potential candidate[22].

TWO-DIMENSIONAL *VS* THREE-DIMENSIONAL CELL CULTURE MODELS

Cell culture is the most basic yet essential process for preclinical drug discovery. Even though the unreliable flaws of monolayer cell culture have been pointed out, 2D cell culture models are still the first option that scientists turn to due to its simplicity in order to obtain preliminary results. Nevertheless, 2D cultures may not sufficiently mimic the physiological conditions in a 3D network where *in vivo* cells reside. Therefore, deceptive data from 2D cell culture model often leads to the irrelevant prediction of drug efficacy and toxicity and finally causes the failure in drug validation and approval processes[23].

One obvious advantage of cell culturing in a 3D manner over 2D cell culture is that it contributes the expression of ECM components as well as the interactions between cell-cell and cell-matrix. The characteristics of 3D cell cultures and the traditional 2D cell culture models are shown in Figure 1. The traditional 2D cell cultures result in a monolayer cell expanding on a flat surface of glass or commercial polystyrene plastic flasks for tissue culture (Figure 1A). In contrast, 3D cell cultures promote cells to form 3D spheroids by utilizing an ECM material (Figure 1B). Cell spheroid is the important characteristic that resembles *in vivo* cells for further replicating cell differentiation, proliferation, and function *in vitro*. Thus, 3D spheroid culture is considered an improved model for predictive *in vitro* cell-based assays and may deliver high physiological relevance for preclinical drug discovery, especially in cancer/stem cell research.

Generally, cells of multicellular organisms capable of forming tissues are in 3D arrangements with complex interactions within cell populations and also between cells and environments. With the dynamics of nutrient and chemical transport between cells in the *in vivo* conditions, cells are hemostatically provided with a relatively constant supply of nutrients with the minimized level of waste products due to the activity of the circulatory system. Therefore, the 3D arrangements of cells are the major employment for 3D cell culture with the optimal spatial organization of cells in the culture environment to be considered[24-26]. When cells are grown in 3D culture systems, cells also induce the formation of aggregates or spheroids within matrix or the culture medium. Even though with cell-cell interactions and cell-matrix interactions are not yet perfectly mimicked in a spheroid culture model, they are close enough to induce the morphological alteration of cells to not be relatively flat but closely resemble its natural shape in the body (Figure 1C).

Furthermore, within the spheroid structure, various stages of cells are established, including proliferating, quiescent, apoptotic, hypoxic, and necrotic cells due to the gradients of nutrients and oxygen level[27,28]. The proliferating cells could be found mainly at the outer layer of the spheroids because they are exposed to sufficient amounts of nutrients from the culture medium[29,30]. Cells at the core of spheroids tend to be in quiescent or hypoxic states because they are faced with the lack of oxygen, growth factors, and nutrients[31]. The cellular heterogeneity within a cell population is quite relevant to *in vivo* tissues, organs, and even tumors. At this point, due to cell morphology, interactions, and heterogeneity of cells grown in 3D culture, it is reasonable to hypothesize that the cellular processes of these cells are also applicable[32].

Comparisons of 3D spheroid culture models and 2D monolayer cell culture models were shown in Table 1. Numerous studies have proven the differences in cell viability, morphology, proliferation, differentiation, cellular responses to stimuli, cell-cell communication, cell stiffness, migrant and invasive properties of tumor cells into surrounding tissues, angiogenesis stimulation and immune system evasion, drug

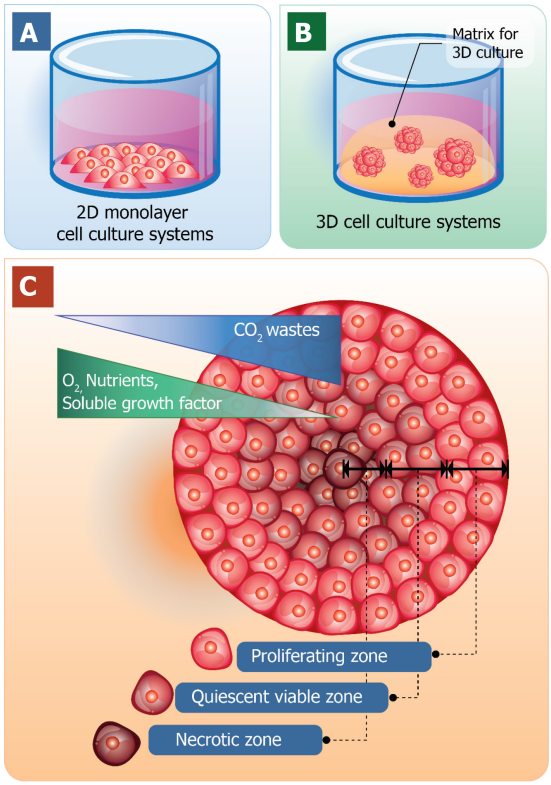

Figure 1 Schematic diagrams of the traditional two-dimensional monolayer cell culture and three-dimensional cell culture systems. A: Traditional two-dimensional monolayer cell culture; B: Three-dimensional cell culture systems; C: The structure of three-dimensional spheroid with different zones of cells with the models of oxygenation, nutrition, and CO_2 removal. Three-dimensional spheroid from inside to outside. The regions are necrotic zone (innermost), quiescent viable cell zone (middle), and proliferating zone (outermost).

responses, transcriptional and translational gene expression, general cell function, and *in vivo* relevance between cells cultured in 2D and 3D models. For example, cell polarization could be more accurate depicted in 3D cell cultures models unlike in 2D models in which the cells can only be partially polarized. Moreover, greater stability and longer lifespans were found in 3D culture models; 3D spheroids can be cultured up to 3 wk, whereas 2D monolayer culture can last for less than a week due to the limitation of cell confluence[33]. Therefore, 3D cell culture models might be more appropriate for handling the long-term experiments and for determining long-term effects of the drug on cellular responses.

THREE-DIMENSIONAL CELL CULTURE TECHNOLOGIES

Because the advantages of 3D culture systems have become widely realized, there have been many studies intensively focused on the development and optimization of 3D cell culture technologies. With the integration of the recent advances in cell biology, microfabrication techniques, and tissue engineering, a wide range of 3D cell culture platforms were constructed, including multicellular spheroid formation (liquid overlay culture and hanging drop method), hydrogel-based culture, bioreactor-based culture, bio-printing, and scaffold-based culture. A summary of the advantages, disadvantages, and research stage of each model are shown in Table 2. Although each 3D culture technique/platform are different in both principle and protocol, the same objectives that they share are to provide the similar features of *in vivo* cells in morphological, functional, and microenvironmental aspects. This section aims to briefly describe the key features of each technique.

Multicellular spheroids formation

Liquid overlay culture: Liquid overlay culture could be the simplest of all 3D cell culture Techniques (Figure 2A). To create 3D culture models, the surface for cell culture is covered with a thin film of inert substrates, such as agar[34], agarose[35], or matrigel[36]. By preventing cell adhesion on the surface and providing the artificial matrix, liquid overlay culture easily promotes the aggregation of cells to become

Table 1 Differences in two-dimensional *vs* three-dimensional cell culture models

Type of culture	2D	3D	Ref.
In vivo-like	Do not mimic the natural structure of the tissue or tumor mass	*In vivo* tissues and organs are in 3D form	Takai *et al*[102]
Proliferation	Tumor cells were grown in monolayer faster than in 3D spheroids	Similar to the situation *in vivo*	Lv *et al*[11]
Polarity	Partial polarization	More accurate depiction of cell polarization	Antoni *et al*[18]
Cell morphology	Sheet-like, flat, and stretched cells in monolayer	Form aggregate/spheroid structures	Breslin *et al*[103]
Stiffness	High stiffness (approximately 3 × 109 Pa)	Low stiffness (> 4000 Pa)	Krausz *et al*[104]
Cell-cell interaction	Limited cell-cell and cell-extracellular matrix interactions and no "niches"	*In vivo*-like, proper interactions of cell-cell and cell-extracellular matrix, environmental "niches" are created	Lv *et al*[11], Kang *et al*[105]
Gene/protein expression	Changes in gene expression, mRNA splicing, topology, and biochemistry of cells, often display differential gene/protein levels compared with *in vivo* models	Expression of genes and proteins *in vivo* is relevantly presented in 3D models	Bingel *et al*[92], Ravi *et al*[106]
Drug responses	Lack of correlation between 2D monolayer cell cultures and human tumors in drug testing.	Tumor cells in 3D culture showed drug resistance patterns similar to those observed in patients	Lv *et al*[11], Bingel *et al*[92]
The culture formation	From minutes to a few hours	From a few hours to a few days	Dai *et al*[33]
Quality of culture	High performance, reproducibility, long-term culture, easy to interpret, simplicity of culture	Worse performance and reproducibility, difficult to interpret, cultures are more difficult to carry out	Hickman *et al*[107]
Access to essential compounds	Unlimited access to oxygen, nutrients, metabolites, and signaling molecules (in contrast to *in vivo*)	Variable access to oxygen, nutrients, metabolites, and signaling molecules (similar to *in vivo*)	Pampaloni *et al*[108], Senkowski *et al*[30]
Cost during maintenance of a culture	Cheap, commercially available tests and media	More expensive, more time-consuming, fewer commercially available tests	Friedrich *et al*[35]

spheroids[37]. This technique is cost-effective and highly reproducible without requirement of any specific equipment[38]. Different cell types can be cocultured with this technique[39]. However, the number and size of formed spheroids are difficult to monitor[40]. Recently, ultra-low attachment plates have been developed and commercialized for the liquid overlay technique. Such plates contain individual wells with a layer of hydrophilic polymer on the surface to overcome the requirement for manual coating, which prevents cell attachment. The specifically designed plates exhibit the capability to produce one spheroid per well and is favorable enough for medium-throughput applications[41].

Hanging drop technique: The hanging drop technique for 3D spheroid production was introduced by Johannes Holtfreter in 1944 for cultivating embryonic stem cells. The technique later became the foundation of scaffold-free 3D culture models capable of multicellular spheroid generation. Resulting spheroids could be generated with consistent size and shape controlled by adjusting the density of cell seeding. As few as 50 cells up to 15000 cells could be varied to obtain the desirable size of spheroids[42]. In the very beginning, the hanging drop technique was carried out in the petri dish lid, by dropping a small volume of cell suspension (15-30 μL) with a specific cell density onto the lid. Then, the lid was subsequently inverted and aliquots of cell suspension turned into hanging drops without dripping due to surface tension. Consequently, cells were forced to accumulate at the bottom tip of the drop, at the liquid-air interface, and further aggregate and proliferate until spheroids were formed (Figure 2B). Recently, bioassay dishes have been used in place of petri dishes for more well-controlled experiments to facilitate the maintenance of moisture levels of the culture system, so that cell culture can be done in the same manner of standard cell culture procedures.

The hanging drop technique is relatively simple and applicable for numerous cell lines, and its reproducibility can be almost 100% for generating one 3D spheroid per drop[42]. The 3D spheroid obtained from this technique tends to be tightly packed

Table 2 Proposed advantages, disadvantages, and research stage of different three-dimensional cell culture methods

Techniques	Advantages	Disadvantages	Research stage
Liquid overlay cultures and Hanging drops	(1) Easy-to-use protocol; (2) No added materials; (3) Consistent spheroid formation; control over size Co-culture ability; (4) Transparent; (5) High reproducibility; (6) Inexpensive; (7) Easy to image/harvest samples	(1) No support or porosity; (2) Limited flexibility; (3) Limited spheroid size; (4) Heterogeneity of cell lineage; (5) Lack of matrix interaction	(1) Basic research; (2) Drug discovery; (3) Personalized medicine
Hydrogel	(1) Large variety of natural or synthetic materials; (2) Customizable; (3) Co-culture possible; (4) Inexpensive; (5) High reproducibility	(1) Gelling mechanism; (2) Gel-to-gel variation and structural changes over time; (3) Undefined constituents in natural gels; (4) May not be transparent	(1) Basic research; (2) Drug discovery
Bioreactors	(1) Simple to culture cells; (2) Large-scale production easily achievable; (3) Motion of culture assists nutrient transport; (4) Spheroids produced are easily accessible	(1) Specialized equipment required; (2) No control over cell number/size of spheroid; (3) Cells possibly exposed to shear force in spinner flasks (may be problematic for sensitive cells)	(1) Basic research; (2) Tissue engineering; (3) Cell expansion
Scaffolds	(1) Large variety of materials possible for desired properties; (2) Customizable; (3) Co-cultures possible; (4) Medium cost	(1) Possible scaffold-to-scaffold variation; (2) May not be transparent; (3) Cell removal may be difficult	(1) Basic research; (2) Drug screening; (3) Drug discovery; (4) Cell expansion
3D bioprinting	(1) Custom-made architecture; (2) Chemical, physical gradients; (3) High-throughput production; (4) Co-culture ability	(1) Require expensive 3D bioprinting machine; (2) Challenges with cells/materials	(1) Cancer pathology; (2) Anticancer drug screening; (3) Cancer treatment; (4) Tissue engineering

Modified from Breslin *et al*[64]; Fang *et al*[47]; Leong *et al*[109].

rather than aggregated loosely, and low variability in sizes were observed. Kelm *et al*[42] reported that 3D spheroids exhibited patho/physiologically relevance because their structures were highly organized along with their produced ECM and turned to be a 'tissue-like' structure. As this technique is based on the tendency of cells to aggregate to each other spontaneously instead of depending on the provided matrices or scaffolds, the problematic concerns regarding the effects from 3D structure formation are reduced. However, the undeniable drawback of the hanging drop technique is the limited volume of the cell suspension. Only up to 50 μL of suspension, including the testing medium, can be accommodated onto the upside down surface unless dripping occurs as the surface tension is not enough to keep liquids attached on the surface[43]. Another limitation is the difficulty in changing culture medium during cultivation without disturbing the spheroids[31].

Hydrogels: Hydrogels are the networks of cross-linked polymeric material, which are generally composed of hydrophilic polymers with high water content (Figure 2C)[44]. There are the swollen structures or microspheres integrated within the network for cell encapsulation and the circulation of nutrients and cellular waste in and out of the hydrogels[45]. Additionally, gels exhibit a soft tissue-like stiffness to potentially resemble natural ECM because they are made from mixtures of natural polymers such as collagen, and alginate, two of the most used substrates in 3D cell culture history[46].

The most common use of hydrogels is to be combined with a reconstituted basement membrane preparation extracted from mouse sarcoma, which has been commercialized by the Matrigel trademark (Corning Life Sciences, Tewksbury, MA, United States). Even though such commercialized hydrogels are rich in ECM proteins, they also possessed some drawbacks, including the deficiency in gelation kinetic control, the undefined and uncontrollable polymer composition, and lack of mechanical integrity. Lot-to-lot variability due to manufacturing mistakes and poorly defined composition also cause difficulty to determine the exact responses of cells to some particular stimuli[47].

Generally, hydrogels are fabricated based on both synthetic and natural polymers, which are water-absorbing, hydrophilic, and highly flexible materials. With the well-controlled fabrication processes and well-defined material composition, hydrogels have become the prominent materials for 3D scaffold development. Because of their structural similarities to natural ECM, they are favorable for *in vivo* chemical delivery in a noninvasive manner[44]. A number of synthetic and natural materials can be incorporated into hydrogel formation, such as hyaluronic acids, polyethylene

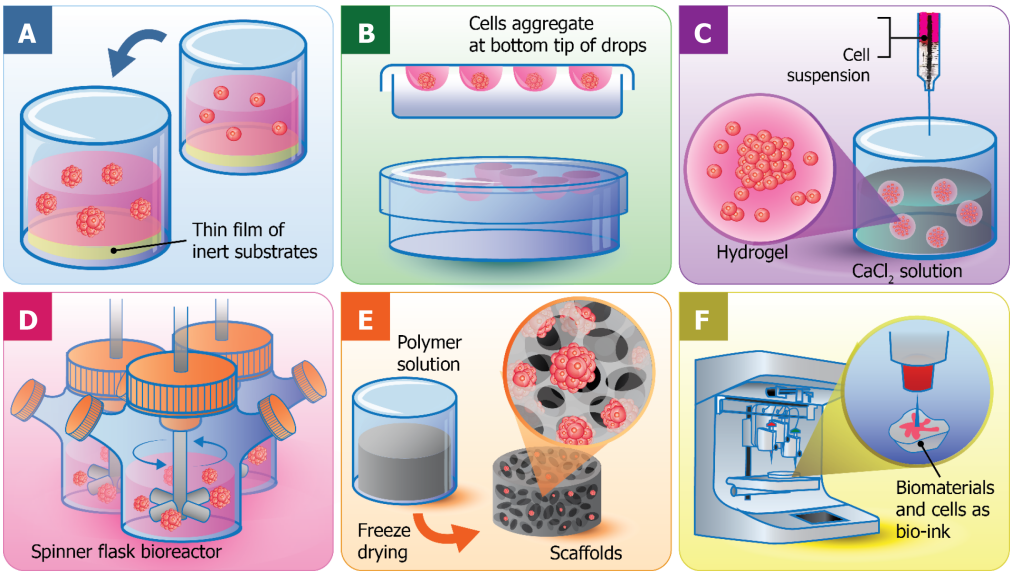

Figure 2 Different techniques used for three-dimensional cell cultures. These techniques include: A: Liquid overlay; B: Hanging drop; C: Hydrogel embedding; D: Spinner flask bioreactor; E: Scaffold; F: Three-dimensional bioprinting.

glycol[48], collagen, gelatin, fibrin, alginate, and agarose[49]. However, the natural hydrogels, like Matrigel and alginate gel, are considered to be more appropriate cell-encapsulated materials due to the great biocompatibility and mild gelling conditions.

The hydrogel technique for cell culture in a calcium alginate hydrogel was first developed by Lim *et al*[50] by mixing the cells with the alginate solution, then cross-linking and forming the hydrogel-based microspheres in an isotonic $CaCl_2$ solution (Figure 2C). The alginate hydrogels are very limited for cell adhesion, which is an advantage for cell encapsulation applications[51] that provide rapid, nontoxic, and versatile immobilization of cells within polymeric networks. In addition, the creation of artificial organs was also consolidated with encapsulating cells or tissue for the treatment of disease. The most well-known example was an artificial pancreas to be used in diabetes therapy[45].

The 3D cell culture can also be carried out in hydrogels and can be integrated with other cell culture models such as cell spheroid cultures, scaffold-based cell cultures, and microchip-based cell cultures[52]. Hydrogels are one potential technique to be used for 3D *in vitro* technology due to their biocompatibility, sufficient water content, and ECM-like mechanical properties[53]. Although hydrogels were not popularly applied to the field of drug screening, they have been widely used for the development of tissue engineering by mimicking cartilage, vascular, bone, and other tissues by mixing particular cells to hydrogel precursors before the gelling process in which cells are distributed evenly and homogeneously throughout the gels.

One reported case was the engineered cardiac tissues obtained from the neonatal rat cardiac myocyte culture in collagen hydrogels that were used for cyclic mechanical stretch research[54]. Hydrogels also facilitate the delivery of soluble or signaling molecules to cells and providing the supportive surroundings for cell growth and function. For example, transforming growth factor β was infused into polyethylene glycol hydrogels to govern the function of smooth muscle cells. In a similar manner, bone morphogenetic protein was covalently attached to alginate hydrogels to govern osteoblast migration and calcification[55]. Despite a variety of hydrogel type applications, Ca-alginate hydrogels are surely a potent candidate system for the delivery of cells to the infarcted heart because they are nontoxic, nonimmunogenic, do not facilitate pathogen transfer, and allow good exchange of waste products and nutrients[56,57]. Ca-alginate hydrogels were primarily implanted into the heart and shown not to induce harmful responses such as thrombosis[56] or fibrosis[58]. The gradual degradation, resulting from the dispersal of calcium crosslinks[59], generated nontoxic alginate polysaccharide degradation products, which can be excreted *via* urinary systems[60]. However, besides a number of advantages of hydrogels, the disadvantages of hydrogel are still present and should not be disregarded. The uncertainty and complexity in composition influenced by gelling mechanism may cause undesirable and nonspecific cellular responses. Additionally, pH based gelling mechanisms can negatively affect sensitive cells[52].

Bioreactors: Because the impact of 3D cell culture models as an appropriate *in vitro*

laboratory platform for the discovery of therapeutics and anticancer agents have been concerned and drawn the attention of scientists, the crucial following step to cope with the increasing demand is the upscale 3D culture system from the laboratory to the industrial level. Bioreactors became the solution for great spheroid formation with a precise control system and guaranteed reproducibility[61]. With specifically designed 3D culture approaches, bioreactors have been adapted in many ways. For example, scaffolds have been added to the large cell culture chambers for high volume cell production.

Normally, a bioreactor for 3D spheroid production can be loosely classified into four categories: (1) Spinner flask bioreactors (Figure 2D); (2) Rotational culture systems; (3) Perfusion bioreactors; and (4) Mechanical force systems[18,62]. The general principle behind the bioreactor-based 3D culture systems is that a cell suspension with the optimal cell density is filled into the chamber with continuous agitation, either by gently stirring, rotating the chamber, or perfusing culture media through a scaffold using a pump system. Bioreactors are equipped with media flowing systems to provide the nutrient circulation, metabolic waste expulsion, and homogeneity of the physical and chemical factors within the bioreactors. Therefore, bioreactor-based cell culture models are appropriate for intensive cell expansion and large-scale biomolecule production, such as antibodies or growth factors.

Although bioreactors are labor-intensive and capable of producing a large number of spheroids[63], the produced spheroids are still distributed heterogeneously in size and number of cell population[31]. Therefore, a manual spheroid selection is required for later replating onto a dish, if the spheroid size needs to be controlled[64]. Even though spheroid generation *via* bioreactors requires expensive instruments[65] and high quality/quantity of culture medium, the bioreactors can still provide greater advantages at the industrial level over other techniques[66].

Scaffolds: The 3D scaffolds are described as the synthetic 3D structures that are constructed from a wide-range of materials and possess different porosities, permeability, surface chemistries, and mechanical characteristics. They are mainly designed to mimic the *in vivo* ECM of the specific tissues for each particular cell type. The 3D scaffold-based cell culture models have been applied to drug screening[11], drug discovery[47], and investigation of cell behaviors[47]. The 3D scaffolds are meant to be porous, biocompatible, and biodegradable, which provides appropriate microenvironments where cells naturally reside, supporting mechanical, physical, and biochemical requirements for cell growth and function[28]. Several biopolymers are used to generate porous scaffolds, which include collagen[11], gelatin[67], silk[68], chitosan[28], and alginate[28,69]. As such, various techniques have been used for the fabrication of scaffolds, such as gas foaming, freeze-drying, phase separation, solvent casting, and particulate leaching. Each technique results in different porosities, pore sizes and shapes, scaffold materials, and features. Among them, freeze-drying is considered the easiest technique to fabricate porous scaffolds[70].

Sequentially, natural or synthetic materials are polymerized, frozen, and freeze-dried. The frozen water embedded in the polymers is sublimated directly without going through the liquid phase resulting in a porous structure formation[71]. The freeze-drying technique for the fabrication of porous biodegradable scaffolds from polylactic and polyglycolic copolymer was first developed by Whang *et al*[72]. With such technique, the porosity and pore dimension of the scaffolds are varied depending on the various parameters such as the ratio of water and polymers and also the viscosity of polymer solution[73]. The porous alginate-based scaffolds can also be easily manufactured by a simple freeze-drying process (Figure 2E). However, it is difficult to generate pores with uniform diameter but can partially be controlled by varying the freezing temperature[74]. Another advantage of this technique is that no rinsing steps are required because dispersed water and polymer solution are removed directly *via* sublimation[72]. Additionally, the biodegradation rates of scaffolds are strongly dependent on polymer components and molecular weight[75].

To date, Ca-alginate copolymer is one of the most prominent materials for freeze-dried scaffolds. Several studies have used 3D Ca-alginate scaffolds as a cell culture platform for screening and efficacy testing of anticancer drugs and tissue engineering. 3D Ca-alginate scaffolds were proposed to allow more realistic cell phenomena, similar to those occurring *in vivo* during cancer formation and progression. Chen *et al*[69] developed a 3D porous Ca-alginate scaffold cell culture system combined with the functionally-closed process bioreactor to form bone-like tissue within the closely mimicked *in vivo* environments. The Ca-alginate scaffolds were reported to support the growth and differentiation of human bone cell clusters, along with the upregulation of bone-related gene expression. Florczyk *et al*[28] developed chitosan-alginate scaffolds using the freeze-drying technique to study cancer stem cells transient behavior *in vitro*. They found that 3D scaffold-based cultures of prostate,

liver, and breast cancer cells exhibited reduced proliferation and tumor spheroid formation and increased expression of cancer stem-like cell associated mark genes (CD133 and NANOG) compared to 2D cell culture. Chitosan-alginate scaffolds were also observed to allow the efficient seeding of human umbilical cord mesenchymal stem cells, promoting the inhabitability of cells throughout the whole volume of the scaffold, which reflected good adhesion and proliferation[76].

3D bioprinting: 3D printing technique is a recently developed technology that, in general, is referred to as the construction of customized 3D structures under computational control in which materials are printed out, solidified, and connected together[51]. 3D printing takes part in a wide-range application, including prototypic and industrial manufacturing, architecture, 3D art and design, and importantly, tissue engineering and regenerative medicine[77]. The 3D tissue printing that the biological constructs composed of cells and biomaterials are printed in a small dimension, ranging from several millimeters to a centimeter. The term is so called because the biocompatible materials, cells and supporting components are used to form a variety of 3D formats instead of any synthetic materials. Therefore, cell function and viability can be sustained within the printed constructs (Figure 2F)[77]. Various 3D bioprinting platforms can already generate vascular-like tubes[78], kidney[77], cartilage[79], artificial skin[80], and a wide range of stem cells including tissue constructs[81]. 3D bioprinting is needed to precisely deposit cells, biomaterials, and biomolecules layer-by layer by computer-aided equipment and software, which has been possibly constructed by integration of modern science and technology knowledge, including cell biology, engineering, material science, and computer science[82].

By using alginate as the main biomaterial in a bio-ink, Zhao *et al*[83] studied the pathogenesis of cervical cancer using the developed cervical tumor model. Alginate, together with gelatin and fibrinogen, was mixed with HeLa cells to initiate gelation prior to printing and resemble the ECM components. The printed constructs were later strengthened by the addition of a calcium chloride solution. Printed HeLa cells subsequently formed spheroids that exhibited more resistance to paclitaxel than 2D monolayer HeLa cells. Correspondingly, Dai *et al*[33] generated 3D bioprinted constructs of glioma stem cells using modified gelatin/alginate/fibrinogen biomaterials printed glioma stem cells. They could survive, proliferate, maintain the inherent characteristics of cancer stem cells, and exhibit differentiation and vascularization potential. In addition, their resistance against temozolomide were higher than those in the 2D cell culture model.

Besides the ability to generate geometric constructs containing viable cells, the 3D bioprinting technique also facilitated high throughput applications with precise reproducibility[84]. However, the main concerns are the requirement of the expensive 3D bioprinting machine and the negative effects on sensitive cells during the printing process. Cells could possibly be damaged due to osmotic, thermal, and mechanical stresses.

APPLICATION OF 3D CELL CULTURE

Recently, the 3D culture models were developed in a specific way to suit each particular cell type rather than to be versatile of different physiological requirements. Despite the great number of reported 3D culture-based studies, they have not been optimized or validated for realistic applications. Advances have been made for cancer and stem cell modeling so far, and prominent studies applied with 3D cell culture systems are summarized in Table 3.

Cancer modeling

Cancer epithelial cells cultured in 3D culture systems were reported to be altered in shape and lose their polarity. Such features are ordinarily found in cancer progression in an *in vivo* environment[22]. Cell proliferation, gene/protein expression, and drug sensitivity of 3D cancer cell models are also more illustrative of *in vivo* cancer cells compared to those cultured as a monolayer[32]. Therefore, to obtain more relevant data, several studies have used 3D cell culture systems as a cancer model.

Peela *et al*[85] revealed novel genetic dependencies linked with breast cancer progression in 3D MCF10 human mammary gland cells. It was found that the alteration in both genetic information and the pattern of gene expression can be disclosed when cells were grown in 3D conditions similar to those *in vivo*. This induced MCF10 progression model therefore represented a suitable system to dissect the potential biomarkers as well as to evaluate therapeutic targets involved in human breast cancer progression.

Zhu *et al*[86] employed the stereolithographic 3D bioprinter with a newly developed

Table 3 Examples of three-dimensional research systems utilized for cancer and stem cell cancer studies

Application/platform	Cells type	3D model	Culture systems/matrix	Results	Ref.
Drug-screening	Breast cancer cells (BT-549, BT-474 and T-47D)	Comparison of 2D- and 3D-culture models as drug-testing platforms in breast cancer	Spheroid formation in 3D-culture plates	Three breast cancer cell lines developed dense multicellular spheroids in 3D-culture and showed greater resistance to paclitaxel and doxorubicin compared to the 2D-cultured cells	Imamura *et al*[89]
Metastasis studies and assessing drug sensitivity	Breast cancer cells (MDA-MB-231 and MCF-7)	Breast cancer bone metastasis	3D bioprinting hydrogel	Breast cancer cells exhibited spheroid morphology and migratory characteristics, then co-culture of breast tumor cells with bone marrow MSCs increased the formation of spheroid clusters	Zhu *et al*[86]
Cancer cell behavior	Breast cancer cells (MCF10)	Breast cancer progression	3D spheroid cultures used U-bottom ultra-low attachment plates	Genetic dependencies can be uncovered when cells are grown in 3D conditions similar to *in vivo*	Peela *et al*[85]
Drug-screening	Human colon cancer cells (HCT116)	Compared gene expression in 2D and 3D systems and identification of context-dependent drug responses	3D spheroid cultures used lowattachment plate (Corning, Amsterdam, The Netherlands)	3D spheroids increased expression of genes involved in response to hypoxia and decreased expression of genes involved in cell-cycle progression when compared with monolayer profiles	Senkowski *et al*[30]
GBM biology, anti-GBM drug screening	Human glioblastoma cells (U87)	Compared gene expression in 2D and 3D systems	3D PLA porous scaffolds	GBM cells in 3D PLA culture expressed, 8117 and 3060 genes were upregulated and downregulated, respectively, compared to 2D cell culture conditions. Further, KEGG pathway analysis showed the upregulated genes were mainly enriched in PPAR and PI3K-Akt signaling pathways while the downregulated genes were enriched mainly in metabolism, ECM, and TGF-β pathways	Ma *et al*[87]
Cancer and tumor cell biology	Human glioblastoma (U-251)	Compared gene and protein expression in 2D and 3D systems	ESPS scaffolds coated with laminin	The results suggested the influence of 3D context on integrin expression upregulation of the laminin-binding integrins alpha 6 and beta 4	Ma *et al*[91]
Cancer and tumor cell biology	Human glioblastoma (U-251) cells	Compared drug-sensitivity in 2D and 3D systems	3D bioprinting of gelatin/alginate/fibrinogen hydrogel	3D bioprinted glioma stem cells were more resistant to temozolomide than 2D monolayer model at temozolomide concentrations of 400-1600 μg/mL	Dai *et al*[33]

Cancer and tumor cell biology	Human glioblastoma (U-251)	Anti-cancer drug screening	3D collagen scaffold	Glioma cells in 3D collagen scaffold culture enhanced resistance to chemotherapeutic alkylating agents with a much higher proportion of glioma stem cells and upregulation of MGMT	Lv *et al*[11]
Cancer and tumor cell biology, development of new therapies and detection of cardiotoxicity	iPSC-derived human cardiomyocytes	Cardiac microtissues	Hanging drops	A 3D culture using iPSC-derived human CMs provided an organoid human-based cellular platform that recapitulated vital cardiac functionality	Beauchamp *et al*[94]
Tissue engineering and toxicity assessment	Human hepatoblastoma (HepG2/C3A)	A liver-on-a-chip platform for long-term culture of 3D human HepG2/C3A spheroids for drug toxicity assessment	Bioprinting of hepatic constructs containing 3D hepatic spheroids	Hepatic construct by 3D bioprinting were functional during the 30 d culture period and responded to acetaminophen that induced a toxic	Bhise *et al*[98]
Brain diseases	Human embryonic stem cells (HUES66), C57	3D neural tissues for use as tractable models of brain diseases	3D hydrogels	3D cocultures of neuronal and astrocytic cells can change expression patterns so that they correlate with specific brain regions and developmental stages	Tekin *et al*[101]
Cancer and tumor cell biology, drug screening	Human neuroblastoma cell lines BE(2)-C (ECACC), IMR-32 (DSMZ)	Compared gene expression profiles in 2D and 3D systems and tumor tissue	Polymeric scaffolds and bioreactor systems	The autophagy-controlling transcription factors, such as TFEB and FOXO3, are upregulated in tumors, and 3D-grown cells have increased expression compared with cells grown in 2D conditions	Bingel *et al*[92]
Cancer and tumor cell biology, neurodegenerative diseases	DPSCs	Differentiation to retinal ganglion-like cells	3D fibrin hydrogel	3D network can mimic the natural environment of retinal cells	Roozafzoon *et al*[95]
Cardiovascular disease	hiPSCs	Cardiomyocytes and endothelial cells, co-differentiated from human pluripotent stem cells	V-bottom 96 well microplates	Human cardiac microtissues were generated in complex 3D structures and differentiation of human pluripotent stem cells into cardiomyocytes and endothelial cells that expressed cardiac markers also present in primary cardiac microvasculature	Giacomelli *et al*[110]
Bioartificial liver support devices, drug screening and	hiPSCs	Differentiation of hiPSCs into hepatocytes	Nanofiber hydrogel 3D scaffold	3D hydrogel culture conditions promote the differentiation of hiPSCs into hepatocytes	Luo *et al*[100]
Ovarian cancer biology, drug sensitivity	Ovarian cancer cell lines (A2780 and OVCAR3)	Compared drug-sensitivity in 2D and 3D systems	Hanging drop	3D tumor spheroids demonstrated greater resistance to cisplatin chemotherapy compared to 2D cultures	Raghavan *et al*[90]
Pathogenesis of prostate cancer, prostate cancer therapy	Prostate cancer cell lines (PC3 and LNCaP)	Simulation of prostate cancer bone metastases	Collagen-based scaffolds	The two cell lines in 3D present increased resistance to docetaxel	Fitzgerald *et al*[111]
Radiosensitivity of cancer cells	Human lung adenocarcinoma cell line (A549)	The metabolic response of lung cancer cells to ionizing radiation	Hydrogels	3D model can help regulate the exposure of oxygen to subpopulations of cells in a tissue-like construct either before or after irradiation	Simon *et al*[112]

Regenerative medicine, drug screening, and potentially disease modeling	HUVECs	Endothelial myocardium construction	3D bioprinting	This technique could be translated to human cardiomyocytes derived from iPSCs to construct endothelial human myocardium	Zhang *et al*[99]
Cancer cell biology, studying and developing therapies against cancer stem cells	Hepatocellular carcinoma (SK-Hep-1), prostate cancer (TRAMP-C2) and breast cancer (MDA-MB-231)	Compared cancer morphogenesis and gene expression in 2D and 3D systems	CA scaffolds	The three cell lines in 3D porous CA scaffolds promote cancer stem-like cell enrichment and increased expression of cancer stem cells genes (CD133 and NANOG)	Florczyk *et al*[28]

2D: Two-dimensional; 3D: Three-dimensional; DPSCs: Dental pulp stem cells; iPSC: Induced pluripotent stem cells; hiPSC: Human induced pluripotent stem cells; HUVECs: Human umbilical vein endothelial cells; CA: Chitosan-alginate; MSC: Mesenchymal stem cell; PLA: Polylactic acid; ESPS: Electrospun polystyrene; TGF-β: Transforming growth factor β; KEGG: Kyoto Encyclopedia of Genes and Genomes; GBM: Glioblastoma multiforme; ECM: Extracellular matrix; CM: Cardiomyocyte.

nano-ink to construct hydrogel-based culture systems infused with hydroxyapatite nanoparticles. This system provided a bone-specific environment for assessing the invasive properties of breast cancer to bone. The breast cancer cultured in the 3D culture system developed spheroid characteristics with a high migratory ability especially when they were cocultured with bone marrow mesenchymal stem cells. The breast cancer cell spheroids also exhibited higher anticancer drug resistance compared with the 2D culture cells. The evidence suggested that the 3D bone matrix mimicked tumor/bone microenvironments serving as a tool for exploring cancer metastasis and assessing anticancer drug sensitivity.

In another report, Senkowski *et al*[30] demonstrated gene expression profiling of 3D multicellular tumor spheroids compared with the 2D monolayer cells. The alteration of gene expression was found to be the upregulation of genes involved in response to hypoxia, and the downregulation of genes involved in cell cycle progression. Further, the mevalonate pathway was upregulated in quiescent cells of the 3D spheroids during oxidative phosphorylation inhibition, which were correlated with the viable deficiency of quiescent spheroids when they were treated with oxidative phosphorylation inhibitors and mevalonate pathway inhibitors. This suggested the context dependence of anticancer drug responses of the 3D tumor spheroids.

Recently, the genome of 3D glioblastoma multiforme (GBM) cells in polylactic acid porous scaffolds were compared to the genome of GBM cells in 2D cell culture conditions. It was found that the 14-d 3D GBM cells upregulated 8117 genes and downregulated 3060 genes compared to the 2D cell culture conditions[87]. The Kyoto Encyclopedia of Genes and Genomes pathway analysis showed that genes involved in PPAR and PI3K-Akt signaling pathways were mainly upregulated, while genes involved in metabolism, ECM receptors, and transforming growth factor β pathway were downregulated. The results acquired from the 3D tumors *in vitro* would be meaningful information for better understanding of both intrinsic and extrinsic features of GBM. Such a 3D tumor model has the potential to serve as a platform for anti-GBM drug screening.

The discovery of anticancer drugs often begins with the lack of suitable medical products for a particular clinical condition[4]. To date, the 3D cancer models have gained recognition in the explication of tumor biology because the conventional 2D cell models are inadequate to solve the unanswered questions. Some of the aforementioned issues, such as indolent cancer pathology, the invasive colonization, and the recurrent and rapid evolution of anticancer drug resistance, were exhibited by 3D cell systems[88].

For example, Imamura *et al*[89] compared the anticancer drug sensitivity between 2D and 3D cells and found that the 3D cancer spheroids contained greater resistance to paclitaxel and doxorubicin than that of the 2D cultured cells. The 3D ovarian cancer spheroids forming by hanging drop technique were 2-fold more resistant to cisplatin compared to the 2D cultures[90]. The ovarian cancer spheroids were uniform in geometry and contained over 85% of cell viability.

The influences of 3D structures and ECM on glioma stemness were examined by Ma *et al*[91]. U251 human glioblastoma cells increased expression of stemness markers (integrin) when cultured on electrospun polystyrene scaffolds coated with laminin. In another study, the 3D tumor cells stimulated autophagic flux and chemotherapy resistance. The key features of cancer, including cell proliferation, cell death, and macroautophagy were modulated by either 3D static or 3D bioreactor systems. The autophagy controlling transcription factors (TFEB and FOXO3) were upregulated in

the 3D tumor spheroids. Altogether, the 3D culture models were a beneficial system to study anticancer drug responses of cancer cells, as these models closely mimic patho/physiology of tumor[92].

Stem cell modeling

Stem cells, particularly pluripotent stem cells (PSCs), contain tremendous potential for generating pure populations of any cell type in the human body and shed light on regenerative medicine. Pure populations of tissue-specific progenitors or terminally differentiated cells could be integrated into healthcare innovations, in particular drug discovery, cell therapy, and tissue regeneration. Major advances have been accomplished in the stem cell arena using 3D cell platforms that recapitulated the development and regulation of cellular signaling in organisms[93]. The development of induced PSC (iPSC)-derived human cardiomyocytes (CMs) by 3D CM spheroids was successfully demonstrated by Beauchamp *et al*[94]. After 4 d of the culture, the iPSC-derived CMs developed cardiac microtissues presenting a uniform structure of mature myofibrils without a necrotic core. Retinal ganglion cells differentiated into incisor dental pulp stem cells when cultured in the 3D scaffolds. The 3D network of biocompatible fibrin hydrogel could resemble the properties of *in vivo* microenvironment for efficient development of retinal ganglion cells, which could be used to tackle neurological disorders, for instance glaucoma[95].

The progress in tissue engineering, cell therapy, and materials research has led to 3D bioprinting, which could generate functional cells, tissues, or organs for transplantation with close similarity to their target graft sites. Nevertheless, the printing of intact tissues or organs still persists as a challenge. The 3D bioprinting of tracheal, bladder, bone, and cartilage was demonstrated to function well *in vivo*[96]. These printed organs can be translated into clinical uses. For instance, Atala *et al*[97] bioengineered a human bladder from autologous urothelial cells and muscle cells prior to culturing the cells *in vitro* onto a biodegradable bladder-shaped scaffolds. After 7 wk of the 3D culture, the artificial bladders were applied for reconstruction and transplantation.

The 3D bioprinting technology was modified for the construction of the liver-like microstructure, exploiting 3D bioprinting of hepatoma cells and gelatin methacryloyl hydrogel[98]. The engineered hepatic constructs were still functional after 30 d as assessed by the production and release of albumin, alpha-1 antitrypsin, transferrin, and ceruloplasmin. Immunostaining of the hepatocyte markers was also performed in order to validate the liver functions, including cytokeratin 18, MRP2, and ZO-1. The treatment of acetaminophen instigated an adverse response in the engineered hepatic-like structure, providing a proof-of-concept of using this artificial liver for toxicity assessment.

The bioprinting strategy was used to print human umbilical vein endothelial cells-laden bio-ink (mainly alginate and gelatin) to fabricate a multi-layered microfibrous construct[99]. The bioprinted human umbilical vein endothelial cells translocated to the periphery and formed a layer of endothelial cells. This 3D endothelial structure was cocultured with human iPSC-derived CMs, fabricating the well-aligned myocardium that could contract in a spontaneous and synchronous manner. These 3D myocardial organoids were then processed into microfluidic perfusion bioreactors in order to develop an endothelial-myocardium chip that was used for the assessment of cardiovascular toxicity. This highlighted the progress of human stem cell technology for cardiovascular disease modeling and testing of relevant drugs.

Another example of 3D culture and stem cell differentiation was presented in a 3D hydrogel, which could promote the differentiation of human iPSCs into functional hepatocytes. The 3D conditions for hepatic differentiation of human iPSCs induced the expression of liver markers, hepatocyte maturation, and metabolic levels. The derivation of hepatocyte-like cells from human iPSCs provided a foundation for an artificial human liver, toxicity screening, and hepatocyte transplantation[100]. Hydrogel encapsulation could generate the 3D neural tissues by coculturing neuronal and astrocytic cells[101]. The transcriptomic profiles proposed that hydrogels could tune the expression patterns of the 3D brain organoids, correlating with those of specific brain regions and developmental stages.

CONCLUSIONS

The 3D cell culture systems are increasingly important in tumor and stem cell biology research. Because of the intrinsic discrepancies in complexity and functionality of tissues and organs, the selection of the 3D cellular model depends on the application, ranging from the simple cell spheroids to the complex 3D bioprinting structures.

Extensive choices of 3D cell culture technologies have been invented in order to fulfill the demand of the pharmaceutical industry. The 3D cell systems hold a great promise for drug discovery, disease simulation, cancer-targeted therapy, and a novel source of tissue replacement materials. The future of 3D cell systems should validate the preclinical outcomes, leading to the replacement of lab animal experimentation. The functional, safe, and transplantable index of the 3D cell cultures will need intensive investigation in order to bring it to clinical use (Figure 3).

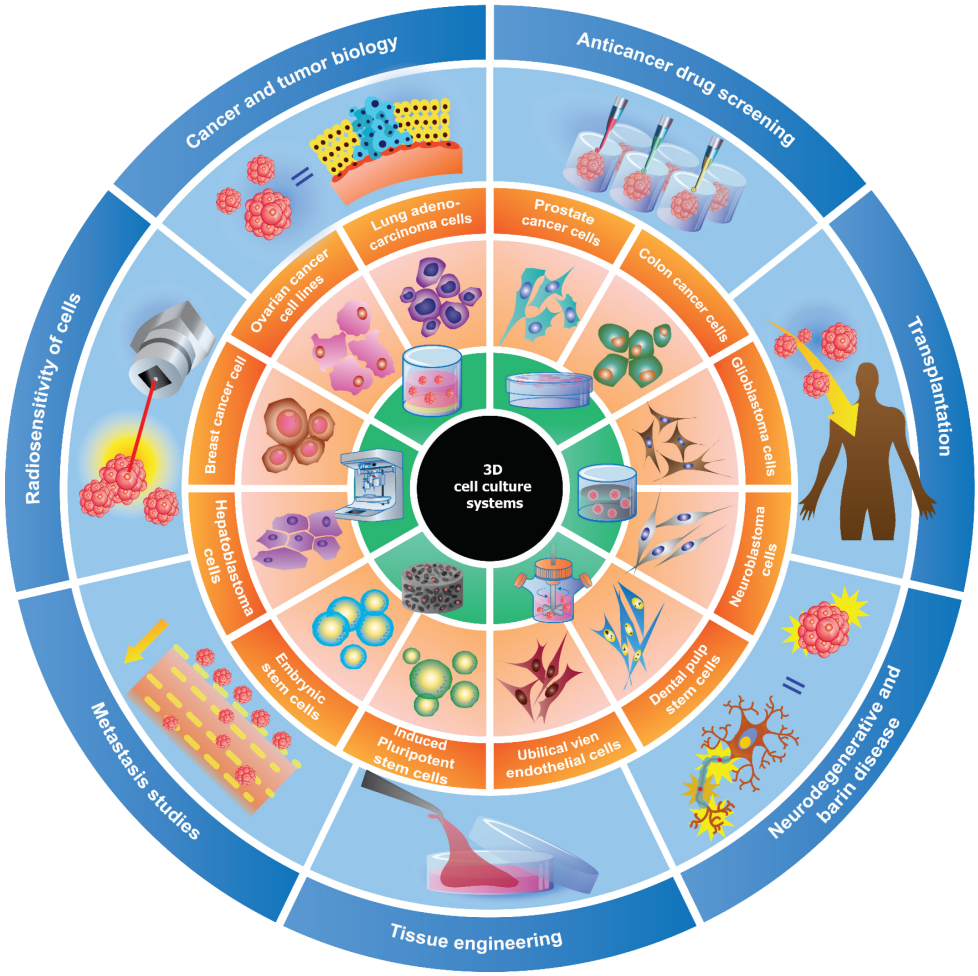

Figure 3 Potential applications of three-dimensional cell culture systems. The invention of three-dimensional cell culture systems could be applied into various aspects, for instance anticancer drug screening, tissue engineering, cancer biology, and clinical uses.

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REVIEW

Small molecules for mesenchymal stem cell fate determination

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Abstract

Mesenchymal stem cells (MSCs) are adult stem cells harboring self-renewal and multilineage differentiation potential that are capable of differentiating into osteoblasts, adipocytes, or chondrocytes *in vitro*, and regulating the bone marrow microenvironment and adipose tissue remodeling *in vivo*. The process of fate determination is initiated by signaling molecules that drive MSCs into a specific lineage. Impairment of MSC fate determination leads to different bone and adipose tissue-related diseases, including aging, osteoporosis, and insulin resistance. Much progress has been made in recent years in discovering small molecules and their underlying mechanisms control the cell fate of MSCs both *in vitro* and *in vivo*. In this review, we summarize recent findings in applying small molecules to the trilineage commitment of MSCs, for instance, genistein, medicarpin, and icariin for the osteogenic cell fate commitment; isorhamnetin, risedronate, and arctigenin for pro-adipogenesis; and atractylenolides and dihydroartemisinin for chondrogenic fate determination. We highlight the underlying mechanisms, including direct regulation, epigenetic modification, and post-translational modification of signaling molecules in the AMPK, MAPK, Notch, PI3K/AKT, Hedgehog signaling pathways *etc*. and discuss the small molecules that are currently being studied in clinical trials. The target-based manipulation of lineage-specific commitment by small molecules offers substantial insights into bone marrow microenvironment regulation, adipose tissue homeostasis, and therapeutic strategies for MSC-related diseases.

Key words: Mesenchymal stem cell; Mesenchymal stromal cell; Cell fate determination; Small molecules; Natural compounds; Signaling pathways

Core tip: Mesenchymal stem cells (MSCs), also called MSCs, are adult stem cells with multilineage differentiation potential. They serve crucial physiological roles, regulating

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the bone marrow microenvironment and adipose tissue remodeling *in vivo*. A complex regulatory network and signaling pathways are involved in governing MSC fate commitment. Much progress has been made in recent years in discovering small molecules and their underlying mechanisms that control the cell fate of MSCs. In this review, we summarize recent findings in applying small molecules to the trilineage cell fate commitment of MSCs, highlighting the underlying mechanisms and the current clinical trials. The small molecules for MSC fate determination offer substantial insights into bone marrow and adipose tissue homeostasis and therapeutic strategies for MSC-related diseases.

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INTRODUCTION

Mesenchymal stem cells (MSCs) are a rare cell population originally identified in the bone marrow stroma[1]. In addition to bone marrow, MSCs have been isolated from a multitude of adult tissues, such as adipose tissue[2], synovial membrane[3], and umbilical cord blood[4]. MSCs exhibit distinctive stem cell properties of self-renewal and multipotency defined by the International Society for Cellular Therapy as the competence of differentiation into three mesodermal lineage cells, which are the osteocytes, the adipocytes and the chondrocytes *in vitro*[5-7]. Beyond well-known trilineage differentiation potential, MSCs have also been reported to be capable of differentiating into other cell types[8], including endothelial cells[9,10], hepatocytes[11-13] and neurons [14,15].

The physiological role of MSCs has been widely investigated in both bone marrow and adipose tissue. The bone marrow MSCs (BM-MSCs) lie in the perivascular region[16,17], and replenish osteoblasts and adipocytes that govern early hematopoiesis with opposing effects[18,19]. An imbalanced ratio of adipocytes and osteoblasts in the bone marrow is found in several pathological conditions, such as aging and osteoporosis, which is the most common bone disorder and presents an increased ratio of marrow fat content[20]. The physiological role of adipose tissue-derived MSCs (AD-MSCs), which are isolated from the stromal vascular fraction of adipose tissue, has also been widely explored[21]. AD-MSCs are the cardinal regulators that govern adipogenesis in adipose tissue and play a critical role in metabolic homeostasis[22]. Impairment of AD-MSCs affects adipose tissue remodeling and expansion, which leads to obesity-associated insulin resistance and increases the risk of cardiovascular diseases[23,24]. Understanding the mechanisms and conceiving a better regimen to control MSC fate *in vitro* and *in vivo* will not only advance the translation of stem-cell-based treatment approaches into clinical treatment but also facilitate the development of novel therapeutic strategies for shaping the bone marrow microenvironment, adipose tissue remodeling and managing MSC-related bone and metabolic diseases.

The fate determination of MSCs is controlled by several intrinsic factors, such as regulation of signaling pathways[20], activation of lineage-specific transcription factors[25-27] and epigenetic modification[28-30], which can be governed by diverse extrinsic factors. The extrinsic factors, including mechanical induction[31,32], growth factors, and small molecules, deliver signal cues and activate downstream signaling pathways to guide the fate commitment of MSCs. Small molecules are one of the earliest approaches that researchers used to modulate cell fate and function of MSCs[33]. Small molecules not only have distinct manipulative features - fast and reversible, providing precise control in compared with genetic or epigenetic strategies[34] but also have discrete functional groups that are modifiable for future large-scale screening and biopharmaceutical application.

Investigations into the effect of small molecules on the fate determination of MSCs will undoubtedly offer insights into bone marrow microenvironment regulation and therapeutic strategies for pathological conditions such as obesity, osteoporosis, and aging. Recent studies based on traditional treatment strategies or large-scale screening allow the identification of many candidates that regulate the cell fate of MSCs. In this article, we review the small molecules that modulate the fate determination of MSCs through the PubMed literature searches (last search conducted on March 23, 2019),

and summarize their underlying mechanisms (Table 1).

SMALL MOLECULES REGULATE MSCS OSTEOGENIC CELL FATE

Osteogenic differentiation of MSCs is commonly induced by the small molecule supplements of ascorbic acid, β-glycerophosphate, and dexamethasone, resulting in an increase in alkaline phosphatase (ALP) activity and calcium deposition[7,35]. Ascorbic acid and β-glycerophosphate increase type I collagen secretion and stimulate the formation of hydroxyapatite-rich mineralized matrix[7]. Dexamethasone induces the expression of osteogenic-associated genes, including runt-related transcription factor 2 (Runx2), Osterix, and bone matrix proteins. Runx2 is the master regulator of osteoblastic commitment. The Runx2 expression is controlled by many signaling pathways; among them, the BMP, Wnt, and Notch signaling pathways are the major cascades that promote both Runx2 expression and osteogenesis[36]. In this section, we will review the small molecules that affect the osteogenic commitment of MSCs based on their mechanisms (Figure 1).

BMP signaling pathway

BMPs are growth factors belonging to the transforming growth factor beta (TGF-β) superfamily. Upon ligand binding, the BMP receptors form an activated quaternary complex, which subsequently phosphorylates and activates intracellular Smad proteins and downstream cascades. The detailed mechanisms of BMP-mediated osteogenesis are not well-characterized; however, BMP triggers MSCs to express downstream osteogenic genes, such as ALP and type I collagen (Col I)[37]. Many small molecules have been identified to exert their osteogenic effect on MSCs by interfering with the BMP signaling pathway. Some examples include genistein, *Solanum muricatum* extract[38], *Herba epimedii* extract[39], malvidin[40], T63 and osthole[41-43].

Genistein is a phytoestrogen enriched in soybean products. Dai *et al*[44] showed that genistein promoted osteogenic differentiation of human BM-MSCs through BMP-dependent SMAD signaling. A concentration ranging from 0.1 to 10 μmol/L Genistein was tested, and the osteogenic stimulations were statistically significant at 0.1 and 1 μmol/L with the highest ALP activity at 1 μmol/L. The gene expression profile showed that osteogenic genes, such as Runx2 and osteocalcin, were highly expressed in genistein-treated cells compared with untreated cells. In addition, the BMP signaling pathway related mediators were upregulated, while BMP signaling pathway inhibitors such as SMAD6 and 7 were downregulated[44]. Soybean products have been reported to prevent bone loss in ovariectomy-induced (OVX) osteoporotic mice in the 1990s, the Dai *et al*[45] study provided a possible explanation for the underlying mechanism. A clinical trial was carried out to assess the effect of genistein on osteopenic postmenopausal women. The results demonstrated an increase in bone mineral density at both the anteroposterior lumbar spine and the femoral neck[46]. However, the study didn't calculate the fracture rate, so more concrete evidence is needed to evaluate the osteoprotective effect of genistein.

Zhao *et al*[47] identified a small molecule, named as T63, by high-throughput screening with the Runx2-responsive 3T3 luciferase cell line. Through ALP activity validation, treatment of T63 showed the most significant increase compared with other screening molecules. The addition of T63 to the osteogenic induction media during C3H10T1/2 cell differentiation showed an upregulation of osteogenic genes, including Runx2, Bglap, and Spp1. When T63 was added to the adipogenic induction medium, the adipogenic markers, including Pparγ2, Srebf1, and Fabp4, were significantly suppressed. Treatment with T63 upregulated the expression levels of the Bmp2, Bmp4 and Bmp7 genes in the BMP signaling pathway and increased the phosphorylation of the BMP downstream mediators Smad1/5/8 in a dose-dependent manner. Meanwhile, the phosphorylation of GSK-3β, an upstream regulator of β-catenin in the Wnt signaling pathway, also increased, indicating that T63 was also involved in the regulation of the Wnt signaling pathway. The addition of the BMP signaling pathway inhibitor Noggin or the Wnt signaling pathway inhibitor DKK-1 reduced the osteogenic effect of T63, confirming that T63 exerted an osteoinductive effect *via* the BMP and Wnt pathways. In the OVX mouse model, after three mo of a dose of 5 mg/kg or 20 mg/kg T63 increased both the bone mineral density and the bone mineral content in femurs and lumbar vertebrae, suggesting that T63 promoted bone formation *in vivo*.

Wnt signaling pathway

The Wnt signaling pathway has been shown to play a critical role in bone formation

Table 1 Selected Small molecules in controlling mesenchymal stem cells fate

Small molecules	Origin/Natural source	Effect	Pathway/Targets	Experimental model and dose	Comments	Ref.
5-azacytidine	Synthetic	Anti-adipogenic	Inhibit DNA methyltransferase	ST2 cells (0.5 μmol/L)	Reduce expression level of PPARγ, aP2, FAS and C/EBPα	[13,8]
8-HUDE	Synthetic	Anti-adipogenic	Activate HO-1 pathway	Human BM-MSCs (1 μmol/L)	Reduce expression level of Fas, Pparγ, and Cebpα	[132]
Albiflorin	Paeonia lactiflora	Beige-Adipoinductive	Activate AMPK, PI3K/AKT/mTOR pathways	Human AD-MSCs (5, 10, 20 μmol/L)/ HFD mice (5 mg/kg per day, in an unknown solvent, orally)	Cell viability decrease when AF exceeds 20 μmol/L. Induce expression of thermogenic marker Ucp1.	[126]
Arctigenin	Arctii fructus, Forsythia fruit	Anti-adipogenic	Activate AMPK pathway	Human AD-MSCs (10, 50, 100 μmol/L)/HFD mice (50 mg/kg per day, in 10% DMSO, orally)	Cell viability was not affected by ARC.	[125]
Atractylenolides	Atractylodis macrocephalae	Chondroinductive	Activate Shh pathway	Rat BM-MSCs (10, 100, 500 μg/mL)	Induce Sox9 collagen type II and aggrecan expression	[149]
AUDA	Synthetic	Anti-adipogenic	Activate HO-1 pathway	Human BM-MSCs (1 μmol/L)	Reduce expression level of Fas, Pparγ, and Cebpα	[132]
Baicalin	Scutellaria baicalensis	Osteoinductive	Activate Wnt pathway	Rat BM-MSCs (0.1, 0.5, 1, 5, 10, 50 μmol/L)/ Radix Scutellariae extract (2 and 50 mg/kg per day, orally)	Enhance ALP activity	[54,55]
Cordycepin	Cordyceps militaris	Osteoinductive	Activate Wnt pathway	Human BM-MSCs with H_2O_2 treatment (1, 5, 10, 20, 40, 80 μg/mL)/ Human BM-MSCs with ethanol treatment/ rat alcohol-induced osteonecrosis of the femoral head model (10 mg/kg per day in saline intraperitoneal)	Induce osteogenic markers osteopontin and collagen type I expression	[59,61]
Cyanidin-3-O-glucoside	Black rice	Anti-adipogenic	Activate Wnt pathway	C3H10T1/2 cells (black rice extract 10, 20, 40, and 80 μg/mL)/ HFD mice (black rice extract 10% corn oil and 90% water, 100 mg/kg per day, orally)	Induce Wnt-specific target genes such as Axin2, Wisp2, and Cyclin d1	[114]
Dihydroartemisinin	Artemisia apiacea	Anti-chondrogenic	Activate Notch pathway	C3H10T1/2 cells (1, 10, 50, 200, 300 μmol/L)	Suppress Sox9, COMP and Col2a1 expression	[151]
Er-Xian Decoction extracts	Er-Xian Decoction	Osteoinductive	Activate MAPK/ERK pathway	OVX mice (30g/kg per day, orally)/ Mice BM-MSCs (isolated from OVX and treated mice)	Elevate ALP activity.	[94]
Ethanol	Alcohol	Anti-osteogenic	Inhibit TGFβ pathway	MSCs (25 mmol/L)/ Tibial fracture mice (20% in saline, intraperitoneal)	Inhibit Col I and Sox9 expression	[62]
Genistein	Soybean	Osteoinductive	Activate BMP pathway	Human BM-MSCs (10 -0.01 μmol/L, MAX at 1 μmol/L)	Induce Runx2 and osteocalcin expression, inhibit BMP pathway inhibitor SMAD6, 7	[44]

Ginkgo biloba extract	Ginkgo biloba	Adipoinductive Osteoinductive	Activate BMP and Wnt pathways	Mouse BM-MSCs (50, 100, 150, 200, 400 μg/mL)/ human BM-MSCs (25, 50, 70, 100)	Induce Runx2, Col 1a1 and BMP-2 experssion	[64,65]
Ginkgolic acid	Ginkgo biloba	Adipoinductive	Inhibit sumoylation	Mouse BM-MSCs (50 μmol/L)	Promote adipogenic commitment and inhibit adipocyte terminal maturation.	[136]
Icariin	Herba epimedii	Osteoinductive	Activate MAPK, BMP, WNT and PI3K/AKT pathway	MC3T3-E1 cells, rat BM-MSCs (0.0001, 0.001, 0.01, 0.1, 1, 10 μmol/L; 5, 10, 20, 40 μmol/L)/ OVX rats (150 mg/kg in saline, orally)	Induce Runx2, BMP4, and Col I expression	[88,89,90,92]
Isorhamnetin	Sea buckthorn	Anti-adipogenic	Activate Wnt pathway	Human AD-MSCs (1, 25 μmol/L)	Downregulate Wnt antagonist Sfrp1 and Dkk1	[113]
Medicarpin	Medicago truncatula	Beige-Adipoinductive	Activate AMPK pathway	C3H10T1/2 cells (10μmol/L)	Induce expression of thermogenic marker Ucp1.	[121]
		Osteoinductive	Activate Wnt and Notch pathways	OVX mice (1 mg or 10 mg/kg per day, orally)/ OVX + Drill hole mice (0.5, 1 mg/kg per day, orally)	Induce Runx2, Osteocalcin and TGF-β expression	[57,58]
Naringin	Rhizoma Drynariae	Osteoinductive	Activate AMPK and AKT pathway	Human BM-MSCs (1, 10, 100 μg/mL)/ OVX mice (0, 0.5, 1, 5 and 10 mg/kg per day, orally)	Induce osteocalcin, collagen type I, osteopontin and ALP expression.	[81,82]
N-methyl pyrrolidone	Synthetic	Anti-adipogenic	Inhibit Brd4	Human BM-MSCs (5, 10 mM)/ OVX mice (10.5 mM/100 g/wk, intraperitoneal injection)	Reduce PPARγ expression level	[140]
Oleanolic acid	Glossy privet	Osteoinductive	Activate Notch pathway	Rat BM-MSCs (1, 10, 100 μmol/L) / OVX mice (2 wks, 3 mo, dissolve in normal saline, 20 mg/kg per day, orally)	KEGG analysis on differential gene patterns	[69]
Peonidin-3-O-glucoside	Black rice	Anti-adipogenic	Activate Wnt pathway	C3H10T1/2 cells (black rice extract 10, 20, 40, and 80 μg/mL)/ HFD mice (black rice extract in 10% corn oil and 90% water, 100 mg/kg per day, orally)	Induce Wnt-specific target genes such as Axin2, Wisp2, and Cyclin d1	[114]
Plastrum testudinis extracts	Plastrum testudinis	Osteoinductive	Activate PI3K/AKT pathway	Rat BM-MSCs (0.03, 0.3, 3, 30, 300 μg/mL)/ Dexamethasone induced osteoporosis rat (30 mg/kg per day, subcutaneous injection)	Induce β-catenin, Runx2 and osteocalcin expression	[76,77]
Platycodin D	Platycodi radix	Anti-adipogenic Beige-Adipoinductive	Activate AMPK pathway	Human AD-MSCs (0.5-5 μmol/L)/ db/db mice (2, 5 mg/kg per day)	Suppress adipogenic genes, such as Pparγ, Cebpα, Fabp4, Adipoq, and Resistin	[127]
Psoralen	Psoralea corylifolia	Anti-adipogenic Osteoinductive	Activate Notch pathway	Rat BM-MSCs (? μmol/L) / OVX mice (2 wks, 3 mo, dissolve in normal saline, 20 mg/kg per day, orally)	KEGG analysis on differential gene patterns	[68]

Psoralidin	Psoralea corylifolia	Anti-adipogenic Osteoinductive	Activate PI3K/AKT pathway	OVX rat (in sesame oil, 10 mg/kg per day, orally)	Promote osteogenic differentiation of BM-MSCs isolated from the treated OVX mice	[78]
Resveratrol	Wine, grape	Osteoinductive	Activate MAPK/ERK pathway	Human BM-MSCs (0.01, 0.1, 1, 10, 100 μmol/L)	Induce Runx2, osterix and osteocalcin expression	[96]
Risedronic acid	Synthetic	Anti-adipogenic	Inhibit PI3K/AKT/mTOR pathway	Human BM-MSCs (1, 5, 10, 25 μmol/L)	Inhibit mTOR1 downstream effector S6 phosphorylation	[118]
Salvianolic acid B	Salvia miltiorrhiza	Osteoinductive	Activate MAPK/ERK pathway	Rat BM-MSCs (50, 100, 500, 1000 nmol/L)/ Steroid induced osteoporotic rat (40, 80 mg/kg per day)	Enhance ALP activity and osteocalcin expression	[101,102]
SKL2001	Synthetic	Osteoinductive	Activate Wnt pathway	ST2 cells (5, 10, 30 μmol/L)	Stabilize β-catenin without affecting expression level	[63]
T63	Synthetic	Anti-adipogenic Osteoinductive	Activate BMP and Wnt pathway	C3H10T1/2 (1-40 μmol/L)/ OVX mice (5 mg/kg and 20 mg/kg, orally)	Induce Runx2, Bglap and, Spp1 expression.	[47]
Tithonia diversifolia extracts	Tithonia diversifolia	Anti-adipogenic	Activate HO-1 pathway	Human AD-MSCs (175 μg/mL)	Oil red staining quantitatively decrease in a dosage-dependent manner	[130]
Tricin	Rice bran	Osteoinductive	Activate Wnt pathway	Human AD-MSCs (5, 10, 15, 20, 25 μmol/L)	Induce bone sialoprotein, osteocalcin, ALP, Runx2, Col I, osterix, osteopontin	[53]

8-HUDE: 12-(3-hexylureido) dodec-8(Z)-enoic acid; ALP: Alkaline phosphatase; AUDA: 12-(3-adamantan-1-yl-ureido)-dodecanoic acid; AD-MSCs: Adipose-derived mesenchymal stem cell; BM-MSCs: Bone marrow mesenchymal stem cell; HFD: High-fat diet; OVX: Ovariectomy; Runx2: Runt-related transcription factor 2; TGF-β: Transforming growth factor beta; Col I: Type I collagen; KEGG: Kyoto encyclopedia of genes and genomes; AMPK: AMP-activated protein kinase.

and osteogenic differentiation of MSCs. Upon Wnt proteins binding to the frizzled (Fzd) receptors, the canonical Wnt signaling pathway is activated, and cytoplasmic β-catenin is stabilized *via* glycogen synthase kinase-3 (GSK3) inhibition[48,49]. β-catenin accumulates in the cytosol and subsequently translocates to the nucleus, where it promotes the transcription of target genes[50]. Activation of the canonical Wnt signaling pathway upregulates the gene expression of the osteogenic regulators Runx2, Dlx5, and Osterix[51] and suppresses the expression of the adipogenic inducers Pparγ and Cebpα[52]. The small molecules that affect the osteogenic cell fate of MSCs *via* the Wnt signaling pathway include medicarpin, cordycepin, SKL2001, tricin[53], baicalin[54,55], Ginkgo biloba extracts (GBE), and Fructus ligustri extracts[56].

Medicarpin (Med), a pterocarpan compound, is present in many leguminous species, such as chickpea and Butea monosperma. Tyagi *et al*[57] demonstrated the osteoprotective effect of Med on OVX osteoporotic rats. Med was given at a dosage of 1mg or 10 mg/kg per day. MicroCT revealed that the osteoporotic phenotype was significantly improved under the Med treatment, giving a higher total trabecular volume and number. With Med administration, BM-MSCs isolated from the treated OVX rats presented a superior mineralization level under osteogenic medium induction compared with the BM-MSCs from the untreated rat[57]. Another study used the OVX drill-hole injury animal model to assess the regenerative effect of Med *in vivo*. The OVX rats underwent the dill-hole procedure at the sites of femur mid-diaphysis and received treatment of 0.5, 1 or 5 mg/kg of Med. The results showed that Med administration not only increased the bone mineral density but also upregulated several osteogenic markers, including Runx2 and osteocalcin. The gene expression profile comparison demonstrated the effect to be mediated by both the canonical Wnt and the Notch signaling pathways, evidenced by the increased expression level of Wnt signaling pathway mediators β-catenin, Dishevelled and Fzd, and the Notch signaling pathway mediators Notch-1 and Jagged-1 at the defect region[58].

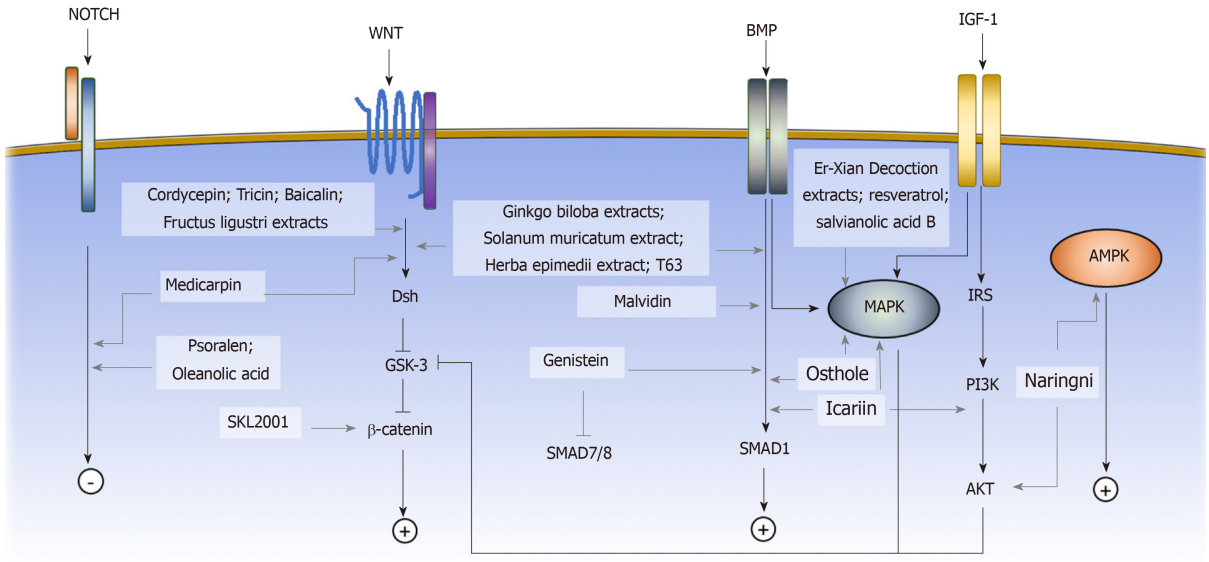

Figure 1 Small molecules guiding mesenchymal stem cell osteogenic cell fate determination. Wnt, BMP, and Notch signaling cascades are the three major pathways governing the expression of the master regulator runt-related transcription factor 2 during mesenchymal stem cell (MSC) osteogenic commitment. The activation of Wnt, Notch and BMP signaling pathways by small molecules promotes the osteogenesis of MSCs. The MAPK and PI3K/AKT signaling pathways interfere with GSK3 phosphorylation and β-catenin degradation and therefore exert an effect on osteogenesis. The AMP-activated protein kinase pathway has also recently been shown to be involved in osteogenic regulation. AMPK: AMP-activated protein kinase.

Cordycepin is one of the major compounds of Cordyceps militaris. Wang *et al*[59] showed that cordycepin prevented oxidative stress-induced inhibition of osteogenesis through activation of the Wnt signaling pathway. The H_2O_2-induced inhibition of human BM-MSC osteogenesis was used as the baseline, and treatment with 10 μg/mL cordycepin reversed the osteogenic dysfunction with the increase in ALP staining and mineralization. The osteogenic genes osteopontin and Col I were upregulated under cordycepin treatment, while the osteoclast promoting agent RANKL was downregulated. The H_2O_2 negatively regulated the Wnt signaling pathway, but cordycepin treatment recovered the downregulation[60]. The addition of the Wnt signaling pathway inhibitor DKK1 reduced the osteoinductive effects of cordycepin on ALP activity, calcium quantification, and Runx2 expression, confirming that Wnt signaling was involved in exerting the osteoprotective effect of cordycepin[59].

The osteoprotective effect of cordycepin has also been examined in alcohol-induced osteonecrosis by using both *in vitro* and *in vivo* models. Chen *et al*[61]demonstrated the osteoinductive effect of cordycepin on human BM-MSCs under ethanol treatment. Previous studies have shown that ethanol treatment impaired the osteogenic differentiation of BM-MSC[62]. The cordycepin treatment at a dose of 1 or 10 μg/mL attenuated the osteogenic inhibitory effect of ethanol. The rat model of alcohol-induced osteonecrosis of the femoral head (ONFH) was established to assess the effect of cordycepin *in vivo*. Intraperitoneal injection of Cordycepin at a dose of 10 mg/kg per day decreased the development rate of ONFH from 70% to 20%, and both trabecular volume and thickness were significantly increased by cordycepin treatment[61].

Gwak *et al*[63] performed a cell-based chemical screening assay with a library of 270000 small molecules on HEK293 reporter cells and identified a compound named as SKL2001 that strongly stimulated the Wnt signaling pathway. When SKL2001 was added, the ST2 BM-MSC cell line expressed higher levels of type I collagen and Runx2 under osteogenic induction. The ALP activity also increased in a dosage-dependent manner. Assessment of Wnt signaling pathway mediators revealed that SKL2001 enhanced the protein expression level of both cytoplasmic and nuclear β-catenin without affecting the RNA expression level, suggesting SKL2001 was involved in the protein degradation of β-catenin. The subsequent findings that SKL2001 inhibited the phosphorylation of β-catenin and hindered the interaction of β-catenin with its degradation mediator β-TrCP confirmed that SKL2001 affected the osteogenic commitment of MSCs by stabilizing β-catenin.

GBE was shown by Wu *et al*[64] to enhance osteogenic differentiation and inhibit adipogenic differentiation in murine BM-MSCs. The addition of 150 μg/mL GBE into osteogenic differentiation medium prominently enhanced both calcium deposits and ALP activity. The expression levels of the osteogenic markers Runx2, Col 1, and BMP-2 were upregulated, whereas GBE treatment decreased lipid accumulation in the

differentiated adipocytes and suppressed the expression levels of the adipogenic genes Pparγ and Fabp4. The osteoprotective effect was also examined in human BM-MSCs, whose results were similar to murine BM-MSCs, showing an increase in ALP activity and upregulation in the expression of the osteogenic genes osteopontin and Col I. A loss-of-function assay was performed to identify the signaling pathway involved in the GBE treatment. When the Wnt or the BMP signaling pathway was inhibited, the ALP activity under GBE treatment decreased significantly, confirming that GBE exerted its effect through regulation of both Wnt and BMP signaling pathways[65].

Notch signaling pathway

Notch signaling is a highly conserved signaling pathway related to cell-fate determination, self-renewal potential, and apoptosis[66]. Induction of Notch signaling enhances the osteogenic differentiation of human BM-MSCs and inhibits adipogenic commitment[67]. The small molecules that are involved in osteogenic differentiation of MSCs by regulating the Notch signaling pathway are psoralen (PSO) and oleanolic acid (OA).

PSO is the active ingredient of *Psoralea corylifolia*, which is commonly prescribed for treating fractures, bone diseases and joint diseases in traditional Chinese medicine. To elucidate the osteoprotective mechanism of PSO, we investigated the effects of PSO on adipogenic and osteogenic differentiation of rat BM-MSCs. In the OVX osteoporotic rats, PSO significantly increased trabecular number and thickness. The *in vitro* assays demonstrated that PSO inhibited adipogenic differentiation and promoted osteogenic differentiation. Using the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis on the microarray data, the differentially expressed genes were highly enriched in the Notch signaling pathway, suggesting that PSO exerts its osteogenic effect *via* the Notch signaling pathway[68].

OA isolated from glossy privet was reported to prevent bone loss by inhibiting osteoclast formation. We discovered that OA not only affected osteoclastogenesis but also stimulated the osteoblastic differentiation of BM-MSCs *in vitro*. In the OVX osteoporotic rats, administration of OA at a dosage of 20 mg/kg per day significantly increased the trabecular number and thickness. The expression levels of both osteocalcin and Runx2, which are markers for bone formation and osteogenic differentiation, also increased compared with untreated mice. KEGG pathway analysis of the differentially expressed genes revealed that the Notch signaling pathway is involved in the osteogenic effect of OA[69].

PI3K/AKT signaling pathway

Insulin-like growth factor 1 (IGF-1) is an important osteogenic regulator during skeletal development. IGF-1 receptor autophosphorylation occurs under IGF-1 stimulation and subsequently activates downstream PI3K/AKT and MAPK signaling pathways[70,71]. Previous studies have shown that loss of IGF-1 receptors could lead to retardation of skeletal development and defects in trabecular bone[72,73]. Evidence has shown that the PI3K/AKT pathway is among the most critical signaling pathways for osteogenic differentiation and bone growth[74,75]. The small molecules that regulate MSC osteogenesis through that PI3K/AKT signaling pathway include *Plastrum testudinis* extracts (PTE) and psoralidin.

Plastrum testudinis is an herbal medication commonly used in traditional Chinese medicine for treating bone diseases. Liang *et al*[76]demonstrated that injecting PTE into the steroid-induced osteoporosis rat at a dosage of 30 mg/kg per day improved not only the histological features, promoting a more orderly trabecular structure, but also the biomechanical properties, promoting bone strength and energy absorption capacity compared with untreated rats. The underlying osteoprotective mechanism of PTE was further investigated by Shen *et al*[77], who demonstrated that PTE promoted BM-MSC proliferation and osteogenic differentiation. Five different concentrations, 0.03, 0.3, 3, 30 and 300 μg/mL, were tested in the study. The ALP activity and mineralization of differentiated cells increased in a dosage-dependent manner. The osteogenic genes, including β-catenin, Runx2, and osteocalcin, were all upregulated. The study also revealed that PTE promoted p-PI3K, p-AKT, and p-GSK3β protein expression during osteogenesis, indicating that the effect of PTE on osteogenic differentiation was dependent on the PI3K/AKT signaling pathway.

Psoralidin is a compound enriched in the seeds of *Psoralea corylifolia*. Zhai *et al*[78] found that administration of 10 mg/kg per day of psoralidin could prevent bone loss in the OVX-induced osteoporosis model, improving both bone density and biomechanical properties. The BM-MSCs were isolated from both treated and untreated rats. Under osteogenic induction, BM-MSCs from the psoralidin-treated rats were prone to undergo osteogenic differentiation, while adipogenic differentiation was suppressed. Psoralidin treatment increased the levels of p-PI3K and p-AKT and

p-GSK3β, which led to an accumulation of β-catenin, confirming that psoralidin promoted MSC osteogenesis through the PI3K/AKT pathway.

AMP-activated protein kinase signaling pathway

The AMP-activated protein kinase (AMPK) signaling pathway has recently been shown to regulate MSC osteogenesis. During osteogenic differentiation, both AMPK expression and phosphorylation increases[79]. Inhibition of the AMPK signaling pathway leads to a reduction in mineral deposition and suppresses the expression of osteogenic genes, including Runx2, ALP, and osteocalcin, indicating that AMPK activation favors MSC osteogenic differentiation[79].

Naringin is a major compound of Rhizoma Drynariae that enhances BMP expression level in osteoblast[80]. Zhang *et al*[81] showed that the treatment with naringin promoted proliferation and osteogenic differentiation of human BM-MSCs. Naringin dose-dependently increased the expression of osteoblast-related markers osteocalcin, Col I, osteopontin, and ALP. The mechanism of naringin was further studied by the Wang group. In the OVX mice, feeding 5 mg/kg per day naringin showed the most significant enhancement in the expression of the osteogenic genes, and improved the total bone density at the distal femur, proximal tibia, and lumbar spine. The addition of AMPK and AKT inhibitor reversed the osteoprotective effect given by naringin, suggesting that the AMPK and AKT signaling pathways could be a possible mechanism for the osteogenic induction of naringin[82].

MAPK signaling pathway

MAPKs are a family of kinases that transmitted extracellular stimuli into intracellular signaling cascade and regulate crucial cell behaviors, including proliferation and differentiation[83]. Conventional MAPK members are the extracellular signal-regulated kinases 1/2 and ERK5, c-Jun amino (N)-terminal kinases 1/2/3, and the p38 isoforms. Activation of the MAPK signaling pathway promotes human MSC osteogenic commitment[84]. The small molecules that regulate MSC osteogenic differentiation through the MAPK signaling pathway include icariin, Er-Xian decoction (EXD) extracts, resveratrol, and salvianolic acid B.

Icariin (ICA) is the main active component of *Herba epimedii*, which is a well-known traditional Chinese medicine for treating osteoporosis[85]. Previous studies have shown that ICA promoted osteogenic differentiation *in vitro*[86,87]. Wu *et al*[88] recently found that the effect of ICA was mainly mediated by MAPK pathway activation, as it increased the phosphorylation of MAPK signaling molecules, including ERK and JNK, upon ICA treatment. Subsequently, the gene expression of osteogenic markers, including Col I, osteocalcin and osteopontin and the ALP activity increased in a dosage-dependent manner. The osteogenic effect of ICA was suppressed by either ERK or JNK inhibitors, suggesting that the MAPK pathway is necessary for the induction of osteogenesis of BM-MSCs by ICA. In addition to the MAPK pathway, ICA is involved in regulating osteogenesis through other osteogenic-associated signaling pathways, including BMP[89], WNT[90], and PI3K/AKT signaling pathways[91]. Cao *et al*[92] showed that the daily intragastric administration of ICA to the fractured OVX rat at a dosage of 150 mg/kg significantly increased bone mineral density and accelerated fracture healing within 5 mo. These findings demonstrated that, following bone fracture in OVX rats, the administration of ICA accelerated bone mineralization and improved fracture healing. A double-blind randomized controlled trial showed that the administration of a daily dose of 60 mg ICA, 15 mg daidzein, and 3 mg genistein for 12 mo or 24 mo significantly reduced bone loss in late-postmenopausal women in comparison with the placebo group, demonstrating a positive effect of epimedium-extract small molecules on preventing bone loss[93].

EXD, which is a common Chinese medicine mixing of six different herbs clinicians prescribed to treat menopausal symptoms. We studied the extracts from EXD and demonstrated their stimulatory effect on the osteoblastic differentiation of murine BM-MSCs[94]. The BM-MSCs isolated from the EXD extract-treated mice showed an increased ALP activity under osteogenic induction compared with those from OVX mice, suggesting the osteoprotective role of EXD extracts. The gene expression profiles showed that the common genes that were upregulated during EXD extract treatment were related to the MAPK signaling pathway, indicating that EXD exerted its effect by regulating the MAPK signaling pathway. A clinical trial showed that EXD improved bone mineral density at both the lumbar spine and the femoral head in postmenopausal women, demonstrating an osteoprotective effect[95]. However, no study has demonstrated its effect on the incidence rate of fracture. Future studies of longer duration with calculation of fracture rates are needed to confirm the clinical benefit of EXD.

Resveratrol (RSVL) is a phenolic compound enriched in wine and grape and famous for its antioxidant effect. Dai *et al*[96] tested the osteogenic regulatory effect of

RSVL by treating BM-MSCs with RSVL at different doses from 0.01 to 100 μmol/L and measured the ALP activity. The results showed that under 1 μmol/L RSVL treatment, the differentiated BM-MSCs presented the maximal increase in ALP activity. The osteogenic genes Runx2, osterix, and osteocalcin were all upregulated during the treatment with RSVL. The addition of the ERK inhibitor PD98059 reversed the expression level of osteogenic markers and ALP activity, confirming that RSVL affects the MSC osteogenesis through the MAPK signaling pathway. In addition to the MAPK signaling pathway, other studies have also shown that RSVL activated SIRT1, a nicotinamide adenine dinucleotide (NAD^+)-dependent deacetylase, and subsequently upregulated FOXO3A protein expression, which promoted SIRT1-FOXFO3A complex formation and upregulated Runx2 expression[97,98]. A clinical trial was performed to assess the osteoprotective effect of RSVL on osteoporotic obese patients. The results showed that RSVL increased lumbar spine bone mineral density in a dose-dependent manner, with a maximal increase of 2.6% in the high-dose RSVL group[99].

Salvianolic acid B (SalB) is the active compound of *Salvia miltiorrhiza*, which is commonly used in treating cardiovascular diseases in Chinese medicine[100]. Cui *et al*[101] demonstrated the association between SalB and osteogenesis by showing that administration of SalB at a dosage of 40 mg/kg per day to the steroid induced osteoporotic rats reversed the osteoporotic phenotype. The rats presented elevated bone mineral density, increased cancellous bone mass, and thicker trabeculae after the treatment. This effect was consistent with the finding that SalB promoted osteogenic differentiation of rat BM-MSCs *in vitro* at the dosages ranging between 100 and 500 nmol/L. The differentiated cells showed a significantly higher ALP activity along with an increase in osteocalcin expression[101]. The underlying mechanism of SalB was studied by the same group in human BM-MSCs. The addition of the ERK inhibitor U0126 diminished the effect of Sal B on osteogenesis, suggesting that the Sal B regulated the osteogenesis of BM-MSCs through the MAPK/ERK signaling pathway[102].

SMALL MOLECULES REGULATE MSCS ADIPOGENIC CELL FATE

The induction of adipogenic differentiation in MSCs *in vitro* is traditionally achieved by the activation of the adipogenic master regulator *Cebpa* and *Pparγ* through a small molecule cocktail of 3-isobutyl-1-methylxanthine (IBMX), indomethacin, dexamethasone, and insulin[7]. IBMX is a phosphodiesterase inhibitor that increases the intracellular cyclic AMP (cAMP) and activates the downstream PKA signaling pathway to induce *Cebpa* and *Pparγ* expression[103-105]. Dexamethasone, on the other hand, binds to the intracellular glucocorticoid receptor and subsequently enhances the expression of the adipogenic transcription factor C/EBPβ[106]. Indomethacin is a well-known COX1/2 inhibitor; however, its adipogenic activity is not due to the inhibition of COX but through activation of PPARγ. Insulin promotes glucose uptake and stimulates triglyceride synthesis in adipocytes. In the past decade, more signaling pathways have been identified to be involved in regulating the adipogenesis of MSCs. Some of the pathways are the Wnt, AKT and AMPK signaling pathways[103]. Activating the AKT signaling pathway promotes the differentiation of adipose stem cells, whereas activating the Wnt[107,108] or AMPK[79] signaling pathways inhibits adipogenesis. Aside from regulating gene expression through a signaling pathway cascade, the post-translational modification also affects adipogenic fate determination through post-translational modification, such as sumoylation and epigenetic modification. In this section, we will review the small molecules that have been reported to affect the adipogenic commitment of MSCs based on their mechanisms (Figure 2).

Wnt signaling pathway

The canonical and noncanonical signaling pathways present different effects on MSC adipogenesis. The canonical signaling pathway mediates signaling through the stabilization of β-catenin, and activation of the Wnt canonical signaling pathway was shown to block the induction of PPARγ and C/EBPα and to inhibit adipogenesis[109]. Wnt also activates noncanonical signaling pathways. Genetic evidence indicates that noncanonical signaling through Wnt5a antagonizes the canonical signaling pathway[110]. Wnt antagonists exert a crucial role during the adipogenic commitment of MSCs[111,112]. Some of the small molecules that have been shown to exert adipogenic regulatory effects *via* the Wnt signaling pathway include isorhamnetin, cyanidin-3-O-glucoside, and peonidin-3-O-glucoside.

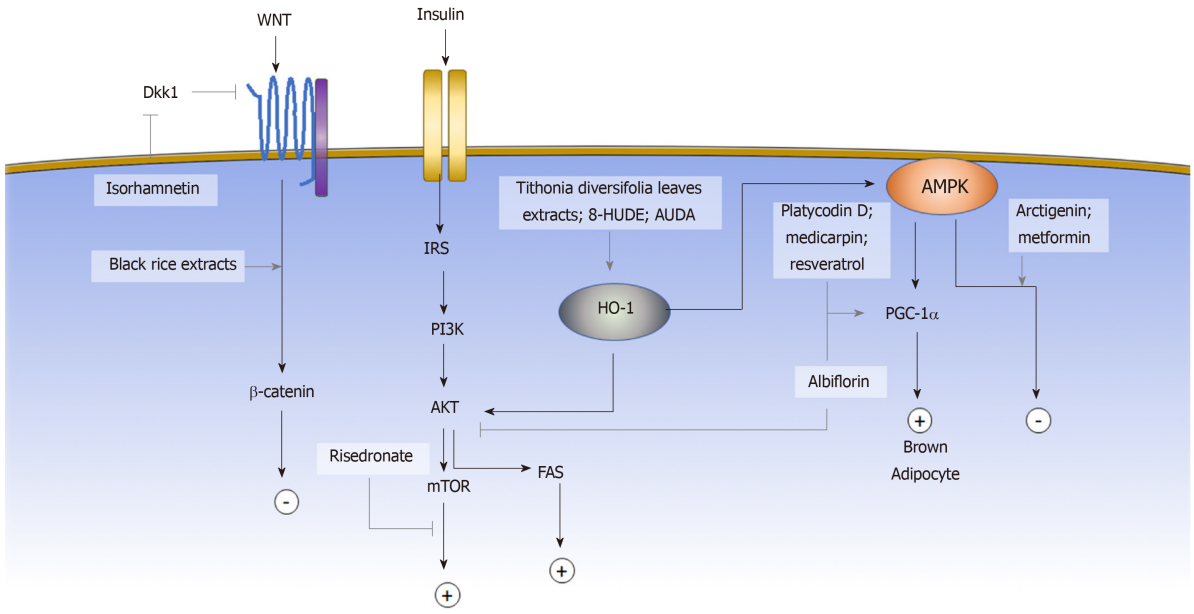

Figure 2 Small molecules guiding mesenchymal stem cell adipogenic cell fate determination. Many pathways are involved in regulating the adipogenesis of mesenchymal stem cells, including the Wnt, AKT and AMP-activated protein kinase (AMPK) pathways. Activating the AKT pathway promotes the differentiation of adipose stem cells, whereas activating the heme oxygenase-1, Wnt or AMPK pathway inhibits adipogenesis. FAS: Fatty acid synthase; 8-HUDE: 12-(3-hexylureido) dodec-8(Z)-enoic acid; AUDA: 12-(3-adamantan-1-yl-ureido)-dodecanoic acid; HO-1: Heme oxygenase-1; AMPK: AMP-activated protein kinase.

Isorhamnetin (IsR) is a flavonoid extracted from sea buckthorn. IsR was first identified by Lee *et al* to inhibit adipogenic differentiation of MSCs. The triglyceride level was significantly lower under treatment of 25 μmol/L IsR. The Wnt signaling pathway antagonists Sfrp1 and Dkk1 were downregulated under IsR treatment, thereby stabilizing and increasing the protein level of β-catenin without affecting the mRNA expression level. The finding indicated that IsR affected adipogenesis through activation of the Wnt signaling pathway[113].

Cyanidin-3-O-glucoside (C3G) and peonidin-3-O-glucoside (P3G) are two anthocyanin components of black rice extract (BRE). Both compounds were shown by Jang *et al*[114] to inhibit adipogenic differentiation of the murine MSC line C3H10T1/2. Under treatment with C3G or P3G, lipid accumulation in the differentiated cells decreased in a dose-dependent manner, and the adipogenic gene Pparγ was significantly suppressed. Although the mechanism of C3G and P3G were not investigated, the original BRE has been shown to activate Wnt signaling and downstream targets, exerting both anti-adipogenic and osteoinductive effects.

PI3K/AKT signaling pathway

The PI3K/AKT signaling pathway can be activated by a range of extracellular factors through the receptor tyrosine kinases (RTKs). Upon RTK activation, the IRS1/2 phosphorylates and activates PI3K. PI3K subsequently activates AKT, which regulates many functional mediators, including GSK3, FoxO, mTOR, which in turn form complex regulatory circuits that govern the manifold response. Among them, activation of mTOR leads to upregulation in Pparγ and promotes adipogenesis[115-117].

Risedronate is a bisphosphonate medication that is used to treat osteoporosis clinically by inhibiting osteoclastic differentiation. Jin *et al*[118] demonstrated that risedronate, in addition to affecting osteoclast development, also inhibited human BM-MSC adipogenesis through the PI3K/AKT signaling pathway. The ratio of adipocyte formation under the adipogenic induction decreased in a dose-dependent manner while increasing the concentration of risedronate from 1 μmol/L to 25 μmol/L. Further exploration of the mechanism showed that phosphorylation of mTOR downstream effectors was inhibited under risedronate, suggesting that its effect on adipogenesis of BM-MSCs was mediated by mTOR signaling pathway regulation[118].

AMPK signaling pathway

AMPK is highly involved in cellular energy homeostasis, and the AMPK signaling pathway has been shown to regulate the adipogenic differentiation of MSC, as inhibition of AMPK signaling pathway promotes lipid droplet formation and adipogenesis[79]. Aside from typical white adipogenesis, the other important cell fate

modulation associated with the AMPK signaling pathway is brown adipogenesis. Unlike white adipocytes, brown adipocytes exert a significant thermogenic effect, which has a promising effect for obesity control. Activation of the AMPK signaling pathway enhances the gene expression of PGC-1α[119] and subsequently mediates MSCs to differentiate into brown adipocytes[120]. Compounds that have been shown to affect adipogenic fate commitment *via* the AMPK signaling pathway include arctigenin (ARC), albiflorin (AF), medicarpin[121], platycodin D (PD), metformin[122] and resveratrol[123,124].

ARC is a major lignan component of *Arctii fructus* and Forsythia fruit. Han *et al*[125] discovered that ARC inhibited adipogenesis in human AD-MSCs. The viability of MSCs did not change between the concentration of 10 and 100 μmol/L. Under ARC treatment, AMPK phosphorylation was significantly increased, suggesting that the adipogenic regulatory effect of ARC was mediated *via* activation of the AMPK signaling pathway. The *in vivo* experiments showed that the administration of ARC at a dose of 50 mg/kg per day reversed the body weight gain in high-fat diet (HFD) induced obesity mice, which is consistent with the *in vitro* findings.

AF is a major monoterpene glycoside compound of *Paeonia lactiflora*. Jeong *et al*[126] demonstrated that AF enhanced brown adipocyte differentiation *via* the AMPK and the PI3K/AKT/mTOR signaling pathways. In the presence of AF, lipid accumulation increased and beige-specific markers, including UCP1, PGC-1α, and CIDEA, were upregulated. After a 6-week administration of AF, body weight gain in HFD mice was significantly reduced, while the worsening of liver function, as measured by total cholesterol, LDL, ALT, and AST, reversed[126].

PD is an active compound of Platycodi radix. Kim *et al*[127] PD showed that administration of PD at a dosage of 5 mg/kg per day reduced the weight gain of db/db mice. However, the anti-obeisty effect of PD was not observed at the lower dosage of 2 mg/kg per day. *In vitro*, PD treatment suppressed the expression of adipogenic genes, such as Pparγ, Cebpα, Fabp4, Adipoq, and resistin, during the adipogenic differentiation of human AD-MSCs, suggesting the anti-adipogenic role of PD. Meanwhile, PD increased the expression of thermogenic factors UCP1 and PGC1 in both db/db mice and *in vitro*, which indicated the beige-adipoinductive role of PD. The phosphorylation of AMPK was significantly elevated under PD treatment, suggesting that PD is involved in regulating the AMPK signaling pathway.

Heme oxygenase-1 signaling pathway

Heme oxygenase (HO) is the enzyme that digests heme proteins and generates carbon monoxide, biliverdin, and iron. HO-1 induction results in increased levels of phosphorylated AMPK and AKT. The upregulation of HO-1 expression inhibits MSC adipogenic differentiation and favors osteogenic differentiation[128,129]. The small molecules that regulate MSC adipogenic cell fate *via* HO-1 activation include Tithonia diversifolia extracts and epoxyeicosatrienoic acid agonists.

Tithonia diversifolia extracts (TDE) are frequently used in traditional medicine for treating diabetes and wound healing. Giacomo *et al*[130] showed that TDE inhibited adipogenesis by inducing the AMPK signaling pathway *via* HO-1 activation. Upon TDE treatment, phosphorylated AMPK and HO-1 protein expression levels were significantly increased. A functional assay showed a dose-dependent effect of TDE on decreasing lipid accumulation in differentiated adipocytes.

Epoxyeicosatrienoic acids (EETs) are the derivative of arachidonic acid and act as an inducer of HO-1 activity[131]. The formed EETs can be metabolized by soluble epoxide hydrolase (sEH) into dihydroxyeicosatrienoic acids (DHETs). Kim *et al*[132] showed that adding the sEH inhibitor, 12-(3-hexylureido) dodec-8(Z)-enoic acid (8-HUDE) and 12-(3-adamantan-1-yl-ureido)-dodecanoic acid (AUDA) activated HO-1 and inhibited human BM-MSC adipogenesis. The inhibitory effects of 8-HUDE and AUDA were reduced by inhibition of HO activity, which demonstrated the role of AUDA and 8-HUDE in regulating adipogenesis of MSCs *via* the HO-1 signaling pathway.

Sumoylation

Sumoylation is a post-translational modification process that is important in regulating the functional features of many proteins. Some of the transcription factors closely related to adipogenesis, such as PPARγ, C/EBPα, and C/EBPβ, are targets of sumoylation. The transcriptional activity of these master regulators can be negatively regulated by sumoylation and affect the commitment of adipogenic cell fate of MSCs[133,134].

Ginkgolic acid (GA), a compound that is enriched in the leaves of *Ginkgo biloba*, impairs SUMOylation by blocking the formation of the E1-SUMO thioester complex and functioning as a sumoylation inhibitor[135]. Liu *et al*[136] investigated the effect of GA on adipogenesis and demonstrated that the addition of GA in the early stage of

adipogenesis promoted the commitment of mouse BM-MSCs into adipocytes, whereas addition at a later stage inhibited adipocyte differentiation.

Epigenetic landscape

DNA and histone methylation are the key components in the epigenetic machinery, regulating gene expression profiles. Some small molecules inhibit acetlyltransferase or methyltransferase and change the epigenetic landscape[137], which is recognized by other proteins that subsequently affect the expression of adipogenic genes. For MSC adipogenesis, these small molecules include 5-azacytidine and N-methyl pyrrolidone.

5-Azacytidine (5-aza) is a DNA methyltransferase inhibitor. Chen *et al*[138] showed that the methylation levels of Wnt10a chromatin regions were significantly reduced under the treatment with 5-aza and subsequently activated the expression of Wnt10a. Wnt10a then downregulated the expression level of adipogenic markers PPARγ, aP2, FAS, and C/EBPα and inhibited MSC commitment to adipogenic lineage.

Brd4 is a member of the bromodomain and extraterminal domain (BET) family that binds to active enhancers through recognition of acetyl-lysine residues of histones and controls PPARγ downstream adipogenic genes[139]. Gjoksi *et al*[140] showed that N-methyl pyrrolidone, a Brd4 inhibitor, reduced transcriptional activation of PPARγ and inhibited adipogenesis of human BM-MSCs. Furthermore, in the OVX rat model, administration of N-methyl pyrrolidone reduced fat accumulation and adipogenesis in the bone marrow tissue further validating the anti-adipogenic effect of Brd4.

SMALL MOLECULES REGULATE MSCS CHONDROGENIC CELL FATE

The chondrogenic differentiation of MSCs is induced with a high cell-density pellet supplemented with transforming growth factor (TGF)-β in a serum-free medium, leading to an elevated production of cartilage-specific proteins, such as proteoglycans and type II collagen (Col 2). In addition to the TGF-β signaling pathway, there are also other signaling pathways involved in regulating the chondrogenesis of MSCs, including includes BMP[141], Wnt[142,143], fibroblast growth factor[144], hedgehog (HH), and Notch signaling pathways.

HH signaling pathway

The HH signaling pathway is known to be important for cartilage development *in vivo*[145]. Implantation of fibroblasts expressing sonic hedgehog protein (Shh) protein or indian hedgehog protein in the nude mice forms cartilage at the donor site[146]. The other important role of HH signaling is the proliferative impact on the chondrocytes. Activation of the HH signaling pathway induces GLI, a transcriptional factor that promotes cell proliferation. Therefore, continuous activation of the HH signaling pathway causes uncontrolled cell proliferation and leads to the development of enchondromatosis[147,148].

Atractylenolides are enriched in *Atractylodis macrocephalae*, a kind of herbal medicine that is commonly prescribed to treat arthritis. Li *et al*[149] demonstrated that the addition of atractylenolides induced the Gli promoter and promoted chondrogenic differentiation in rat BM-MSCs. The chondrogenic markers Sox9, Col 2 and aggrecan were upregulated compared with the untreated group. When Shh signaling was inhibited by the signaling pathway inhibitor cyclopamine, the effect of atractylenolides on promoting chondrogenic differentiation was reduced, confirming that the chondrogenic effect of Atractylenolides was dependent on the Shh signaling pathway.

Notch signaling pathway

The Notch intracellular domain is the main mediator for regulating the chondrogenesis of the MSCs in the Notch signaling pathway. Upon ligand stimulation, the NOTCH protein undergoes proteolytic cleavage and releases the intracellular domain. The intracellular domain subsequently translocates into the nucleus and induces the expression of the HES gene family. The HES gene family, including HES-1 and HEY-1, acts on the Sox9 binding site at the Col2a1 enhancer and consequently prevents Sox9-mediated transcriptional activation of Col2a1, suppressing the chondrogenesis of MSC[150].

Dihydroartemisinin (DHA) is a major compound derived from *Artemisia apiacea*. Cao *et al*[151] showed that DHA inhibited chondrogenic differentiation of the C3H10T1/2 cell line *in vitro*. After treatment with DHA containing chondrogenic medium for 14 d, the chondrogenic-specific markers Sox9, COMP and Col2a1 were significantly suppressed compared with the untreated cells. The key factors in

different signaling pathways were subsequently assessed, revealing upregulation of Hey1 expression, indicating that the Notch signaling pathway is involved in the DHA inhibition of chondrogenesis of the MSCs.

CONCLUSION

The regulation of MSC differentiation is multifaceted, governed by multiple signaling pathways, epigenetic regulation, and post-translational modification. Beyond the above small molecules illustrated, there were many others that have recently been identified to affect the lineage commitment of MSCs, but the underlying mechanisms are still elusive. For instance, the *Cornus walteri* extracts, the *Oryza sativa* extracts, and piceatannol (enriched in *Aiphanes horrida*) were shown to inhibit adipogenesis of MSC[152-154]. *Ajuga decumbens* extracts were shown to stimulate osteogenesis of MSCs[155]. Honokiol improved the chondrogenesis of MSCs[156]. However, the underlying mechanisms of all the above compounds have not yet been explored. Understanding the mechanism by which different small molecules affect MSC cell determination will benefit the application utility of small molecules as precisive modulators, offering researchers a useful probe in guiding MSC differentiation.

Some of the small molecules identified have been investigated in clinical trials for the treatment of MSC-related diseases, while many others identified recently remained unexplored. Even though the *in vitro* cell culture experiments and the animal studies offered promising results, there are several aspects that can hinder investigators from translating these small molecules into clinical use. One of the major obstacles to success is that the cells and animal models cannot fully reflect the effect of small molecules in humans for many reasons, such as differences in physiological background, length of administration and subjective endpoints. The other obstacle is that, for those small molecules identified from a complex compound, one kind of small molecule may not be sufficient to have the maximal effect, but require other small molecules from the original compound to synergize with it.

Overall, the ongoing discovery of new small molecules facilitating MSC cell fate commitment will continue to play critical roles in basic science research and potentially become novel therapeutic agents in treating various MSC-related diseases.

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REVIEW

Mechanoresponse of stem cells for vascular repair

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Abstract

Stem cells have shown great potential in vascular repair. Numerous evidence indicates that mechanical forces such as shear stress and cyclic strain can regulate the adhesion, proliferation, migration, and differentiation of stem cells *via* serious signaling pathways. The enrichment and differentiation of stem cells play an important role in the angiogenesis and maintenance of vascular homeostasis. In normal tissues, blood flow directly affects the microenvironment of vascular endothelial cells (ECs); in pathological status, the abnormal interactions between blood flow and vessels contribute to the injury of vessels. Next, the altered mechanical forces are transduced into cells by mechanosensors to trigger the reformation of vessels. This process occurs when signaling pathways related to EC differentiation are initiated. Hence, a deep understanding of the responses of stem cells to mechanical stresses and the underlying mechanisms involved in this process is essential for clinical translation. In this the review, we provide an overview of the role of stem cells in vascular repair, outline the performance of stem cells under the mechanical stress stimulation, and describe the related signaling pathways.

Key words: Stem cells; Shear stress; Strain stress; Vascular repair

Core tip: Stem cells and biomechanical stresses are very important for the success of stem cell-based therapy. In this review paper, we first summarize the application of stem

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cells for vascular repair, then discuss the response of stem cells to the biomechanical stresses in blood vessels, and finally describe the underlying mechanisms. This paper should be very beneficial to researchers in this field, as it provides a deeper understanding of the interactions between stem cells and biomechanical stresses for vascular repair.

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INTRODUCTION

Adult stem cells derived from the same precursors have potential functions for tissue development and regeneration including bone regeneration, wound healing, and vessel repair[1,2]. Traditionally, the damaged endothelium in vessel wall was thought to be replaced by nearby endothelial cell (EC) replication. However, recent findings challenge this notion and point out that stem cells also participate in the process of vascular repair. In fact, the promising role of stem cells in vascular repair has been well determined by numerous studies *in vitro* and *in vivo* experimental settings[3,4].

The repair processes includes relative signaling pathway activation, gene expression, oxidative balance and alignment of cytoskeletal filaments[5]. Based on these scientific outcomes, it is feasible for scientists to use stem/progenitor cells with or without scaffolds to create bioengineered vessels *in vitro* that are suitable for grafting clinically[6]. However, the key factors influencing the successful utilization of bioengineered vessel is the replication of different physical forces generated by blood flow during the cardiac cycle and the understanding of how these physical forces affect the biological behaviors of the grafted and resident stem cells[7].

The biomechanical patterns of blood flow in vessels are very complex[8]. Among these types of biomechanical stress, shear stress and strain stress are the major components[9]. Many studies have determined that these two stresses contribute to the repair of vessel lesion, as well as vessel injury and remodeling[10,11]. Importantly, they are involved in the process of rearrangement of vascular ECs and smooth muscle cells (SMCs), and they also regulate the differentiation of several types of stem cells, including resident or circulating progenitor cells[12] and stem cells derived from other sources[13]. To more deeply understand the response of stem cells to biomechanical stress and the underlying mechanisms, in this review paper, we summarize the use of stem cells for vascular repair, outline the role of biomechanical stress for vascular injury and repair, and emphasize how shear stress and strain stress regulate the behavior of stem cells for vascular repair. Furthermore, the transduction of biomechanical signals into stem cells are discussed.

STEM CELLS FOR VASCULAR REPAIR

In the formation of blood vessels of angioblasts during embryogenesis, the peripheral endothelial progenitor cells (EPCs) and inner hematopoietic stem cells (HSCs) form blood islands and they participate in vascular repair[14]. In the process of vascular repair, quiescent stem cells such as HSCs and mesenchymal stem cells (MSCs) mobilize from bone marrow into the circulation, differentiate into EPCs (as circulating EPCs), and are home to the area of lesion (where they can contact and sense the blood flow) to participate in neovessel formation[15] (Figure 1). This phenomenon indicates that neovascular formation in adults may be the result of proliferation, migration, and remodeling of stem cells.

Several studies have shown that $CD34^+CD133^+$ EPCs can differentiate into ECs and enhance angiogenesis in injury vessels, as well as grafted vessels[16,17]. Endothelial colony-forming cells as a rare population of ECs, can be isolated from peripheral blood mononuclear cells and share characteristics with EPCs, including expression of the endothelial marker and vessel regeneration ability[18]. In addition, MSCs derived from bone marrow can differentiate into a variety of cell types and contribute to vascular reconstruction[3]. Beyond that, MSCs derived from umbilical cord, adipose tissue, dental pulp, and hair follicle also have the potential to differentiate into EPCs

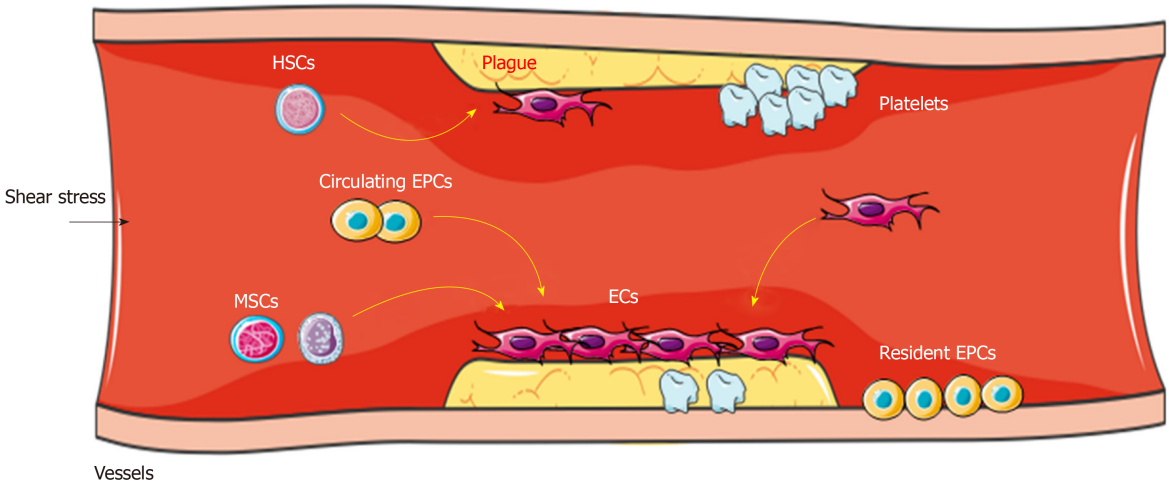

Figure 1 In pathological status, several types of stem cells contribute to the process of vascular repair. Stem cells such as hemopoietic stem cells, MSCs, circulating EPCs (derived from bone marrow), and resident EPCs are able to sense the activation signals (such as altered shear stress, acute ischemic, and anoxic injury). Then they move to the sites of lesion, differentiate into ECs, produce paracrine signals such as VEGF, PDGF, and SCDF; these actions take part in the vessel regeneration. Importantly, the resident ECs can be activated to proliferation types with a high turnover rate. HSCs: Hemopoietic stem cells; MSCs: Mesenchymal stem cells; EPCs: Endothelial stem cells; ECs: Endothelial cells; VEGF: Vascular endothelial growth factor; PDGF: Platelet-derived growth factor; SCDF: Stromal cell-derived factor.

in injury conditions[18-20].

At the beginning of vessel repair, a part of EPCs directly incorporate into vessel intima and differentiate into ECs with active angiogenesis, while the other part of EPCs display a proliferative potential[21]. The mechanism of EPCs promoting the angiogenesis varies greatly, including the direct formation of neovessels and the production of paracrine signals such as vascular endothelial growth factor (VEGF), stromal cell-derived factor, and platelet-derived growth factor (PDGF), which further activate the proliferation and vascular repair of ECs[22]; this process depends on the recognition of markers on the cell surface. These findings suggest that vascular repair is probably induced by the interaction between stem cells and the certain microenvironment of injured vessels such as biomechanical stresses. Recently, stem cells combined with mechanical forces have been used in research and the clinic. For instance, the functional vessels constructed with scaffold and stem cells have the potential to promote stem cell differentiation into ECs during vessel grafting or damage[23]. *In vitro* experiments have found that the decellularized vessel scaffold surrounded by stem cells on both inner surface and the adventitial side can sense biomechanical forces under the pressure-driven perfusion with medium. Then these cells differentiate into both ECs and SMCs, which are induced by shear stress and strain stress respectively, in the bioengineered vessels[23-25]. A study reported that vascular grafts *via* EPCs seeding and maturation can rely on a controllable flow formed by bioreactor[26]; this strategy may be beneficial for utilizing EPCs in vascular repair.

BIOMECHANICAL STRESS FOR VASCULAR INJURY AND REPAIR

Studies on biomechanical forces have focused on their role in balancing the microenvironment of vessel, which is closely related to the vascular injury and repair[27]. Blood flow consists of two types: laminar and turbulent flows. There are three types of laminar flow, namely steady flow, pulsatile flow, and oscillatory flow. Among them, steady flow does not occur in arteries, while pulsatile and oscillatory flows are unsteady. In straight arterial areas, ECs are exposed to pulsatile shear stress generally between 10-20 and 40 dynes/cm^2 as maximum[28]. In branch points, bifurcations, and curvatures, ECs are exposed to oscillatory shear stress of ± 4 dynes/cm^2, where they easily develop atherosclerosis[28,29] (Figure 2). In physiological conditions, vessel intima is subjected to a fluid shear stress (average 10-20 dynes/cm^2) caused by blood flowing[28].

The ECs display a fast turnover rate in certain regions such as bifurcations, branch

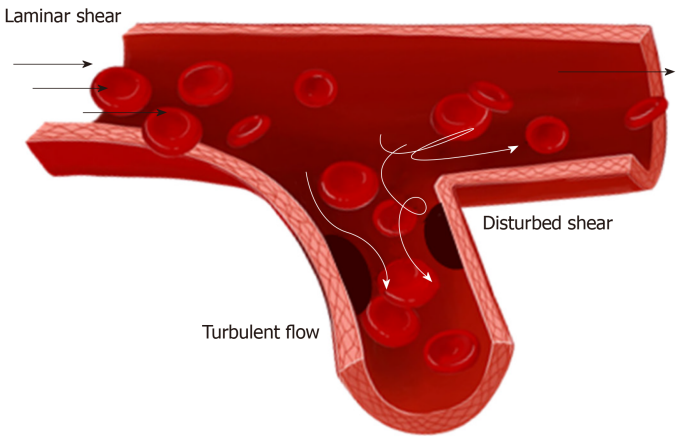

Figure 2 Different patterns of blood flow in vessels. Arterial flow patterns are exquisitely sensitive to vessel geometry including branching, bifurcation, and curvature. The ECs are exposed to turbulent flow or disturbed shear in the branch points where the atherosclerosis easily developed.

points, and curved regions, while overall rates of cell turnover in the artery are very low because ECs experience various flow patterns[30]. Indeed, areas of low shear stress in human arteries have a relatively high rate of endothelial death, supporting the statement that high turnover rate of ECs is crucial to maintain vessel homeostasis[31]. Low and oscillatory shear stress is thought to play a causative role in endothelial dysfunction[32]. It is generally believed that endothelial dysfunction/loss is a common characteristic of vascular injury, which may cause severe cardiovascular disease such as atherosclerosis, hypertension, thrombosis, and ischemia/reperfusion injury[32,33].

ECs with a variety of receptors can sense the altered flow and transmit mechanical signals through mechanosensitive signaling pathways, then activating a series of signaling cascades and cell events. Several potential mechanosensors including ion channels, cell surface or cytoplasmic receptors, integrins, kinases, and extracellular matrix components have been well determined[34,35]. When the blood flow changes, the mechanosensors quickly sense the signal, transduce to the downstream, and activate a series of cascades, finally triggering the physiological response including atherosclerosis, proliferation, angiogenesis, and inflammation[5,36]. Recently, the influence of biomechanical stresses in EPCs, MSCs, and other types of stem cells has been investigated. Mechanical stresses have been shown to increase the proliferation, differentiation, motivation, and recruitment of EPCs in the process of vascular repair[37] (Figure 3). In addition, cell functions are influenced by biomechanical stress including the activation of flow-sensitive ion channels, increased cell membrane permeability, release of several types of agonists (adenosine triphosphate, acetylcholine, and nitrous oxide), and mobilization of intracellular calcium (Ca^{2+}), which keep the homeostasis of vascular system[38,39]. Subsequently, the following responses are triggered: increased cyclic guanosine monophosphate levels, cytoskeletal deformation, activation of mitogen-activated protein (MAP) kinase signaling cascades, transcription factors nuclear factor-kappa B (NF-κB) and nuclear factor activator protein-1[40]. Meanwhile, mechanical stresses regulate the expression of critical vasoactive and growth factors such as endothelin-1, nitric oxide synthase, PDGF A and B chains (PDGF-A and PDGF-B), and transforming growth factor β1, which have protective roles against atherosclerosis[41].

THE RESPONSE OF STEM CELLS TO SHEAR STRESS

Over the past several years, it has been suggested that EPCs and other types of stem cells are home to the area of vascular damage to re-establish an intact endothelial layer after endothelium injury or damage[42]. Among various mechanical stimuli, shear stress is a critical factor to stimulate stem cells and activate downstream signaling. As a physical stimulus, shear stress plays a crucial role in signal transduction at focal adhesions, where the cell-extracellular matrix contacts[43]. In recent decades, researchers have explored the potential signaling pathways, but the precise mechanism is still unclear.

Once cells are stimulated by shear stress, at the early stage of response to the shear stress, several kinds of cells transform it to biochemical signals, and transmit into the nucleus to tune their physiological response. Several mechanosensitive molecules

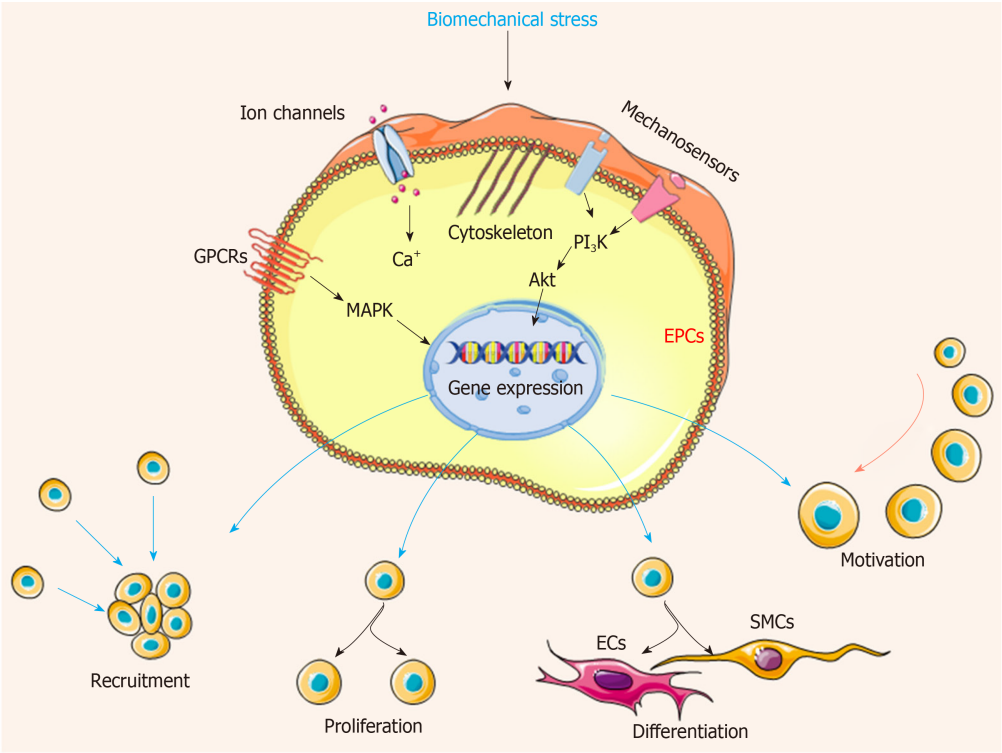

Figure 3 Biomechanical stress stimulates the mechanosensitive molecules on the EPC surface, and then induces cytoskeleton rearrangement and activates series of downstream signaling pathway. The following cascades lead to change of gene expression, ultimately influencing the cell fundamental activities including cell proliferation, differentiation, recruitment, and motivation, depending upon the need of vessel repair. EPCs: Endothelial progenitor cells; ECs: Endothelial cells; SMCs: Smooth muscle cells. The black arrow points out the direction of stress.

and/or compounds serve as "gatekeepers" in the whole process. The overall mechanosensors and the downstream are descripted in Figure 4.

Integrin has been verified to work as a mechanosensor[44]. It can be activated by shear stress and it accumulates at the vascular peripheral areas and is located along the stress fibers. Integrin and the associated RhoA small GTPase have been confirmed to participate in the process of sensing the shear stress and converting it to cascades of molecular signaling, which modulate gene expression[45]. These changes are involved in the process of anti-apoptosis, arrest of cell cycle, and morphological remodeling[46]. Inhibition of integrin β1 suppresses the formation of focal adhesions, which reversely verifies its role in vascular repair[20,47]. As one of the small G proteins, Ras is the earliest link between mechanical perception and transduction; Ras affects the downstream signal transduction cascades, which can be activated by integrin β1-related signals[45]. Then the above process is mediated by G protein-dependent activation of extracellular signal-regulated kinase (ERK) and JNK[48]. It has been demonstrated that Rho, Cdc42, and Rac (belonging to the Ras super-family of proteins) can modulate cytoskeletal rearrangement, EPC differentiation, and permeability of the endothelium after shear stress is applied[45].

Junctional adhesion receptors also play important roles in mechanoresponses[49,50]. VEGF receptor-2 (VEGFR-2) is required for the activation of most biomechanical stress-dependent signaling pathways[51,52]. VEGFR-2 can be activated by shear stress in a ligand-independent manner without the involvement of VEGF *via* two distinct signaling pathways in ECs: the phosphoinositide 3-kinase (PI3K)-Akt pathway and the protein kinase C-mitogen-activated protein kinase-ERK (PKC-MAPK-ERK). The PI3K-Akt pathway is a key regulator in shear stress-induced endothelial differentiation of EPCs[53,54]. In addition, suppression of the shear stress-induced phosphorylation of VEGFR2 by the VEGFR kinase inhibitor SU1498 abolishes the induction of EPC differentiation into vascular ECs; the inhibitor SU1498 blocks the shear stress-induced Notch cleavage in EPCs and suppresses expression of ephrinB2, which exerts a functional role in vascular repair[54]. It has also been reported that VE-cadherin works as an adapter, which is polarized activation of Rac1 in response to shear stress[55]. These results indicate the ability to repair damage vessels of EPCs by differentiation, and this ability has been verified using animal models[56].

PKC-MAPK-ERK is regulated by glycocalyx, which works as soon as the blood flow is initiated, and this signal activation is required for normal vascular

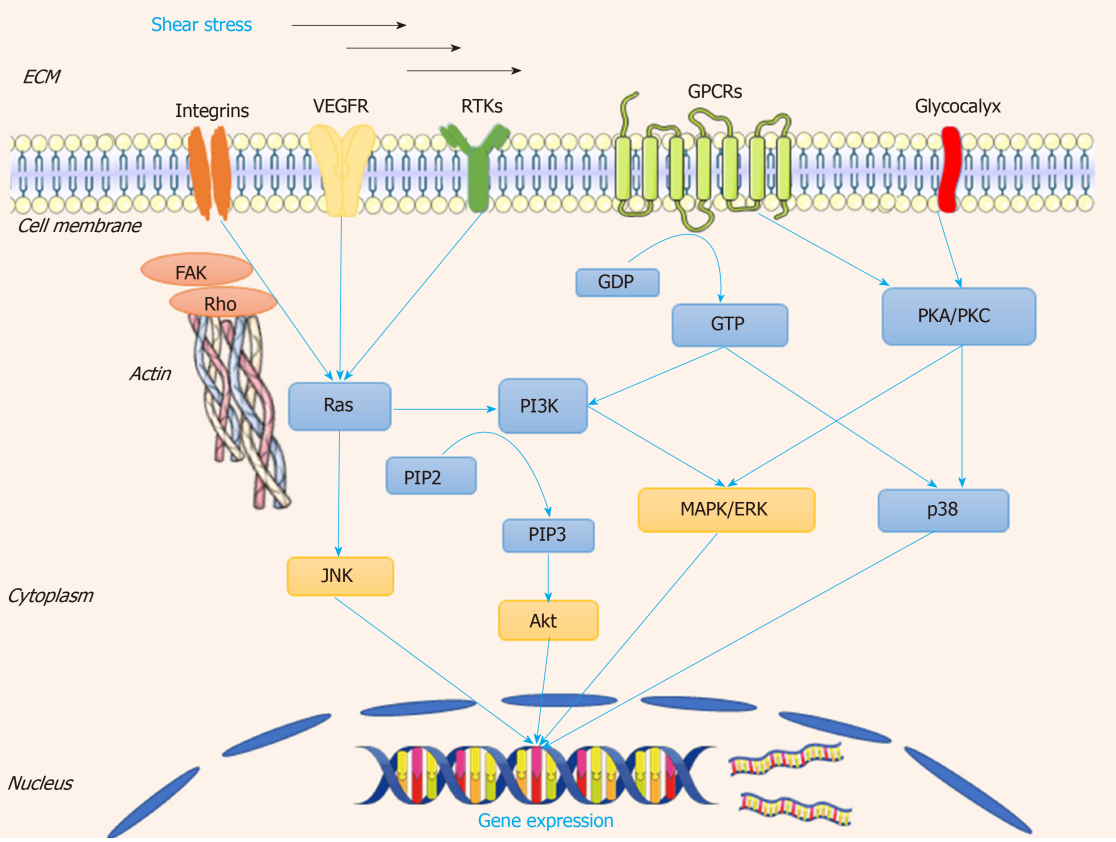

Figure 4 Transduction patterns of mechanical signals into the biological signals, when the shear stress is applied to the cellular surface. This figure describes how the mechanosensitive molecules such as integrins, VEGFR, RTKs, and GPCRs sense the shear stress, induce cytoskeleton reorganization, and activate various signaling pathways including Ras-PI3K-Akt, Ras-JNK, and PKC/PKA-MAPK-Akt. These signaling cascades influence the gene expression of EPC, ultimately regulating vessel maintenance and reformation. VEGFR: Vascular endothelial growth factor receptor; GPCR: G protein-coupled receptor; RTKs: Receptor tyrosine kinases; PKC: Protein kinase C; MAPK: Mitogen-activated protein kinase; ERK: Extracellular signal-regulated kinase.

development[57]. Previous studies have shown that in arteries, glycocalyx components are synthesized much more quickly under high shear stress than under low shear stress[58,59]. In a model of three-dimensional collagen-1 gel culture of SMCs, the flow-induced mechanotransduction could be sensed by glycocalyx biosynthesis, and then activated FAK-ERK1/2-C-Jun signaling pathway, leading to the up-regulation of MMP expression, cell migration and motility[60].

G protein-coupled receptors (GPCRs) show the ability to sense fluid shear stress, and the precise molecular mechanisms of mechanotransduction has been extensively studied[61,62]. Several GPCRs such as angiotensin II receptor type1 and bradykinin receptor B2 work as mechanosensors in vascular physiology[63]. It has been demonstrated that acute shear stresses induce various downstream signaling pathways such as phospholipase C, which further increases the intracellular Ca^{2+} concentration[64].

Endothelial injury is associated with activation of the coagulation system and recruitment of platelets[65]. The areas abundant with platelets promote recruitment and homing of EPCs which further lead to vessel formation[66]. Platelet EC adhesion molecule contains an immunoreceptor tyrosine-based inhibitory motif, which becomes the phosphorylated form when responding to shear stress, and directly induces the activation of ERK[67]. It was found that shear stress regulates the expression of endothelial markers von Willebrand factor and platelet EC adhesion molecule 1 in late EPCs, resulting in cytoskeletal arrangement, cell differentiation, and the activation of various mechanosensitive molecules including integrin β1, Ras, ERK1/2, paxillin, and FAK[68,69]. Mechanosensitive PPAP2B, an integral membrane protein involved in maintaining vascular integrity and EC rearrangement, is reduced as the result of low shear stress caused by vessel plaques[70].

Except EPCs, there are other types of stem cells involved in the process of vascular repair, for instance MSCs and EPCs derived from adipose[18], liver[71] and muscle[72]. It was found that when exposed to laminar shear stress at 0.5 dynes/cm^2 with 30 min, MSCs contribute to the lack of polarity and upregulation of β-catenin downstream proteins, which are associated with cardiovascular development, EC protection, and

angiogenesis[73]. When vascular injury occurs, MSCs resident in the medial intima of a healthy vessel can migrate to damaged areas and differentiate into SMCs[12]. Additionally, ECFCs isolated from the white adipose possess large expansion potential, stable differentiation, and robust *in vivo* vessel-forming capacity[18].

THE RESPONSE OF STEM CELLS TO STRAIN STRESS

The vascular wall is subject to cyclic stretch of about 100-150 kPa, which is generated by the pulsatile blood pressure[74]. The excessive and pathological mechanical stretch occurring during hypertension is harmful as these high magnitude strain stress perturbs the vascular tone and causes improper cellular response of vascular wall, leading to cardiovascular diseases[32].

Venous bypass grafting is one of the most commonly used surgery for atherosclerosis patients; the insertion of a grafted vein into the arterial system probably exposes the vascular wall to the new hemodynamic environment, which has been considered to be a critical stimulus for vascular remodeling[74]. Cyclic strain stress generated after venous bypass grafting have been reported to regulate and change the functions of vascular smooth muscle cells (VSMCs) such as excessive proliferation, differentiation, and apoptosis[75].

The cyclic strain is generated by the pulsatile of flow blood throughout one cardiac cycle to ensure SMCs within the wall maintain an active and contractile status[76]. Several membrane proteins or compounds have been found to be mechanosensitive to stretch, consisting of integrins, G-proteins, receptor tyrosine kinases (RTKs), and ion channels[77]. The overall signaling pathway is described in Figure 5.

Similarly, under the condition of shear stress, integrin molecules are involved in the pathway of extracellular matrix-integrin-cytoskeleton[78]. Normally, the strain stress applied to focal adhesion activates integrins and its downstream cascades including the focal adhesion kinase, G-proteins, Rho, and various signaling pathways related to stem cell differentiation[79]. A previous study showed that strain stress could induce stem cell-derived Sca-1$^+$ progenitors to differentiate into SMCs *via* collagen IV-integrin-FAK-PI3-kinase-MAP kinase and PDGF receptor-beta signaling pathways[80].

G-proteins are another type of important mechanosensors in response to biomechanical stresses. Strain stress on cells allows structure changes in the G-proteins receptors, transducing mechanosignal into chemical signal and activating further signaling cascades[62]. These alternations may be related to iron channels and RTKs, which are considered to be the regulators for the development of stem cells. Thompson *et al*[81] found a novel mechanical target-mTORC2, which is critical for the proliferation, adipogenic, and cytoskeletal architecture of MSCs. Activation of mTORC2 requires focal adhesions and the binding of Fyn and FAK in vascular repair through the similar signaling pathway[81].

Ion channels also regulate the transduction of cyclically mechanical strain. As a ubiquitous secondary messenger, Ca^{2+} connects the inside and outside of cell, to maintain the homeostasis of cellular microenvironment. A study reported that VEGF-induced Ca^{2+} oscillations promote EPC growth and tubulogenesis by activating NF-κB[82,83]. It has been revealed that the ion channels stimulated by stretch induce Ca^{2+} influx in VSMCs and active the PKC signaling pathway, enhancing VSMC migration to lesion areas and accelerating the wound closure[84]. Interactions are present between these mechanosensors to strengthen the impacts of strain stress on activating the stem cells. TPRV4 ion channels can be activated by cyclic stretch, leading to cytoskeletal remodeling and cell reorientation *via* integrin-PI3K signaling[85]. Thus, strain stress can stimulate several types of mechanosensors, transmit biomechanical signaling into the nucleus, and regulate the related gene expression.

SUMMARY AND PERSPECTIVES

In this review, we first summarized the role of stem cells in vascular repair and then discussed the responses of stem cells to biomechanical stress and the underlying mechanisms. As the direct stimuli of vessel walls, mechanical forces play a crucial role in vascular injury and repair, which can directly activate the mechanosensing molecules. Mechanosensors of stem cells such as integrins, ion channels, GPCRs, RTKs, and VEGFR are able to sense the mechanical stresses and then are involved in the cytoskeleton rearrangement and finally regeneration of the endothelium. Manipulation of stem cells' mechanosensors should be beneficial for vascular repair in clinics and the development of new therapeutic strategies. Therefore, identification of the mechanosensors and a full understanding of the molecular mechanism are

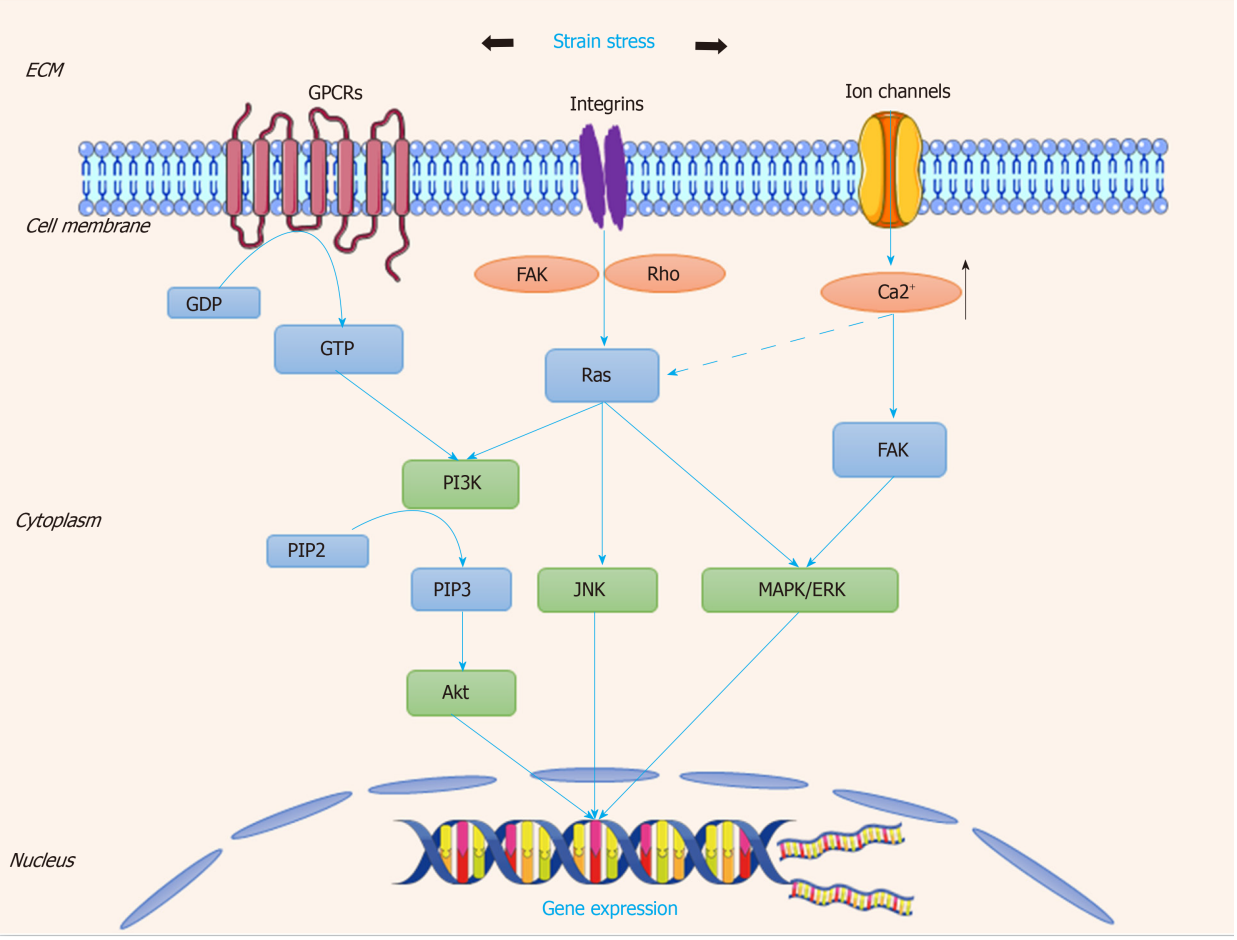

Figure 5 Transduction patterns of mechanical signals into the biological signals, when the strain stress is applied onto the cellular surface. This figure describes how the mechanosensitive molecules such as integrins, GPCRs, and ion channels sense the strain stress and trigger downstream signaling cascades, then initiate transcription and translation of various genes for cell events in the process of vessel repair. GPCR: G protein-coupled receptor; RTKs: Receptor tyrosine kinases; PKC: Protein kinase C; MAPK: Mitogen-activated protein kinase; ERK: Extracellular signal-regulated kinase.

essential to design effective treatments.

Many authors have proposed that increasing the number of stem cells is necessary to achieve sufficient vascular recovery and regeneration; hence, the safe and effective strategies to obtain enough number of stem cells which maintain the mechanical sensing potential are still a major challenge for the basic scientists and surgeons. Stem cells represent a promising tool for mechanical stresses sensing in the vasculature, but the methods to activate the resident and circulating stem cells and the underlying mechanisms for vascular repair remain unclear. The deeper understanding of how stem cells respond to mechanical forces should open a new dimension for the treatment of vascular disease, and enhance the clinical translation of stem cell-based strategy.

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ORIGINAL ARTICLE

Basic Study

Anti-osteoarthritis effect of a combination treatment with human adipose tissue-derived mesenchymal stem cells and thrombospondin 2 in rabbits

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Author contributions: Shin K, Cha Y, and Ban YH performed animal experiments and assembled histological samples; Seo DW performed the cell differentiation study; Choi EK and Park D collected and analyzed the data; Kang SK prepared and characterized the stem cells; Ra CJ and Kim YB designed and interpreted the study, and wrote the manuscript.

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Abstract

BACKGROUND

Osteoarthritis (OA), a chronic age-related disease characterized by the slowly progressive destruction of articular cartilage, is one of the leading causes of disability. As a new strategy for treatment of OA, mesenchymal stem cells (MSCs) have the potential for articular cartilage regeneration. Meanwhile, thrombospondin 2 (TSP2) promotes the chondrogenic differentiation of MSCs.

AIM

To investigate whether TSP2 induces chondrogenic differentiation of human adipose-derived MSCs (hADMSCs) and potentiates the therapeutic effects of hADMSCs in OA rabbits.

METHODS

We investigated the chondrogenic potential of TSP2 in hADMSCs by analyzing the expression of chondrogenic markers as well as NOTCH signaling genes in normal and TSP2 small interfering RNA (siRNA)-treated stem cells. Anterior cruciate ligament transection surgery was performed in male New Zealand white rabbits, and 8 wk later, hADMSCs (1.7×10^6 or 1.7×10^7 cells) were injected into the injured knees alone or in combination with intra-articular injection of TSP2 (100 ng/knee) at 2-d intervals. OA progression was monitored by gross, radiological, and histological examinations.

Institutional Animal Care and Use Committee of Laboratory Animal Research Center at Chungbuk National University (Chungbuk, Korea).

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RESULTS

In hADMSC culture, treatment with TSP2 increased the expression of chondrogenic markers (SOX9 and collagen II) as well as NOTCH signaling genes (JAGGED1 and NOTCH3), which were inhibited by TSP2 siRNA treatment. *In vivo*, OA rabbits treated with hADMSCs or TSP2 alone exhibited lower degree of cartilage degeneration, osteophyte formation, and extracellular matrix loss 8 wk after cell transplantation. Notably, such cartilage damage was further alleviated by the combination of hADMSCs and TSP2. In addition, synovial inflammatory cytokines, especially tumor-necrosis factor-α, markedly decreased following the combination treatment.

CONCLUSION

The results indicate that TSP2 enhances chondrogenic differentiation of hADMSCs *via* JAGGED1/NOTCH3 signaling, and that combination therapy with hADMSCs and TSP2 exerts synergistic effects in the cartilage regeneration of OA joints.

Key words: Osteoarthritis; Anterior cruciate ligament transection; Human adipose tissue-derived mesenchymal stem cell; Thrombospondin 2; Notch signaling; Cartilage regeneration

Core tip: We demonstrated the role of thrombospondin 2 (referred to as TSP2) in the chondrogenic differentiation of human adipose-derived mesenchymal stem cells *in vitro* and in osteoarthritis therapy with the cells *in vivo*. TSP2 induced chondrogenic differentiation *via* JAGGED1/NOTCH signaling, and potentiated the cartilage-restoring efficacy of human adipose-derived mesenchymal stem cells.

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INTRODUCTION

Osteoarthritis (OA) of the knee is the most common form of arthritis, which causes pain, stiffness, and decreased function. OA is characterized by the degeneration of articular cartilage, mainly due to change in the activity of chondrocytes in favor of catabolic activity as well as reduced cartilage cellularity[1,2]. The capacity of adult articular chondrocytes to regenerate the normal cartilage matrix architecture declines with aging, due to cell death and abnormal responsiveness to anabolic stimuli. OA chondrocytes lose their capacity to secrete the specific components of the extracellular matrix (ECM), such as type II collagen (collagen II) or aggrecan. Currently, no treatment capable of markedly altering the progression of OA exists and therapeutic options are essentially pain management and surgical intervention[3]. Indeed, new innovative therapeutic strategies for cartilage protection/repair are currently being evaluated mainly based on stem cell-mediated approaches.

Mesenchymal stem cells (MSCs) isolated from various tissues such as bone marrow, adipose tissue, and umbilical cord blood are capable of self-renewal and can differentiate into chondrogenic lineage cells *in vitro* and *in vivo*[4,5]. Besides this property, MSCs produce diverse secretory factors such as growth factors (GFs) and neurotrophic factors, cytokines, and chemokines that mediate paracrine functions including tissue protection/regeneration and immunomodulation[6,7].

Compared with bone marrow-derived MSCs (BMMSCs), adipose tissue-derived MSCs (ADMSCs) are more genetically stable in a long-term culture, and display a lower senescence ratio and higher proliferative capacity[8]. Recently, some studies have shown that autologous ADMSCs improve the function and pain of knee joints without causing adverse events and reduced cartilage defects by chondrocyte

regeneration in animal models of OA and in humans[9-11].

Thrombospondin 2 (TSP2) is well known as a multi-functional protein with anti-angiogenic property that interacts with the ECM and various binding partners[12,13]. Recent studies have indicated that TSP2 is also involved in cartilage biology. TSP2 is expressed in chondrocytes[14], and its deficiency results in connective tissue abnormalities in mice[15]. In embryonic and adult mice, TSP2 is distributed in the areas of chondrogenesis. Moreover, it was previously demonstrated that TSP2 from human umbilical cord blood-mesenchymal stem cells (hUCBMSCs) promote chondrogenic differentiation of chondroprogenitor cells through paracrine action[16].

Meanwhile, various studies have shown that NOTCH signaling plays a role in chondrogenic differentiation[17-21], and that NOTCH receptors are expressed in articular cartilage and are involved in chondrogenesis[22,23]. Recently, several studies have characterized signaling pathways involved in chondrogenic differentiation of MSCs[24,25]. Indeed, chondroprogenitor cells and MSCs express NOTCH signaling components. Systemic inhibition of NOTCH leads to reduced cartilage formation *in vivo*[26], and NOTCH inhibition by N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT) results in blockage of chondrogenesis *in vitro*[27,28]. Similarly, it has been observed that TSP2 enhances chondrogenic differentiation of chondroprogenitor cells *via* NOTCH signaling, which is inhibited by DAPT treatment[16].

In the present study, the effect of TSP2 on chondrogenic differentiation of human ADMSCs (hADMSCs) was confirmed using TSP2 small interfering RNA (siRNA)-treated hADMSCs *in vitro*. Furthermore, based on the effectiveness of hADMSCs and TSP2 in cartilage regeneration, their synergistic effects on clinical improvement in a rabbit model of experimental OA were investigated.

MATERIALS AND METHODS

Preparation of hADMSCs

All human tissues were obtained with approval of the Institutional Review Board of K-Stem Cell (Seoul, Korea) (Protocol No. KS-2015-01-001). hADMSCs were prepared under Good Manufacturing Practice conditions (Biostar Stem Cell Research Institute of R-BIO Co., Ltd., Seoul, Korea). In brief, human abdominal subcutaneous fat tissues were obtained by simple liposuction from a 53-year-old female donor after obtaining informed consent[29]. The adipose tissues, after digestion with collagenase I, were suspended in Dulbecco's modified Eagle's medium (Invitrogen, Grand Island, NY, United States) containing 0.2 mM ascorbic acid and 10% fetal bovine serum (FBS). After overnight culture, the cell medium was changed to Keratinocyte-SFM (serum-free keratinocyte medium; Invitrogen) containing 0.2 mM ascorbic acid, 0.09 mM calcium, 5 ng/mL recombinant epidermal growth factor, and 5% FBS. When the cells reached 90% confluency, they were subculture-expanded in Keratinocyte-SFM medium until passage 3. FBS from cultured stem cells was completely removed by several washes with phosphate-buffered saline (PBS), which was verified by testing the albumin level below the measurement limit using a bovine albumin enzyme-linked immunosorbent assay (ELISA) quantification kit (Bethyl Laboratories, Montgomery, TX, United States).

siRNA treatment

TSP2 siRNAs were used to knock down TSP2 expression in hADMSCs during chondrogenic differentiation. TSP2 siRNAs (Thermo Fisher Scientific, Lafayette, CO, United States) at a final concentration of 25 nM were transfected into hADMSCs using DharmaFECT reagent 1 (Thermo Fisher Scientific) according to the manufacturer's instructions. Transfected hADMSCs were maintained for 24 h in 10% FBS-containing medium.

In vitro chondrogenic differentiation

Chondrogenesis was performed in micromass pellet cultures. hADMSCs (2×10^5 cells) were harvested, resuspended in STEMPRO® Chondrogenesis Differentiation Kit medium (Life Science, Carlsbad, CA, United States) in a 15 mL polypropylene tube, and centrifuged at 500 g for 5 min at room temperature. TSP2 siRNA-transfected hADMSCs were mixed with various concentrations (0, 10 or 100 ng/mL) of recombinant human TSP2 (R&D Systems, Minneapolis, MN, United States). The cell pellets were cultured in chondrogenic medium and maintained (37 °C, 5% CO_2) for 14 d, in which the medium was replaced with fresh medium every 3 d.

Real-time reverse transcription-PCR

Total RNA was isolated from hADMSCs culture pellets using TRIzol® reagent (Life

Technologies, Carlsbad, CA, United States). RNA (1 μg) was reverse-transcribed into complementary DNA using SuperScript® III First-Strand Synthesis System (Life Technologies). Glyceraldehyde-3-phosphate dehydrogenase was used as a reference gene. Real-time PCR was performed using Power SYBR® Green PCR Master Mix (Life Technologies). The primers used for real-time PCR are described in Supplementary Table 1 (Genotech, Daejeon, Korea).

Immunohistochemistry

Chondrogenic pellets of hADMSCs and TSP2 siRNA-treated hADMSCs were fixed for 24 h in 4% paraformaldehyde, frozen in OCT compound, and 5-μm cryosections were prepared. For immunohistochemistry, non-specific antibody-binding sites were blocked by incubating the slides in PBS with 5% bovine serum albumin (BSA) for 1 h. The slides were incubated overnight at 4 °C with primary antibody against SOX9 (1:200, rabbit monoclonal; EMD Millipore, Temecula, CA, United States), JAGGED1 (1:200, mouse monoclonal; Santa Cruz, Dallas, TX, United States), NOTCH3 (1:200, mouse monoclonal; Abnova, Taipei, Taiwan) or collagen II (1:200, mouse monoclonal; EMD Millipore) diluted in 0.1% Tris-buffered saline with Tween-20 with 1% BSA. Secondary antibodies conjugated with Alexa Fluor-488 or -594 (1:200, mouse polyclonal; Life Technologies) for 2 h at room temperature. All pellets were examined immediately after staining and photographed with a laser-scanning confocal microscope (LSM710; Zeiss, Oberkochen, Germany).

In vivo experimental design

Adult male New Zealand white rabbits weighing 3.2 ± 0.4 kg were obtained from Daehan-Biolink (Eumseong, Korea). The animals housed in a room with a constant temperature (22 ± 2 °C), relative humidity of 55 ± 10%, and a 12-h light/dark cycle. The rabbits were fed standard chow and purified water *ad libitum*.

OA was induced surgically *via* anterior cruciate ligament transection (ACLT) of right hind knee, except sham control rabbits, under inhalation anesthesia with isoflurane (Sigma-Aldrich, St. Louis, MO, United States). For the ACLT procedures, a 4-cm skin and capsular incision was carried out and right ACLs were exposed through a medial para-patellar cut. To achieve optimal visualization of the ACLs, the patellar bone was displayed laterally and the knee was placed in full flexion. ACL removal was performed by cutting its attachment on the medial aspect of the lateral femoral condyle. The stifle was moved in a drawer test to ensure that the entire cruciate ligament had been excised. The incision was sutured in a routine fashion. After each operation, antibiotic (Foxolin®; Samjin Pharm, Seoul, Korea) and analgesic (Maritrol®; Jeil Pharm, Daegu, Korea) treatments were given immediately after the surgery and for 3 d thereafter. All surgical procedures were performed under general anesthesia and sterile conditions.

After ACLT surgery, the rabbits (n = 6/group) were subjected to a forced-exercise (walking) for 15 min every day 5 d a week for 8 wk to aggravate OA. The OA rabbits were randomly divided into five groups, and treated with hADMSCs (1.7×10^6 or 1.7×10^7 cells/0.5 mL/knee) and/or TSP2 (100 ng/0.1 mL/knee). hADMSCs were transplanted once, and TSP2 was injected intra-articularly at 2-d intervals into the hind limb joints underwent ACLT for 8 wk, during which the joints were X-ray-photographed and synovial fluid was collected. Animals were sacrificed 8 wk after hADMSCs administration to investigate the effects of stem cells and TSP2 on the different compartments of the knee joint. Femoral condyles and tibial plateau were isolated for gross and microscopic examinations. All protocols and procedures of animal experiments complied with the Institutional Animal Care and Use Committee of Laboratory Animal Research Center at Chungbuk National University, Korea (Approval No. CBNUA-743-14-01).

Analysis of proinflammatory cytokines in synovial fluid

At 1, 2, 4, and 8 wk after cell transplantation, synovial fluid was collected from ACLT knees of rabbits using sterile techniques. After centrifugation to remove cellular debris, the samples were analyzed for tumor-necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and IL-6 by commercially available ELISA kits (TNF-α: E-EL-RB0011, Elabscience, St. Charles, MO, United States; IL-1β: E-EL-RB0013, Elabscience; IL-6: E-EL-RB0014, Elabscience), according to the manufacturer's instructions.

Radiological evaluation

Knee joint radiographs were taken with a portable X-ray machine (Rextar EXO1414; Posdion, Seoul, Korea) before and 2, 4, and 8 wk after cell transplantation. For antero-posterior view, rabbits were placed in dorsal recumbency with legs extended caudally. Radiological OA was assessed by means of the Kellgren and Lawrence (KL) grading system[30]. This system is based on radiological features including the

presence of osteophyte (0-5), deformity of subchondral bone (0-5), and sharpening of tibial plateau (0-5).

Gross examination

After sacrifice, macroscopic assessment of the knee joint surfaces of the rabbits was performed to score cartilage lesions. The scored was based on the protocol of the International Cartilage Research Society (ICRS) for cartilage repair[30,31] using criteria of roughness of surface (0-8), adhesion with meniscus (0-8), hypertrophy of joint (0-8), and osteophyte (0-8).

Microscopic examination

After gross examination on the joint surfaces, distal femurs were excised with the knee joints intact. All samples were fixed in 10% neutral buffered formalin and decalcified in 10% EDTA-buffered saline (pH 8.0). Five-μm paraffin-embedded sections were stained with hematoxylin and eosin (H&E) or Safranin O/Fast green to assess general morphology and proteoglycan contents, respectively. Separately, collagen II, accounting for about a half of cartilage ECM, was immuno-stained with a specific antibody. The evaluation was scored based on the criteria of the Osteoarthritis Research Society International (OARSI) that include intensity of ECM staining (0-6), structure (0-11), cellularity (0-4), and cluster formation (0-3)[32].

Data and statistical analysis

Data are presented as mean ± standard error of the mean. Statistical significance between groups was determined by one-way analysis of variance followed by post-hoc Tukey's multiple-comparison tests using SAS program (version 6.12). $P < 0.05$ was considered statistically significant.

RESULTS

TSP2-mediated chondrogenesis via NOTCH signaling

The correlation between TSP2 expression and chondrogenic potential of hADMSCs was analyzed by inhibiting the expression of TSP2 with siRNA. Treatment with TSP2 siRNA greatly decreased the expressions of JAGGED1 and NOTCH3 to 12% and 18% of the control levels, respectively (Figures 1-3). Furthermore, the expressions of SOX9 (a chondrogenic transcription factor) and collagen II (a cartilage-specific marker) also markedly decreased in TSP2 siRNA-transfected hADMSCs.

Next, the chondrogenic potential of TSP2 siRNA-transfected hADMSCs pellet cultures was analyzed in the presence of exogenous recombinant human TSP2 during differentiation. Treatment with 10 ng/mL TSP2 significantly recovered the expression of GAGGED1, NOTCH3, SOX9, and collagen II to 53%-71% compared to their control levels. A higher dose (100 ng/mL) of TSP2 fully recovered the expression of NOTCH3, SOX9, and collagen II. The change in mRNA expression was confirmed by immunohistochemical analysis of proteins, *i.e.*, the production of chondrogenic proteins blocked by siRNA was reconstituted by exogenous TSP2 treatment (Figures 2 and 3). From these results, it was suggested that chondrogenic differentiation of hADMSCs should be mediated by TSP2 through NOTCH signaling.

Change in proinflammatory cytokines

In order to evaluate the inflammatory reaction caused by OA progression, the levels of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 in synovial fluid were quantified using ELISA. In ACLT knees, the cytokines markedly increased during 8-wk OA-induction and lasted for an additional 8 wk (Figure 4). Notably, the increases in TNF-α and IL-1β levels in ACLT rabbits were markedly attenuated as early as 1 wk after hADMSC treatment, reaching normal levels with a high dose (1.7×10^7) of hADMSCs. TSP2 also attenuated the increase in TNF-α level. Interestingly, IL-6 was fully suppressed in a short time (1 wk) by hADMSCs or TSP2 treatment. In the combination treatment, TSP2 further enhanced the effect of 1.7×10^6 hADMSCs on TNF-α, but did not affect the effects on IL-1β and IL-6 levels. Taken together, these data indicate that TSP2 lower the levels of TNF-α and IL-6, and hADMSCs have inhibitory capacity on pro-inflammatory cytokines including TNF-α, IL-1β, and IL-6.

Radiological findings

At 8 wk after surgery before treatment with hADMSCs and TSP2, all knees subjected to ACLT showed radiological signs of OA including osteophytes in medial femoral condyle, deformity of medial tibia, and sharpening of the tibial spine. Based on the modified KL grading system, the total lesion scores ranged from 4.8 to 6.5 (maximum score = 15), and further increased in ACLT rabbits without treatment up to a score of

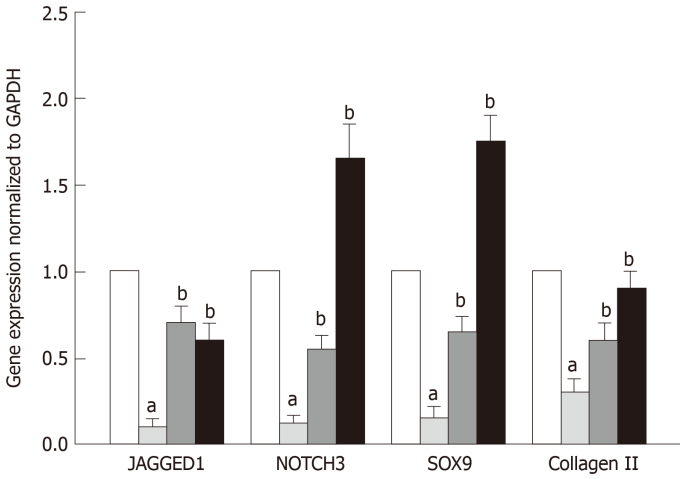

Figure 1 Expression of mRNA of NOTCH signaling and chondrogenic genes in pellet cultures. White: Normal hADMSCs; Light grey: TSP2 siRNA-transfected hADMSCs; Dark gray: TSP2 siRNA-transfected hADMSCs + 10 ng/mL TSP2; Black: TSP2 siRNA-transfected hADMSCs + 100 ng/mL TSP2. [a]Significantly different from normal hADMSCs, $P < 0.05$. [b]Significantly different from TSP2 siRNA-transfected hADMSCs, $P < 0.05$. hADMSCs: Human adipose-derived mesenchymal stem cells; TSP2: Thrombospondin 2.

9.3 ± 0.6 during the additional 8 wk (Figure 5). Representative images of articular osteophytes and deformities and each score of lesions 8 wk after cell treatment are shown in Figure 6 and Table 1, respectively. However, treatment with hADMSCs remarkably suppressed the progression of OA signs in a dose-dependent manner to a score of 7.2 ± 0.3 at a high dose (1×10^7 cells). Notably, TSP2 alone significantly attenuated the OA lesions (score 7.2 ± 0.8), and further enhanced the effects of hADMSCs following combination treatment (score 6.0 ± 0.4).

Gross findings

At 8 wk post-treatment, exposed subchondral bone exhibited large fissures and erosions on lateral femoral condyle and tibial plateau in ACLT rabbits (Figure 7). In addition, severe deformities including osteophytes as well as adhesion of meniscus to cartilage surface were observed, leading to a total ICRS score of 20.0 ± 3.1 (Table 2). In contrast, treatment with a high dose (1.7×10^7) of hADMSCs greatly attenuated the ACLT-induced osteoarthritic lesions to a mean score of 8.5 ± 2.5, although a low dose (1×10^6) of cells did not exert a significant effect. It is of interest to note that TSP2 also significantly protected against OA gross lesions (score, 11.5 ± 4.5), and further improved when co-administered with hADMSCs (score, 7.5 ± 0.5).

Microscopic findings

Histological observations were quantified according to the modified OARSI scale. In sham control cartilage, H&E and Safranin O/Fast green staining as well as collagen II immunostaining demonstrated normal features, *i.e.*, the surfaces of articular cartilage were not fissured nor cracked, and the ECM and cartilage remained intact and dense from superficial to deep zones (Figure 8). In contrast, the articular cartilages in ACLT knees displayed severely rough surface, decreased chondrocytic density, clusters of chondrocytes, and loss of ECM such as proteoglycan (stained red with Safranin O) and collagen II (stained brown with collagen II antibody). The total modified OARSI score of ACLT knees without treatment reached 23.0 ± 5.0 (maximum score = 30) (Table 3). In parallel with the gross lesions, treatment with a high dose (1.7×10^7) of hADMSCs drastically attenuated the ACLT-induced microscopic lesions to a score of 8.5 ± 3.5, in comparison with a minimal effect of a low dose (1×10^6) of the cells. Noteworthily, TSP2 also exhibited a potent activity (score 10.5 ± 2.5), and synergistically enhanced the efficacy of hADMSCs (score 7.5 ± 0.6). Indeed, following treatment with a high dose of hADMSCs or co-administration of a low dose of the cells and TSP2, the improved tissues were hyaline-like and showed thicker and smoother surface, in addition to intact integrity comparable to sham-operated cartilages.

DISCUSSION

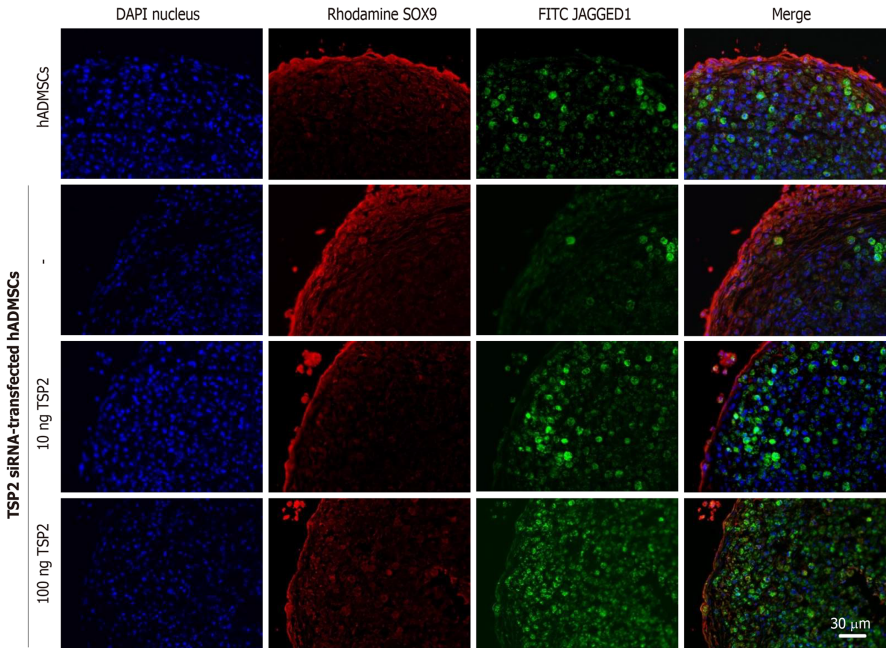

Figure 2 Production of SOX9 (chondrogenic transcription factor) and JAGGED1 (NOTCH ligand) proteins in normal hADMSCs and TSP2 siRNA-transfected hADMSCs treated with TSP2 (10 or 100 ng/mL). hADMSCs: Human adipose-derived mesenchymal stem cells (200 ×); TSP2: Thrombospondin 2.

OA is one of the major chronic diseases leading to musculoskeletal morbidity and functional loss[33]. The etiology of OA is not completely understood. However, trauma, age, and genetic factors have been considered risk factors[34]. The ACLT model is widely validated in investigating OA disease, because it determines similar biomechanical and pathological changes to those seen in humans[35]. Previous studies have proven that 8-20 wk after ACLT in rabbits, the knee joints show diverse signs of OA with cartilaginous damage and osteophytes[36-38]. In the present study, however, we applied the ACLT to adult rabbits followed by daily 15-min forced exercise to induce mechanical inflammation in a relatively short-term period. At 8 wk after ACLT surgery, the rabbit knees displayed moderate osteoarthritic lesions according to the KL grading system in radiological analysis, compared to mild injury without exercise (data not shown).

Many cytokines have been implicated in the pathogenesis of OA. IL-1β inhibits the synthesis of ECM of hyaline cartilage and promotes resorption of the cartilage matrix by stimulating the synthesis of matrix metalloproteases (MMPs), which digest ECM components[39,40]. IL-1β also stimulates chondrocytes and synoviocytes to produce IL-6, which contributes to various manifestations of inflammatory arthropathies[41]. In addition, IL-1β-induced IL-6 secretion may be a required cofactor for the inhibition of proteoglycan synthesis by IL-1β[42]. TNF-α is a macrophage-derived cytokine that, like IL-1β, promotes the degradation of cartilage and suppresses the synthesis of the primary building blocks of cartilaginous matrix, proteoglycans[43]. TNF-α increases IL-1β and MMP synthesis by synovial cells[43]. TNF-α, therefore, promotes cartilage catabolism and decreases matrix synthesis directly or by inducing other cytokines.

In OA knees, all the cytokines markedly increased after ACLT surgery and 8-wk exercise, and were maintained at high levels for an additional 8 wk, which probably facilitated degradation of cartilaginous matrix as shown in the decreased safranin O-stained proteoglycans and collagen II immunoreactivity. Notably, the increases in TNF-α, IL-1β, and IL-6 levels in OA rabbits were drastically attenuated by hADMSCs to near normal levels as early as 1 wk after cell transplantation. Such an early suppression of inflammatory cytokines might be related to the effective protection against the progress of bone and cartilage lesions and deformities as observed by radiological observations. Although we injected the cells when the OA lesions were already formed (8 wk after ACLT surgery) to investigate their therapeutic potential rather than preventive-mode activity, it was demonstrated that earlier injection of MSCs was more beneficial in a donkey model[44].

Interestingly, TSP2 also attenuated the increase in TNF-α level, in addition to full blocking of IL-6 secretion, indicating that TSP2 itself has OA-protective potential *via* anti-inflammatory activity. In the combination treatment, TSP2 further enhanced the effect of low dose (1.7×10^6) hADMSCs on TNF-α without affecting the effects on IL-

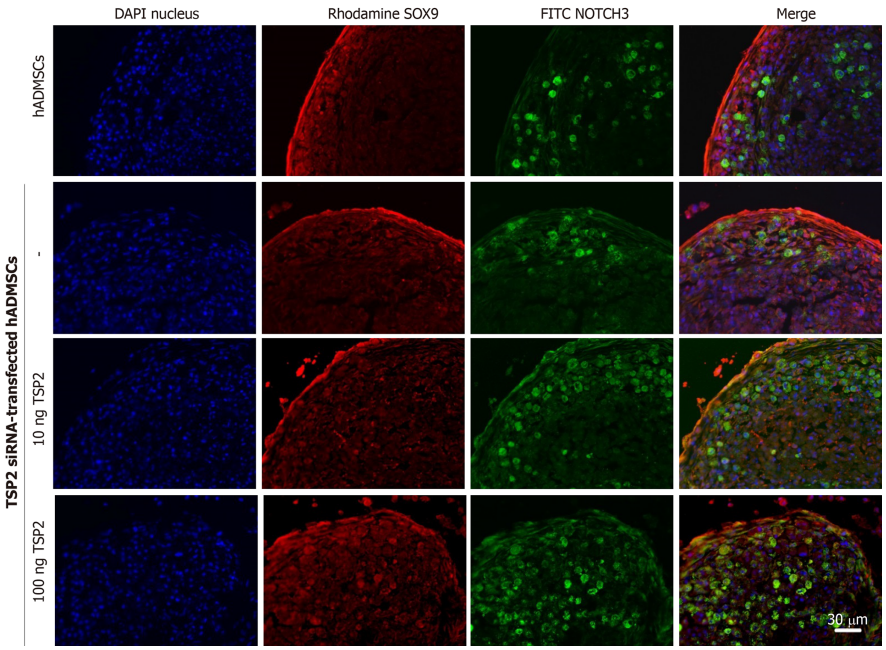

Figure 3 Production of SOX9 (chondrogenic transcription factor) and NOTCH3 (NOTCH receptor) proteins in normal hADMSCs and TSP2 siRNA-transfected hADMSCs treated with TSP2 (10 or 100 ng/mL). hADMSCs: Human adipose-derived mesenchymal stem cells (200 ×); TSP2: Thrombospondin 2.

1β and IL-6 levels. These data suggest that the combination therapy of hADMSCs and TSP2 may be effective in inhibiting the digestion of cartilage ECM by reducing inflammatory cytokines, especially TNF-α.

ADMSCs can be isolated from various organs that have a limited amount of adipose tissue, and are easy to be culture-expanded. Therefore, obtaining autologous ADMSCs, compared to those derived from bone marrow or other tissues, is believed to be less aggressive and quantitatively advantageous[45,46]. In the meantime, the number of ADMSC-based Investigational New Drug applications submitted to the US Food and Drug Administration as well as clinical trials registered worldwide significantly increased from 2011, indicating that adipose tissue is becoming an attractive cell source for MSC-based therapeutic application[47].

It is believed that proliferation of remaining host synovial chondrocytes as well as chondrocytic differentiation of transplanted stem cells are key factors for the restoration of OA cartilages. It is well known that among these GFs, transforming growth factor-β, insulin-like growth factor, and fibroblast growth factor are potent regulators of proliferation and differentiation of chondrocytes[44,45]. Notably, it was recently revealed that TSP2 contributes to the chondrogenic potential of hUCBMSCs for the regeneration of cartilage defects[48]. Moreover, TSP2 is released from hBMMSCs and hUCBMSCs themselves[16], and potentiates NOTCH signaling for self-chondrogenic differentiation[18,20,49,50]. Therefore, it has been hypothesized that the chondrogenic potential of hADMSCs may be enhanced by TSP2 *via* NOTCH signaling.

Indeed, we showed that TSP2 regulated the chondrogenic differentiation of hADMSCs, which was mediated by the NOTCH signaling pathway. This hypothesis was elucidated in TSP2 siRNA-transfected cells, in which the expression of JAGGED1 and NOTCH3 was blocked, and eventually, the genes and proteins of SOX9 and collagen II were downregulated. However, the blocking of chondrogenic differentiation of hADMSCs by TSP2 siRNA was fully recovered by exogenous TSP2, confirming the involvement of TSP2 in MSC chondrogenic differentiation as previously reported[18,20,49,50].

According to *in vitro* studies, it was expected that combination treatment with hADMSCs and TSP2 may lead to enhanced efficacy in the recovery of OA. hADMSCs alone improved the articular lesions in radiological, gross, and microscopic observations in a dose-dependent manner, *i.e.*, 1.7×10^7 cells were enough for full recovery in 8 wk, but 1.7×10^6 cells displayed a partial effect. More specifically, the high-dose (1.7×10^7) hADMSCs greatly restored the cartilage structure as well as the integrity of ECM including proteoglycan and collagen II. It is of interest to note that co-administration of TSP2 and hADMSCs exhibited synergistic effects, *i.e.* repeated injections of TSP2 (100 ng/knee at 2-d intervals) increased the efficacy of low-dose

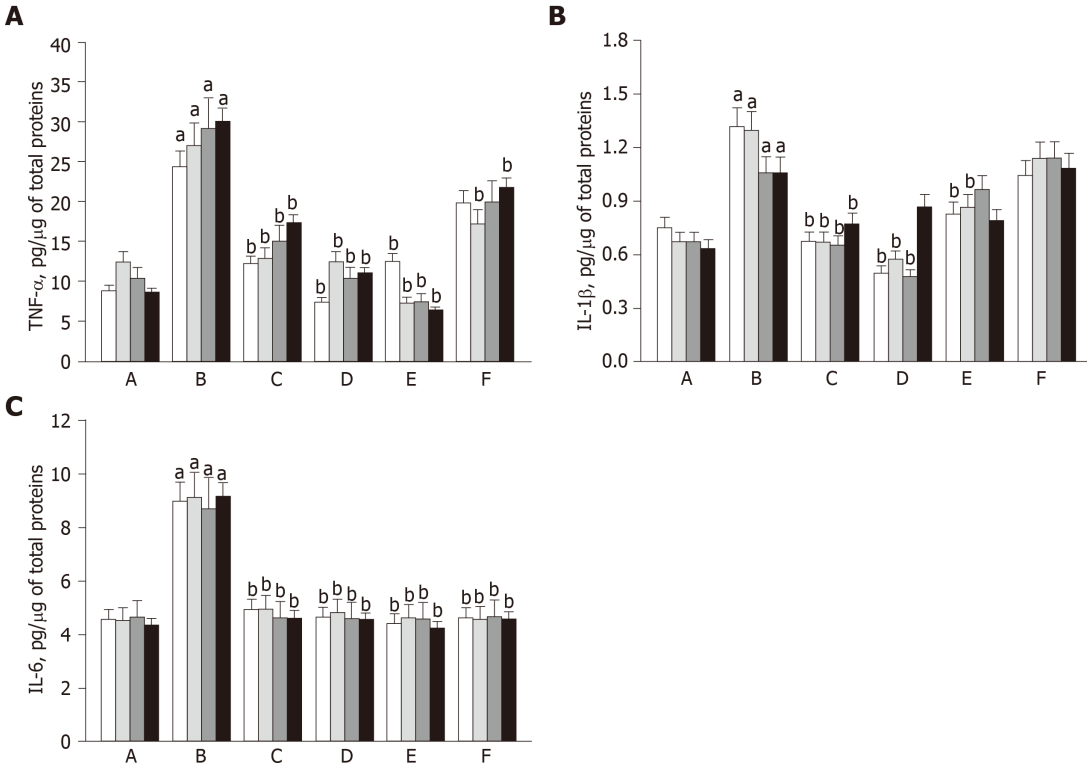

Figure 4 Concentration of pro-inflammatory cytokines in the synovial fluid 1 wk (white), 2 wk (light grey), 4 wk (dark gray), and 8 wk (black) after cell transplantation. A: Sham control; B: ACLT alone; C: Low-dose (1.7×10^6) hADMSCs; D: High-dose (1.7×10^7) hADMSCs; E: Low-dose (1.7×10^6) hADMSCs + TSP2 (100 ng); F: TSP2. [a]Significantly different from sham control, $P < 0.05$. [b]Significantly different from ACLT alone, $P < 0.05$. hADMSCs: Human adipose-derived mesenchymal stem cells; TSP2: Thrombospondin 2.

(1.7×10^6) cells similar to or higher than that of 10-fold higher-dose (1.7×10^7) cells alone. In fact, however, TSP2 alone exerted beneficial effects to some extent, which may come from its paracrine action for the differentiation and proliferation of remaining host chondroprogenitor cells[16], in addition to anti-inflammatory potential *via* cytokine regulation.

Actually, there are controversies on the long-term survival, attachment to the injured articular surface, and differentiation into chondrocytes of transplanted autologous or allogeneic human stem cells due to limited clinical studies on those issues. However, it has been demonstrated that autologous equine BMMSCs survive longer than 6 mo and integrates within the existing articular cartilage[44]. Therefore, it is anticipated that similar long-term survival and participation of human stem cells in the reparative process of human OA joints would be practical. In our recent study, heterologous human ADMSCs transplanted to rabbits' synovial cavity were also found to attach on the injured articular surface, survive some periods, and recover OA cartilages by secreting GFs, as inferred from the fact that direct injection of conditioned medium of hADMSCs exerted OA-improving activity to some extent (unpublished results). Despite the high activities of hADMSCs shown in this study, the potential, especially in proliferation and chondrogenic efficiency, of MSCs from individuals are somewhat different according to the age and health conditions. Thus, we selected healthy stem cells from a single individual to obtain consistent results.

Taken together, in this study, ACLT plus exercise were carried out to induce moderate to severe OA conditions to explore the efficacy of hADMSCs and TSP2. Our investigations demonstrated enhanced beneficial effects of the combination treatment of hADMSCs plus TSP2, showing well-organized tissues with low cytokine contents, smooth articular surfaces, and abundant ECM contents in diverse radiological, gross, and microscopic evaluations. As an underlying mechanism on the role of TSP2, we propose its potential to stimulate the chondrogenic differentiation of hADMSCs as well as anti-inflammatory activity.

In conclusion, TSP2 enhanced chondrogenic differentiation of hADMSCs *via* JAGGED1/NOTCH3 signaling, and the combination therapy with hADMSCs and TSP2 exerted synergistic efficacy in the cartilage regeneration of OA joints of rabbits. It is suggested that the beneficial effects of human ADMSCs plus TSP2 combination treatment could be obtained in human patients.

Table 1 Radiological evaluation scores 8 wk after cell transplantation

Treatment (dose)	Osteophytes in medial femoral condyle (0-5)	Deformity of medial tibia (0-5)	Sharpening of tibial spine (0-5)	Total score (0-15)
ACLT alone	3.5 ± 0.2	3.2 ± 0.4	2.7 ± 0.3	9.3 ± 0.6
hADMSCs (low)	3.0 ± 0.4	3.0 ± 0.4	2.7 ± 0.2	8.7 ± 0.4
hADMSCs (high)	2.7 ± 0.2	2.3 ± 0.2	2.2 ± 0.2	7.2 ± 0.3[1]
hADMSCs (low) + TSP2	2.3 ± 0.3	1.5 ± 0.3	2.5 ± 0.3	6.0 ± 0.4[1]
TSP2 (100 ng)	2.5 ± 0.3	2.5 ± 0.3	2.3 ± 0.3	7.2 ± 0.8[1]

[1]Significantly different from ACLT alone, $P < 0.05$. ACLT: Anterior cruciate ligament transection; hADMSCs: Human adipose-derived mesenchymal stem cells; TSP2: Thrombospondin 2.

Table 2 Gross evaluation scores

Treatment (dose)	Roughness of surface (0-8)	Adhesion with meniscus (0-8)	Hypertrophy of joint (0-8)	Osteophyte (0-8)	Total score (0-32)
ACLT alone	5.5 ± 0.5	4.5 ± 0.5	6.0 ± 0.4	4.0 ± 2.0	20.0 ± 3.1
hADMSCs (low)	4.0 ± 1.0	4.0 ± 1.0	5.0 ± 1.0	4.0 ± 1.2	17.0 ± 2.2
hADMSCs (high)	2.0 ± 1.1	2.5 ± 0.5	2.5 ± 1.5	1.5 ± 0.5	8.5 ± 2.5[1]
hADMSCs (low) + TSP2	3.0 ± 0.2	1.5 ± 0.6	1.0 ± 0.4	2.0 ± 0.6	7.5 ± 0.5[1]
TSP2 (100 ng)	1.5 ± 1.5	3.5 ± 0.5	3.5 ± 1.5	3.0 ± 1.1	11.5 ± 4.5[1]

[1]Significantly different from ACLT alone, $P < 0.05$. ACLT: Anterior cruciate ligament transection; hADMSCs: Human adipose-derived mesenchymal stem cells; TSP2: Thrombospondin 2.

Table 3 Microscopic evaluation scores

Treatment (dose)	Structure (0-11)	Cellularity (0-4)	Cluster (0-3)	Proteoglycan (0-6)	Collagen II (0-6)	Total score (0-30)
ACLT alone	7.5 ± 1.5	3.0 ± 1.1	2.0 ± 1.0	6.0 ± 0.3	4.5 ± 1.5	23.0 ± 5.0
hADMSCs (low)	8.0 ± 0.5	1.0 ± 1.0	1.5 ± 1.5	4.5 ± 0.5	3.0 ± 0.4	18.0 ± 2.0
hADMSCs (high)	2.5 ± 1.5	2.0 ± 1.0	0.0 ± 0.0	2.5 ± 0.4	1.5 ± 0.5	8.5 ± 3.5[1]
hADMSCs (low) + TSP2	1.0 ± 0.2	3.0 ± 0.5	0.0 ± 0.0	2.0 ± 0.3	1.5 ± 0.5	7.5 ± 0.6[1]
TSP2 (100 ng)	1.5 ± 0.5	2.0 ± 0.2	1.5 ± 1.5	4.0 ± 0.7	1.5 ± 1.5	10.5 ± 2.5[1]

[1]Significantly different from ACLT alone, $P < 0.05$. ACLT: Anterior cruciate ligament transection; hADMSCs: Human adipose-derived mesenchymal stem cells; TSP2: Thrombospondin 2.

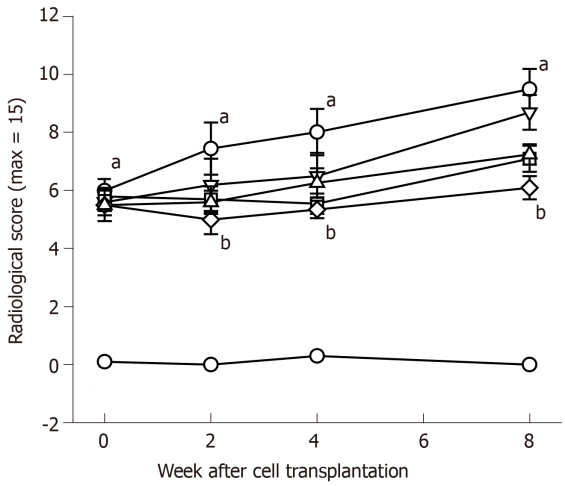

Figure 5 Time-course radiological evaluation on knee joints. Open circle: Sham control; Filled circle: Anterior cruciate ligament transection alone; Inverted triangle: Low-dose (1.7×10^6) hADMSCs; Square: High-dose (1.7×10^7) hADMSCs; Lozenge: Low-dose (1.7×10^6) hADMSCs + TSP2 (100 ng); Triangle: TSP2. [a]Significantly different from sham control, $P < 0.05$. [b]Significantly different from ACLT alone, $P < 0.05$. hADMSCs: Human adipose-derived mesenchymal stem cells; TSP2: Thrombospondin 2.

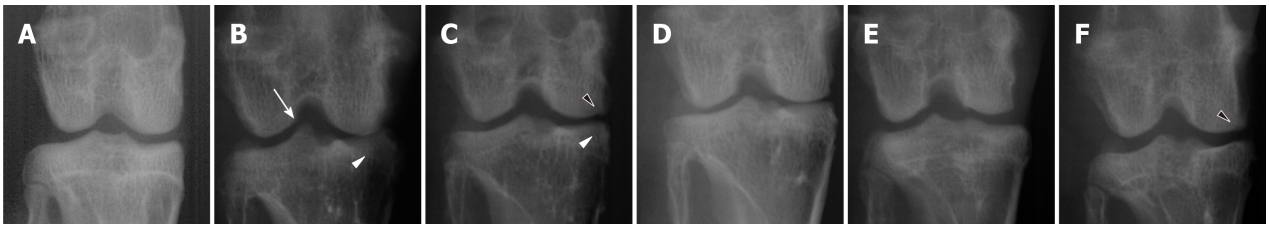

Figure 6 Representative radiological images 8 wk after cell transplantation. A: Sham control; B: Anterior cruciate ligament transection alone; C: Low-dose (1.7×10^6) hADMSCs; D: High-dose (1.7×10^7) hADMSCs; E: Low-dose (1.7×10^6) hADMSCs + TSP2 (100 ng); F: TSP2. Black arrowhead: Osteophytes in medial femoral condyle; White arrow: Deformity of medial tibia; White arrowhead: Sharpening of tibial spine. hADMSCs: Human adipose-derived mesenchymal stem cells; TSP2: Thrombospondin 2.

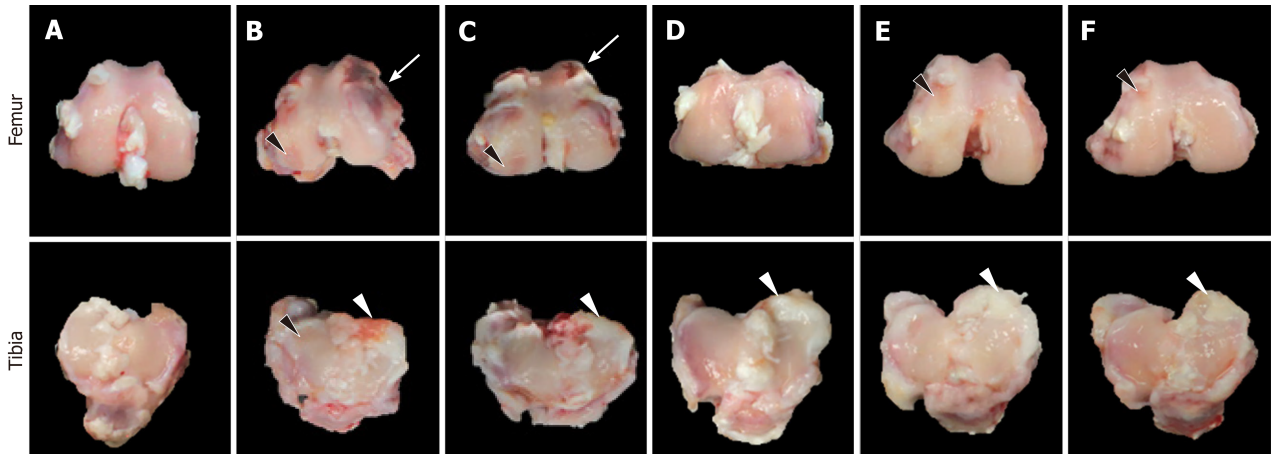

Figure 7 Representative gross findings. A: Sham control; B: Anterior cruciate ligament transection alone; C: Low-dose (1.7×10^6) hADMSCs; D: High-dose (1.7×10^7) hADMSCs; E: Low-dose (1.7×10^6) hADMSCs + TSP2 (100 ng); F: TSP2. Black arrowhead: Fissure and erosion; White arrowhead: Adhesion with meniscus; White arrow: Osteophyte. hADMSCs: Human adipose-derived mesenchymal stem cells; TSP2: Thrombospondin 2.

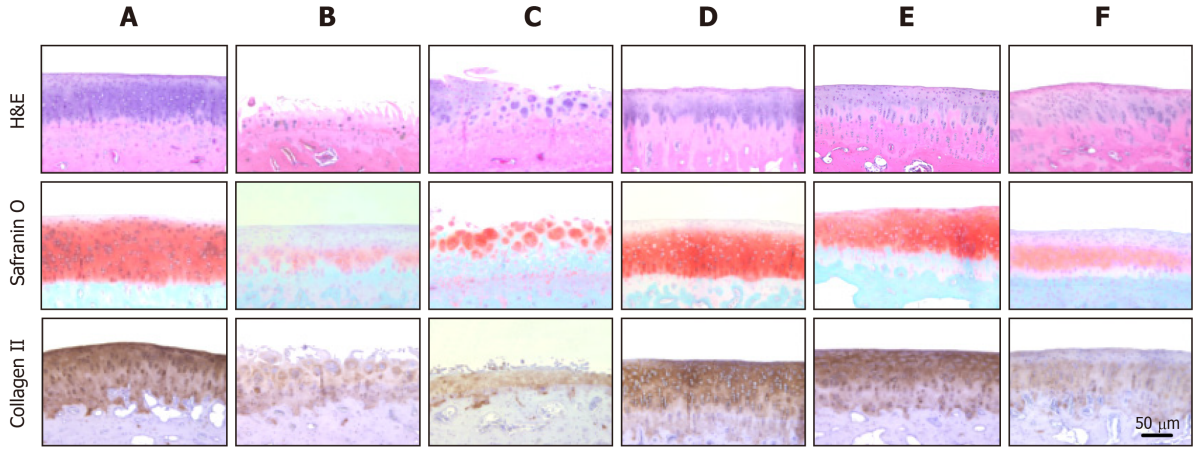

Figure 8 Representative microscopic images stained with H&E (for structure, chondrocyte density, and cluster formation), safranin O (for proteoglycan contents) or collagen II antibody (for extracellular matrix). A: Sham control; B: ACLT alone; C: Low-dose (1.7×10^6) hADMSCs; D: High-dose (1.7×10^7) hADMSCs; E: Low-dose (1.7×10^6) hADMSCs + TSP2 (100 ng); F: TSP2 (100×). hADMSCs: Human adipose-derived mesenchymal stem cells; TSP2: Thrombospondin 2.

ARTICLE HIGHLIGHTS

Research background

Mesenchymal stem cells (MSCs) reportedly differentiate into chondrocytes and have a potential for articular cartilage regeneration. Therefore, they may be a novel strategy for osteoarthritis (OA) treatment.

Research motivation

Since MSCs can be differentiated into osteocytes, adipocytes, and other cells, in addition to chondrocytes, the efficiency for OA treatment is limited. We tried to increase the ratio of chondrocytic differentiation of human adipose-derived MSCs (hADMSCs) with thrombospondin 2 (TSP2), thereby enhancing OA therapeutic efficacy.

Research objectives

The present study investigated whether TSP2 induces the chondrogenic differentiation of hADMSCs and potentiates the therapeutic effects of hADMSCs in OA rabbits.

Research methods

In vitro, the chondrogenic potential of TSP2 in hADMSCs was investigated by analyzing the expression of chondrogenic markers as well as NOTCH signaling genes in normal and TSP2 siRNA-treated stem cells. *In vivo*, hADMSCs were injected into the injured knees of OA rabbits alone or in combination with TSP2, and OA progression was monitored *via* gross, radiological, and histological examinations.

Research results
In hADMSC culture, TSP2 increased the expression of chondrogenic markers as well as NOTCH signaling genes, which were inhibited by TSP2 siRNA treatment. *In vivo*, combination treatment with hADMSCs and TSP2 not only attenuated cartilage degeneration, osteophyte formation, and extracellular matrix loss, but also decreased synovial inflammatory cytokines.

Research conclusions
TSP2 enhances chondrogenic differentiation of hADMSCs *via* JAGGED1/NOTCH3 signaling, and combination therapy with hADMSCs and TSP2 exerts synergistic effects in the cartilage regeneration of OA joints.

Research perspectives
We demonstrated the positive roles of TSP2 in the chondrogenic differentiation of hADMSCs and in OA therapy. TSP2 could be an adjunct therapeutic for enhancing the cartilage-restoring efficacy of hADMSCs.

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ORIGINAL ARTICLE

Basic Study

MiR-301a promotes embryonic stem cell differentiation to cardiomyocytes

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Abstract

BACKGROUND

Cardiovascular disease is the leading cause of death worldwide. Tissue repair after pathological injury in the heart remains a major challenge due to the limited regenerative ability of cardiomyocytes in adults. Stem cell-derived cardiomyocytes provide a promising source for the cell transplantation-based treatment of injured hearts.

AIM

To explore the function and mechanisms of miR-301a in regulating cardiomyocyte differentiation of mouse embryonic stem (mES) cells, and provide experimental evidence for applying miR-301a to the cardiomyocyte differentiation induction from stem cells.

METHODS

mES cells with or without overexpression of miR-301a were applied for all functional assays. The hanging drop technique was applied to form embryoid bodies from mES cells. Cardiac markers including GATA-4, TBX5, MEF2C, and α-actinin were used to determine cardiomyocyte differentiation from mES cells.

RESULTS

High expression of miR-301a was detected in the heart from late embryonic to neonatal mice. Overexpression of miR-301a in mES cells significantly induced the expression of cardiac transcription factors, thereby promoting cardiomyocyte

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differentiation and beating cardiomyocyte clone formation. *PTEN* is a target gene of miR-301a in cardiomyocytes. PTEN-regulated PI3K-AKT-mTOR-Stat3 signaling showed involvement in regulating miR-301a-promoted cardiomyocyte differentiation from mES cells.

CONCLUSION
MiR-301a is capable of promoting embryonic stem cell differentiation to cardiomyocytes.

Key words: miR-301a; Mouse embryonic stem cells; Differentiation; Cardiomyocytes

Core tip: MiR-301a was identified as a miRNA highly enriched in the heart from late embryonic to neonatal mice. Overexpression of miR-301a significantly induced the expression of cardiac transcription factors and promoted cardiomyocyte differentiation of mouse embryonic stem cells. These findings will help improve the efficiency of cardiomyocyte differentiation from stem cells, and strengthen the potential of cell therapeutics to treat heart failure caused by myocardial infarction.

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INTRODUCTION

Cardiovascular disease is the leading cause of death worldwide[1]. After myocardial infarction (MI) in adults, the regenerative ability of the differentiated cardiomyocytes is very limited due to the "terminated" cell proliferative ability and the lack of cardiac stem cells[2,3]. Although the cell proliferative potential of cardiomyocytes in adults is occasionally reported[4], this ability cannot produce enough cardiomyocytes for functional recovery of the injured heart. Tissue repair after pathological injury in the heart, including that after MI, is still a major clinical challenge.

MI occurs mostly due to coronary artery disease in which the heart blood flow is blocked, causing damage to the cardiomyocytes. MI may cause heart failure, heart arrhythmia, cardiogenic shock, or cardiac arrest. Although progress has been made in the pharmacologic and device management and gene or cell therapy of heart failure, the mortality in heart failure patients remains significant. All of the current pharmacologic or surgical approaches have limited effects on heart function recovery. Two novel strategies have been suggested to restore the lost cardiomyocytes caused by MI. One is to apply cardiomyocytes differentiated from stem cells or derived from cardiospheres (CDCs)[5,6], and the other is to induce cell cycle reentry in cardiomyocytes[4,7].

Stem cell-based therapy tests in chronic heart failure[8] and preclinical studies applying transplantation of embryonic stem (ES) cell-derived cardiac progenitors in an animal model of MI[9] have suggested strategies to compensate for the lost cardiac cells in damaged hearts. A phase I clinical trial showed that patients treated with CDCs had a reduction in scar mass and an increase in both viable heart mass and systolic wall thickness[6]. As such, stem cells, stem cell-derived cardiomyocytes, and CDCs provide a promising source for cell transplantation-based treatment of injured hearts. In addition, modification of approaches to induce cardiac cell differentiation from stem cells with high efficiency will be crucial to improve the therapeutic effect.

ES cells have been applied to differentiate into cardiomyocytes in the treatment of MI *in vivo*[9]. During embryogenesis, the heart is derived from mesodermal cells. The mesothelial pericardium forms the outer lining, and the endothelium forms the inner lining and lymphatic and blood vessels of the heart. Myocyte differentiation begins from E7.5 in mouse embryos and day 15 in human embryos. Before birth, cardiomyocytes undergo the hyperplastic to hypertrophic transition. The majority of cardiomyocytes withdraw from the cell cycle and stop proliferation shortly after birth.

Here, mouse ES (mES) cells were applied for the induction of cardiomyocyte differentiation *in vitro* to determine the therapeutic potential of ES cell-based cell transplantation in the treatment of heart failure.

MicroRNAs (miRNAs) have been shown to regulate diverse biological processes, including cell fate decision, organ formation, and stem cell self-renewal and differentiation[10-12]. The aberrant expression of miRNAs in tissues has been closely connected to tissue-related disease. MiRNAs are involved in regulating the development and progression of cancer, cardiovascular disease, and other conditions[11,13-15]. To the best of our knowledge, miR-1 and miR-133 are the most important miRNA families regulating cardiac development and heart function[16,17]. Muscle-specific miR-1 and miR-133a both promote mesoderm formation from ES cells and suppress ectoderm and endoderm fates[18], but later, during further differentiation into cardiac muscle progenitors, these miRNAs show opposing regulatory functions[12,19]. Other miRNAs, including miR-206, miR-708, miR-208a, miR-208b, and miR-499, have also been reported to regulate heart development and heart diseases[20].

In the current study, we identified miR-301a as a highly enriched miRNA in embryonic and neonatal cardiomyocytes. Although overexpression of miR-301a is frequently observed in diverse tumor types, promoting cell proliferation, invasion, and metastasis of cancer cells[21-23], the functional properties of miR-301a in the heart remain unclear, except one recent report indicating that miR-301a is a novel cardiac regulator of Cofilin-2 in cardiomyocytes[24]. In contrast to its function in tumors, miR-301a may have tissue-specific functions in the heart. Here, we for the first time demonstrated that overexpression of miR-301a significantly induced the expression of cardiac transcription factors in mES cells, thereby promoting cardiomyocyte differentiation and beating cardiomyocyte clone formation. Our findings will be beneficial in the development of an approach with high efficiency to induce stem cell differentiation to cardiomyocytes and strengthen the potential of cell therapeutics for heart failure.

MATERIALS AND METHODS

Animals

Animal studies were approved by the Institutional Animal Care and Use Committee of the Tongji University School of Medicine. Male C57BL/6J mice were purchased from Silaike Animal Company (Shanghai, China). The hearts were collected from mouse embryos at E11.5, 13.5, 15.5, 17.5, and 19.5 and from neonatal and adult mice and placed into TRIzol for total RNA isolation using a tissue homogenizer.

Cells and cell culture

The murine embryonic stem cell line ES-D3 was originally from ATCC and maintained in "feeder free" culture conditions as described previously[25]. The mES cell culture plates were coated with fetal bovine serum (FBS). The DMEM/F12 medium containing Neurobasal Medium was supplemented with 0.5% N2, 1% B27, 2 mM L-glutamine, 0.055 mmol/L β-mercaptoethanol, 0.05% bovine serum albumin (BSA; Fraction V), 0.1% insulin, 100 U/mL penicillin, 100 μg/mL streptomycin, 3 μmol/L CHIR99021, 0.4 μmol/L PD0325901, and 1000 U/mL LIF. All cells were cultured at 37 °C in a 5% CO_2 environment unless stated otherwise.

Oligos and transfection

All primers and miR-301a mimic and negative control oligos were synthesized by GenScript (Nanjing, China). Forward primer sequences for miRNA amplification are as follows: MiR-301a: 5′-CCAGTGCAATAGTATTG-3′; 5S rRNA: 5′-AGTACTTGGATGGGAGACCG-3′. The double-strand miRNA mimic sequence for miR-301a is 5′-CAGU GCAAUAGUAUUGUCAAAGC-3′, and the negative control for the miRNA mimic is 5′-UGGGCGUAUAGACGUGUUACAC-3′. Lipofectamine RNAiMAX (Invitrogen) was applied for oligo transfection, following the manufacturer's instructions. A final concentration of 50 nM of miRNA mimic or negative control was used. The cells were applied for further assays 24 h after transfection.

Quantitative real-time PCR analysis

Total RNA was extracted with TRIzol reagent (#15596026, Invitrogen, Thermo Fisher Scientific). Then, 500 ng of purified total RNA was applied to prepare the first strand cDNA of miRNA using an M and G miRNA Reverse Transcription Kit (miRGenes, Shanghai, China) following the manufacturer's instructions. The cDNA was diluted 1:1000 for real-time PCR analysis of miRNAs. For mRNA analysis, a regular approach and random primer were used for reverse transcription. The SYBR Green Master Mix

(Applied Biosystem, Thermo Fisher Scientific) and 7900 HT Sequence Detection System (Applied Biosystem, Thermo Fisher Scientific) were used for real-time PCR assays. GAPDH was used for mRNA normalization, and 5S rRNA was used for miRNA normalization. Primer information for all tested genes is shown in detail in Supplemental Table 1.

Western blot analysis

Cell lysates (50 μg) prepared with RIPA buffer containing protease inhibitor cocktail (Roche Diagnostics) were separated by 10% SDS-PAGE. The proteins were transferred to PVDF membranes. Then, 5% nonfat milk (w/v) was used for the blocking step. The following primary antibodies (1:2000) were used: PTEN (sc-7974, Santa Cruz), p-STAT3 (Tyr705, 9145T, Cell Signaling Technology), p-AKT (Ser473, 4060T, Cell Signaling Technology), p-mTOR (Ser2448, 5536T, Cell Signaling Technology), total STAT3 (4904, Cell Signaling Technology), total AKT (4691, Cell Signaling Technology), total mTOR (2983T, Cell Signaling Technology), MEF2C (sc-365862, Santa Cruz), NKX2.5 (sc-376565, Santa Cruz), GATA4 (sc-25310, Santa Cruz), CTNT (sc-20025, Santa Cruz), α-actinin (Sigma, A7811), and GAPDH (sc-47724, Santa Cruz). HRP-conjugated anti-rabbit IgG (7074S, Cell Signaling Technology) and HRP-conjugated anti-mouse IgG (7076S, Cell Signaling Technology) were used as secondary antibodies (1:3000).

Immunofluorescence

Cells were fixed with 4% paraformaldehyde for 15 min and permeabilized with 0.5% Triton X-100 (Sigma) in 1 × PBS for 10 min at room temperature. Next, 1% BSA was used for blocking for 1 h at room temperature. Then, cells were incubated with the primary antibodies (1:10 to 1:100 dilution), including anti-OCT4 (2750S, Cell Signaling Technology) and anti-NANOG (4903S, Cell Signaling Technology), overnight at 4 °C, and the secondary antibody conjugated to Alexa Fluor 555 (Invitrogen, A21428) was added for 1 h at room temperature. The nucleus was stained with 6-diamidino-2-phenylindole (DAPI, Sigma, D9542) for 30 min at room temperature. All slides were photographed using a fluorescence microscope (Leica, Germany).

Embryoid body (EB) formation and cardiomyocyte differentiation

The hanging drop technique was applied to form EBs from ES cells. Iscove's modified Dulbecco's medium (IMDM, Gibco) containing 20% FBS, 1% penicillin-streptomycin, 2 mM L-glutamine, 0.055 mmol/L β-mercaptoethanol, and 1% MEM nonessential amino acids (Gibco, 11140050) was used for induction of mES cell differentiation. Briefly, a single cell suspension (5×10^4 mES cells/mL) in differentiation medium was split to cell droplets with ~1000 cells in 20 μL for each drop and hung from the bottom of bacterial-grade dishes upside down to culture for 2 d, followed by suspension culture for two more days in 10 mL of differentiation medium still using bacterial-grade dishes on a shaking platform at 40 rpm. This step is for the maturation of EBs. After that, the EBs were moved to 0.1% gelatin-coated plates for adherent culture for an additional 8 d. This step is for cardiomyocyte differentiation.

Alkaline phosphatase staining

Alkaline phosphatase activity of the mES clones was examined using a BCIP/NBT alkaline phosphatase color development kit according to the manufacturer's protocol (Beyotime Institute of Biotechnology, China). The clones were fixed with 70% ethanol and incubated with BCIP/NBT staining work solution for 30 min, followed by washing with ddH_2O.

Statistical analysis

Data are presented as the mean ± SE. A standard two-tailed Student's *t*-test with SPSS 21.0 software was used for statistical analyses, in which $P < 0.05$ was considered significant.

RESULTS

High expression of miR-301a in the heart from late embryonic to neonatal mice

Our previous work demonstrated the enrichment of a subset of miRNAs, including miR-708 and miR-301a, in the cardiomyocytes of neonatal rodents[26]. To further determine the expression pattern and function of miR-301a during heart development, we collected mouse embryo hearts at days 11.5, 13.5, 15.5, 17.5, and 19.5, as well as hearts from 3-day-old neonatal and 6-wk-old adult mice. The expression analysis of miR-301a indicated much higher levels in the hearts of late-stage embryos and 3-day-old postnatal mice than adult hearts. In particular, the levels

in the hearts from E17.5 to newborn mice were over 10-fold higher than those in adult hearts (Figure 1), suggesting the potential function of miR-301a in regulating heart development, cardiomyocyte differentiation, and cardiomyocyte proliferation.

MiR-301a does not regulate the formation of early EBs

To explore the role of miR-301a during heart development, we established a cell-culturing system using the hanging drop technique to induce mES cell differentiation into cardiomyocytes *in vitro* (Figure 2A), which can partly mimic the development of the embryonic heart *in vivo*. We compared the expression of miR-301a in mES cells before differentiation and mES cell-derived cardiomyocytes at day 12 after differentiation, demonstrating a ~10-fold upregulation of miR-301a in differentiated cardiomyocytes compared to mES cells (Figure 2B). To further determine the expression of miR-301a at different stages of EBs during cardiac differentiation, we performed a quantitative analysis of miR-301a in EBs at days 4, 8, and 12. As shown in Supplemental Figure S1, a ~5-fold increase in miR-301a was observed in EBs at both days 8 and 12 compared to day 4.

As shown in Figure 2A, spheroid structured EBs were formed from day 2 to day 4 at the beginning of mES cell differentiation. EB formation is often considered to initiate differentiation toward the three germ lineages. Embryonic organoids derived from EB culture show remarkable parallels to embryonic development. Immunofluorescence staining with the stem cell markers OCT4 and NANOG was applied to ES clones, confirming the stemness maintenance at the early stage (day 2 under adherent culture) of ES cell differentiation (Figure 2C). To determine the regulatory effect of miR-301a on stem properties and EB formation, we performed alkaline phosphatase staining of mES clones and did not find a difference in stemness between the two groups with or without overexpression of miR-301a (Supplemental Figure S2).

MiR-301a-overexpressing mES cells were cultured in parallel with a negative control (NC) under differentiation conditions using the hanging-droplet method to develop EBs (Figure 2A). A quantitative analysis of gene expression of stem cell markers, including *SOX2*, *OCT4*, *NANOG*, and *KLF4*, was applied to EBs at day 2 and did not show a significant difference between the NC and miR-301a groups (Figure 2D). Subsequent analysis of EBs at day 4 did not show any changes in morphology, diameter, or amount between the NC and miR-301a groups, as shown in Figure 2E and 2F. These observations suggest the very limited effect of miR-301a on the self-renewal and stemness of mES cells during the early period of EB formation.

MiR-301a promotes mES cell differentiation to cardiomyocytes

MiR-301a overexpression in mES cells does not regulate stemness, but can it control cardiac differentiation? To address this question, we assessed several cardiac-specific markers and cardiac-specific transcription factors at day 4 (EB formation, corresponding to E7.5 mouse embryos), day 8 (cardiac differentiation, corresponding to E10.5-E16.5 mouse embryos), and day 12 (formation of immature cardiomyocytes, corresponding to E17.5 and thereafter mouse embryos) after mES cell differentiation following the procedure in Figure 2A. As shown in Figure 3A, mid-stage cardiac markers, including GATA-4, TBX5 and MEF2C, and late-stage cardiac markers, including α-actinin, α-sarcomeric myosin heavy chain (α-MHC), and myosin light chain (MLC), showed higher levels at the stages of cardiac differentiation (days 8 and 12) compared to the stage of early EB formation (day 4) (fold changes are indicated in Figure 3A, days 8 and 12 *vs* day 4 in the NC group). Meanwhile, compared to the control, miR-301a overexpression induced the expression of these cardiac markers during mES differentiation (Figure 3A). Proteomic analysis further demonstrated the increased expression of the cardiac markers by miR-301a, including GATA-4, MEF2C, NKX2.5, CTNT, and α-actinin, at day 12 after mES cell differentiation (Figure 3B). A semiquantitative analysis (cardiac markers normalized on GAPDH) of the Western blot results clearly showed the upregulation of these cardiac markers by miR-301a (Figure 3B). Does this kind of regulation occur in mES cells? To address this question, a gene expression analysis of these cardiac markers in mES cells with or without overexpression of miR-301a was performed. Meanwhile, EBs undergoing differentiation at day 8 were used as positive controls. As shown in Supplemental Figure S3, very low or undetectable levels of these cardiac markers were detected in mES cells. Their expression in mES cells was not affected by miR-301a.

During cardiac differentiation from mES cells, beating of the cardiac clones was observed from day 9 to day 12 and is shown in Supplemental Videos 1-5. Quantitative analysis of the percentage of beating EBs at different time points indicated a greater number in the miR-301a group compared to the control group (Figure 3C). Furthermore, the beating EBs were first observed in the miR-301a group at day 9, one day earlier than that in the control group. At day 11, more than 90% of EBs were

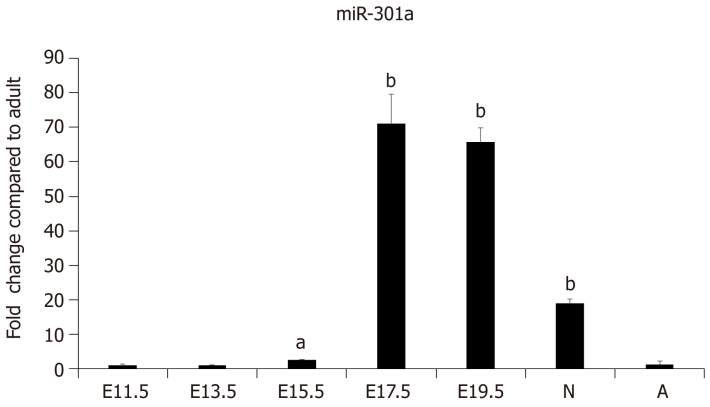

Figure 1 MiR-301a is enriched in the heart of late-stage mouse embryo and neonatal mouse heart tissues. Tissues were collected from mouse embryos at days 11.5, 13.5, 15.5, and 17.5 and from 3-d-old postnatal and 6-wk-old adult mice. The expression level of miR-301a in the hearts was analyzed using quantitative real-time PCR. 5S rRNA was used for normalization. Data are presented as the mean ± SE (n = 3). $^{a}P < 0.05$, $^{b}P < 0.01$.

beating in the miR-301a group, while 75% were beating in the control group (Figure 3C). Taken together, these findings demonstrated the promotion of cardiac differentiation from mES cells by overexpression of miR-301a.

MiR-301a activates mTOR-STAT3 signaling by targeting PTEN

We next determined the mechanism by which miR-301a induces cardiac differentiation from mES cells. Both bioinformatics analysis and literature[27] suggested *PTEN* as a potential target gene of miR-301a in cardiomyocytes. As shown in Figure 4A, three binding sites to miR-301a are predicted in the highly conserved 3′ untranslated region of human and rodent *PTEN* mRNAs. Considering the function of PTEN in regulating cell survival and cell differentiation, the downstream PI3K-AKT and mTOR-Stat3 signaling pathways were further analyzed during mES cell differentiation. As shown in Figure 4B, activation of the PI3K-AKT signaling pathway was accompanied by inhibition of PTEN by miR-301a overexpression. A semi-quantitative analysis (normalized on GAPDH) clearly indicated the upregulation of p-AKT, p-mTOR, and p-Stat3 in the miR-301a-mES-derived cardiac cells at day 12 after cell differentiation, while total AKT, total mTOR, and total STAT3 did not show differences between the two groups (Figure 4C). These findings suggest that the activation of PI3K-AKT-mTOR-Stat3 signaling by miR-301a may contribute to the promoted cardiac differentiation shown in Figure 3.

DISCUSSION

Regulation of cardiac differentiation in embryos

The cardiovascular system is the first system developed in an embryo, and the heart is the first functional organ developed during embryogenesis. The earliest heart precursor cells are detectable in an embryo as early as E6.5 in mice and days 18-19 following fertilization in humans. Cardiac cell differentiation from the precursor cell population is initiated and guided by transcription factors, such as NKX2.5, GATA4, GATA6, and TBX5. Thereafter, signaling pathways, including fibroblast growth factors and bone morphogen proteins, modulate the function of these transcription factors and induce early progenitor cells to further differentiate into mature ventricular or atrial cardiomyocytes[28].

Noncoding genomes play important roles during mES cell differentiation and cell fate determination. A group of miRNAs, including miR-1, miR-133, miR-208a, miR-208b, and miR-499, have been demonstrated to regulate heart development, cardiovascular diseases, and cardiac remodeling[13,16-20]. MiR-1 is enriched in embryonic cardiac cells and mES cell-derived cardiomyocytes, and it promotes not only mesoderm formation from mES cells but also further differentiation into cardiac muscle progenitors[12,16]. In this study, we observed enrichment of miR-301a in the hearts from embryos and neonatal mice. Functional assays demonstrated the induction of cardiac differentiation from mES cells by miR-301a overexpression. Our results contribute to knowledge of the noncoding genome in regulating stem cell differentiation to cardiomyocytes. We demonstrated the potential of miR-301a as a novel target miRNA candidate to induce cardiomyocyte differentiation.

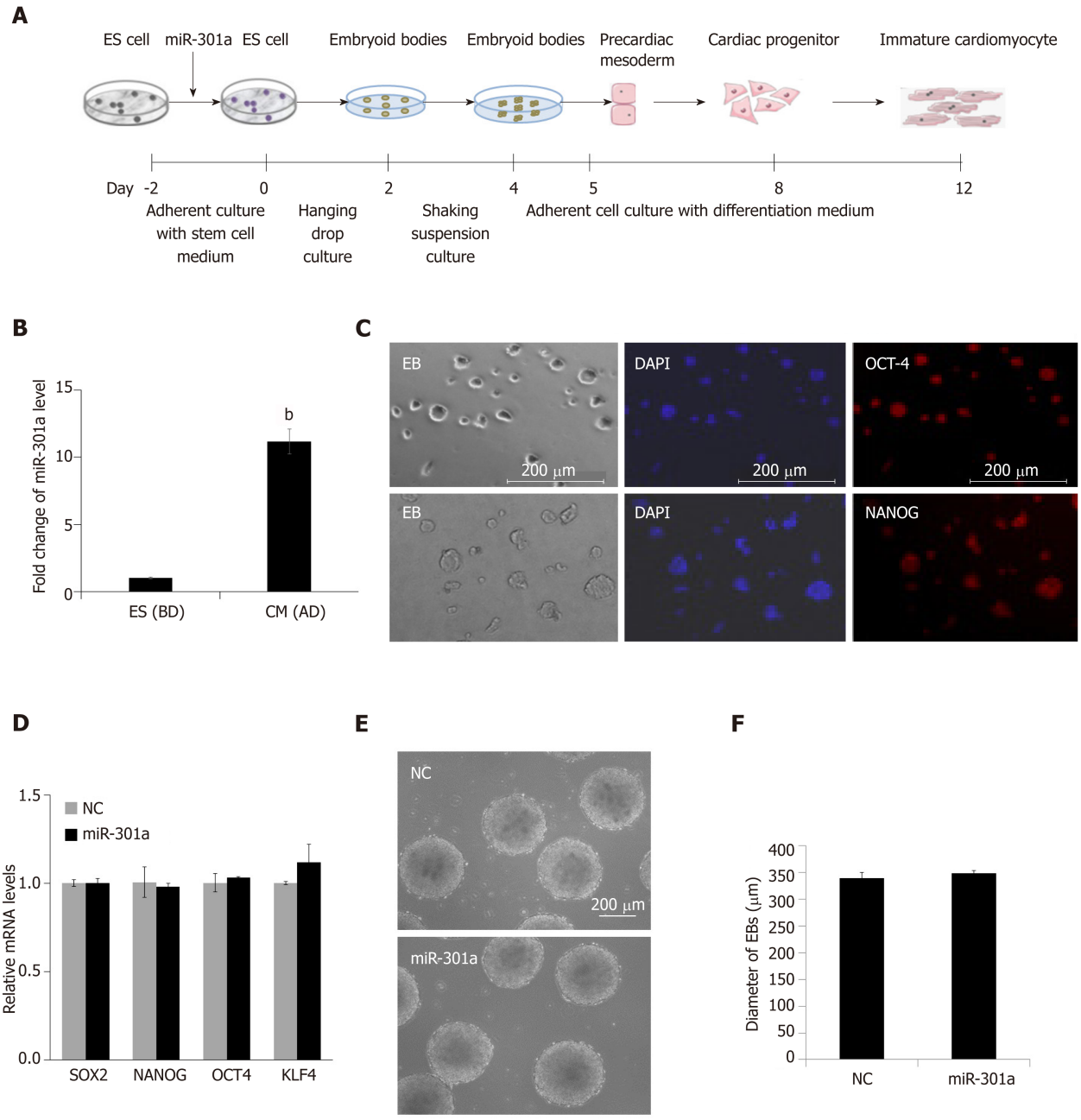

Figure 2 MiR-301a does not affect mouse embryonic stem cell properties or the formation of early embryoid bodies. A: Schematic representation of the procedure to induce cardiomyocyte differentiation from mouse embryonic stem cells; B: The expression level of miR-301a in mouse embryonic stem cells before differentiation (BD) and cardiomyocytes at day 12 after differentiation (AD). Fold change was calculated by dividing the value at AD by the value at BD. Data are the mean ± SE (n = 3), ^{b}P < 0.01; C: Immuno-fluorescence staining for the stem cell markers OCT4 and NANOG in mouse embryonic stem cell clones at day 2 under adherent culture; D: Gene expression detection of stem cell markers, including *SOX2*, *OCT4*, *NANOG*, and *KLF4*, in embryoid bodies at day 2 with or without overexpression of miR-301a. A quantitative real-time PCR method was applied. Data are presented as the mean ± SEM (n = 3). E: Representative images of embryoid bodies formed from mouse embryonic stem cells at day 4 with or without overexpression of miR-301a; F: Average diameter of embryoid bodies at day 4 with or without overexpression of miR-301a. Data are presented as the mean ± SEM (n = 10).

Regulation of cardiomyocyte differentiation from mES cells

ES cell differentiation, cell fate determination, and organoid morphogenesis are closely related to studies of developmental biology and mammalian embryogenesis. ES cells are derived from the early blastocyst stage of embryo development. Because of the similarities between embryogenesis and ES cell differentiation, the process of EB formation and ES cell differentiation can partly resemble the development of embryonic organoids during embryogenesis. The formation of EBs is the typical first step for ES cell differentiation, from which differentiation begins, and three types of tissues are formed, including mesodermal tissues (muscle, bone, connective tissue, *etc.*), ectodermal tissues (nervous system, hair, eyes, *etc.*), and endodermal tissues (epithelium, gastrointestinal tract, *etc.*). The induced cardiomyocyte differentiation

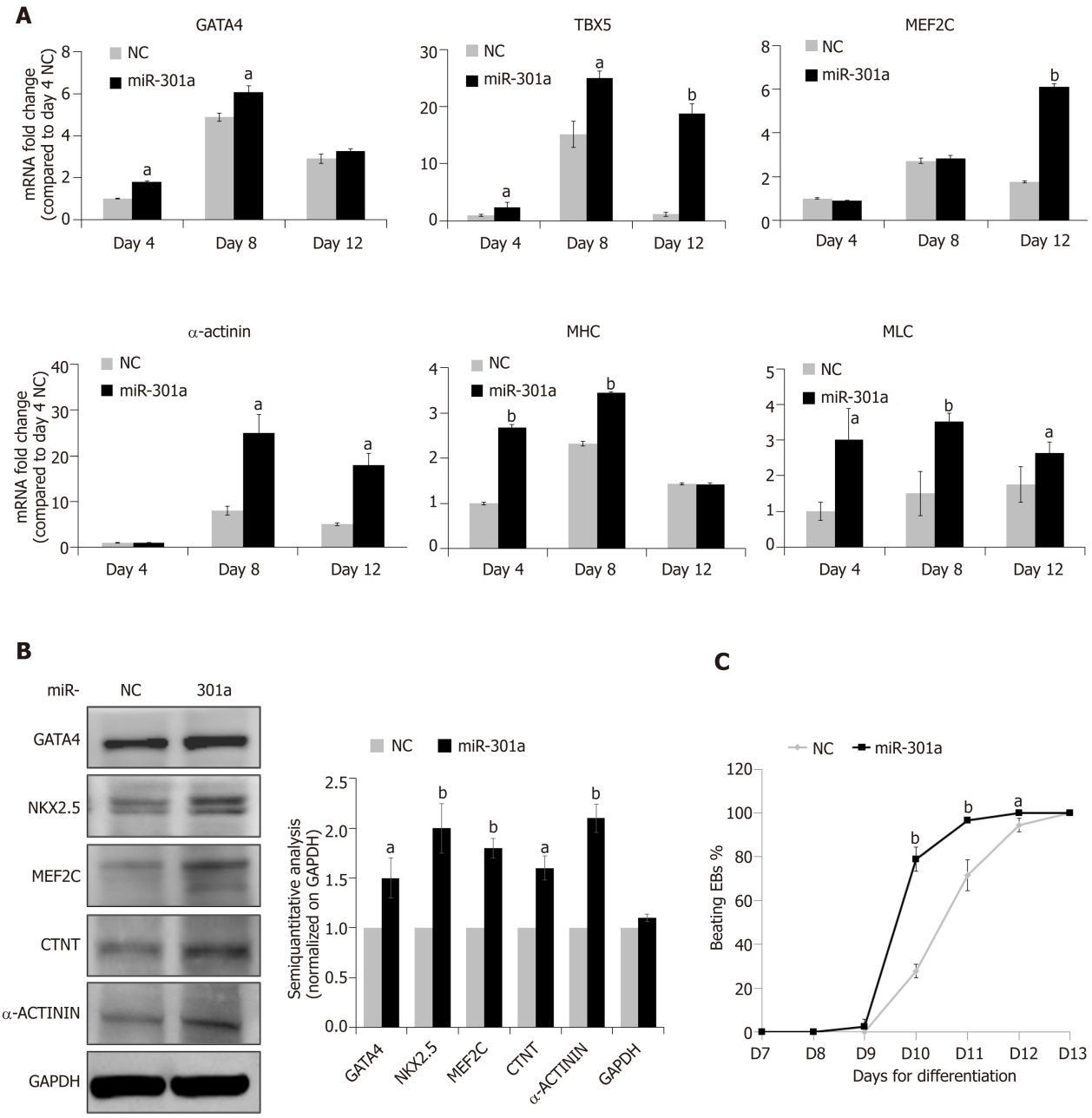

Figure 3 MiR-301a promotes mouse embryonic stem cell differentiation to cardiomyocytes. A: Quantitative real-time PCR analysis of the gene expression for mid-stage cardiac markers, including *GATA-4*, *TBX5*, and *MEF2C*, and late-stage cardiac markers, including α-actinin, sarcomeric *MHC*, and *MLC*, in cells at day 4 (embryoid body formation), day 8 (cardiac differentiation), and day 12 (immature cardiomyocytes) during mouse embryonic stem cell differentiation as indicated in Figure 2A. The gene expression levels are shown as the fold change compared to control cells at day 4. Data are presented as the mean ± SEM (n = 3); B: Western blot analysis of the cardiac markers, including GATA-4, MEF2C, NKX2.5, CTNT, and α-actinin, in cells at day 12 after mouse embryonic stem cell differentiation with or without overexpression of miR-301a. A semiquantitative analysis (cardiac markers normalized on GAPDH, miR-301a group *vs* control group) was applied and shown; C: Quantitative analysis of the percentage of beating embryoid bodies at different time points demonstrated earlier initiation and more embryoid body beating in the miR-301a group than in the control group. All of the embryoid bodies in three independent dishes in each group (141 embryoid bodies in the control group and 131 embryoid bodies in the miR-301a group) were calculated at each time point. Data are presented as the mean ± SE (n = 3). $^{a}P < 0.05$, $^{b}P < 0.01$.

method from EBs has been well developed[9,25].

Stem cell-derived cardiomyocytes represent a good source of cells for studying early cardiac development as well as cell-based therapies in postnatal pathologies. The protocol for cardiomyocyte differentiation was first developed for ES cells[29] and then adapted to iPS cells[30]. In the past decade, cardiomyocyte differentiation protocols have been modified and improved to be more reliable and more efficient, which has contributed to preclinical trials of stem cell-derived cell transplantation therapeutics to treat heart diseases, including heart failure. Herein, we demonstrated that the small RNA molecule miR-301a can induce the expression of cardiac transcription factors and promote cardiomyocyte differentiation from mES cells. As such, miR-301a showed potential to be applied towards the modification of the

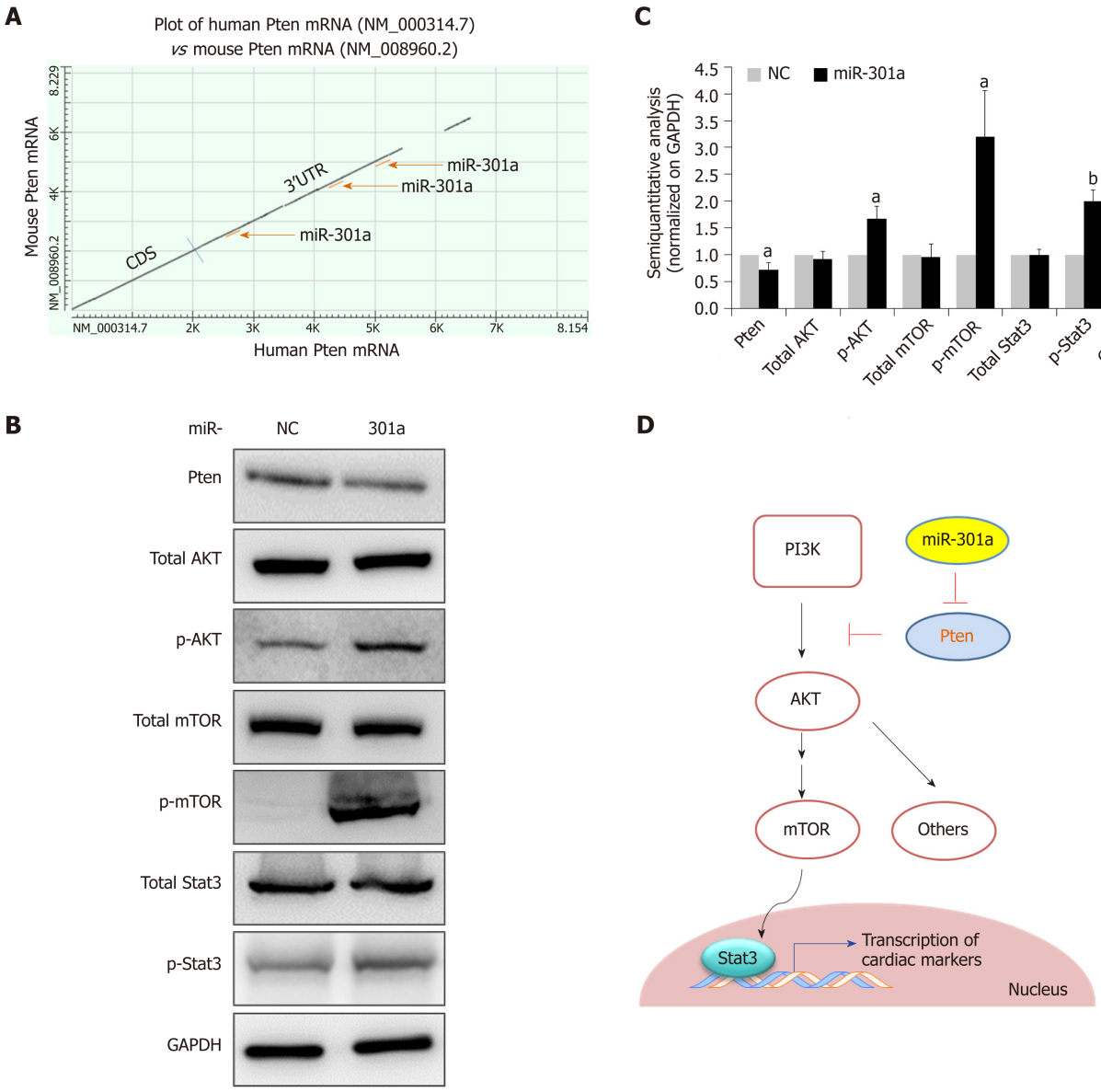

Figure 4 MiR-301a targets PTEN and activates the mTOR-STAT3 signaling pathway in the regulation of mouse embryonic stem cell differentiation. A: Plot of human *PTEN* mRNA *vs* mouse *PTEN* mRNA showing three predicted binding sites to miR-301a at the highly conserved 3' untranslated region; B: Western blot analysis showing decreased expression of PTEN and increased expression of p-AKT, p-mTOR, and p-STAT3 by miR-301a overexpression in mouse embryonic stem cell-differentiated cardiomyocytes. Total AKT, total mTOR ,and total STAT3 did not show differences between the two groups; C: A semiquantitative analysis (normalized to GAPDH, miR-301a group *vs* control group) was applied to protein expression in B; D: Schematic representation of the hypothetical mechanism by which miR-301a regulates mouse embryonic stem cell differentiation to cardiomyocytes by targeting PTEN. Data are presented as the mean ± SE ($n = 3$). $^{a}P < 0.05$, $^{b}P < 0.01$.

current cardiomyocyte differentiation protocols.

The enrichment of miR-301a in active cardiomyocytes was originally determined from our miRNA screening study in the hearts of neonatal rodents[26], which was recently confirmed by Rangrez *et al*[24], who detected much higher expression of miR-301a in isolated cardiomyocytes than in fibroblasts. That study also found that miR-301a negatively regulates SRF signaling through inhibiting the expression of the target gene Cofilin-2 in cardiomyocytes, suggesting the therapeutic potential of miR-301a in the treatment of cardiac disorders caused by the deregulation of Cofilin-2[24]. Here, we first showed that miR-301a has a high level in the hearts of late-stage embryos, while it is low in undifferentiated ES cells and cardiomyocytes in early-stage embryos. Subsequent functional assays demonstrated the induction of cardiomyocyte differentiation from mES cells by miR-301a, suggesting a cell-type specific function for this miRNA. Our findings add to knowledge of miR-301a in the treatment of heart disease by ES cell-based strategies. Notably, the development of a stem cell-specific gene expression system or a cardiomyocyte-targeted local delivery system for miR-301a will be required given the possible oncogenic side effects of miR-301a. As

discussed above, the upregulation of miR-301a has been reported in multiple tumor types[21-23].

mTOR-STAT3 signaling regulates cardiomyocyte differentiation

The activation of mTOR-STAT3 signaling by the PI3K-AKT pathway has been validated in cardiomyocytes[31,32]. STAT3 is essential for cardiomyocyte differentiation, directly promoting the expression of cardiac markers, including TBX5, NKX2.5, and GATA4[33]. PTEN dephosphorylates PIP3 [phosphatidylinositol (3,4,5)-trisphosphate] to from PIP2 [phosphatidylinositol (4,5)-bisphosphate], thereby inhibiting the PI3K/AKT pathway and regulating cell proliferation and cell differentiation in cardiomyocytes[34]. PTEN is a key regulator of the PI3K/AKT pathway. PTEN/PI3K/AKT signaling-mediated miRNA regulation of ES cell differentiation to cardiomyocytes has been reported for miR-1[35]. Target interactions between miR-301a and PTEN have been demonstrated in cervical cancer[36] and pancreatic cancer[37]. In the current study, we demonstrated that miR-301a activates the PI3K-AKT-mTOR-STAT3 signaling pathway, promoting cardiomyocyte differentiation from mES cells, which was mediated by the target interaction between miR-301a and PTEN. These findings further demonstrated the importance of miRNAs and AKT signaling in regulating mES cell differentiation to cardiomyocytes.

In conclusion, we demonstrated that miR-301a promotes transcriptional activation of the cardiomyocyte-driving genes during mES cell differentiation to cardiomyocytes, and PTEN is a target gene of miR-301a in cardiomyocytes. PTEN-regulated PI3K-AKT-mTOR-STAT3 signaling is involved in regulating miR-301a-promoted cardiomyocyte differentiation from mES cells (Figure 4D). As discussed above, application of a heart-specific local delivery system for miR-301a administration will be required to avoid potential side effects of miR-301a. These findings will shed light on the induction of stem cell-derived cardiomyocyte differentiation and strengthen the potential of miR-301a in cell therapeutics in the treatment of heart disease.

ARTICLE HIGHLIGHTS

Research background

After myocardial infarction (MI) in adults, the regenerative ability of the differentiated cardiomyocytes is very limited due to the "terminated" cell proliferative ability and the lack of cardiac stem cells. Tissue repair after pathological injury in the heart, including that after MI, is still a major clinical challenge. Two novel strategies have been suggested to restore the lost cardiomyocytes caused by MI. One is to apply cardiomyocytes differentiated from stem cells or derived from cardiospheres, and the other is to induce cell cycle reentry in cardiomyocytes.

Research motivation

Stem cells and stem cell-derived cardiomyocytes have been demonstrated to be a promising source for cell transplantation-based treatment of injured hearts. Modification of approaches to induce cardiac cell differentiation from stem cells with high efficiency will be crucial to improve the therapeutic effect.

Research objectives

To explore the function of miR-301a in regulating cardiomyocyte differentiation of mouse embryonic stem cells, and provide experimental evidence for applying miR-301a to the cardiomyocyte differentiation induction from stem cells.

Research methods

The hanging drop technique was applied to form embryoid bodies from mouse embryonic stem cells with or without overexpression of miR-301a. Cardiac markers including GATA-4, TBX5, and MEF2C, and α-actinin were used to determine cardiomyocyte differentiation from mouse embryonic stem cells.

Research results

MiR-301a was identified as a miRNA highly enriched in the heart from late embryonic to neonatal mice. Overexpression of miR-301a in mouse embryonic stem cells significantly induced the expression of cardiac transcription factors, thereby promoting cardiomyocyte differentiation and beating cardiomyocyte clone formation. *PTEN* was demonstrated to be a target gene of miR-301a in cardiomyocytes. PTEN-regulated AKT-mTOR-Stat3 signaling was involved in regulation of miR-301a-induced cardiomyocyte differentiation.

Research conclusions

MiR-301a is capable of promoting embryonic stem cell differentiation to cardiomyocytes. As such, miR-301a has potential as a novel target gene to induce cardiomyocyte differentiation.

Research perspectives

These findings will be beneficial in development of an approach with high efficiency to induce stem cell differentiation to cardiomyocytes, and strengthen the potential of cell therapeutics to treat heart failure caused by myocardial infarction.

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