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## REVIEW



## ORIGINAL ARTICLE

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REVIEW

# Effects of environmental stressors on stem cells

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## Abstract

Environmental toxicants are ubiquitous, and many are known to cause harmful health effects. However, much of what we know or think we know concerning the targets and long-term effects of exposure to environmental stressors is sadly lacking. Toxicant exposure may have health effects that are currently mischaracterized or at least mechanistically incompletely understood. While much of the recent excitement about stem cells (SCs) focuses on their potential as therapeutic agents, they also offer a valuable resource to give us insight into the mechanisms and risks of toxicant effects. Not only as a response to the increasing ethical pressure to reduce animal testing, SC studies allow us valuable insight into the true effects of human exposure to environmental stressors under controlled conditions. We present a review of the history of publications on the effects of environmental stressors on SCs, followed by a consolidation of the literature over the past five years on a subset of key environmental stressors of importance to human health and their effects on both embryonic and tissue SCs. The review will make constructive suggestions as to areas of toxicant research where further studies are needed, as well as making indications of the potential utility for advancing knowledge and directing research on environmental toxicology.

**Key words:** Environmental substances; Toxic; Stem cells; Endocrine disruptors; Alcohols; Tobacco smoking; Metals; Heavy; Particulate matter; Volatile organic compounds; Ozone



**Core tip:** Environmental toxicants can cause health effects. While most research and discussion of stem cells focuses on their potential as therapeutic agents, they also offer a valuable resource to give us insight into the mechanism and incidence of the effects of environmental toxicants. We present a review of the history of relevant publications, followed by a consolidation of the literature over the past five years on a subset of key environmental stressors of importance to human health. Constructive suggestions as to the areas of toxicant research where further studies are needed, and indications of the

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## INTRODUCTION

Humans are chronically exposed to environmental stressors, pollutants of natural or anthropic origin which can have the effect of altering normal biological processes. While we have long known that many environmental toxicants, such as Lead (Pb) and Mercury (Hg), have deleterious effects on human health, the mechanisms by which these occur are not fully understood[1]. Further compelling the imperative to more thoroughly understand the biological mechanisms behind these environmental toxicants are recent findings that their effects are transgenerational, echoing resulting outcomes far beyond initial exposure[2].

While much attention has been given to the the-rapeutic potential of stem cells (SCs)[3], their ability to serve as barometers of the toxic effects of environmental stressors should not be understated[4]. As the body's raw materials, SCs and their responses to environmental insult serve as windows into the pathways of disease. In this review, we highlight this much overlooked intersection of the study of environmental stressors and their impact on SC health.

Both tissue and embryonic SCs (ESCs) are seen as resources for the repair and regeneration of human tissues[5]. SCs are also thought to maintain these tissues for the lifespan of the individual through their key characteristics of self-renewal and differentiation into specialized cells. Healthy SC function includes balanced cell proliferation and sufficient capacity for appropriate differentiation. We focus on the effects of environmental stressors that impact these operations. We also examine cell viability to analyze toxicity and provide a fuller picture of a toxicant's effect, by correlating cell number to cell behavior[6,7].

We began by a careful search to numerate the publications examining the effects of environmental stressors on SC populations. The goal was to achieve a better understanding of the history of this research as well as understand how research on individual stressors has changed over that time. We then made a more focused search of PubMed for research in the last five years on known environmental toxicants and SCs.

A list of known environmental agents whose exposure is known to cause adverse health effects in humans was drawn from the National Institute of Environmental Health Sciences (NIEHS)[8]. These environmental toxicants were cross-referenced with the United States Comprehensive Environmental Response, Compensation, and Liability Act Priority List of Hazardous Substances as outlined in the Agency for Toxic Substances and Disease Registry (ATSDR) 2017 substance priority list (SPL)- "the government's list, in order of priority, of substances most commonly found at waste facility sites on the National Priorities List (NPL) that are determined to pose the most significant potential threat to human health due to their known or suspected toxicity and potential for human exposure"[9]. Each substance on the list is given an impact score, derived from an algorithm, with higher scores denoting those substances that most frequently appear at NPL sites, their known toxicity, and potential for human exposure, and then ranked by order of highest to lowest impact. Findings were summarized into a list of 20 environmental toxicants, including four heavy metals, ten endocrine disruptors, and six other important substances.

Table 1 outlines these toxicants with the corresponding highest rank of each substance within its class in the 2017 ATSDR SPL. For example, Arsenic (As) is listed at a rank of #1, with a score of 1674, derived from an algorithm that factors its ubiquity at NPL sites, its high toxicity, and the large risk for human exposure. The heavy metals, Pb, As, and Hg claim the top three spots on the list while the Volatile Organic Compound (VOC), Vinyl Chloride, ranks #4 on the list. Several other VOCs, like Benzene and Trichloroethylene, are also included on the ATSDR SPL. However, we record the highest ranked substance (Vinyl Chloride) for each toxicant class in our table, along with their score.

## LITERATURE SEARCH

A keyword search was then performed in PubMed including the name of the environmental toxicant and SCs, *e.g.*, "Cadmium and stem cells" or "Particulate matter and stem cells" that were published from 2014 - June 2019. For each query, counts were recorded of total number of articles, review articles, and original research articles, and recorded in Table 2. On the topic of radiation and SCs, only those articles which treated radiation as an environmental exposure, as opposed to a tool for directed differentiation, were included. Our findings are classified again into three different classes: heavy metals, endocrine disruptors, and other environmental toxicants known to have deleterious human health effects. A separate PubMed search was

**Table 1 Toxicants and their highest rank on the 2017 ATSDR substance priority list1**

| Toxicant | Rank of highest scored class member | ATSDR score | Toxicant | Rank of highest scored class member | ATSDR score |
|---|---|---|---|---|---|
| Heavy metals: | | | | | |
| Lead (Pb) | 2 | 1531 | Mercury | 3 | 1458 |
| Arsenic | 1 | 1674 | Cadmium | 7 | 1320 |
| EDs: | | | | | |
| PAHs | 8 | 1306 | Organotins | Not Rated | - |
| OCs | 37 | 1049 | DDT | 13 | 1183 |
| BPA | Not Rated | - | DES | Not Rated | - |
| Dioxins | 72 | 941 | PCBs | 5 | 1345 |
| Phthalates | 58 | 995 | PFAS | 143 | 788 |
| Other environmental toxicants: | | | | | |
| Radiation | Not Rated | - | Particulate Matter | Not Rated | - |
| Alcohol | Not Rated | - | Ozone | Not Rated | - |
| Tobacco Smoking | Not Rated | - | VOCs | 4 | 1358 |

[1] Toxicants listed in order of number of PubMed counts listed in Table 2. ATSDR: Agency for Toxic Substances and Disease Registry; EDs: Endocrine disruptors; PAHs: Polycyclic aromatic hydrocarbons; OCs: Organophosphorus compounds; BPA: Bisphenol A; DDT: Dichlorodiphenyltrichloroethane; DES: Diethylstilbestrol; PCBs: Polychlorinated biphenyls; PFAS: Per- and polyfluoroalkyl substances; PM: Particulate matter; VOCs: Volatile organic compounds.

**Table 2 Counts from PubMed1 searches from January 2014 - June 2019**

| Toxicant | Total number of articles | Review articles | Original research articles | Toxicant | Total number of articles | Review articles | Original research articles |
|---|---|---|---|---|---|---|---|
| Metals: | | | | | | | |
| Lead (Pb) | 4436 | 1239 | 3197 | Mercury | 22 | 0 | 22 |
| Arsenic | 108 | 11 | 97 | Cadmium | 44 | 2 | 42 |
| EDs: | | | | | | | |
| PAHs | 944 | 22 | 922 | Organotins | 24 | 0 | 24 |
| OCs (Pesticides) | 430 | 31 | 399 | DDT | 11 | 1 | 10 |
| BPA | 84 | 15 | 69 | DES | 10 | 5 | 5 |
| Dioxins | 44 | 9 | 35 | PCBs | 8 | 1 | 7 |
| Phthalates | 36 | 2 | 34 | PFAS | 2 | 0 | 2 |
| Other environmental toxicants: | | | | | | | |
| Radiation | 4302 | 589 | 3713 | PM | 61 | 3 | 58 |
| Alcohol | 1760 | 112 | 1648 | Ozone | 17 | 0 | 17 |
| Tobacco Smoking | 188 | 34 | 154 | VOCs | 11 | 0 | 11 |

[1]PubMed.gov, the United States National Library of Medicine National Institutes of Health. ED: Endocrine disruptors; PAHs: Polycyclic aromatic hydrocarbons; OCs: Organophosphorus compounds; BPA: Bisphenol A; DDT: Dichlorodiphenyltrichloroethane; DES: Diethylstilbestrol; PCBs: Polychlorinated biphenyls; PFAS: Per- and polyfluoroalkyl substances; PM: Particulate matter; VOCs: Volatile organic compounds.

conducted for each of the 20 toxicants, including the substance name and SC, *e.g.*, "Phthalates and stem cells" without a time limitation. Key class members' data are presented in Figure 1-3: Separated as Figure 1. Heavy metals, Figure 2. Endocrine disruptors, and Figure 3. Other environmental toxicants. The figures illustrate PubMed publication counts for the substances plotted against time in years, from 1953, the earliest publication on SCs and one of the listed environmental toxicants, up until June 2019. The findings of our PubMed literature review from 2014 - June 2019 are presented in Table 3-5, with toxicants organized again into three classes: Table 3; Heavy metals, Table 4; Endocrine disruptors, and Table 5. Other environmental toxicants. The results are presented by mechanism of action as pertaining to SC viability, differentiation, and proliferation. Each table is then followed by a more focused discussion of key references, the mechanism of action and their associated health outcomes.

## HEAVY METALS

Heavy metals are ubiquitous in the human environment and their toxicity is associated with varied adverse health effects depending on the dose, route and duration of exposure. Of the top ten chemicals on the 2017 ATSDR SPL, four of these are heavy metals: As, Pb, Hg, and Cadmium (Cd)[9].

Pb is a persistent environmental toxin that for more than a hundred years has been known to have harmful human health effects[10]. Pb exposure in neural SCs (NSCs) was shown to slightly reduce cell proliferation[11]. Pb exposure was also shown to induce changes in microgliosis and astrogliogenesis in the hippocampus of mice, interfering with normal

**Table 3 Heavy metals and their effects on stem cells**

| Environmental Toxicant | Type of stem cell | Model | *In vivo/ In vitro* | Parameters[1] | Ref. | Environmental Toxicant | Type of stem cell | Model | *In vivo/In vitro* | Parameters[1] | Ref. |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Lead | Fetal germ | H | *In vivo* | ↑DNA methylation changes | [2] | Arsenic | Adipose-derived MSC | M | *In vivo* | ↓Differentiation | [15] |
| Lead | Neural progenitor | H | *In vitro* | ↓Proliferation | [11] | Arsenic | Induced pluripotent stem cell | H | *In vitro* | ↓Viability, ↑DNA damage | [16] |
| Lead | ESC | H | *In vitro* | ↑Neuronal differentiation changes | [77] | Mercury | HSC | M | *In vivo* | ↓Proliferation at high-doses ↑Proliferation at low-doses | [17] |
| Lead | Bone marrow-derived MSC | R | *In vitro* | ↓Osteogenesis | [13] | Mercury | Neural progenitor | M | *In vivo* | ↓Differentiation | [18] |
| Lead | Neural stem | M | *In vitro* | ↑Astrogliogenesis, ↑Microgliosis | [12] | Cadmium | Neural progenitor | H | *In vitro* | ↓Proliferation, ↑Apoptosis | [19] |
| Arsenic | ESC | M | *In vitro* | ↓Differentiation | [78] | Cadmium | HSC | M | *In vivo* | ↓Differentiation potential, ↑Myelopoiesis | [20] |

[1]For detailed information on parameters, see text below. H: Human; R: Rat; M: Mouse; ESC: Embryonic stem cells; HSC: Hematopoietic stem cells; MSC: Mesenchymal stem cells.

**Table 4 Endocrine disruptors and their effects on stem cells**

| Environmental Toxicant | Type of stem cell | Model | *In vivo/In vitro* | Parameters[1] | Ref. | Environmental Toxicant | Type of stem cell | Model | *In vivo/In vitro* | Parameters[1] | Ref. |
|---|---|---|---|---|---|---|---|---|---|---|---|
| PAHs | Neural progenitor | R | *In vitro* | ↑Proliferation, ↓Cell size | [23] | Dioxins | HSC | M | *In vivo* | ↑Cell number, ↓Lymphocyte differentiation | [33] |
| PAHs | HSC | H | *In vitro* | ↓Osteoblast differentiation, ↓Self-renewal | [24] | Dioxins | HSC | M | *In vitro* | ↓Long-term self-renewal | [34] |
| PAHs | Spermatogonial stem | M | *In vivo* | ↑Mutations | [25] | Phthalates | HSC | H | *In vitro* | ↓Viability | [6] |
| PAHs | Adipose-derived MSC | C | *In vitro* | ↓Adipocyte differentiation potential | [26] | Phthalates | Neural progenitor | M | *In vitro* | ↓Viability, ↑ROS, ↑Apoptosis | [36] |
| PAHs | Skeletal muscle-derived progenitor | H | *In vitro* | ↓Myogenic differentiation | [27] | Phthalates | ESC | M | *In vitro* | ↓Viability | [37] |
| OCs | Neural progenitors derived from human embryonal carcinoma stem | H | *In vitro* | ↓Viability | [28] | Organotins | Spermatogonial stem | H | *In vitro* | ↑Apoptosis | |
| Bisphenol A | Mammary epithelial stem | H | *In vitro* | ↑Proliferation, ↑Sphere-forming capability | [30] | Organotins | Bone marrow MSC | M | *In vitro* | ↑Adipogenesis, ↓Osteogenesis | [38] |
| Bisphenol A | Prostate epithelial stem | R | *In vivo* | ↑Proliferation | [31] | Organotins | Bone marrow MSC | M | *In vitro* | ↑Adipogenesis | [39] |
| Bisphenol A | Bone marrow MSC | H | *In vitro* | ↑Cytotoxicity | [79] | DDT | Bone marrow MSC | H | *In vitro* | ↑Proliferation, ↑Differentiation, ↓Morphological changes | [41] |
| Dioxins | Umbilical cord blood–derived iPSC | H | *In vitro* | ↑Differentiation | [80] | DES | Spermatogonial stem | M | *In vitro* | ↑DNA damage, ↑Apoptosis | [43] |
| Dioxins | Cord blood derived HSC | H | *In vitro* | ↓Lymphopoiesis | [32,81] | PCBs | Liver epithelial stem-like | R | *In vitro* | ↑Alterations in gene signaling | [46] |
| Dioxins | Bone marrow MSC | M | *In vitro* | ↓Osteogenesis | [35] | PFAS | Spermatogonial stem | H | *In vitro* | ↓Expression of spermatogonial markers | [48] |

[1]For detailed information on parameters, see text below. PAHs: Polycyclic aromatic hydrocarbons; OCs: Organophosphorus compounds; BPA: Bisphenol A; DDT: Dichlorodiphenyltrichloroethane; DES: Diethylstilbestrol; HSC: Hematopoietic stem cells; MSC: Mesenchymal stem cells; iPSC: Induced pluripotent stem cells; ESC: Embryonic stem cells; PCBs: Polychlorinated biphenyls; PFAS: Per- and polyfluoroalkyl substances; H: Human; R: Rat; C: Canine; M: Mouse; ROS: Reactive oxygen species.

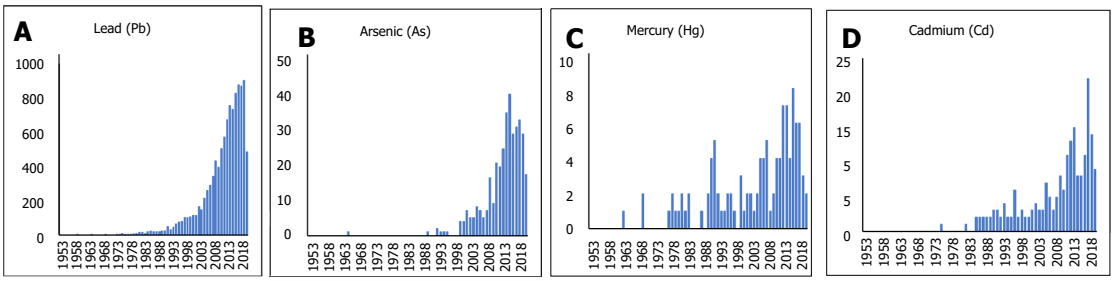


**Figure 1 Heavy metal PubMed publication counts from 1953 - June 2019**. A: Counts resulting from a PubMed search of the term, "Lead Pb and Stem Cells" plotted against time in years; B: Counts resulting from a PubMed search of the term, "Arsenic and Stem Cells" plotted against time in years; C: Counts resulting from a PubMed search of the term, "Mercury and Stem Cells" plotted against time in years; D: Counts resulting from a PubMed search of the term, "Cadmium and Stem Cells" plotted against time in years.

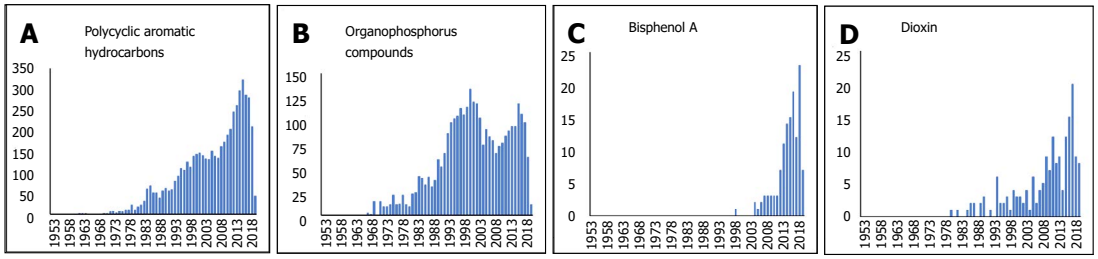


**Figure 2 Endocrine disruptor PubMed publication counts from 1953 - June 2019**. A: Counts resulting from a PubMed search of the term, "Polycyclic Aromatic Hydrocarbons and Stem Cells" plotted against time in years; B: Counts resulting from a PubMed search of the term, "Organophosphorus Compounds and Stem Cells" plotted against time in years; C: Counts resulting from a PubMed search of the term, "Bisphenol A and Stem Cells" plotted against time in years; D: Counts resulting from a PubMed search of the term, "Dioxins and Stem Cells" plotted against time in years.

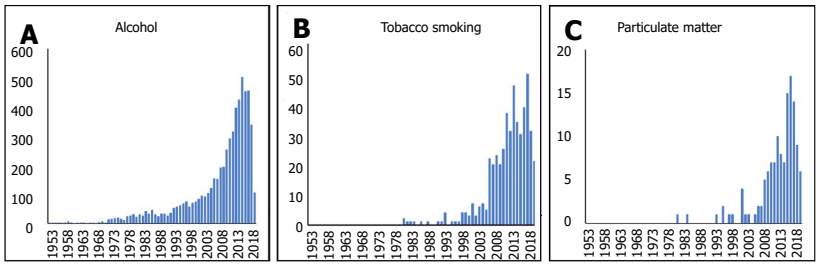


**Figure 3 Other environmental toxicants PubMed publication counts from 1953 - June 2019**. A: Counts resulting from a PubMed search of the term, "Alcohol and Stem Cells" plotted against time in years; B: Counts resulting from a PubMed search of the term, "Smoking and Stem Cells" plotted against time in years; C: Counts resulting from a PubMed search of the term, "Particulate Matter and Stem Cells" plotted against time in years.

neurogenesis[12]. Bone marrow-derived mesenchymal SCs (BM-MSCs) in rats showed an inversely proportional relationship between osteocalcin expression, a gene key to osteogenesis and Pb intake[13]. In a striking finding, DNA methylation changes in the fetal germ cells of pregnant mothers exposed to Pb were carried over to her grandchildren[2].

As is a known human carcinogen and is rated as the number one substance of concern on the ATSDR's 2017 SPL. It is a naturally occurring element that when combined with oxygen, chlorine, or sulfur can form inorganic As compounds. In embryonic mouse SCs, As inhibited differentiation into neurons and myotubes[14]. Differentiation, specifically osteogenesis and chondrogenesis, was also decreased in murine adipose-derived MSCs (AD-MSCs) after exposure to inorganic As[15]. In human induced pluripotent SCs, As exposure was shown to create a dose-dependent sequence of morphology changes, a decrease in viability, and induced genotoxicity[16].

Hg is a heavy metal with known associations to neuroinflammation, immunotoxicity, behavioral disorders, and adverse kidney effects. Mice exposed to 50 μmol of $HgCl^2$ experienced an increase in hematopoietic SC (HSC) proliferation, while those exposed to the higher dose of 100 μmol $HgCl^2$ saw HSC suppression[17]. Hg exposure suppressed embryonic murine NSCs neural differentiation at as low as 10 pmol concentration within 7 d and inhibited neural and glial differentiation by day 14. Moreover, Hg concentrations over 100 pmol suppressed NSC differentiation to motor or dopaminergic neurons[18].

Cd is a naturally occurring toxic metal in the earth's crust, typically extracted as a byproduct in the mining for other metals. It is commonly found in batteries, dyes, and some metal and plastic products. Low levels of Cd exposure decreased cell number and proliferation and induced apoptosis in adult human neural progenitor cells (NPCs)[19]. HSCs exposed to Cd over three months experienced an increase in long-term HSCs, a loss in long-term potential, and promoted

**Table 5 Other environmental toxicants and their effects on stem cells**

| Environmental Toxicant | Type of stem cell | Model | *In vivo/In vitro* | Parameters[1] | Ref. | Environmental Toxicant | Type of stem cell | Model | *In vivo/In vitro* | Parameters[1] | Ref. |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Radiation, Ionizing | HSC | H | *In vitro* | ↑ROS, ↑Apoptosis, ↑Senescence, ↓Long-term renewal | [49,50] | Particulate Matter | Bone marrow MSC | M | *In vivo* | ↑ROS, ↓Proliferation | [61] |
| Radiation, Radiofrequency | HSC | H | *In vitro* | ↓DNA damage | [51] | Particulate Matter | HSC | H | *In vivo* | ↓Telomere length | [62] |
| Alcohol | HSC | M | *In vivo* | ↑DNA double stranded breaks, ↑Chromosome rearrangement, ↑Myelopoiesis | [52] | Ozone (O3) | Adipose-derived MSC | H | *In vitro* | ↑ROS, ↑Lipid accumulation | [64] |
| Alcohol | Intestinal stem | M | *In vivo* | ↓Differentiation | [53] | VOCs | Bone marrow HSC | M | *In vivo* | ↑Apoptosis, ↓Nucleated bone marrow cells | [67] |
| Alcohol | ESC | H | *In vitro* | ↑Differentiation | [54] | VOCs | Enhanced eosinophil/ basophil progenitor | H | *In vivo* | ↑Differentiation | [65] |
| Cigarette smoke | ESC | M | *In vitro* | ↑Apoptosis, ↓Viability | [59] | VOCs | Neural progenitor | M | *In vitro* | ↑Cytotoxicity | [66] |
| Cigarette smoke | Bone marrow MSC | H | *In vitro* | ↓Differentiation, ↓Morphological changes | [58] | | | | | | |

[1]For detailed information on parameters, see text below. HSC: Hematopoietic stem cells; ESC: Embryonic stem cells; MSC: Mesenchymal stem cells; VOCs: Volatile organic compounds; H: Human; M: Mouse; ROS: Reactive oxygen species.

myelopoiesis[20].

Endocrine disruptors are chemicals or chemical mixtures that interfere with the proper function of hormones and can be naturally occurring, such as phytoestrogens, or synthesized as in plastics, plasticizers, pesticides, fungicides, and pharmaceuticals[21]. In this review, we include organophosphorus compounds (OPs), polycyclic aromatic hydrocarbons (PAHs), bisphenol A (BPA), dioxins, phthalates, organotins, dichlorodiphenyltrichloroethane (DDT), diethylstilbestrol (DES), polychlorinated biphenyls (PCBs), and per- and polyfluoroalkyl substances (PFAS).

PAHs are highly persistent organic compounds primarily released through both naturally occurring and man-made combustion, such as smoking or burning of fuel[22]. NSCs exposed to the PAH, benzo(a)pyrene (BaP), showed impairment in the transition from cell replication to neurodifferentiation, resulting in higher cell number, but reduced cell size and damaged neuronal features such as neurite formation and the development of dopamine and acetylcholine phenotypes[23]. BaP also decreased self-renewal and osteoblast differentiation of human BM-MSCs[24]. Mice exposed orally to BaP experienced spermatogonial SC (SSC) mutations with different phases of spermatogenesis exhibiting varying sensitivities to BaP[25]. In AD-MSCs, BaP did not inhibit cell proliferation, but did significantly inhibit adipocyte differentiation potential[26]. In human skeletal muscle-derived progenitor cells, low doses of BaP repressed myogenic differentiation without causing cell toxicity. When BaP exposure was withdrawn, the inhibitory effects on myogenesis were reversed[27].

OPs include the highly toxic nerve agent, sarin, as well as commonly used pesticides because of their inhibition of acetylcholinesterase. NPCs exposed to the OP pesticides paraoxon and mipafox during retinoic acid-induced differentiation showed reduced cell viability at high concentrations. Only paraoxon was shown to alter the process of neurodifferentiation[28].

The concern over the harmful health effects of BPA, a xenoestrogen used in the making of plastics, has been widely popularized in the media, leading the United States Food and Drug Administration to abandon its endorsement of its use in baby bottles[29] BPA is also commonly found in sports equipment, food and beverage packaging, and thermal paper products. In BM-MSCs, BPA exposure led to a dose-responsive increase in cytotoxicity, along with increased lipid peroxidation. BPA altered the response of proteins key in the regulation of fate and differentiation of human mammary epithelial SCs[30]. Low level BPA exposure altered differentiation of prostate epithelial SCs toward basal progenitors, reducing commitment to luminal progenitor cells, while increasing SC size and proliferation[31].

Dioxins include chlorinated dibenzo-p-dioxins, chlorinated dibenzofurans and certain PCBs. Dioxins are highly toxic, persistent compounds that are typically released into the environment through industrial incineration and bleaching processes. Exposure to the dioxin 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD), the most toxic dioxin, was shown to impair human B cell development by reducing lineage commitment in HSCS[32]. TCDD was also

shown to suppress hematopoietic progenitor cells and accelerate their differentiation to mature lineages[32]. HSCs in the fetal livers of offspring of TCDD-exposed mice experienced increased HSC proliferation, but B and T lymphocyte differentiation was significantly decreased[33]. TCDD exposure activated the aryl hydrocarbon receptor (AHR) in the fetus of pregnant mice, leading to impairment of the long-term self-renewal of HSCs[34]. In BM-MSCs, AHR activation by TCDD decreased osteoblast differentiation, possibly by suppressing the expression of the protein, β-catenin[35].

Phthalates are a group of chemicals primarily used to soften plastics and due to the prevalence of plastic products, exposure is widespread in the United States population. HSCs were exposed to four phthalates: dibutyl phthalate (DBP), benzylbutyl phthalate, diethyl phthalate (DEP), and diethylhexyl phthalate (DEHP), and all four compounds were shown to reduce cell viability[6]. NSCs exposed to DEHP also showed reduced cell viability, along with increased apoptosis due to reactive oxidative stress[36]. DEP and DBP also reduced cell viability in the ESCs of mice[37].

Organotins include tributyltin (TBT) and triphenyltin, substances with strong antibacterial and fungicidal properties that were historically used as marine anti-fouling additives in paint. Baker *et al*[38] showed that in BM-MSCs, TBT activated adipogenesis and reduced osteogenesis. This finding was also supported in primary BM-MSCs for both TBT and tryphenyltin[39].

DDT, an organochlorine developed as an insecticide was banned in the United States in 1972 after public outcry over its health effects, most notably outlined in Rachel Carson's famous work, *Silent Spring*[40]. However, DDT is a persistent organic pollutant, maintaining long-term exposure to organisms in the soil. When BM-MSCs were exposed to DDT, they exhibited altered morphology and inhibited self-renewal capacity, along with dose-dependent increased proliferation and differentiation[41].

DES is a synthetic estrogen that was prescribed by the United States physicians from 1938-1971, to prevent miscarriages and avoid other pregnancy problems, but was later found to cause a rare vaginal cancer to girls exposed in utero[42]. DES exposure in SSCs increased DNA damage, induced apoptosis, and increased intracellular superoxide anions[43].

PCBs are intentionally produced, stable aromatic chlorinated hydrocarbons commonly used as coolants in electrical equipment, as lubricants and plasticizers. Some PCBs are "dioxin-like" (DL-PCBs) and others are non-dioxin-like (NDL-PCBs), with the distinction owing to the site of chlorine substitution on the phenyl rings. DL- PCBs have congeners with no or only one chlorine substitution in the ortho position, have toxic effects similar to dioxins and bind strongly to the AHR[44]. The remaining NDL-PCB congeners, who have been linked through epidemiological studies with prostate cancer, have unique toxic effects and thus we examine them as a separate category of toxicant[45]. Human exposure occurs from improper storage and spillage of PCBs, where they bind strongly to soil and enter food sources. Liver progenitor cells exposed to the NDL-PCB, PCB153, experienced significant changes in the S1P/ceramide (Cer) ratio, known to be crucial in determining cell fate[46].

PFAS are a group of manmade chemicals that are used in including stain- and water-repellent fabrics, nonstick coatings, polishes, paints, and fire-fighting foams. They are very persistent compounds and thus can be found in water, soil, and organisms. Epidemiological studies have linked PFAS exposure to increased cholesterol levels[47]. SSCs exposed to PFAS did not experience a decrease in germ cell viability, an increase in reactive oxygen species (ROS), or reduced cell viability[48].

## RADIATION

Ionizing radiation, a common treatment for cancer, is known to induce ROS by altering cellular metabolism and is known to reduce numbers of bone marrow HSCs and alter differentiation[49,50]. However, the lower-intensity radiofrequency radiation, now rampant in society due to increased cell-phone and electronic use was shown to have only a mild effect on DNA damage and no effect on HSC apoptosis, ROS, cell cycle or DNA repair[51].

## ALCOHOL

Alcohol, known by its chemical name, ethanol, is mainly consumed via liquor, wine, and beer in order to create a psychoactive effect. Acetaldehyde, an endogenous and alcohol-derived metabolite, was shown to create DNA double-stranded breaks in HSCs. These breaks altered homeostasis by stimulating recombination repair, causing chromosome rearrangements, and inducing myelopoiesis[52]. Chronic alcohol consumption in mice was also shown to disrupt homeostasis in intestinal SCs (ISCs), in part, through the β-catenin pathway, suppressing proliferation of ISCs[53]. In human ESCs, alcohol was shown to stimulate differentiation by increasing the influx and metabolism of retinol[54].

## TOBACCO SMOKING

Cigarette smoking and the associated nicotine exposure is a notorious carcinogen and is the leading cause of preventable death in the United States[55]. While tobacco smoke is also considered a VOC and its smoke is known to contain PAHs, we treat it separately here due to its known severe health implications. Smoking is known to induce oxidative damage in SCs[56]. AD-MSCs exposed to cigarette smoke extract had significant impairment to cell viability, proliferation, and experi-

enced genetic level variations in differentiation[57]. Moreover, electronic cigarette extract exposure showed detrimental effects on BM-MSC morphology and proliferation[58]. ESCs exposed to cigarette smoke condensate produced altered gene expressions, reduced viability, and induced apoptosis[59].

It is important to note that PAHs are known to be present in cigarette smoking. However, for the purpose of this review, we have examined the effects of cigarette smoking and PAH exposure on SCs separately.

## PARTICULATE MATTER

Particulate matter (PM) are fine particles of air pollution whose potential to harm health is directly related to their size and capacity for inhalation[60]. Cui and colleagues showed that PM induces ROS, causing a suppression of *in vivo* proliferation in BM-MSCs[61]. Moreover, more recent and higher concentrations of $PM_{2.5}$ exposure in workers *via* welding fumes were shown to significantly reduce telomere length in HSCs[62].

## OZONE

Ozone ($O_3$) is a highly unstable toxic gas present in low levels in the atmosphere known to cause oxidative stress. However, there has been recent mechanistic evidence to suggest that low concentrations of $O_3$ may be therapeutic in some diseases[63]. AD-MSCs exposed to high concentrations of $O_3$ experienced cell damage *via* ROS, but low concentrations (5, 10 μg $O_3$/mL) had no effect on viability. Further $O_3$ exposure promoted adipogenesis[64].

## VOCS

VOCs are a large group of organic chemicals who disperse easily into surrounding air due to their high vapor pressure at standard air pressure. They are abundant in building materials, paints, and are produced in the burning of fossil fuels, and include third-hand smoke, the residue left behind on surfaces after smoking.

Infant eosinophil/basophil progenitor cell (Eo/B) viability was positively associated with VOC exposure, which contrasted from maternal Eo/B cells, which showed few to no associations[65]. This increase of HSCs in infants due to environmental exposure suggests an enhanced risk of the development of respiratory outcomes. NSCs exposed to acrolein, a VOC present in third hand smoke, experienced high rates of cytotoxicity, altered regulatory gene expression, inhibited proliferation at low doses, and cell death at high doses[66].

Formaldehyde (FA), a VOC commonly found in building materials and paints, significantly reduced nucleated bone marrow cells, and increased apoptosis in HSCs. These results suggest that FA's toxic effects operate by altering myeloid progenitor growth and survival through oxidative damage and reduced gene expression levels[67].

## DISCUSSION

The figures illustrating PubMed publication counts for key environmental toxicants attempt to describe the onset and subsequent pattern of research interest. There was little interest in Pb from the 1960s until the late 80s' and then a precipitous explosion of publications that is still climbing. The capture of the public's attention with the water supply crisis which started in Flint, Michigan in 2014, coupled with the headline grabbing work exploring the transgenerational effects[2], guarantee that Pb will remain a highly investigated toxicant for the foreseeable future. Compare now the recent onset of research on the heavy metal with an even higher ATSDR substance priority score, As. At the peak of publication number, six years ago in 2013, there was not even a twentieth of the number of publications on Pb. Hg and Cd have even fewer publications, with almost as high ATSDR substance priority scores as Pb and As. The difference is even more striking when we switch attention to publication in just the last five years. In that time, no review articles exist on the effect of Hg on SCs compared to over a 1000 on the effects of Pb.

Research on PAHs and OCs began in the late 60s' and show an interesting hump of activity peaking in the late 90s' followed by another peak in 2012-2013. BPA has only very recently begun to capture public attention and we confidently predict a steep increase in publications over the next few years. It is less clear why the dioxins on which research has existed for longer has yet to see an increase in publication activity.

Publications on the effects of alcohol on SC populations began in the 50s and show a broadly similar increase to that of Pb until a peak in 2015. More surprising is that the research on tobacco smoking garners only a tenth of the publications of alcohol in spite of constant public attention to the deleterious effects, with research appearing to have plateaued as of 2013. No reviews have been published on the effects of $O_3$ or VOCs on SCs in the last five years. To further emphasize the disparity in publication activity, Figure 4 plots the number of PubMed Original Research Articles published between January 2014 to June 2019 against the toxicant's ATSDR substance priority score. The regression line hopefully helps indicate those substances that are under-researched.

There are a number of reasons that contribute to the disparity including, but not limited to: Difficulty of isolation and maintenance of a given toxicant; Issues related to organic *vs*. inorganic form; Difficulty of administration in an *in vitro in vivo* preparation; and of course,

**Table 6 Proposed under-researched opportunities for stem cell models on environmental exposures**

| Environmental toxicant | Health outcomes | Stem cell model | Environmental toxicant | Health outcomes | Stem cell model |
|---|---|---|---|---|---|
| Heavy metals | | | | | |
| Pb | Decreased child cognition | Neural progenitor and SC-derived organoids | Cd | Kidney | Renal epithelial stem Nephron progenitor |
| Pb | Adult liver function | SC-derived organoids | Cd | Lung damage | Alveolar epithelial progenitor |
| As | Carcinogen: all tissues | Epigenetic analysis of different tissue SC populations | Cd | Lower bone strength | MSC |
| Hg | Cognitive function | Neural progenitor and SC-derived organoids | | | |
| Endocrine disruptors | | | | | |
| PAHs | Carcinogen: lung, skin | Epigenetic analysis of different tissue SC populations | Organotins | Liver | SC-derived organoids |
| OCs (Pesticides) | Cognition | Neural progenitor and SC-derived organoids | | Kidney | Renal epithelial stem Nephron progenitor |
| BPA | Unclear | | DDT | Carcinogen | Epigenetic analysis of different tissue SC populations |
| Dioxins | Carcinogen | Epigenetic analysis of different tissue SC populations | PCBs | Immune system | HSC derived populations |
| Phthalates | Carcinogen | Epigenetic analysis of different tissue SC populations | PCBs | Carcinogen | Epigenetic analysis of different tissue SC populations |
| | Cognition | Neural progenitor and SC-derived organoids | PCBs | Cognition | Neural progenitor and SC-derived organoids |
| Organotins | Carcinogen | Epigenetic analysis of different tissue SC populations | PFAS | Unclear | |
| Organotins | CNS | Neural progenitor and SC-derived organoids | | | |
| Other toxicants | | | | | |
| Particulate matter | Unclear | | VOCs | Unclear | |
| Ozone | Constricted breathing | SC-derived smooth muscle | | | |

PAHs: Polycyclic aromatic hydrocarbons; OCs: Organophosphorus compounds; Pb: Lead; As: Arsenic; Cd: Cadmium; Hg: Mercury; BPA: Bisphenol A; DDT: Dichlorodiphenyltrichloroethane; DES: Diethylstilbestrol; PCBs: Polychlorinated biphenyls; PFAS: Per- and polyfluoroalkyl substances; VOCs: Volatile organic compounds.

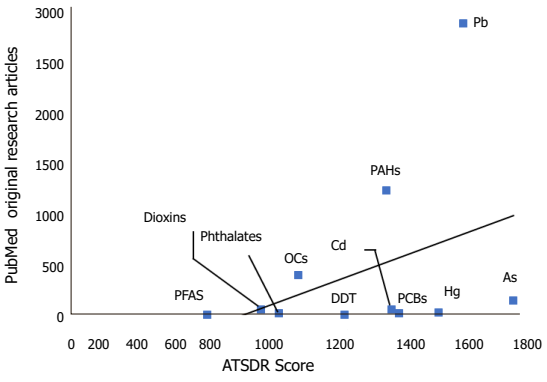


**Figure 4 Agency for toxic substances and disease registry score *vs* number of PubMed original research articles 2014 - June 2019.** ATSDR: Agency for Toxic Substances and Disease Registry; PAHs: Polycyclic Aromatic Hydrocarbons; OCs: Organophosphorus Compounds; Pb: Lead; As: Arsenic; Cd: Cadmium; Hg: Mercury; DDT: Dichlorodiphenyltrichloroethane; PCBs: Polychlorinated Biphenyls; PFAS: Per- and polyfluoroalkyl substances.

obtaining of funding to pursue research on a given toxicant.

Finally, in Table 6, we consider what if any are the clear and most prevalent health outcome(s) associated with each toxicant exposure and identify the most appropriate SC or SC-derived model for further research given the phase of life associated with that health outcome.

The tables of the toxicants and their effects on SCs represent a comprehensive consolidation of the references on the effects of environmental toxicants on SCs over the last five years. A note of qualification is required. It is tempting to conclude that any effect of a toxicant on a cell population is negative. Anyone who has worked in this field for any length of time likely, like one of us, has entire data sets that could not attract funding for further analysis because the interpretation of the data suggested that the "toxicant" exposure had what prima facie appeared a positive effect on the population under examination (Parker, Unpub. Obsn.)[68]. However, it is important to note that, for example, an increase in proliferation in a cell population does not necessarily mean the exposure's effect is beneficial. The result without a proper developmental investigation defies proper interpretation. But for the purposes of this review, and for the field of toxicology, that the toxicant has an effect at a level that can be reasonably be expected to be experienced by the target tissues is sufficient to indicate that the toxicant requires further study.

One of the major challenges to environmental toxicology today is quantifying the joint impact of environmental mixtures on health outcomes to more closely resemble real-world exposure[69]. We have tried to be as structured as possible in our classifications and groupings but inevitably certain of our "environmental toxicants" are themselves a mixture of active ingredients. An excellent recent review on the suitability of *in vitro* SC preparation for high throughput screening of mixtures was published by Liu *et al*[70].

A relatively recently acknowledged challenge is studying the combined effects of chemical and social stressors[71]. Such issues appear to be a problem not tractable by an *in vitro* cell preparation. However, one can imagine comparing cell samples obtained from carefully selected subject populations to begin to ask questions of how socioeconomic status, proximity to industrial pollutants, and occupation, affects response to a subsequent stressor. Further, how cells obtained from subjects during specific stages of life may usefully inform particular risk.

Finally, the effects of radiation and SC populations rightly have focused on the role of radiation in the treatment of patients with cancer. However, particularly as attention shifts again to exploration of low earth orbit travel and beyond, researchers are turning their attention to how such travel and potential settlement will affect human physiology[72,73].

Almeida-Porada *et al*[74] explored how the effects of radiation on the bone marrow niche can negatively impact hematopoiesis due to changes in MSC function independently of direct effect on HSCs.

It is worth emphasizing that our intent is not to say that all environmental toxicant effects on human health are mediated by SCs. But understanding the effects, or lack thereof, are an important part of the process of dermining mechanism, and potential interventions to ameliorate or prevent deleterious health impacts of exposure. Such models might in this regard be the "canary in the coal mine" as a more sensitive test for toxicity than other cell populations, *e.g.*, fibroblasts[75]. In this direction, Liang, Yin, and Faiola present a comprehensive review on developmental neural toxicity and environmental toxicants[76].

Industries manufacturing chemicals have a financial and legal duty to their shareholders to develop their technologies to maximize profit. The rapidity with which new chemicals appear in our environment outpaces the ability of interested parties to test and demonstrate potential deleterious impact using existing models. SC models offer a fast and robust model that can be at least a first indicator of the need for more laborious, time consuming and resource-expensive testing.

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REVIEW

# Suitability and limitations of mesenchymal stem cells to elucidate human bone illness

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## Abstract

Functional impairment of mesenchymal stem cells (MSCs), osteoblast progenitor cells, has been proposed to be a pathological mechanism contributing to bone disorders, such as osteoporosis (the most common bone disease) and other rare inherited skeletal dysplasias. Pathological bone loss can be caused not only by an enhanced bone resorption activity but also by hampered osteogenic differentiation of MSCs. The majority of the current treatment options counteract bone loss, and therefore bone fragility by blocking bone resorption. These so-called antiresorptive treatments, in spite of being effective at reducing fracture risk, cannot be administered for extended periods due to security concerns. Therefore, there is a real need to develop osteoanabolic therapies to promote bone formation. Human MSCs emerge as a suitable tool to study the etiology of bone disorders at the cellular level as well as to be used for cell therapy purposes for bone diseases. This review will focus on the most relevant findings using human MSCs as an *in vitro* cell model to unravel pathological bone mechanisms and the application and outcomes of human MSCs in cell therapy clinical trials for bone disease.

**Key words:** Mesenchymal stem cells; Bone illness; Osteoporosis; Osteogenesis; Osteoanabolic therapies; *In vitro* cell models; Cell therapy



**Core tip:** Human mesenchymal stem cells (hMSCs) have emerged as an encouraging therapeutic strategy for the treatment of bone diseases. Moreover, certain limitations of

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animal models for the study of bone disorders highlight the suitability and benefits of hMSCs for the elucidation of these pathologies. The current review explains the available strategies based on hMSCs for bone illness, new treatment development, and future directions in the field for more accurate knowledge of the cause underlying these human pathologies.

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## INTRODUCTION

In humans, the structural maintenance of the skeleton during adulthood is ensured by the continuous self-regeneration of bone tissue in a process called bone remodeling. The entire skeleton is renewed approximately every 10 years[1] by a sequentially coordinated action of two coupled processes performed in bone remodeling units at distinct locations all throughout the skeleton: Bone resorption and bone formation. Bone resorption, in which old and damaged bone is removed by osteoclasts, is a relatively fast process that can last 4-6 wk; whereas, new bone formation orchestrated by osteoblasts, which produce collagen and mineralized bone matrix, takes approximately 4-5 mo[2]. Osteoclasts and osteoblasts are differentiated cells originating from two separate cell lineages: Osteoclasts differentiate from hematopoietic precursors, and osteoblasts are cells of mesenchymal origin. Thus, osteogenic differentiation and the generation of new osteoblasts are driven by a sequential cascade of processes performed by mesenchymal stem cells (MSCs). First by the recruitment of MSCs to bone remodeling sites and subsequent proliferation, then lineage commitment with expression of lineage-specific markers, and finally with collagen secretion and extracellular matrix (ECM) mineralization[3].

Bone remodeling is a continuous process throughout life; however, the balance between bone formation and bone resorption is age-dependent. Thus, bone formation predominates for the first three decades until peak bone mass occurs[4]. Thereafter, when the growth period is complete in adulthood, there is a remodeling balance in which the previously achieved bone mass is maintained, and the amount of resorbed bone equals that which is subsequently formed. Later, in aging, the bone loss common to this period of life is due to an imbalance between bone resorption and bone formation: Accelerated osteoclastic bone resorption occurs compared to the amount of new bone formed by osteoblasts. Moreover, aged MSCs show a shift of lineage commitment to adipogenesis at the expense of osteogenesis[5] and a concomitant reduction in self-renewal capacity[6]. This dysfunction of MSCs, which contributes to the remodeling imbalance, lies at the root of bone loss due to aging. As a consequence, bone aging is the leading risk factor for primary osteoporosis, a progressive systemic skeletal disease characterized by a reduction in bone mineral density, predisposing the elderly population to an increased risk of fractures. In this scenario, the use of MSCs (osteoblast progenitor cells) for bone disease modeling emerges as a suitable approach to perform mechanistic studies, devise drug discovery by high throughput screenings, and test cell-based therapies. This review will focus on the current benefits and limitations of MSCs for two different goals related to bone illness: As *in vitro* disease models to study the pathogenic mechanisms of bone disease in order to screen and/or develop new therapeutic drugs, and as treatments based on cell therapies.

## THE SOCIO-ECONOMIC IMPACT OF BONE DISEASES

Age-related osteoporosis is the most prevalent bone disease, especially among postmenopausal women and older men, affecting over 200 million worldwide and causing more than 9 million fractures per year[7]. Improvements in socioeconomic and health-related factors have resulted in an increase in population life expectancy making osteoporosis a global and growing public health challenge. Osteoporotic fractures cause a 20% increase in mortality within 1 year of the broken bone and also result in poor quality of life, functional impairment, and loss of independence leading

to an increased financial burden in health care systems[8]. In addition to osteoporosis, more than 450 skeletal dysplasias have been described that affect primarily bone and cartilage, most of them with limited treatment options[9]. Abnormal bone formation directed by osteoblasts, abnormal bone resorption by osteoclasts, or both may be among the underlying pathological cellular mechanisms of these heritable diseases. Studying these rare genetic bone disorders is clinically highly relevant, and although individually they affect a small percentage of the population, their overall frequency is high: Two to five per 10000 live births[10]. Importantly, many of these diseases become apparent early in life and are present throughout the patient's entire life implying tremendous burdens in disability and suffering and requiring extensive medical and surgical treatments. Research focusing on these genetic skeletal disorders is not only beneficial for future treatment of patients but significantly contributes to the knowledge of key concepts of bone biology.

Pharmacologic therapies for osteoporosis can be categorized as either antiresorptive or anabolic; both strategies focus on reducing the risk of fractures[11]. Current treatments are mainly based on antiresorptive agents, including estrogen, selective estrogen receptor modulator, bisphosphonates, and a monoclonal antibody to receptor activator of NF-κB ligand (RANKL) (denosumab)[12]. These therapies decrease the generation, function, and survival of osteoclasts thereby reducing the rate of bone resorption. However, because bone resorption and formation are coupled processes, this inhibition in bone resorption also results in lower bone formation. Although antiresorptive drugs are effective in reducing fracture risk[13], there are concerns about side effects accompanying their continued use, such as increased cardiovascular events, increased breast cancer risk due to estrogen use[14], and more rare side effects, such as atypical femur fractures[15] and osteonecrosis of the jaw[16].

Moreover, bisphosphonates are known to accumulate in the skeleton and continue to be released for long periods of time following treatment[17]. Given that osteoporosis is a chronic disease, treatments for osteoporosis should theoretically be administered throughout the patient's life. However, due to the aforementioned side effects of antiresorptive drugs, they are generally not administered for more than 5 years. Taking into account both increased life expectancies and these limitations regarding the continued use of antiresorptive agents, there is an urgent need to develop new drugs for osteoporosis focused on osteoanabolic goals (to increase bone formation).

Currently, the two available anabolic drugs are teriparatide and abaloparatide, both recombinant human parathyroid hormone (PTH) analogs, which have been demonstrated to increase bone formation when given intermittently in small doses[18]. However, there were initial concerns regarding the long-term administration of these therapies as well because extended exposure to analogs of PTH in preclinical (animal) studies was associated with a higher risk of osteosarcoma[19]. However, a later long-term surveillance study of adult cases of osteosarcoma did not show an increased risk of osteosarcoma associated with teriparatide treatment[20]. Overall, these observations evidence that the range of current anabolic treatment is quite limited, making it imperative to identify, characterize, and develop novel effective and safe osteoanabolic therapies.

## ADVANTAGES AND FLAWS OF ANIMAL MODELS OF BONE DISEASES

Several animal models have been developed in order to study the different molecular mechanisms underlying bone-related diseases and to serve as fundamental tools in which to test and develop new therapeutic strategies. The biggest challenge when choosing the appropriate animal model is not knowing the exact cause of the disease. Rodents are the most commonly used animal model for research, despite the fact that large animals show bone development resembling the human process more accurately than rodents[21]. We will briefly summarize the advantages and disadvantages of different animal models used for the study of various bone diseases, and we will focus here on the success and failures of murine models to mimic different types of the bone disorder called osteogenesis imperfecta (OI).

Some rodent models successfully resemble the human characteristics of several bone-related diseases. A mouse model of Paget's disease in which the normal bone recycling process is affected shows increased bone resorption and bone formation and increased numbers of osteoclasts that are larger and multinucleated, a finding similar to human patients suffering from this disease[22,23].

Osteoporosis is distinguished by low bone mass and structural deterioration of bone tissue, occasioning bone fragility, and increased risk of fractures[24]. Osteoporosis has been studied in different animal models; however, none of these models

satisfactorily resembles the characteristics of the disease in humans[25]. The most extensively used model is the ovariectomized rodents (mouse or rat). This process induces a loss of bone mass and strength due to the reduction of estrogen, similar to the loss of estrogen in postmenopausal women. Despite the low costs and easy handling, rodents lack the Harvesian canal system of the cortical bone present in humans[25]. This is the initial animal system for identifying possible therapies. Potential drugs or treatments are subsequently replicated and tested on larger animals, such as primates, rabbits, sheep, and pigs[25,26].

Hypophosphatasia (HPP), or deficiency of the alkaline phosphatase (ALP) enzyme[27], has been investigated in various murine models. ALP knock-out mice have been largely used to identify mechanisms underlying the disease since affected mice adequately mimic the phenotype of children with HPP[28-31].

OI is a genetic disease with high heterogeneity at both the genotypic and phenotypic levels[32-34]. OI patients are classified into different OI types according to their phenotype and genetic mutation causing the disease. The majority of the mutations are autosomal dominant and are located in the *COL1A1* and *COL1A2* genes (Type I-IV), while some less frequent mutations are recessive and located in different genes involved in the osteogenic process (*IFITM5*, *CRTAP*, *LEPRE1*, *SERPINF1*, *PPIB*, and *FKBP10* among others)[32-34]. As the genetic causes of the OI phenotype are so diverse, several different models have been described for the study of the different OI types. Various models have been useful for the elucidation of OI pathology, while some models have shown effects opposite to those observed in OI patients. Here we present some of the murine models and their effectiveness in reproducing human OI phenotypes/symptoms (Table 1).

The low prevalence of certain types of OI (IX[35,36], XII[37,38], XIII[39], XIV[40,41], XV[42], and XVI[43]) makes it difficult to develop an exact diagnosis of symptoms and causes of these types of OI. Therefore, it is difficult to assess the suitability of the models even though such models could be a useful tool for gaining basic knowledge of these OI types. In contrast, several OI types have been successfully described for which the suitability of the animal models can be evaluated. Murine models for OI types I[44-46], II[44,47], III[44,48-51], IV[52-56], VI[57], VII[58-61], and XI[62,63] positively mimic human phenotypes. Models developed for OI type V[64-66], VI atypical[67], VIII[60,68,69], and X[70,71] show differences in the mechanisms underlying those mutations with diverse grades of severity when compared to humans and different signaling pathways involved in the process.

Despite murine models being the most utilized animal models for the study of human bone-related diseases, mice and humans diverged at some stages of the skeletal regulatory process[72]. More than half of the signaling pathways and bone development-related genes are expressed in both species. These include *BMP*, *Hedgehog*, *FGF*, and *Notch* and transcriptional regulators of osteogenesis like *RUNX2* and *SOX9*[72]. On the other hand, divergent genes comprise various members of the WNT signaling pathway, such as *SOST*, *CXXC4*, and deoxyribonucleic acid *(DNA)JB6*[72]. This fact should be kept in mind when trying to extrapolate results from murine models to patients.

In summary, animal models are a useful and necessary tool when elucidating the molecular mechanisms underlying disease with low prevalence, but are not sufficient to properly understand the human pathophysiology of the disease.

## MSCs AS EXPERIMENTAL HUMAN DISEASE MODELS

### *In vitro bone disease modeling by primary MSCs*

The failure of some animal models to resemble the features of many human diseases led to the development of a field focused on the creation of *in vitro* cell models using primary cells isolated from patients and healthy cohorts. These disease-relevant cell types recapitulate the majority of the pathological phenotypes observed in patients, providing new opportunities to study the cell biology and pathophysiology of the disease.

An example of such models focusing on prematurely aging cells is based on either human MSCs (hMSCs)[73] or induced pluripotent stem cells (iPSCs)[74]. MSCs are characterized by multipotency, self-renewal capacity, and the ability to differentiate into different cell lineages, *e.g.*, an osteogenic lineage[75-78]. The osteogenic potential of MSCs has been demonstrated in MSCs expanded culture[79] making them a perfect cell type for the study of molecular mechanisms regulating bone disorders, especially those disorders caused by osteoblast alterations[76-78,80]. Thus, MSCs, which are the context-related cell type for modeling diseases with mesenchymal defects, have emerged as an essential tool to unravel the molecular and cellular mechanisms

**Table 1 Mouse models developed for OI**

| OI type | Mutations at gene | Human phenotype | Ref. | Mouse model | Mouse phenotype | Effectiveness | Ref. |
|---|---|---|---|---|---|---|---|
| I | COL1A1/2 | α1 chain collagen haplo-insufficiency; vertebral compression fractures; short height; low lumbar spine bone mineral density | [32,33] | Col1a1+/Mov13 | Decreased type I collagen in mineralized tissue, weakened bone strength; abnormal shape of long bones; alterations of the mechanical properties of long bones | + | [44-46] |
| II | COL1A1/2 | Perinatal lethal | [32,33] | BrtlII; Aga2/b | Perinatal lethal | + | [44,47] |
| III | COL1A1/2 | High bone turnover; decreased mineralization; increased osteoclastic activity; small size; fractures; osteopenia; bone deformities | [32,33] | COL1A2 KO | Increased bone formation rate; fractures; reduced size; osteopenia; decreased mineralization; abnormal bone shape | + | [44,48-51] |
| IV | COL1A1/2 | Increased bone fragility; growth deficiency; weak bone geometry; impaired bone remodeling; decreased bone volume | [32,33] | 349G->C COL1A1 | Decreases in severity with age; increased bone brittleness; reduced bone size; abnormal bone shape; impaired bone remodeling | + | [52-56] |
| V | IFITM5 | Increased mineralization; increased osteoblast markers; decreased COL1A1 expression, secretion and deposition in the matrix; hyperplastic callus; calcification of the forearm interosseous membrane; radial-head dislocation; subphyseal metaphyseal radiodense band | [32,33] | 14C->T IFITM5 | Severe skeletal defects; perinatal lethality; decreased mineralization; reduced expression of osteoblast markers | - | [64-66] |
| VI Atypical | IFITM5 | Decreased levels of PEDF; decreased mineralization | [32,33] | IFITM5 Knock-Down | Reduced skeletal size less extreme in adults; no abnormal osteoclastogenesis; no abnormal osteoblasto-genesis | - | [67] |
| VI | SERPINF1 | Decreased mineralization; decreased trabecular bone | [32,33] | PEDF KO | Decreased ECM mineralization; reduced trabecular bone volume | + | [57] |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| VII | CRTAP | Growth delay; osteopenia; decreased bone formation; decreased mineralization; multiple fractures | [32,33] | CRTAP KO | Growth underdevelopment; osteopenia; decreased osteoblastogenesis; decreased mineralization; no spontaneous fractures | + | [58-61] |
| VIII | LEPRE1 | Lethal; severe growth deficiency; bone fragility; poorly mineralized skull; scoliosis; decreased mineralization | [32,33] | LEPRE1 Knock-Down | No lethality; abnormal collagen fibril ultrastructure in bone, tendon and skin | - | [60,68,69] |
| IX | PPIB | Lethality; severe bone mass reduction; extreme bone strength reduction | [32,33] | PPIB KO | Bone mass reduction; bone strength reduction | No enough information | [35,36] |
| X | SERPINH1 | Embryonic lethality; delayed type I collagen secretion; collagen accumulation in Golgi apparatus; osteopenia; dentinogenesis imperfecta; thin bones | [32,33] | HSP47 KO | Delayed type I collagen secretion; collagen accumulation in the endoplasmic reticulum | - | [70,71] |
| XI | FKBP10 | Growth delay; neonatal lethality; bone fragility | [32,33] | FKBP10 KO | Bone brittleness; underdeveloped growth; lethality | + | [62,63] |
| XII | OSX | Skeletal deformities; fractures; osteoporosis | [32,33] | Osx KO | No bone formation; decreased mineralization | No enough information | [37,38] |
| XIII | BMP1 | Skull defects; reduced bone mass; reduced bone strength | [32,33] | BMP1 KO | Reduced ossification of certain skull bones | No enough information | [39] |
| XIV | Tric-b | Reduced bone mass | [32,33] | Tric-b | No incorporation of collagen in the matrix; matrix insufficiency | No enough information | [40,41] |
| XV | WNT1 | Reduced bone mass; reduced bone strength; fractures; increased ductility | [32,33] | sw/sw | Bone fragility; low bone mass | No enough information | [42] |
| XVI | CREB3L1 | Reduced bone mass and fractures | [32,33] | CREB3L1 KO | Severe osteopenia; reduced type I collagen | No enough information | [43] |

+/- stand for positive mimicry of the OI type symptoms in humans (+) or negative mimicry of OI type symptoms in humans (-). OI: Osteogenesis Imperfecta; KO: Knock-out; ECM: Extracellular matrix.

involved in normal and pathological bone biology. Physiological aging is known to be accompanied by a switch of MSCs differentiation to the adipogenic lineage at the expense of osteogenesis, which leads to osteoporosis[81]. MSCs used as *in vitro* disease models of aging have been essential to elucidate various mechanisms that account for the osteogenic differentiation impairment exhibited in the context of aging, such as dysregulation of transcription factors and microRNAs, autophagy impairment, alterations of the nuclear lamina, and epigenetic modifications of DNA[82].

MSCs isolated from patients with particular bone disorders have also been essential in deciphering the underlying molecular mechanisms of the associated bone diseases.

**HPP:** MSCs isolated from pediatric patients suffering from HPP showed a premature entry into senescence and a differentiation switch to adipogenesis at the expense of osteogenesis, both of which are typical features of aging MSCs. These results indicated that the *ALPL* gene contributes to controlling MSC lineage differentiation and prevents cell senescence[83].

**Hutchinson-Gilford progeria syndrome (HGPS):** Also known as progeria, is a devastating rare genetic disorder characterized by dramatic premature aging in children, and the disease primarily affects tissues of mesenchymal origin[84]. Skeletal defects are among the HGPS phenotypes, including abnormalities in bone morphology and alterations in bone structure, which result in a unique skeletal dysplasia[85]. MSCs differentiated from patients iPSCs recapitulate some aspects of the syndrome, including abnormal nuclear architecture, progerin expression, defects in the DNA repair process, and premature differentiation into the osteoblastic lineage[74].

Recently, two simultaneous works based on a high throughput drug screening in progeria-MSCs showed the usefulness of this cell model to decipher the functional effects of drugs that are currently used in HGPS patients and to identify new potential pharmacological drugs to treat the disease[86]. Both works evaluated the capacity of already known and new screened drugs to restore the impaired osteoblastic differentiation exhibited by progeria-MSCs. Moreover, paracrine signaling appears to be impaired in aged MSCs, a hypothesis supported by results in which an *in vitro* aged hMSCs model has a secretome enriched in osteogenesis-related proteins that can trigger accelerated early osteogenesis in normal MSCs[87]. Among the increased secreted factors, insulin-like growth factor binding protein 7 (IGFBP7) was identified. Subsequent experiments silencing IGFBP7 expression revealed an essential and unknown role for IGFBP7 to maintain the viability of MSCs during the first steps of osteogenesis in which MSCs and pre-osteoblasts proliferate actively. Moreover, sheets of hMSCs overexpressing IGFBP7 improved bone healing in a rat tibial osteotomy model[88].

### *2D versus 3D culture of MSCs*

Although experimental modeling of human bone disorders represents a breakthrough to provide outstanding insight into the cellular and molecular mechanisms involved in bone pathologies, there are several drawbacks regarding the use of MSCs models that must be taken into account. The limited availability and extreme heterogeneity of MSCs from patients as well as limited proliferation capacity and loss of functionality are among the most common pitfalls when using MSCs *in vitro*.

In addition, the main cell culture approach used in research is 2D cell cultures in which cells adhere to the culture dish forming monolayers, a situation that does not reflect the *in vivo* cellular display where cells grow in a complex 3D disposition[89]. The conditions of the natural environment *in vivo* are poorly mimicked by 2D cell cultures since they do not preserve normal physiological shape and function. In other words, the morphology and physiology of 2D cultured cells highly diverge from *in vivo* grown cells[89,90]. Due to the complex architecture of bone tissue, the use of 2D cell cultures does not adequately mimic the actual mechanisms involved in bone tissue development and repair[90] making it a limited approach to the study of bone-related diseases. Furthermore, bone remodeling consists of a highly regulated balance between bone resorption and bone formation mediated by osteoclasts and osteoblasts, respectively. Osteoclasts are phagocytic cells derived from circulating macrophages in charge of bone degradation while osteoblasts differentiate from MSCs and are in charge of bone formation[91,92]. Osteoblast and osteoclast activity is tightly coupled positively influencing the osteogenic differentiation and matrix deposition in the same way as osteoclast development[91,92]. Therefore, osteoblast and osteoclast activity is directly regulated by the crosstalk between both cell types leading to an increased matrix deposition in osteoblast and osteoclast co-culture experiments[91,92]. Moreover, conventional 2D cultures have limited cell-cell and cell-matrix interactions, which are especially relevant in bone tissue such as the direct crosstalk between osteoblasts and osteoclasts, highlighting the need for more realistic 3D *in vitro* models of bone disease[90]. 3D cultures have been proposed as a bridge between 2D cell cultures and *in vivo* models, and therefore have been used in the study of bone diseases[93] as a consequence of their higher structural complexity and cellular homeostasis, which is more closely comparable to that of tissues and organs[89].

Due to the importance of the ECM in bone microarchitecture, a wide range of scaffolds have been developed for 3D culture of bone tissue[94]. The purpose of these scaffolds is to serve as extracellular support for adhesion of growing cells in a 3D structure[89]. Scaffolds used for bone tissue culture can be formed by different materials, such as collagen, bioceramics, titanium, gelatin, chitosan, polymers, hydrogels, and others[94-96]. The ideal scaffold should have similar mechanical

properties to bone; therefore, hydrophobicity and porosity are two essential features to keep in mind when engineering the scaffold[94,96,97]. Scaffolds have been used for different purposes such as basic research tools for *in vitro* and *in vivo* studies. Certain bone pathologies require therapeutic grafts due to the necessity of extensive bone regeneration[96]. Autografts are the best choice when compared to allografts; however, both have certain disadvantages. Autografts are size restricted and could create infections or morbidity in the healthy tissue from which the graft is taken. On the other hand, allografts lack the cellular content to assist tissue regeneration and could carry diseases[96]. Nevertheless, engineered scaffolds are considered a promising solution for bone grafts.

Several studies on animal models have been performed showing positive results for bone regeneration using engineered scaffolds and MSCs[80,98-100]. 3D scaffolds could also be used for drug delivery into bone tissue[93,101]. However, several disadvantages have been described when using scaffolds, such as cell adhesion, degradability of the scaffold, appropriate communication between cell types, and the simple 3D architecture of scaffolds[93]. Given these challenges, bioprinting has emerged as a potential solution to develop more sophisticated, complex, and accurate architectures of bone tissue *in vitro*[93].

Bioprinting is the latest tool in tissue engineering. This technology is based on a computer-aided design to create a 3D construct assembling biocomposite materials and living cells[93,102,103]. This strategy has the advantage of more accurate control of cell distribution, higher resolution, ability of cell deposition, spatial complexity in cell types and tissue organization, scalability, and lower cost when compared to 3D cultures using scaffolds. In addition, bioprinting provides a better cell-cell interconnection, oxygen diffusion, nutrient transportation, appropriate attachment, proliferation, and tissue formation factors[102-105]. Several studies have described the possibility of 3D-bioprinted bone substitutes for tissue regeneration[102,103,106]. In these studies, osteogenic differentiation of MSCs is possible allowing successful bone repair processes *in vitro* and *in vivo*[102,106,107]. Vascularization of the tissue is a crucial limitation[108]. Bioprinting of MSCs along with a functionalized vascular endothelial growth factor allows vascularization of the tissue leading to a successful proliferation,differentiation and generation of the mineralized ECM *in vitro*[108].

The classical methods for osteogenic differentiation of MSCs in culture are based on the addition of chemical and growth factors although environmental properties influence the *in vivo* osteogenic differentiation of MSCs. Osteogenic differentiation of 3D-bioprinted MSCs could be performed by the classical addition of chemical and growth factors or by the use of the appropriate bioink containing these stimuli. In fact, environmental effects can be mimicked through 3D bioprinting by the addition of soluble factors and additives into the chosen bioink[109]. Accessibility of nutrients and osteogenic stimuli are problems recognized in 3D cultures on scaffolds. Thus, stimulation of the cells through components of the bioink allows for a homogeneous distribution of the osteogenic stimulus reaching all the seeded cells[109].

On the other hand, 3D co-cultures of osteoblasts and osteoclasts have been described in which cells are able to deposit mineral matrix[91,92,110]. Most of the literature describing 3D co-cultures of bone cells is based on human and murine cell lines, which are barely exportable to human primary cells lines. However, recently a 3D co-culture system has been successfully described using patient MSCs for the study of jawbone osteonecrosis[110], which would be exportable to other bone-related diseases. This system means advancement in the elucidation of the pathogenic mechanisms and the discovery of novel therapeutic strategies for the treatment of bone-related diseases[110].

## MSCs AS THERAPEUTIC TOOLS FOR BONE DISEASES

MSCs are fibroblast-like cells that exist in almost all tissues, including bone marrow, fat, and the umbilical cord among others. They comprise a heterogeneous population of cells with differentiation and self-renewal ability ensuring a replacement mechanism for cells that die due to normal aging, injury, or disease[111]. Three criteria were proposed by the International Society for Cellular Therapy to define hMSCs as a cell type: (1) Plastic adherence when grown in standard culture conditions; (2) Expression of the cell surface molecules CD73, CD90, and CD105 and lack expression of hematopoietic markers CD34, CD45, CD14, CD19, CD11b, and HLA-DR; and (3) Multilineage differentiation potential into osteoblasts, adipocytes, and chondrocytes[112]. MSCs also exhibit immunosuppressive properties and express a broad range of chemokine receptors and therefore can migrate in response to many chemotactic factors[113].

On account of the aforementioned features of MSCs, they are thought to be ideal candidates for cell therapy purposes. However, from a clinical point of view, it must be considered that MSCs show huge variability in terms of functional capacities depending on different factors: Donors, tissue sources, clonal subpopulations, and even at the single-cell level[114]. When focusing on bone diseases, it would be recommended to screen those MSCs with a higher osteogenic potential to enhance the efficacy of cell therapy applications. With this regard, a striking paper recently described the identification of a human skeletal stem cell population that gave rise to the progenitors of bone and cartilage by the differential expression of four surface markers: PDPN, CD146, CD73 and CD164[72]. Importantly, these human skeletal stem cells were also shown to be locally amplified in response to skeletal injury. We anticipate that further characterization, isolation and amplification of human skeletal stem cells would be of special interest to obtain better outcomes in the treatment of skeletal disorders by future cell therapy approaches[72].

For most clinical indications, hMSCs are administered intravenously despite a post-infusion febrile reaction, which is a unique adverse effect associated with their use[115]. It was initially thought that, upon administration, the cells would home to the sites of injury, engraft, and differentiate into functional cells and then replace affected tissues. However, after administration, especially if they are systemically infused, MSCs engraftment levels are low, and their numbers decrease rapidly with time. The greater cell size of MSCs relative to the pulmonary microvasculature causes the vast majority of infused MSCs to be transiently trapped in the lungs upon the first pass through the circulation; the cells then become undetectable within hours[116]. This low survival and homing capacity of exogenous MSCs after administration raised the question of the underlying mechanisms responsible for the reported therapeutic benefits of MSCs therapy. Currently, there is growing evidence suggesting that the beneficial effects of MSCs come mainly from their paracrine properties. MSCs are known to secrete a wide range of bioactive factors and extracellular vesicles (exosomes and/or microvesicles) containing proteins, microRNAs, and hormones in response to the local environment, which affects the biology of nearby and distant responder cells and tissues[117]. Whether the observed beneficial effects of MSCs infusions are directly induced by their secreted factors, or if these factors initiate a cascade of signaling in the resident cell population, which then perform tissue repair, is currently under intense investigation.

### *MSCs-based therapies for skeletal dysplasias*

MSCs infusion has already been tested in clinical trials for two types of skeletal dysplasias, OI and HPP.

OI, or brittle bone disease, is a highly heterogeneous group of genetic disorders mainly caused by autosomal dominant mutations in one of the two genes (*COL1A1* or *COL1A2*) that encode type I collagen. These mutations can affect collagen structure (more severe phenotypes) or collagen quantity (milder phenotypes)[118]. In addition, severe additional non-collagenous genes have been described recently that cause severe forms of OI, including genes involved in post-translational modification, bone matrix mineralization, and osteoblast differentiation and function[32]. At this time, there is no cure for OI, and current treatments are focused on inhibiting bone resorption in these patients thus preventing bone loss.

The first proof of principle with allogenic MSCs infusions in the context of human OI was performed in 2002 by Horwitz *et al*[119]. They based their approach on a previous preclinical study that showed successful MSC engraftment into a murine model of OI, which produced a small but appreciable improvement in the disease phenotype[120]. Horwitz's study included six children, who had received bone marrow transplantation in a previous clinical trial that were given two infusions of adult MSCs. Although MSC engraftment was minimal (< 1% in osteoblasts), an increase in linear growth velocities was observed. Thus, it was established that allogeneic MSC infusion was not only safe in those pediatric patients affected by OI but also resulted in an increase in growth velocity albeit for a limited period of time[119]. A later investigation from this group of children indicated that the observed benefits could not be attributed to the direct differentiation of surviving infused MSCs into osteoblasts. The authors showed that infusion of MSCs conditioned medium in a mouse model stimulated chondrocyte proliferation suggesting that the secreted factors from MSCs could be responsible for the observed benefits in patients[121].

Gotherstrom and collaborators demonstrated the safety and efficacy of prenatal transplantation of human fetal MSCs in two fetuses affected by OI, with the premise that the administration of MSCs before birth would be more effective in alleviating OI symptoms[122]. However, both studies showed that the benefits from a single transplant of MSCs, regardless of the stage of life at administration, are transient, and subsequent infusions with the same donor-MSCs are needed to maintain the

beneficial effects.

HPP is a rare metabolic disorder resulting from a loss-of-function mutation in the *ALPL* gene that codes for the tissue-nonspecific ALP (TNSALP). There is no curative therapy for the disorder[123]. Impaired function of TNSALP leads to increased concentration of inorganic pyrophosphate in bone ECM; the deposition of this pyrophosphate hampers mineralization of bone and teeth and leads to pathological fractures. Due to the fact that current therapies for HPP have shown limited clinical improvements, hMSCs transplantation offers an attractive therapeutic option for these patients since MSCs, as well as osteoblasts, express high levels of TNSALP in their cell membrane, where it functions as an ectoenzyme[124].

Two studies have been carried out in which hMSC therapy has been administered to children suffering HPP showing improvements in bone mineralization in patients. In both of these studies, an hMSC infusion was given after previous transplantation of allogeneic bone marrow[125]. Moreover, chimerism analysis of the *ALPL* gene in the latest study revealed both the expression of wild type and mutant *ALPL* gene products suggesting that donor-derived MSCs were engrafted[126].

### MSCs-based therapies for delayed fracture healing

Non-unions are complications that imply a permanent failure of healing 6 mo after the fracture[127]. *In vitro* studies showed a decreased functionality of the pool of hMSCs in patients affected by nonunions likely due to a decreased serum expression level of chemokines and growth factors required for their recruitment and proliferation[128]. However, there was no impairment in the osteogenic capacity of these hMSCs once they were committed to osteogenic differentiation. Taking into account these previous results, a very recent prospective study described the treatment of fracture non-unions in patients with autologous culture expanded bone marrow-derived MSCs. A total of 35 patients received cell therapy, and fracture union was observed in 21 patients. Interestingly MSCs doubling time as well as age, diabetes, and multiple surgeries arose as significant predictors for the outcome of fracture unions[129].

### Cell-free therapies based on the secretome of MSCs

A concentrated secretome of MSCs, *i.e.* the paracrine factors secreted by MSCs mixed with beta-tricalcium phosphate scaffold have been used as a treatment in a recent clinical study for alveolar bone regeneration with encouraging outcomes. In this clinical study, authors showed an enhancement in vascularization, and early bone formation in patients treated with grafts impregnated with MSCs conditioned medium when compared to control patients, which were treated only with beta-tricalcium phosphate scaffolds. Moreover, the presence of MSCs conditioned medium shortened the time needed for degradation and replacement of beta-tricalcium phosphate scaffolds[130].

## CONCLUSION

In summary, primary MSCs isolated from patients in comparison with established cell lines efficiently resemble the pathological mechanisms of bone disease *in vivo*. Secondly, co-cultures offer a greater opportunity to mimic the *in vivo* intercellular crosstalk occurring in patients affected by bone diseases. Lastly, 2D cultures are easier to handle but are quite limited in mimicking the 3D architecture of bone *in vivo*; therefore, 3D cultures are more appropriate to resemble the *in vivo* cellular phenotype in the pathological conditions.

Moreover, MSCs are demonstrating their potential as human experimental models, as essential tools to develop new pharmacological and cell-based treatment strategies, and specifically as a therapeutic modality for bone disorders. Still, there are many questions to be elucidated regarding MSCs therapeutic effects and action mode on human pathologies. A better characterization of the pro-osteogenic MSCs will enable the development of more efficient cell therapies focused on the skeletal disorder.

The advances in using MSCs for therapeutic purposes indicate the extreme relevance of MSC in addressing bone disorders, and the unanswered challenges also suggest many opportunities for further research in this intensive field.

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*REVIEW*

# Breast cancer stem cells: The role of sex steroid receptors

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## Abstract

Breast cancer (BC) is the most common cancer among women, and current available therapies often have high success rates. Nevertheless, BC might acquire drug resistance and sometimes relapse. Current knowledge about the most aggressive forms of BC points to the role of specific cells with stem properties located within BC, the so-called "BC stem cells" (BCSCs). The role of BCSCs in cancer formation, growth, invasiveness, therapy resistance and tumor recurrence is becoming increasingly clear. The growth and metastatic properties of BCSCs are regulated by different pathways, which are only partially known. Sex steroid receptors (SSRs), which are involved in BC etiology and progression, promote BCSC proliferation, dedifferentiation and migration. However, in the literature, there is incomplete information about their roles. Particularly, there are contrasting conclusions about the expression and role of the classical BC hormonal biomarkers, such as estrogen receptor alpha (ERα), together with scant, albeit promising information concerning ER beta (ERβ) and androgen receptor (AR) properties that control different transduction pathways in BCSCs. In this review, we will discuss the role that SRs expressed in BCSCs play to BC progression and recurrence and how these findings have opened new therapeutic possibilities.

**Key words:** Breast cancer; Steroids; Sex steroid receptors; Cancer stem cells; Therapeutic implications



**Core tip:** Many studies have reported the presence of cancer stem cells (CSCs) in breast cancer (BC), highlighting the correlation between CSCs and BC aggressiveness. Sex steroids and steroid receptors play a pivotal role in BCSCs. By controlling different pathways, BCSCs are able to influence both BC recurrence and drug resistance. Therefore, better knowledge of BCSC features and behavior would be useful to employ

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these cells as BC prognostic factors, and develop new promising therapies targeting these cells and improving BC recurrence.

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## INTRODUCTION

Breast cancer (BC) is the most common cancer in women worldwide and the second most common cancer overall[1]. Although it is considered to be a postmenopausal disease, genetic predisposition, aging, gender, age of menarca, null parity, late age menopause and familial history of BC still represent the leading risk factors for BC[2].

Transformation of breast stem/progenitor cells is involved in breast carcinogenesis[3] and many studies have reported the presence of cancer stem cells (CSCs) in malignant BC[4-6]. CSCs might positively affect tumor survival, metastatic spreading and therapy escape[7]. Specifically, secretion of interleukins 6 and 8 (IL-6 and IL-8) by tumor associated fibroblasts, mesenchymal stem cells and macrophages promote CSCs self-renewal in BC, further pointing to the role of tumor micro-environment in cancer progression[7]. Estradiol also influences the breast cancer stem cells (BCSCs) population in a paracrine manner, as well as other factors, including metalloproteases (MMPs), insulin growth factor (IGF), platelet growth factor (PDGF) released by cancer-surrounding cells, which might affect proliferation, invasiveness and metastatic spreading of BC cells[8,9].

The presence and frequency of CSCs are, however, related to BC type, and many findings have shown a strong correlation between CSCs and BC aggressiveness. Meta-analyses from twelve published studies have shown that BCSCs are significantly associated with high histological grade, human epidermal receptor-2 (Her-2) positivity, estrogen receptor (ER) and progesterone receptor (PR) negativity, as well as the absence of any correlation with tumor size or nodal status[10]. Moreover, BCSCs are resistant to classical therapies. By enriching for the BCSC population, anti-cancer treatments often fail. Chemo- or radio-resistance of BCSCs has been attributed to different factors. As it occurs in SCs, they persistently remain in a quiescent state (G0 phase), while the cancer cells quickly replicate. Therefore, the standard therapies, which only act on rapidly dividing cells, are ineffective against BCSCs[11]. Again, BCSCs have enhanced expression of ATP-binding cassette (ABC) transporters and aldehyde dehydrogenase (ALDH), both capable of reducing the drug concentration inside cells[12]. Lastly, BCSCs exhibit an altered response to DNA damage, which protects them from apoptosis[11]. All of these properties make them resistant to the currently available antineoplastic therapies.

The role of sex steroids (estrogens, progestins and androgens) as well as SSRs in BC is largely recognized[13]. It is also currently accepted that sex steroids sustain the stem cell population in normal and malignant breast. An increase in the stem cell population might lead to cancer susceptibility in normal breast, while an increase in BCSCs influences both drug resistance and tumor recurrence[14,15]. Taken together, findings collected thus far suggest that CSCs represent a very promising prognostic factor in BC, although additional studies are required to confirm their importance in clinical practice.

In this review, we will present the recent findings on the role of sex steroid receptors (SSRs) in BCSCs. The therapeutic implication of these studies will also be discussed, since BCSC-targeted therapies seem very promising in the clinical management of BC patients.

## BCSCs

Mammary gland morphology continuously changes throughout life. At birth, human mammary gland epithelium is made up of a network of ducts. During puberty, mammary ducts form side branches, while also forming numerous lobulo-acinar structures containing the milk-secreting alveolar cells during pregnancy and lactation.

By activating massive apoptosis and tissue remodeling, the mammary gland then reduces its dimensions at the end of lactation[16]. To do this, a group of cells with high proliferative potential and differentiation ability have to be localized within the mammary tissue. Despite different studies demonstrating the presence of SCs in mammary tissue, these cells have not yet been identified and isolated to date[3]. Mammary (Ma)SCs are undifferentiated and their cell division can be symmetric, resulting in the production of two self-renewing or asymmetric cells. As such, a variety of pluripotent differentiated cells, including luminal and basal SCs as well as pluripotent progenitors, might differentiate into ductal, alveolar and myoepithelial cells. Consistent with the CSCs theory, both MaSCs and progenitor cells can give rise to BCSCs during these cell divisions, thereby fostering carcinogenesis[17]. A different theory claims that BCSCs are derived from de-differentiated cancer cells induced by changes in the tumor microenvironment, or chemotherapy or other targeted therapies. Through genetic or epigenetic modifications, transformed cells might acquire a stem-like phenotype[17-20].

BCSCs are more resistant than MaSCs, and are characterized by the expression of specific cell surface markers, such as high levels of cluster of differentiation 44 (CD44) and low levels of cluster of differentiation 24 (CD24). Particularly, high expression of CD44 maintains BCSC multipotency, while low levels of CD24 maintain cell stemness[21]. More recently, additional markers have been identified, including ALDH1, which oxidizes retinol to retinoic acid, thereby playing a role in the first step of BCSC differentiation. Elevated expression of ALDH1 identifies BCSCs and correlates with poor prognosis in receptor-negative BCs[22,23]. Again, other cell surface markers, such as the cluster of differentiation 133 (CD133), 49f (CD49f), and 90 (CD90) have been identified as CSC markers and are associated with drug resistance, poor prognosis, and reduced BC survival[24].

These findings, summarized in Table 1, have made it possible to design and synthesize specific antibodies to target these BCSC markers and create more efficacious therapies for aggressive BCs. To make this landscape more intricate, a plethora of pathways activated in MaSCs are deregulated in BCSCs. These include the Notch, Wnt, Hedgehog and Hippo pathways that, in addition to cross-reacting with each other, intersect with the main signaling pathways (PI3-K/Akt; MEK-dependent pathway) in BCSCs. As such, their successful targeting is very ambitious, since inhibition of one circuit frequently induces up-regulation and/or hyper-activation of the other pathways[24]. Unfortunately, less is known about the classical and non-classical pathways commonly activated by SSRs in BC cells. In the subsequent sections of this review, we will discuss the scant data in the literature that integrates and improves our knowledge about this topic.

### *ER in BCSCs*

Two isoforms of ER, ERα and ERβ, are expressed in BCs[25-28], with ERα representing the most important hormonal biomarker in this cancer. ERα is expressed in almost 75% of BCs, and its presence positively correlates with response to endocrine therapy[29]. In some studies, ERβ has also been associated with improved survival in tamoxifen-treated patients[30,31]. The two ER subtypes are encoded by genes on different chromosomes, and differentially activate common estrogen response elements (ERE) in gene reporter assays[32,33]. In target cells, both ER isoforms act through transcriptional and non-transcriptional mechanisms, thereby controlling cell cycle progression, invasiveness and metastatic phenotypes[34-36]. Recently, a new 36KDa truncated variant of ERα (ERα36) has been identified, which is expressed in both ERα-positive and negative BC cells. ERα36 lacks both of the transactivation ER domains, localizes to plasma membrane as well as cytoplasm, and responds to estrogens and anti-estrogens. It also regulates BC cell proliferation and contributes to BC aggressiveness[37].

The expression and role of each ER isoform in BCSCs, however, still remains under debate. The majority of studies points to the absence of ERα in BCSCs[38]. It has been consistently reported that $CD44^+/CD24^-/ALDH^+$ CSCs lack ER or express it at very low levels[15,39]. Although considered ERα-negative, the number of both BCSCs and MaSCs can be increased by stimulation with estradiol[38], likely because other receptors (for instance, G-protein coupled receptor 30, ERα36 or ERβ) might mediate estrogen action in these cells. These findings will be extensively discussed below.

Additional studies also argue that BCSCs do not harbor ERα, and that the receptor rather arises from the original BC. As a result, ERα would be expressed in BCSCs derived from ERα-positive BCs, while it would be absent in BCSCs derived from ERα-negative BCs[40]. As it occurs in prostate CSCs[41,42], these quite divergent findings may be due to experimental differences, such as ER assays, cell culture conditions and BC cell populations.

It is, however, currently accepted that estrogens act on BCSCs *via* non-genomic

**Table 1 Breast cancer stem cell biomarkers**

| Biomarkers | Expression | Role | Ref. |
|---|---|---|---|
| CD44 | Positive/high | Maintenance of breast cancer stem cell multipotency, cell proliferation and cell migration | Schabath *et al*[21], 2006 |
| CD24 | Negative/low | Cell migration and metastases | Jaggupilli *et al*[82], 2012 |
| ALDH1 | Positive/high | Stemness, cell migration, invasion, and tumor metastases | Ma *et al*[28], 2017 |
| CD133 | Positive | Cellular differentiation | Sin *et al*[83], 2017 |
| CD49f | Positive | Tumor initiation and metastases | Sin *et al*[83], 2017 |
| CD90 | Positive | Drug-resistance and poor prognosis | Schabath *et al*[21], 2006 |

CD44: Cluster of differentiation 44; CD24: Cluster of differentiation 24; ALDH1: Aldehyde dehydrogenase 1; CD133: Cluster of differentiation 133; CD49f: Cluster of differentiation 49f; CD90: Cluster of differentiation 90.

signaling, by activating GPR30, a seven domain trans-membrane receptor expressed in both ER-positive and ER-negative breast cancers[43]. It has been reported that this influences the Hippo pathway *via* tafazzin (TAZ) activation. In BCSCs, TAZ activation is responsible for BC metastatic features[44]. Again, elevated levels of TAZ combined with its increased activation can be detected in poorly differentiated BCs, where it confers self-renewal capacity to non-CSCs[45]. Other reports indicate that estrogens act by activating ERα or its variant, ERα36. In ERα-positive, MCF-7-derived tumor spheres collected on day 21 (tertiary tumor spheres), when they possess high levels of stemness markers and self-renewal ability, estrogen stimulation increases the levels of PI-9, a granzyme B inhibitor. Such an effect impairs immune surveillance, and increases both the number and size of tumor spheres[46]. ERα36, which lacks transcriptional activity and exclusively acts through non-genomic action, could mediate these responses since estrogen treatment of tertiary tumor spheres increases ERα36 levels and decreases the level of the full-length ERα[46]. In spite of ERα36 being predominantly a plasma membrane-based receptor and lacks both the AF-1 and AF-2 transactivation domains of ERα66 (ERαwt), it also acts as a negative regulator of genomic estrogen signaling mediated by both ERα wt and the ERβ[47]. A small amount of ERα36 is located in nuclei where it competes with the two receptors for DNA binding sites (ERE, [47]).

Again, upon estrogen stimulation, ERα36 rapidly activates the MAPKs/ERK pathway, thus triggering cellular proliferation[47]. The MAPK/ERK pathway is activated not only by estrogens but also by the antiestrogen tamoxifen in a stronger and prolonged way[47]. These findings might explain the pivotal role of ERα36 in anti-estrogen BC resistance.

ERβ and stem cell marker expression have been recently studied in mammospheres derived from fresh primary BC specimens and BC cells. In about 50% of cases, ERβ was upregulated in BCSCs. More importantly, it was co-expressed with CD44 and ALDH1 in the absence of ERα. Again, ERβ was responsible for the growth of mammospheres and the upregulation of glycolysis. Thus, ERβ might actually be considered as a stemness marker in BC cells[28]. This study offers new hints for a better understanding of ERβ function in BC and, in contrast with the concept that BCSCs respond to estradiol *via* paracrine signaling, it proposes that estrogens directly challenge BCSCs through ERβ activation. At last, identification of ERβ-enriched BCSCs offers new therapeutic possibilities based on the use of ERβ antagonists, combined with classical drugs (antiestrogens or aromatase inhibitors) routinely employed in the clinical management of BC.

Altogether, the data discussed thus far show that ERα and ERβ can both be detected in BCSCs. Depending on the specific context, they can be targeted to limit the proliferative and invasive rate of BCSCs. Although these cells are usually resistant to the classical therapies that target ER, the presented data support the idea that ER acts in an unconventional manner in BCSCs, paving the way for the exploration of new GPR30[48] or ERβ [28] inhibitors or drugs/peptides that specifically inhibit the non-genomic action induced by ERs in BC[25,35]. Some of the principal pathways operating in BCSCs are sketched in Figure 1.

### *PR in BCSCs*

Progesterone and its receptor play a pivotal role in mammary gland side branching that occurs during puberty, as well as lobular-alveolar development during pregnancy. PR exists in two isoforms, PR-A (PR-A, 94KDa) and PR-B (PR-B, 114KDa). The same gene encodes for the two PR isoforms, but PR-A lacks the first 164 amino acids of the PR-B, and might act as a trans-repressor of PR-B transcriptional activity, although it might even trans-repress the activity of ER, androgen receptor (AR), and

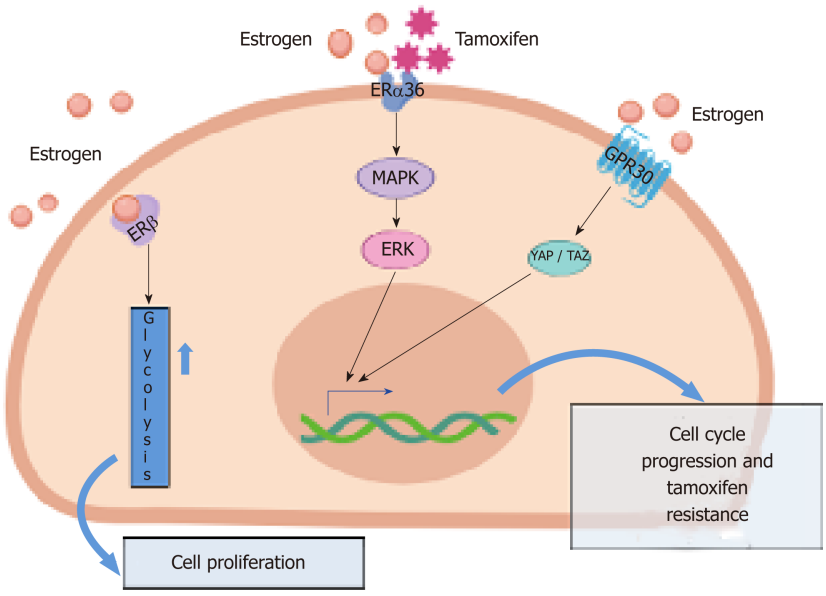


**Figure 1 The main pathways activated by different estrogen receptor isoforms in breast cancer stem cells, responsible for cell proliferation and tamoxifen-resistance.** GPER: G-protein coupled receptor; ERα36: estrogen receptor alpha 36; ERβ: estrogen receptor beta; MEK: Mitogen activated protein kinase; ERK: extracellular signal-regulated kinase; YAP: Yes-associated protein; TAZ: Tafazzin.

glucocorticoid and mineralcorticoid receptors[49]. The two isoforms are co-expressed at similar levels in normal breast cells, but this balance is altered in cancer cells, where one of the two isoforms, PR-A, is commonly overexpressed[50].

By enhancing SC proliferation and increasing the number of progenitor cells, progesterone influences mammary gland growth[50] and induces mammary tumor formation[38]. As it occurs for ER and AR, the ligand-activated PR works in BC cells through genomic and non-genomic mechanisms, thus controlling transcriptional machinery, epigenetic modifications and rapid signaling pathways depending on Src or PI3-K activation[51]. This is, however, a simple picture of progesterone action in target cells. We now appreciate that rapid activation of signaling cascades by ligand-bound PR fuels chromatin remodeling and gene transcription, on the one hand[52]. On the other, the progestin-activated transcriptional machinery might regulate cytoplasmic events, which impinges on signaling activation[53].

In women with pre-existing BC, progestins are responsible for the re-activation of ER-/PR- cancer stem-like cells[54]. Progesterone stimulation of differentiated cancer cells (ER+, PR+, CK5-) increases the number of stem-like colony cells (ER-, PR-, CD44+, CK5+) within the tumor. Ligand activation of PR does not modify the cell number, but rather de-differentiates the more abundant ER+/PR+/CK5- cells into ER-/PR-/CK5+ cells harboring stem-like properties[54]. Specifically, activated PR binds two putative progesterone response elements localized within the CK5 promoter. This transcriptional regulation finally leads to an increase in CK5 expression and is more effective in small, almost undetectable BCs, allowing their recurrence.

PRs are commonly considered as an indicator of the transcriptionally intact ER axis[55]. In BC-derived T47D cells, which express the two PR isoforms under basal conditions, PR-A is the principal driver of CSC expansion, while PR-B regulates anchorage-independent growth. Specifically, expansion and biochemical features of CSCs (ALDH1, $CD44^+/CD24^-$, $CD49f^+/CD24^-$) are linked to PR-A phosphorylation at the Ser 294 residue. $PR\text{-}A^+$ tumor spheres are, hence, small but express an enriched basal-like CSC phenotype ($CD49f^+/CD24^-$), which is suggestive of increased malignancy and metastatic potential. On the other hand, $PR\text{-}B^+$ tumor spheres are larger than the PR-A+ ones and exhibit a $CD49f^+/CD24^+$ luminal phenotype. Cells expressing a PR-A mutant that cannot be phosphorylated at the Ser 294 residue display an impaired CSC phenotype associated with an enhancement of anchorage-independent growth[55].

Taken together, the data presented thus far highlight the role of the progestin/PR axis in sustaining the survival and growth of BCSCs, and emphasize the role of each PR isoform in these processes. A better understanding of the role of each PR isoform in BCSCs might open new perspectives in the therapeutic approach of this cancer type, particularly in its recurrent forms.

### *AR in BCSCs*

AR expression is closely associated with a group of hormone-related diseases, including cancers of the prostate, breast, ovary, pancreas, liver and lung. It is also linked to various diseases that include muscle atrophy, osteoporosis, diabetes and neurodegenerative disorders[56-58].

AR is expressed in both ER-positive and ER-negative BCs[59]. In ER-positive BCs, AR correlates with a more favorable prognosis, while it is commonly considered to control progression and drug resistance in triple negative BCs[2,60]. It is largely accepted that AR activation by androgens regulates important changes in gene transcription or signaling pathway activation (*i.e*. Src/Ras/MAPKs, PI3K/Akt, filaminA/Rac). These actions control different processes, including proliferation, migration, and invasiveness of normal and cancer cells[25,58,61,62].

The role of androgens and AR in BCSCs is poorly explored, and few data have been published in the literature. By perusing the United States National Library of Medicine (https://www.ncbi.nlm.nih.gov/pubmed/), we found only 43 results matching with our analysis. In a recent paper[63], AR expression has been correlated with "stemness" markers (*i.e*. CD44, CD24 and ALDH1) in 166 BC patients. A significant correlation between AR and CD24 has been observed in stage I-III invasive BC. Such a phenotype correlates with favorable clinicopathological features, and delineates a subgroup of patients with better disease-free survival[63]. However, AR expression in CSCs might foster BC invasiveness. Forced suspension culture of AR-positive MDA-MB453 with SUM195pt cells induces an increase in the BCSC-like population, and protects cells from anoikis. Such effects depend on AR, as shown by experiments with the anti-androgen enzalutamide[64].

Again, dihydrotestosterone treatment increases the $CK5^+$ population in MCF-7 but not T47D cells. Notably, $CK5^+$ cells are therapy resistant, have increased tumor-initiating potential, and express the SC marker CD44[65]. The finding that androgens exert different actions in the two BC-derived cell types might be related to the different intersection of AR with other SSRs occurring at the transcriptional or non-transcriptional level in breast and prostate cancer-derived cells[41,42,66]. Furthermore, AR maintains the BCSC population in AR-positive TNBCs, since its knockdown or treatment with enzalutamide reduces the number of $ALDH1^+$ cells as well as mammosphere formation[67]. It should be noted that synthetic progestins activate AR[68]. Therefore, progestin-induced BCSC enrichment might be due to AR activation[69]. In addition to reinforcing the concept that SRs substitute each other in mediating important biological effects[25,70,71], such a mechanism might take place in BCs expressing high levels of AR in association with low or undetectable PR levels. Consistent with this hypothesis, it might also be argued that progestins launch a double hit by acting on both AR and PR. Altogether, these considerations account for the clinical correlation between progestin-treated women with increased BC risk, and highlight the complexity of AR's role in BC pathogenesis. The contribution of the androgens/AR axis in BCSC regulation, however, still remains uncertain.

## STEROID RECEPTOR-REGULATED MIRNAs IN BCSCs

In BCSCs, steroid receptors are also able to control miRNA levels. ERα regulates microRNA (miRNA) expression, thereby controlling the ability of BCSCs to affect proliferation, death, adhesion and cell-cell communication[72]. In BCSCs, activated ERα binds a specific ERE flanking the promoter region of miRNA-140, thereby suppressing miRNA-140 transcription and enhancing the expression of SOX2, a stemness marker, which maintains SCs[73].

PR regulates different miRNAs in BC. Among them, miR-29 and mi-R 200 families are involved in BCSC formation. The miR-29 family includes three members, miR-29 a, b and c, which are all down-regulated by progestins in BC. Such down-regulation is linked to an increase in the transcription factor KLF4, as well as CD44 and CK5, with the subsequent de-differentiation of cells[74]. It has also been shown that the progestin-induced increase of GATA3 results in miR-29b down-regulation, and a subsequent increase in the BCSC population[75]. Again, the miR-200 family includes miR-141, which is down-regulated by PR. miR-141 increases the CD44+ and CK5+ cell population, while reducing PR and Stat-5 levels, two important transcription factors implicated in the control of mammary cell fate[76].

There are no studies about miRNA regulation by AR in BCSCs. Few obtained data have shown that AR is responsible for miRNA down-regulation[77]. In ER-/PR-/AR+ cells, AR up-regulates the pro-differentiation of miRNA let7a, which, in turn, inhibits cell proliferation by downregulating c-MYC and K-Ras[78].

Altogether, the findings reported here indicate that ER and PR upregulate miRNA

levels involved in CSCs formation and differentiation. As such, they represent excellent targets to impair CSCs formation and, likely, BC recurrence.

## CONCLUDING REMARKS AND FUTURE DIRECTIONS

A growing number of studies is trying to clarify the role of BCSCs in BC pathogenesis and progression. Although interest in the study of BCSCs is currently high, it is not yet well known how these cells work within the cancer, and the identity of the engaged pathways.

Based on the stem cell hypothesis, cancer might arise from a cell population with the stem property of self-renewal. Such a property can already be owned by cells or can be acquired. As such, cancers originating from these cells are organized in a hierarchical fashion, in which SCs or stem-like cells drive the malignant process and generate a population of non-renewing cells that regulate the cancer bulk[79].

Less is known about the role of SSRs in SCs. Despite the fact that some reports claim that ERs are not expressed in BCSCs, many studies concerning the expression and role of this receptor have been published, with very conflicting data. The classical isoform of ERα acts, for instance, through a genomic pathway that regulates miRNA expression and SC phenotype, while the ERα variant, ERα 36 or GPR30, may act through non-genomic pathways, thereby contributing to cell dedifferentiation, tumor metastases and therapy resistance. Surprisingly, ERβ is commonly considered a marker of stemness in BCSCs. Its targeting by specific antagonists can be envisaged as mono or combinatorial therapy in the clinical management of BC.

Both PR isoforms seem to play a pivotal role in BCSC expansion and proliferation, and are tightly linked to BC metastatic and malignant properties. In this way, deepened knowledge of the machinery controlled by PR in BCSCs might be a big step forward to predict BC relapse and inhibit the growth of BCs resistant to currently employed therapies. The role of AR remains uncertain, and data about its behavior in BCSCs are very scant. Therefore, it is very difficult to draw any conclusions concerning the role of this receptor in BCSCs.

In conclusion, the data discussed thus far points to PR isoforms and ERβ as the more convincing targets to reduce the BCSCs population within human BC. Therefore, a better and more exhaustive understanding of other SSRs is required in order to develop new treatments of BC and control drug resistance, which is often imputable to BCSCs.

Preclinical and clinical evidence indicates that BCSCs control progression, invasion, metastasis as well as drug and radiation therapy resistance. Therefore, eradication of BC strictly depends on the elimination of BCSCs. New molecules such as GDC0449 or eribulin have entered clinical trials for their anticancer stem cell activity [80,81]. Further preclinical and clinical studies are needed to elucidate the relevance of CSCs signaling in BC recurrence and therapy resistance.

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REVIEW

# Immunomodulatory properties of dental tissue-derived mesenchymal stem cells: Implication in disease and tissue regeneration

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## Abstract

Mesenchymal stem cells (MSCs) are considered as an attractive tool for tissue regeneration and possess a strong immunomodulatory ability. Dental tissue-derived MSCs can be isolated from different sources, such as the dental pulp, periodontal ligament, deciduous teeth, apical papilla, dental follicles and gingiva. According to numerous *in vitro* studies, the effect of dental MSCs on immune cells might depend on several factors, such as the experimental setting, MSC tissue source and type of immune cell preparation. Most studies have shown that the immunomodulatory activity of dental MSCs is strongly upregulated by activated immune cells. MSCs exert mostly immunosuppressive effects, leading to the dampening of immune cell activation. Thus, the reciprocal interaction between dental MSCs and immune cells represents an elegant mechanism that potentially contributes to tissue homeostasis and inflammatory disease progression. Although the immunomodulatory potential of dental MSCs has been extensively investigated *in vitro*, its role *in vivo* remains obscure. A few studies have reported that the MSCs isolated from inflamed dental tissues have a compromised immunomodulatory ability. Moreover, the expression of some immunomodulatory proteins is enhanced in periodontal disease and even shows some correlation with disease severity. MSC-based immunomodulation may play an essential role in the regeneration of different dental tissues. Therefore, immunomodulation-based strategies may be a very promising tool in regenerative dentistry.

**Key words:** Mesenchymal stem cells; Dental tissue; Immunomodulation; Peripheral blood mononuclear cells; Oral diseases; Tissue regeneration

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**Core tip:** In the present review, the complex mechanisms of interactions between dental-tissue derived mesenchymal stem cells (MSCs) and immune cells are considered. Potential implication of MSC-mediated immunomodulation into progression of periodontal disease and dental tissue regeneration is discussed.

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## DENTAL TISSUE-DERIVED MESENCHYMAL STEM CELLS AND THE BASIC MECHANISMS OF MESENCHYMAL STEM CELL-MEDIATED IMMUNOMODULATION

Mesenchymal stem/stromal cells (MSCs) are defined as cells that fulfil the following minimal criteria: First, they adhere to culture plastic under standard cell culture conditions; second, they express the mesenchymal surface markers CD29, CD73, CD90, and CD105 and do not express the haematopoietic surface markers CD11b, CD14, CD34, CD45, and HLA-DR; third, they are able to differentiate into osteoblasts, adipocytes, and chondrocytes under certain conditions *in vitro*[1]. MSC-like cells were found in different postnatal tissues, inclusive of numerous dental tissues such as the dental pulp, human exfoliated deciduous teeth, the periodontal ligament, the apical papilla, the dental follicles and the gingival tissue[2-8]. Most dental tissue-derived MSCs express several neural lineage markers, which may be due to their neural crest origin[9,10].

Similar to MSCs from other tissues, dental MSCs possess a strong immunomodulatory ability[11-13]. Potential mechanisms of the immunomodulatory effects of MSCs include the expression of enzymes, the production of soluble factors, and cell-to-cell contact, and these mechanisms are reviewed in detail elsewhere[14-16]. MSC-mediated immunosuppression in humans is largely mediated by indoleamine-2,3-dioxygenase-1 (IDO-1), which catalyses the catabolism of L-tryptophan into L-kynurenine. As a result, depletion of tryptophan leads to the suppression of different immune cells[17]. Further important soluble factors involved in the MSC-dependent immunomodulation are prostaglandin E2 (PGE-2), tumor necrosis factor α-stimulated gene 6 (TSG-6), hepatocyte growth factor (HGF), transforming growth factor (TGF)-β, interleukin (IL)-10, galectins, and human leukocyte antigen (HLA)-G5[18]. PGE-2, a metabolic product of the arachidonic acid cascade, is produced by cyclooxygenase 2 (COX-2) and influences both the innate and adaptive immune systems[19]. MSCs continuously produce the potent immunomodulatory cytokines TGF-β and its production can be enhanced by other immunomodulatory cytokines, such as IL-4 and IL-13[20]. The anti-inflammatory cytokine IL-10 can be produced either by MSCs themselves or MSC-regulated immune cells[21]. Additionally, direct cell-to-cell contact accounts for at least some of the immunosuppressive effects of MSCs, and these effects are mediated mainly through programmed cell death ligand 1 (PD-L1), PD-L2 and membrane-bound HLA-G1[22].

## IMMUNOMODULATORY EFFECTS OF DIFFERENT DENTAL TISSUE-DERIVED MSCS

Dental tissue-derived MSCs, similar to MSCs from other tissues, modulate the activity of different immune cell subsets. Our knowledge in this field arises mainly from *in vitro* cell culture studies. Usually, these studies have used different co-culture models of MSCs with various subsets of immune cells and can be relatively easily controlled. Some studies have used a so-called direct co-culture model, in which the immune cells are added directly to tissue culture plastic-adherent dental MSCs. Other studies have used an indirect co-culture model in which the immune cells and MSCs are separated by a liquid-permeable membrane. In most studies, dental MSCs have been co-cultured with peripheral blood mononuclear cells (PBMCs), followed by the analysis of specific markers and/or functional characteristics of different immune cell

subsets. These experimental approaches have some advantages and limitations. PBMCs are a heterogeneous population of different immune cells, with a composition of 70%-90% lymphocytes (T cells, B cells, and NK cells), 10%-20% monocytes, and 1%-2% dendritic cells[23]. These co-culture models are relatively easily controlled and are convenient for studying the mechanisms of MSCs' immunomodulatory effects. However, such co-culture models hardly mimic any known *in vivo* interaction. Furthermore, this approach does not allow for the evaluation of the direct effects of MSCs on different subpopulations of PBMCs. In some studies, the co-culture of dental MSCs with isolated immune cell subsets has been performed. In most co-culture experiments, immune cells have been activated with different stimuli, such as concanavalin A (Con A), phytohemagglutinin (PHA), anti-CD3/CD28 antibodies, lipopolysaccharide, etc. These stimuli are crucial for activating immune cell proliferation and/or differentiation and, as we discuss in chapter 3, for stimulating the immunomodulatory ability of dental MSCs. However, the activation of PBMCs with most of these stimuli is rather artificial and hardly representable for the situation *in vivo*.

### Dental pulp stem cells

In 2002, the first characterized dental-derived mesenchymal stem cells were isolated from the dental pulp by Gronthos *et al*[4]. These cells were located in the perivascular region and fulfilled all minimal criteria for mesenchymal stem cells[4,24]. Several studies have investigated the interaction of dental pulp stem cells (DPSCs)with various components of both the innate and adaptive immune systems like T cells, natural killer cells, and macrophages, as well as the complement system. A pioneering study by Wada *et al*[25] showed that DPSCs suppress the proliferation of allogeneic PHA-activated PBMCs in a cell-to-cell contact-independent manner. The same study showed that the conditioned medium from interferon (IFN)-γ pre-treated DPSCs inhibits PBMC proliferation[25]. Another study investigated the effect of DPSCs on PHA-activated $CD4^+$ T cells. This study showed that IFN-γ-primed DPSCs inhibit T cell proliferation, reduce IL-17 production and stimulate regulatory T cell ($T_{reg}$) differentiation[26]. A recent study demonstrated that DPSCs inhibit PHA-induced PBMC proliferation but have no effect on $T_{reg}$ differentiation[27]. The same study also showed that DPSCs in co-culture with anti-CD3/CD28 antibody-activated PBMCs inhibit $CD8^+$ T cell proliferation and B cell immunoglobulin production[27]. The inhibitory effects of DPSCs on T cells and B cells are enhanced by IFN-γ and mediated by TGF-β[27]. One study reported that DPSCs also induce T cell apoptosis, which is supposed to have an anti-inflammatory effect *in vivo*[28]. DPSCs in co-culture with PHA-activated $CD3^+$ T cells inhibit T cell proliferation, induce T cell apoptosis and stimulate $T_{reg}$ formation[29]. A recently published study showed that osteogenic-differentiated DPSCs also inhibit the proliferation of PHA-activated PBMCs[30]. DPSCs isolated from healthy and inflamed pulp tissue suppress LPS-triggered TNF-α secretion by macrophages *via* an IDO-dependent mechanism but have no effect on IL-1β production[31]. DPSCs also influence macrophage polarisation *in vivo*. In particular, transplanting DPSCs into the unilateral hindlimb skeletal muscle triggers macrophage M2 polarization and suppresses sciatic nerve inflammation[32]. DPSCs can sometimes be susceptible to NK cell-mediated cytotoxicity[33]. The resistance of DPSCs to NK cell-mediated lysis is substantially increased after DPSC differentiation, the overexpression of hypoxia-inducible factor 1 or monocyte co-culture[34,35]. Furthermore, DPSCs activate the complement system. In particular, lipoteichoic acid-treated dental pulp progenitor cells express almost all factors necessary for complement system activation[36]. Furthermore, the complement system seems to influence DPSC proliferation and mobilization by activating the C3a and C5a complement system receptors, which are expressed by DPSCs[37,38].

### Periodontal ligament stem cells

A heterogeneous population of periodontal ligament stem cells (PDLSCs) was isolated for the first time from the periodontal ligament, a highly specialized connective tissue located between the alveolar bone and cementum, and characterized by Seo *et al*[6] in 2004. To date, these cells have been shown to exhibit immunomodulatory effects *in vitro* and/or *in vivo* on T cells, B cells, dendritic cells, macrophages and polymorphonuclear neutrophils (PMNs). Wada *et al*[25] showed that human PDLSCs, similar to DPSCs, suppress PBMC proliferation by a paracrine mechanism and this ability is enhanced by pre-treatment with IFN-γ. A later study reported that IFN-γ-primed PDLSCs in co-culture with PHA-stimulated PBMCs inhibit T cell proliferation, stimulate $T_{reg}$ differentiation and decrease IL-17 production by T cells[39]. The same study showed that human PDLSCs isolated from inflamed tissue suppress Th1 differentiation and IFN-γ secretion by T cells, which are effects that have not been observed with human PDLSCs isolated from healthy tissue[39]. Human PDLSCs inhibit

proliferation and IFN-γ production by Con A-stimulated PBMCs *via* both indirect soluble mediators and direct cell-to-cell contact[40]. Human PDLSCs inhibit proliferation and IL-2 and IFN-γ production in PHA-stimulated PBMCs[41]. A further study investigated the effect of human PDLSCs on the proliferation of $CD3^+$ T cells primed by monocyte-derived dendritic cells[42]. This study showed that the $STRO1^+$ $CD146^+$ subpopulation of human PDLSCs inhibits T cell proliferation by suppressing the expression of the non-classical major histocompatibility complex-like glycoprotein CD1b on dendritic cells[42]. One study showed that human PDLSCs negatively regulate the proliferation, differentiation and chemotaxis of differently stimulated B cells *in vitro*, mainly through cell-to-cell contact mechanisms mediated by PD-L1, and inhibit B cell apoptosis *via* an IL-6-dependent mechanism[43]. Furthermore, the transplantation of allogenic human PDLSCs suppresses humoral immunity in a minipig periodontitis model[43]. The effect of human PDLSCs on macrophages is controversial in the literature. One study reported that medium from PDLSCs suppresses TNF-α expression in the murine monocyte/macrophage RAW 264.7 cell line[44]. In contrast, another study did not find any effect of conditioned medium from PDLSCs on the polarisation of the human monocyte/macrophage THP-1 cell line[45]. Moreover, the same study showed that extracellular vesicles from LPS-pre-treated PDLSCs promote macrophage polarization towards an inflammatory M1 phenotype[45]. A study on periodontal ligament cells (PDLs), which share many features with PDLSCs[46], demonstrated that these cells downregulate TNF-α production by THP-1 macrophages in the presence of *Porphyromonas gingivalis* (*P. gingivalis*) through cell-to-cell contact and the secretion of IL-6 and IL-10[47]. The same study showed that periodontal ligament fibroblasts increase the phagocytosis of *P. gingivalis* by macrophages[47]. There is some evidence that human PDLSCs modulate the function of PMNs. One study showed that human PDLSCs reduce apoptosis and enhance the antimicrobial activity of human PMNs *via* both cell-cell interactions and paracrine mechanisms[48]. Another study found that human PDLSCs reduce PMN apoptosis *via* an IL-6-dependent mechanism[49]. A very recent study demonstrated that reactive oxygen species produced by neutrophil-differentiated leukemic HL-60 cells are inhibited by conditioned medium from resting PDLSCs but stimulated by that from *P. gingivalis*-treated PDLSCs[50]. In addition to the effect on different immune cell subsets, human PDLSCs can also influence their tissue recruitment. A very recent study reported that LPS-stimulated PDLSCs reduce CD29 expression in PBMCs and inhibit the transendothelial migration of PBMCs *in vitro*[51].

### Gingival mesenchymal stem cells

The gingiva is a specialized oral tissue attached to the alveolar bone that is considered as a mucosal barrier and is of essential importance for oral mucosal immunity. Currently, gingival mesenchymal stem cells (GMSCs) are assumed to be the best stem cell source for cell-based therapies and regenerative dentistry[52]. The isolation and characterization of GMSCs and their immunomodulatory properties were first described by Zhang *et al*[2] in 2009. In this study, GMSCs were shown to suppress PHA-induced PBMC proliferation[2]. Human GMSCs inhibit proliferation and Th1/Th2/Th17 differentiation in mouse $CD4^+$ T cells[53]. Furthermore, GMSCs promote the polarization of PBMC-derived macrophages towards the M2 phenotype[54]. Similarly, a recent study using THP-1 macrophages showed that human GMSCs suppress the activation of M1 macrophages and promote their polarization into the M2 phenotype[55]. GMSCs have also been shown to inhibit the maturation and differentiation of monocyte-derived dendritic cells through a PGE-2-dependent mechanism[56]. The same study also showed that GMSCs suppress the release of inflammatory cytokines by the human mast cell line HMC-1 through a PGE-2-dependent mechanism but have no effect on the proliferation of HMC-1 cells[56]. There are also some studies showing that gingival fibroblasts (GFs), which are isolated from the gingival tissue and share many characteristics with GMSCs, possess an immunomodulatory ability[57]. In particular, human GFs suppress the Con A-induced proliferation of PBMCs, and this effect is quantitatively similar to that of DPSCs on PDLSCs[25]. Both primary human GFs and the HGF-1 cell line suppress LPS-induced TNF-α production by THP-1 macrophages[47].

### Stem cells of human exfoliated deciduous teeth

In 2003, mesenchymal stem cells were first isolated from human exfoliated deciduous teeth by Miura *et al*[5] and termed stem cells of human exfoliated deciduous teeth (SHEDs). In particular, these cells were obtained from the pulp of deciduous teeth and show a higher proliferation rate, faster cell-proliferation doubling time and higher osteoinductive capacity than DPSCs isolated from permanent teeth[5]. A study using anti-CD3/CD28 antibody-activated PBMCs and naïve $CD4^+$ T cells showed that SHEDs inhibit Th17 differentiation, and the effect of SHEDs was stronger than that of

bone marrow MSCs[58]. Furthermore, the differentiation, maturation, and T cell-activation ability of monocyte-derived DCs have been shown to be influenced by SHED[59]. Particularly, DCs exhibit decreased production of the inflammatory cytokines IL-2, TNF-α, and IFN-γ and increased production of anti-inflammatory IL-10 protein after the exposure to SHEDs. Further, DCs have been observed to exhibit enhanced ability of$T_{reg}$ cells induction under the influence of SHEDs[59]. Recently, polarization of mouse bone marrow-derived macrophages toward M2 phenotype has been shown to be promoted by human SHEDs[60].

### *Dental follicle stem cells and stem cells from apical papilla*

MSCs from dental follicles [dental follicle stem cells (DFSCs)] were first isolated from the ectomesenchymal tissue surrounding the developing tooth germ and characterized in 2005[8]. MSCs from human root apical papilla tissue [stem cells from apical papilla, (SCAP)] obtained from the exterior of the root foramen area were first isolated and characterized in 2006[7]. Only a few studies have addressed the immunomodulatory ability of these dental MSCs. After priming with toll-like receptor (TLR)-3 or TLR-4 agonists, DFSCs inhibit the PHA-stimulated proliferation of PBMCs, and this inhibition is mediated by IDO and TGF-β[61]. Human DFSCs infected with the periodontal pathogen *Prevotella intermedia* or *Tannerella forsythia* reduce neutrophil chemotaxis, phagocytic activity and NET formation[62]. SCAP in co-culture with PHA-stimulated porcine PBMCs inhibit the proliferation of $CD3^+$ T cells[63].

## RECIPROCAL REGULATION OF MSC IMMUNOMODULATORY PROPERTIES BY THE IMMUNE SYSTEM

The immunomodulatory properties of dental MSCs are determined by the surrounding microenvironment and are usually low in quiescent MSCs. The activation of MSCs with inflammatory cytokines such as IFN-γ, TNF-α and IL-1β, which are produced in high amounts by activated immune cells, drastically enhances their immunomodulatory potential[64]. Thus, MSCs and activated immune cells reciprocally regulate each other. Moreover, MSCs might adopt either an immunosuppressive or immunostimulatory phenotype depending on the level of inflammation[65]. Similar to that of MSCs from other sources, the immunomodulatory activity of dental MSCs largely depends on activation by inflammatory cytokines, which are usually produced by immune cells. A pioneering study by Wada *et al*[25] showed that the proliferation of Con A-stimulated PBMCs is inhibited by direct co-culture with PDLSCs, DPSCs and GFs but not by conditioned medium collected from resting dental MSCs. This finding suggests that the activation of the immunosuppressive abilities of different dental tissue-derived MSCs requires several factors produced by activated PBMCs. A recent study of human DPSCs showed that their ability to inhibit PBMC proliferation and B cell immunoglobulin production was significantly enhanced by IFN-γ and inhibited by anti-IFN-γ antibodies[27]. In a recent study, GFs were co-cultured with PBMCs without any activating stimuli. Under these conditions, the GFs induced the survival and selective proliferation of different lymphocytes but had no immunosuppressive effects[66]. These facts suggest that activated immune cells play a crucial role in inducing the immunomodulatory potential of dental MSCs and suggest tight reciprocal regulation between these cell types.

Activated immune cells induce the upregulation of the expression of various immunomodulatory proteins in dental MSCs. Thus, the expression of IDO, TGF-β1 and HGF in PDLSCs, DPSCs, and GFs is upregulated upon co-culture with Con A-activated PBMCs[25]. Another study found that the expression of IDO, COX-2, TSG-6, and IL-10 in human PDLSCs is upregulated after co-culture with Con A-activated PBMCs[40]. Conditioned medium from PBMCs stimulated with various stimuli, such as phorbol methyl acetate/ionomycin, LPS, Con A, and anti-CD3/CD28 antibodies, upregulates IDO and COX-2 expression in human PDLSCs[67]. The expression of HGF, HLA-G5, IL-6 and TGF-β is upregulated in human DPSCs after co-culture with PHA-activated $CD3^+$ T cells[29]. A recent study showed that the production of PGE-2, TGF-β and IL-10 by human DPSCs is enhanced by PHA-activated PBMCs[30]. The expression of PD-L1 and PD-L2 in human PDLSCs is upregulated after co-culture with activated B cells[43]. COX-2 expression and PGE-2 production by GMSCs are upregulated by activated mast cells through a TNF-α-dependent mechanism[56].

The expression of various immunomodulatory factors in dental MSCs is also upregulated by different inflammatory cytokines. The expression of IDO is drastically upregulated by IFN-γ in human PDLSCs, DPSCs, and GMSCs/GFs[25,68-71]. In GFs, the

gene expression levels of IDO induced by IFN-γ are significantly higher than those of IL-1β and TNF-α; moreover, only IFN-γ is able to enhance the enzymatic activity of IDO[71]. The expression of HLA-G in human PDLSCs is enhanced by IL-12 mediated by autocrine IFN-γ signalling[72]. Another study showed an upregulation of HLA-G5 expression in human PDLSCs induced by IFN-γ[67]. The surface expression of PD-L1 in human PDLSCs is upregulated by different inflammatory cytokines, including TNF-α, IL-1β, and IFN-γ, but the effect of TNF-α is significantly greater than that of other cytokines[73]. Moreover, the effect of TNF-α on PD-L1 expression is further enhanced by the simultaneous application of other cytokines[73]. IL-1β and IFN-γ but not IL-17A enhance the gene expression of PD-L1 in periodontal ligament cells (PDLs)[74]. Interestingly, the expression of other immunomodulatory factors, such as HGF and TGF-β, in human PDLSCs, DPSCs, and GFs is not affected by IFN-γ[25,67]. One study found that the expression of TGF-β in human PDLs is upregulated only by the simultaneous application of IL-1β, IL-17A, and IFN-γ but not by separate stimulations with these cytokines[74]. Thus, one can assume that certain inflammatory cytokines activate only specific immunomodulatory parameters. In this case, priming with different cytokines might hypothetically activate only specific immunomodulatory functions in dental MSCs.

## REGULATION OF THE IMMUNOMODULATORY PROPERTIES OF DENTAL TISSUE-DERIVED MSCS BY BACTERIAL PATHOGENS

The oral cavity is a habitat for different microorganisms, and host-microbial homeostasis is a crucial factor for maintaining oral health[75,76]. Oral diseases are often associated with the disruption of this homeostasis and bacterial invasion into the oral tissues. The mobilization of MSCs to the inflamed area is assumed to be an important factor contributing to the progression of the inflammatory response, but the exact role of these cells in inflammatory processes *in vivo* still needs to be clarified. During inflammatory processes, dental MSCs are exposed to different bacterial and viral products. The expression of TLR family members in different dental MSCs is well described, but their contribution to immunomodulation by dental MSCs is not yet well understood[77-79]. Initially, it was believed that the priming of MSCs with TLR-2 or TLR-4 agonists conferred a pro-inflammatory phenotype that allowed these primed MSCs to stimulate the immune response; in contrast, MSCs primed with TLR-3 agonists were thought to adopt an anti-inflammatory phenotype and exhibit immunosuppressive properties[80]. However, a recent study showed that the ability of bone marrow MSCs to induce $T_{reg}$ differentiation is enhanced by both TLR-3 and TLR-4 activation[81]. Another study reported that TLR-3 and TLR-4 activation abolishes the ability of MSCs to suppress T cell activation[82]. These findings imply that the roles of different TLRs in MSC-mediated immunomodulation still need to be clarified. Unfortunately, there are only a limited number of studies in which the effects of different TLR agonists on the interactions of dental MSCs with different immune cell subsets have been investigated.

One report investigated the effect of TLR-3 and TLR-4 agonists on the ability of DPSCs and DFSCs to suppress the proliferation of PBMCs[61]. This study found that the TLR-3 agonist enhances the inhibitory effects of both types of MSC on PBMC proliferation. In contrast, the TLR-4 agonist augmented the immunosuppressive properties of DFSCs but inhibited those of DPSCs[61]. The treatment of human PDLSCs with the TLR-4 agonist LPS did not influence the inhibitory effect of the PDLSCs on the PHA-stimulated proliferation of $CD4^+$ T cells or the ratio of $CD4^+$ $CD25^{high}$ /$CD4^+$ $CD25^{low}$ lymphocytes[51]. The LPS-treated PDLSCs did not change the frequencies of $CD34^+$ and $CD45^+$ cells but decreased the frequencies of $CD33^+$ and $CD14^+$ myeloid cells within the PBMC population[51]. The pre-treatment of GFs with *P. gingivalis*-derived LPS stimulated their ability to suppress PBMC proliferation, but this effect was rather small compared to the effects of IFN-γ-primed cells[70]. Interestingly, *P. gingivalis*-derived LPS enhanced the IFN-γ-induced immunosuppressive ability of GFs[70].

As shown by some studies, different TLR agonists and bacterial compounds may stimulate the expression of various immunomodulatory proteins in dental MSCs. The expression of IDO seems to be influenced by different TLR agonists, but there are some controversies in the existing data. In human PDLs, *Escherichia coli* (*E. coli*)-derived LPS enhances *IDO* gene expression levels and IDO activity, as measured by kynurenine production[83]. In contrast, another study showed that the TLR-2 agonist Pam3CSK4 only increases the gene expression of IDO and does not have any effect on protein expression, as measured by intracellular staining[68]. In addition, Pam3CSK4

was able to enhance IFN-γ-induced IDO protein expression[68]. The same study did not find any effect of the TLR-4 agonist *E. coli*-derived LPS on the expression of IDO at either the gene or protein level[68]. A study of DPSCs found that Pam3CSK4 and *E. coli*-derived LPS have no effect on IDO protein expression but are able to enhance IFN-γ-induced IDO expression[69]. Another study found that the expression of IDO in DPSCs is enhanced by *E. coli*-derived LPS at both the gene and protein levels after prolonged stimulation for up to 48 h[31]. In GFs, *P. gingivalis*-derived LPS induces a significant increase in the gene expression of IDO but has no effect on IDO activity[71]. In a further study, the same group reported that the mRNA expression of IDO in gingival cells can be induced by bacterial LPS, the TLR-3 agonist Poly I:C, and the TLR5 agonist flagellin but not by the TLR-7 agonist loxoribine, and the effect of Poly I:C was significantly higher than that of the other TLR agonists[70]. In STRO-1$^+$ GMSCs, IDO mRNA expression is induced only by a TLR-3 agonist and not by agonists of TLR-1, 2, 4, 6, or 7[77]. The effect of TLR agonists on IDO expression in dental MSCs might also depend on the origin of the MSCs. For example, IDO expression is not affected by LPS in DFSCs but is inhibited by LPS in DPSCs[61]. In addition to their direct effects, different bacterial products can induce IDO expression in MSCs indirectly through the activation of different immune cells. For example, conditioned medium from LPS-stimulated PBMCs induces the expression of IDO and COX-2 in PDLSCs[67].

The expression of other immunomodulatory factors in dental tissue-derived MSCs is also influenced by bacterial products and TLR agonists. LPS enhances the production of PGE-2 by PDLSCs[51]. The periodontal pathogens *P. gingivalis* and *Fusobacterium nucleatum* (*F. nucleatum*) induce the production of IL-10 by DFSCs under anaerobic conditions[84]. Interestingly, *F. nucleatum*-induced IL-10 production in DFSCs is significantly higher than that in BM-MSCs[84]. The expression of PD-L1 in human PDLSCs is upregulated by various periodontal pathogens, such as *P. gingivalis*, *P. intermedia* and *F. nucleatum*, as well as LPS[73]. It should be noted that bacterial products and TLR agonists also induce the expression of inflammatory mediators such as IL-1β, IL-6, IL-8 and monocyte chemoattractant protein 1 in various craniofacial MSCs[51,68,85-88]. Thus, TLR agonists might activate both the pro-inflammatory and anti-inflammatory properties of dental MSCs, and the exact role of these cells in the inflammatory response is determined by other factors, such as the degree of inflammation and the microenvironment.

## POTENTIAL ROLE OF IMMUNOMODULATION MEDIATED BY DENTAL TISSUE-DERIVED MSCS IN ORAL DISEASES

Although the immunomodulatory ability of dental MSCs is widely recognized, its exact roles in the pathogenesis of various oral diseases remain rather obscure and only hypothetical. MSCs are located in a region that can occasionally be exposed to different bacterial challenges, and this inflammatory milieu might have a substantial effect on their immunomodulatory properties. PDLSCs isolated from inflamed tissue exhibit higher proliferative and migratory capacities than those isolated from healthy tissue[41,89]. Additionally, "inflamed" PDLSCs exhibit impaired abilities to promote $T_{reg}$ induction and suppress Th17 differentiation compared to cells isolated from healthy tissue[39]. PDLSCs from inflamed tissue are less effective in suppressing PBMC proliferation than those isolated from the healthy tissue of matched donors after 72 h of co-culture but not after 24-48 h of co-culture[41]. The same study found higher levels of IL-2, TNF-α and IFN-γ but similar levels of IL-10 in a co-culture of PBMCs and PDLSCs isolated from inflamed tissue compared to a co-culture containing healthy tissue cells[41]. "Inflamed" PDLSCs induce less PBMC apoptosis than "healthy" PDLSCs. In contrast to the above-mentioned studies, one study did not find any differences in the ability to suppress PBMC proliferation or in the expression of COX-2 and IL-10 between PDLSCs isolated from healthy tissue and those from inflamed periodontal tissue[40]. The same study found that upon co-culture with Con A-activated PBMCs, "healthy" PDLSCs exhibited lower IDO and significantly higher TSG-6 expression than "inflamed" PDLSCs[40]. DPSCs derived from teeth with pulpitis fail to suppress PBMC proliferation, but this ability might be restored with IFN-γ treatment[90]. In contrast to the above studies, no difference is found in the ability to modulate macrophage functions between DPSCs isolated from healthy and inflamed tissues[31].

Only a few *in vivo* studies have investigated the expression of immunomodulatory factors in dental tissue under inflammatory conditions. In a mouse model, the severity of periodontal disease induced by the injection of *P. gingivalis* was negatively correlated with the expression of PD-L1 in the periodontal tissue[73]. IDO expression is significantly augmented in macrophages and MSCs in inflamed human pulp tissue[31].

In rats with experimental periodontitis, topical and systemic application of MSCs transfected with TSG-6 leads to significantly lower bone loss, osteoclast formation and systemic levels of the inflammatory cytokines IL-1β and TNF-α[12]. These observations suggest a potential role for immunomodulation mediated by dental tissue-derived MSCs in the progression of different oral diseases, such as pulpitis, gingivitis and periodontitis, and this topic still needs to be intensively explored in future studies.

## CONTRIBUTION OF THE IMMUNOMODULATORY EFFECT OF DENTAL TISSUE-DERIVED MSCS ON TISSUE REGENERATION

Although the multilineage differentiation ability of MSCs *in vitro* is largely recognized, the mechanisms of their differentiation *in vivo* are not yet understood, and their clinical application for tissue regeneration is still limited. Transplanted MSCs have a rather short lifespan; for example, after intravenous injection MSCs accumulate in the lungs and disappear within 24 h[91]. The interaction of transplanted MSCs with the host immune system seems to be one of the key elements in the regeneration process[92]. Immunomodulatory and tropic capacity of transplanted MSCs contribute to the creation of a microenvironment, promote the activation of endogenous tissue repair mechanisms and are now considered to be the major mechanism of their therapeutic effect *in vivo*[93].

The healing and regeneration of different dental tissues, similar to the healing and regeneration of other tissues, consists of four overlapping phases: Haemostasis, inflammation, proliferation, and maturation/remodelling[94]. A recent review appreciated the essential role of inflammatory cytokines in the different stages of periodontal wound healing[95]. Inflammatory cytokines might have both positive and negative impacts on the tissue regeneration process[95]. Immune cells are involved in each stage of tissue regeneration, and modulating the immune system is considered a promising approach to promote tissue regeneration[96]. Therefore, the immunomodulatory properties of dental MSCs are hypothesized to be involved in tissue regeneration[97].

Several studies have investigated the levels of different inflammatory cytokines after MSC application for the regeneration of craniofacial tissue. The transplantation of human PDLSC-conditioned medium improves the healing process of surgically created periodontal defects in rats and is accompanied by a decreased mRNA level of TNF-α in the healing periodontal tissue[44]. The local administration of allogenic bone marrow MSCs into periodontal defects is not only beneficial for tissue regeneration but is also accompanied by decreased local levels of IL-1β, TNF-α and IFN-γ[98]. The transplantation of SHEDs into periodontal defects after experimentally induced periodontitis stimulates the regeneration of the periodontal tissue, which is accompanied by an increased proportion of M2 macrophages[60]. Moreover, SHED application is characterized by lower levels of IL-1β and higher levels of IL-10 in the gingival crevicular fluid[60]. Although these studies do not provide any evidence that the immunomodulatory ability of MSCs is necessary for tissue regeneration, they unequivocally suggest that the MSC-based regeneration of dental tissues is accompanied by the modulation of the inflammatory response.

In addition, few experimental studies have investigated the effect of immunomodulatory factors on periodontal tissue regeneration. PDLSCs transfected with HGF improve periodontal bone regeneration in swine[99]. Intragingival injection of TSG-6 promotes early wound healing after gingival resection in rats and results in lower levels of the inflammatory markers IL-1β and myeloperoxidase[100]. The transplantation of IFN-γ-pre-treated bone marrow-MSC sheets into mouse calvarial bone defects induces bone regeneration, which is not observed with non-treated cell sheets[101]. Moreover, the transplantation of non-treated bone marrow-MSC sheets induces T cell infiltration into the grafted area[101]. Some studies have also implied that the activation of the complement system by DPSCs can contribute to pulp regeneration[37,38].

The regenerative and immunomodulatory abilities of MSCs are supposed to be tightly interconnected, but the exact relationship between these two functions still needs to be established. Some factors mediating the immunomodulatory effects of MSCs also influence their differentiation potential. Particularly, osteogenic, adipogenic and neural differentiation in human MSCs are altered upon IDO activation by IFN-γ[102]. The differentiation ability of MSCs is also influenced by TSG-6[103,104]. Both, regenerative and immunomodulatory functions of MSCs are influenced by their TGF-β production[105]. One *in vitro* study showed that the differentiation potential of human PDLSCs depends on the inflammatory microenvironments and

correlates with their immunomodulatory properties[106]. TSG-6 production in hPDLCS is induced by BMP-2, which is widely used for bone regeneration in clinic[107]. Furthermore, BMP-2 decreases the inflammatory response in the human macrophage THP-1 cell line[108].

## CONCLUSIONS, OPEN QUESTIONS AND FURTHER PERSPECTIVES

Dental tissue-derived MSCs, similar to MSCs from other tissues, influence the properties of both the innate and adaptive immune systems. Numerous *in vitro* studies have shown that dental MSCs influence the functional activities of key components of the immune system, namely, T cells, dendritic cells, natural killer cells, B cells, macrophages, and neutrophils. However, knowledge about MSC-dependent immunomodulation *in vivo* is limited and originates mostly from animal models, which are usually mouse models. Although the principles of immune defence are quite similar among all mammalian species, there are some differences between the mouse and human immune systems[109]. Differences have been found for almost all components of both innate and acquired immunity, and therefore, the translation of data obtained in mice to humans must be done cautiously. Moreover, there are also some differences in the mechanisms of MSC-mediated immunomodulation between humans and mice. Nevertheless, animal studies will be especially important for deepening our knowledge of the *in vivo* mechanisms of MSC immunomodulation. Studies with conditional knockout mice, in which the expression of different proteins involved in immunomodulation can be eliminated in a tissue-specific manner, are especially important.

The interaction of MSCs with the immune system is reciprocal, and the immunomodulatory ability of MSCs is highly regulated by different inflammatory cytokines. The priming of dental MSCs with IFN-γ, TNF-α and IL-1β usually enhances their immunosuppressive ability and could be considered a feedback mechanism that dampens exacerbated immune responses (Figure 1). This reciprocal interaction between immune cells and MSCs could be considered an important mechanism contributing to tissue homeostasis and regeneration as well as the progression of different inflammatory diseases. However, the interaction between MSCs and immune cells seems to be more complex. An interesting paradox regarding the immunomodulation mediated by dental MSCs is that this ability might be impaired in cells isolated from an inflammatory environment. This is rather surprising because MSCs in inflamed areas are continuously exposed to numerous inflammatory mediators, which should enhance the immunosuppressive properties of the MSCs. Therefore, the immunomodulation mediated by MSCs could be influenced by other factors and/or chronic inflammation, and these issues need to be further explored.

It is also possible that the impairment of MSC immunomodulation might contribute to the progression of various inflammatory diseases. Dental MSCs express numerous TLRs, and different TLR ligands are thought to influence the immunomodulatory ability of MSCs. Furthermore, bacterial ligands can influence MSCs indirectly through the activation of inflammatory cytokine production in different immune cells. Nevertheless, there is still an insufficient number of experimental studies investigating the effects of different TLR ligands and bacterial products on the ability of MSCs to modulate different immune cell subsets. Importantly, the regulation of immunity by MSCs in different inflammatory diseases might play dual roles and influence both pathogen elimination abilities and collateral tissue damage. The activation of MSCs by TLRs has been shown to have both immunoactivating and immunosuppressive effects, and the physiological and pathophysiological relevance of these effects still needs to be understood.

In addition, the regulation of the immune system by MSCs is thought to largely contribute to tissue regeneration processes. However, the interplay between the immunomodulatory function of MSCs and their regenerative potential in different dental tissues still needs to be investigated. There is already a first report showing that priming MSCs with IFN-γ can enhance their regenerative potential, presumably through the activation of their immunomodulatory potential. As mentioned above, different inflammatory mediators might differentially activate various immunomodulatory proteins in MSCs and thus their immunomodulatory activity. Different populations of immune cells are involved in different time-separated stages of wound healing. Therefore, the activation of a specific "immunomodulatory profile" by MSCs may enhance the efficiency of their application at different stages of the healing process and should be tested in further studies.

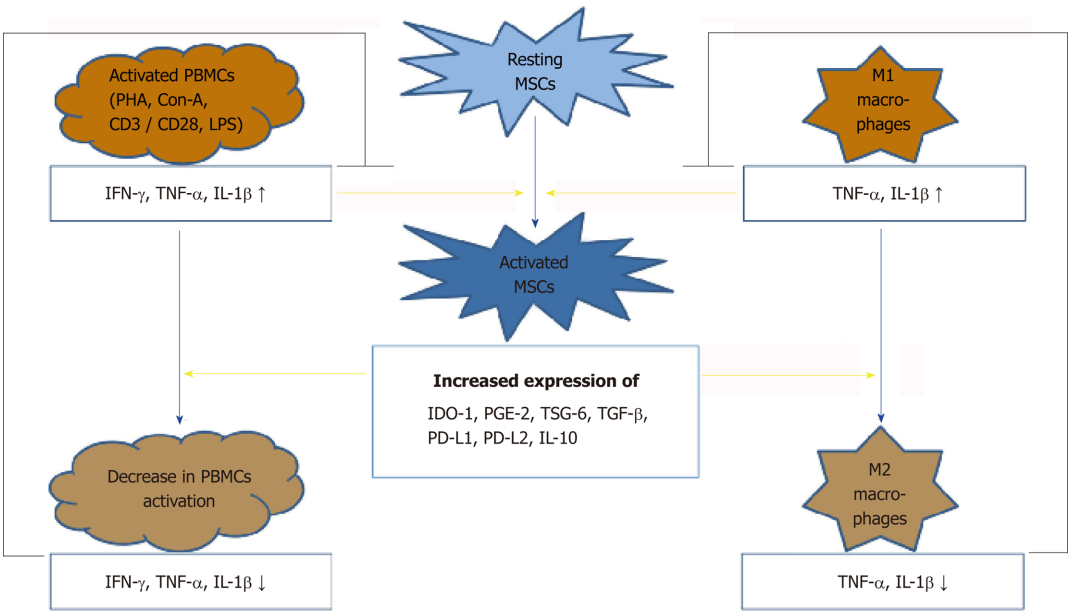


**Figure 1 Reciprocal interaction between dental tissue-derived mesenchymal stem cells and immune cells.** While the immunomodulatory ability of resting mesenchymal stem cells (MSCs) is usually low, inflammatory cytokines such as interferon-γ, tumour necrosis factor-α, and interleukin-1β lead to strong activation of this ability. Large amounts of these cytokines are produced by immune cells, such as peripheral blood mononuclear cells (PBMCs) or macrophages, under inflammatory conditions. *In vitro* cytokine production can be activated by either mitotic stimuli or bacterial pathogens. Inflammatory cytokines increase the expression of different immunomodulatory proteins in MSCs, which leads to the suppression of the activity of PBMCs or directs macrophage polarization towards the M2 phenotype *via* paracrine mechanisms or direct cell-to-cell contact. The resulting lower levels of the inflammatory cytokines produced by PBMCs or macrophages diminish the ability of these cells to activate MSC-dependent immunosuppression. Thus, the continuous interaction between immune cells and dental tissue-derived MSCs determines the intensity of the immune response and hypothetically plays an important role in tissue homeostasis.

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*REVIEW*

# Cell membrane and bioactive factors derived from mesenchymal stromal cells: Cell-free based therapy for inflammatory bowel diseases

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## Abstract

Inflammatory bowel diseases (IBD) are chronic inflammatory disorders of the gastrointestinal tract associated with multifactorial conditions such as ulcerative colitis and Crohn's disease. Although the underlying mechanisms of IBD remain unclear, growing evidence has shown that dysregulated immune system reactions in genetically susceptible individuals contribute to mucosal inflammation. However, conventional treatments have been effective in inducing remission of IBD but not in preventing the relapse of them. In this way, mesenchymal stromal cells (MSC) therapy has been recognized as a promising treatment for IBD due to their immunomodulatory properties, ability to differentiate into several tissues, and homing to inflammatory sites. Even so, literature is conflicted regarding the location and persistence of MSC in the body after transplantation. For this reason, recent studies have focused on the paracrine effect of the biofactors secreted by MSC, especially in relation to the immunomodulatory potential of soluble factors (cytokines, chemokines, and growth factors) and extracellular vehicles that are involved in cell communication and in the transfer of cellular material, such as proteins, lipids, and nucleic acids. Moreover, treatment with interferon-γ, tumor necrosis factor-α, and interleukin-1β causes MSC to express immunomodulatory molecules that mediate the suppression *via* cell-contact dependent mechanisms. Taken together, we present an overview of the role of bioactive factors and cell membrane proteins derived from MSC as a cell-free therapy that can improve IBD treatment.

**Key words:** Bioactive factors; Cell membrane; Mesenchymal stem cells; Cell-free therapy; Inflammatory bowel diseases

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**Core tip:** Recent experimental studies have suggested that both bioactive factors and surface proteins of mesenchymal stem cells demonstrate great therapeutic potential for overcoming the deficiencies of current therapies for inflammatory bowel diseases. Our goal in this review is to describe cell-free therapy based upon the therapeutic potential of mesenchymal stem cells, while avoiding the practical issues associated with the use of living cells.

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## INTRODUCTION

Inflammatory bowel diseases (IBD) comprise ulcerative colitis (UC) and Crohn's disease (CD), both of which are chronically multifactorial inflammatory disorders of the gastrointestinal system. Although the pathogenesis of IBD remains unclear, mounting evidence suggests that abnormal immune regulation in genetically susceptible individuals and/or environmental factors contribute to mucosal inflammation[1-3]. Conventional treatments for IBD involve immunosuppressive drugs that lead to the remission of intestinal inflammation and related symptoms. However, there is no known medical/surgical cure for IBD[4,5]. Therefore, additional therapeutic strategies, such as cell-based therapy, are required for unresponsive patients.

In this way, mesenchymal stromal cells (MSC) are a promising strategy for treating inflammatory diseases, immune disorders, and tissue regeneration due to their immunomodulatory properties, ability to differentiate into several tissues, and homing to inflammatory sites, by which they control inflammation and the production of cytokines[6-8]. However, there are conflicts in the literature regarding the location and persistence of MSC in the body after transplantation. Cell-tracking studies have shown that most MSC are localized in the lungs after intravenous infusion and have a short-term survival span[9-12]. After 24 h of infusion, MSC tend to disappear from the lungs, suggesting that they probably transmit their effects to resident cells[9]. Based on this, MSC may interact with resident cells through the secretion of paracrine factors or cell-cell communication[13-15].

Recent studies have focused on the paracrine effect of the biofactors secreted by MSC, especially in relation to the immunomodulatory potential of soluble factors (cytokines, chemokines, and growth factors)[16-18]. Also, MSC alter their immune function in response to the inflammatory environment, especially by the stimulation of proinflammatory cytokines interferon-γ (IFN-γ) and the tumor necrosis factor (TNF)-α[19,20]. After activation, MSC upregulate the expression of interleukin (IL)-6, IL-10, indoleamine 2,3 dioxygenase (IDO), transforming growth factor (TGF), prostaglandin E2 (PGE-2), hepatocyte growth factor (HGF), nitric oxide, and heme oxygenase-1[20-23]. It is also known that MSC are capable of releasing extracellular vehicles (EVs) that are involved both in cell communication and in the transfer of cellular material, such as proteins, lipids, and nucleic acids[24-26]. Moreover, MSC express immunomodulatory molecules in their cell membrane such as ATPases, CD73, and Toll-like receptors (TLRs)[27-29], and, under inflammatory conditions, they express the programmed death ligand 1 (PD-L1) and the Fas ligand[22,30-34]. For this reason, both biofactors secretion and cell contact may be required for efficient MSC immunomodulation[14,35].

Therefore, the study of bioactive factors and cell membrane molecules of MSC becomes important in the search for new cell-free therapeutic methodologies that aim to reduce the complications associated with the administration of MSC but while preserving the immunological properties of these cells.

## IBD

IBD are chronic inflammatory disorders of the gastrointestinal system associated with multifactorial conditions, such as UC and CD. Although the underlying mechanisms of UC and CD remain uncertain, growing evidence has shown that failure of the epithelial barrier and dysregulation of the immune system in genetically predisposed individuals contribute to mucosal inflammation[1-3]. These diseases are characterized by the dysfunction of mucosal T cells, alteration in the production and secretion of cytokines, and cellular inflammation affecting the digestive tract, especially the distal small intestine and colon mucosa[36]. While CD may affect any part of the gastrointestinal segment and is characterized by an inflammatory process that recruits macrophages and forms granulomas, UC is generally limited to the colon and rectum, characterized by neutrocytic infiltrate with formation of cryptic abscesses and epithelial ulceration[37]. Patients with UC experience continuous inflammation confined to the mucosal layer. In CD, the inflammation is discontinuous and affects all intestine layers[2].

At the clinic, patients with IBD have recurrent episodes of abdominal pain, diarrhea, bloody stools, and weight loss. Moreover, they may present extra intestinal manifestations in the skin, joints, eyes, and less frequently in the abdominal organs, for example, the biliary tract[38]. Treatment of IBD is based on the severity, site, clinical manifestations, and complications of the disease in each case. Several cellular and molecular pathological pathways have been identified as targets for IBD treatment[39]. The exacerbated progression of IBD requires a scale ranging from anti-inflammatory treatment to biological agents, usually with limited success[40]. Furthermore, medical treatments are expensive and common drugs are toxic and ineffective for most patients, making surgical resection of the parts of the intestine necessary in many cases[2,41]. Regarding the progress achieved by intensive clinical-drug treatment, approximately 20% of patients with UC and 50% of patients with CD require surgical intervention within 10 years of diagnosis[41]. Accordingly, some studies have addressed MSC therapy as a promising approach for treating IBD[42].

## MSC

MSC are multipotent cells capable of differentiating into mesodermal lines, particularly osteoblastic, adipogenic, and chondrogenic strains[43,44]. There is controversy over the naming and definition of MSC in the scientific community. The term "mesenchymal stromal cell" is used in parallel with the terms "mesenchymal stem cell" and "multipotent mesenchymal stromal cell"[45]. MSC are indeed a heterogeneous population of cells characterized immunophenotypically by the expression of CD73, CD90, and CD105 and do not present the expression of CD45, CD34, CD11, CD14, CD19, CD79A, and human leukocyte antigen (HLA)-DR hematopoietic lineage markers[46]. Most of the knowledge about these cells was generated from bone marrow-derived MSC. However, the origin of MSC production has been expanded to other tissues, including muscle, adipose, and neonatal tissues[47,48]. Recently, Soontararak *et al*[49] demonstrated that MSC derived from induced pluripotent stem cells are equivalent to MSC derived from adipose tissue in terms of improving the intestinal healing in IBD model.

MSC exhibit great therapeutic potential in regenerative medicine owing to their ability to differentiate *in vitro*, homing (the process in which cells are able to migrate and graft to the tissues) to inflamed tissues after *in vivo* infusion, and the secretion of various bioactive molecules[8]. In addition, the immunomodulatory properties of MSC suggest that even MSC of the incompatible HLA may be suitable for a wide variety of new therapeutic applications, especially for cellular therapy of inflammatory and autoimmune diseases[50]. Traditionally, MSC are isolated from bone marrow, but other cellular sources may be of greater benefit due to the higher number of MSC or easier accessibility[13]. Moreover, MSC from different tissue sources share several phenotypic and functional features. Even so, there are subtle peculiarities in the expression and differentiation abilities of specific markers on cell surface[13,45].

Studies have compared the ability of MSC from different tissues to suppress peripheral blood cells, and adipose tissue-derived MSC have demonstrated a greater immunomodulatory effect than MSC from other sources[51,52]. Although adipose and bone marrow-derived MSC share several properties, there are variation in gene expression and growth factor secretion profiles[53]. In addition, various types of adipose tissue may have distinct properties. MSC isolated from abdominal and mammary adipose tissue, for example, present discrepancy in the expression of fibroblast growth factor and receptor, suggesting variability in angiogenic potential[54].

Neonatal tissues (cord blood, umbilical cord, placenta, amnion, and chorion) have been an alternative for MSC isolation. These tissues are usually discarded as a

residual product after delivery and can be obtained in large quantities in an easy and noninvasive way[55]. Another relevant advantage of neonatal tissues is that they supply immature cells, which present a lower risk of mutations and superior cell activity, such as increased differentiation, homing, and grafting ability[56,57]. Previous studies have shown that neonatal sources exhibit superior proliferation and immunosuppressive and regenerative potential when compared to adult tissues, for example, bone marrow and adipose tissue[47,58].

### *Immunological properties of MSC*

MSC have demonstrated low levels of HLA or major histocompatibility complex (MHC) class I and insignificant levels of HLA class II. Moreover, they do not express co-stimulatory molecules such as CD40, CD40L, B7-1 (CD80), and B7-2 (CD86)[22,51,59-61]. Consequently, MSC present low immunogenicity and may "escape" the immune system due to their surface phenotypes that are not recognized by T cells. Absence of MHC II or T cell co-stimulatory molecules make the MSC immune-privileged cells and may explain the mechanism by which MSC are not recognized by T cells[62]. Interestingly, both syngeneic and allogeneic MSC are immunotolerable by the receptor[63].

MSC have presented a high immunosuppressive capacity and interaction with immune cells through several mechanisms. Studies have shown that MSC-mediated immunosuppression may occur through the secretion of soluble factors and the presence of MSC membrane protein[51,64]. Regarding soluble factors, important anti-inflammatory molecules, such as transforming TGF-β, PGE-2, and HGF, have been enrolled[21,23]. Through secretion of TGF-β and other factors, MSC promote the induction of regulatory cells, including T cells[65], macrophages[66], and B cells[67] and thus transmit their immunosuppressive effects to different types of cells that exert several mechanisms of immune suppression. MSC can also be induced to produce IDO enzyme, which has an effective ability to inhibit lymphocyte proliferation by metabolism of L-tryptophan to L-kynurenine. Thus, low levels of L-tryptophan and high levels of L-kynurenine impose a block of lymphocyte proliferation[68].

Moreover, MSC exert their immunomodulatory function through molecules in their cell membrane, such as ATPases and CD73 (ecto-5'-nucleotidase, Ecto5'NTase)[28,69]. ATPase converts ATP to ADP and then to AMP. CD73 further dephosphorylates AMP to adenosine, which exhibits immunosuppressive properties[69,70]. Also, the ability of MSC to modify the immune system response can be enhanced by treatment with proinflammatory cytokines, TNF-α and IFN-γ in particular[19-21]. Under inflammatory conditions, MSC express membrane proteins with an immunological regulatory function, such as PD-L1 and the Fas ligand, through which they direct to the target cells and prevent their activation/function[6,22,32-34].

### *Interaction between MSC and immune cells: Direct cell contact or secretion of molecules?*

Secreted bioactive molecules as well as the membrane proteins of MSC demonstrate an ability to mediate immunosuppression. Nevertheless, there are controversies in the literature about the interaction of MSC with immune cells[71-73]. For MSC treatments to reach their full potential, a broader understanding of how cells exert their immunosuppression must be developed. Then it will be possible to define the relevant pathways for improving MSC treatment for specific inflammation or immune disorders.

Factors secreted by MSC have modulated the expression profile of cytokines in macrophages, inducing the polarization of these cells to an anti-inflammatory phenotype (M2)[74,75]. However, González *et al*[63] reported a partial dependence of the cell-cell contact mechanism by the induction of secretion of immunosuppressive factors. In their study, different co-culture systems of adipose tissue-derived MSC and macrophages were assessed and high levels of IL-10 were only observed in the co-culture system that promoted cell-cell contact. Furthermore, macrophages that phagocyte MSC acquire regulatory properties[35]. In this sense, bioactive factors and/or cell-cell interaction promote distinct types of regulatory macrophages that can change their function in response to the proinflammatory signals of the micro-environment[76]. MSC may also affect populations of monocytes, precursors of macrophages and dendritic cells, through the secretion of HGF that induces monocytes to an immunomodulatory phenotype ($CD14^+CD16^-$) with IL-10 production[27].

However, some authors suggest that activation of T cells by MSC is independent of cell contact[77-79]. Several soluble factors have been associated with the immunomodulatory ability of MSC to affect the activation or proliferation of T cells, such as the secretion of HGF, TGF-β, IDO, and PGE-2[21,23,68,71,73]. Saldanha-Araujo *et al*[80] demonstrated an additional mechanism by which MSC suppress T cell proliferation. This study demonstrated that, during the interaction between MSC and T cells, there

is adenosine production by MSC, which reduce T cell proliferation by flagging the adenosine receptor on the membrane surface. Still, other authors suggest that cell-cell contact is essential for an intense immunosuppressive effect[78]. In fact, MSC express integrins, intercellular adhesion molecules, and vascular cell adhesion protein on their surface and can bind to T cells with high affinity[71,81]. Quaedackers *et al*[82] cultivated MSC with peripheral blood mononuclear cells and indicated that such interaction is a specific process, since the subpopulations of lymphocytes that interacted with MSC were distinct from cells that remained in suspension. The T cell fraction that adhered to MSC was regulatory T cells exhibiting an immunosuppressive phenotype. In inflammatory environment, the expression of the molecules PD-L1 and PD-L2 on the MSC surface enhance, restricting the function of T cells through PD-1 ligands[83]. The high levels of these inhibitory molecules on the MSC membrane confirm that one of the mechanisms of MSC immunosuppression is also by cell–cell contact-mediated responses.

MSC can also immunomodulate other cell types of the innate and the adaptive immune system, such as B lymphocytes, dendritic cells, and natural killer (NK) cells[72,84]. Luk *et al*[14] have demonstrated that MSC, under normal culture conditions, promote B cell survival and induce the formation of regulatory B cells, but negligible interference on B cell proliferation and immunoglobulin G production was observed. However, after pre-treatment with IFN-γ, MSC appear to inhibit B cell proliferation and reduce immunoglobulin G production, even though these primed cells lose the ability to induce the formation of regulatory B cells. Moreover, the authors evaluated distinct mechanisms as key factors for MSC-mediated immunomodulation. First, they demonstrated that the effects of MSC on B cells do not depend only on soluble factors, since no production of regulatory B cells or IL-10 was induced when MSC were cultured in a Transwell system with separated chambers. Second, they assessed that the presence of heat-inactivated MSC (dead cells not capable of secreting factors but phenotypically intact) was not sufficient to induce IL-10 producing B cells. These data suggest that B cell modulation by MSC is mediated by an active metabolic process and requires B cell and MSC contact.

Another mechanism of immunosuppression exerted by MSC has been observed in the inhibition of the differentiation and maturation of dendritic cells derived from $CD14^+$ monocytes[85]. Co-culture with Transwell suggests that IL-6 and macrophage colony stimulating factor are partially involved in the inhibition of dendritic cell differentiation by MSC. MSC may also alter the cytokine secretion profile of dendritic cells[86]. Co-culture with MSC demonstrates decreased secretion of TNF-α by mature myeloid dendritic cells, while increasing IL-10 secretion by plasmacytoid dendritic cells[87]. Moreover, MSC are sensitive to lysis by activated NK cells but are resistant to naive NK cells[88]. Spaggiari *et al*[89] observed that MSC treated with IFN-γ are resistant to lysis by NK cells. In addition, they found that some inhibitory effects of MSC on NK cells demand cell-cell contact, while others are regulated by soluble factors, including PGE-2 and TGF-β1. Therefore, these data together suggest that the mechanism of immunoregulation of MSC depends on the types of cell populations, the inflammatory conditions, and the presence or absence of cell-cell contact.

## MSC THERAPY IN IBD

Autologous and allogeneic MSC have been evaluated in clinical trials in two different modalities: Local injection of MSC to treat fistulizing CD and intravenous (IV) infusion of MSC to treat UC or luminal colitis[42,90-93]. Published work demonstrates that to date 117 IBD patients with refractory disease or intolerant to standard treatment have received one or more IV infusions of autologous or allogeneic MSC[94]. Currently, results of clinical trials are particularly encouraging in terms of safety and efficacy; however, due to different study designs regarding tested groups, short follow-up, and a lack of endoscopic data and unified primary outcomes, no final conclusions can be made. In fact, MSC demonstrated their ability to repair perianal fistulas in CD patients, refractory to conventional or biological therapy in several controlled trials[95,96]. Still, MSC need to demonstrate their clear efficacy on luminal CD and UC[97,98].

In this sense, experimental studies in animal models have contributed to a better understanding of cellular, molecular, and immunological mechanisms of IBD associated with cell therapy. Recent studies have demonstrated a clinical and histopathological improvement of colitis after an infusion of MSC, such as decreased inflammation and increased survival[99,100]. In addition, much has been researched regarding the homing of exogenous MSC infused by different pathways in response to an inflammatory insult. A comparative study between the IV and intraperitoneal

(IP) routes in a trinitrobenzene sulfonic acid (TNBS)-induced colitis model concluded that, in systemic administration, MSC accumulated preferentially in the lungs, with no evidence of migration to the colon. On the other hand, MSC injected *via* IP were located in the inflamed colon[101]. Another study in an experimental model of sodium dextran sulfate (DSS)-induced colitis demonstrated that MSC migrate towards the lung, liver, and spleen, and even a small amount to the inflamed colon after 24 h of IV infusion. In contrast, IP and intracolonic routes showed more cell grafting in the colon and fewer cells trapped in the pulmonary alveoli[100]. However, a previous study conducted by our research group also evaluated the effect of MSC administered by different routes, IV and IP, in DSS-induced acute colitis. We demonstrated that infusion of MSC by IV route decreased intestinal inflammation, modulated serum cytokines, and induced apoptosis of T cells of the intestinal mucosa. Meanwhile, the same effect was not found in animals treated by IP route[102].

In this way, there are controversial studies about the location and persistence of MSC in the body after cell transplantation. The efficiency of cellular delivery is dependent on the route of administration[101,102]. IV infusion, for example, has been used as a cellular delivery route for preclinical studies[9,10,102] and for recent clinical trials[103] due to its wide distribution and easy access. However, cell-tracking studies have shown that most MSC are localized in the lungs and have a short-term survival in the body after IV infusion[9-12]. The entrapment of MSC in the lungs is occasioned by their size[24], which exceeds the width of the pulmonary micro-capillaries[25,26]. In patients, respiratory discomfort has been reported after transfusion of MSC[10]; and high and subsequent doses of cells are usually required to observe some effect in animal models[104]. Interestingly, in experiments of Lee *et al*[105], MSC retained in the lungs improved cardiac function after myocardial infarction by releasing the anti-inflammatory protein TSG-6[105].

After 24 h of infusion, MSC tend to disappear from the lungs, and their cellular debris are distributed to other sites, particularly the liver, suggesting that these cells pass their effects to the resident immune cells[106]. Then, the resident cells possibly mediate the immunomodulatory effects induced by transplanted MSC[9]. Moreover, immune system cells are likely to play a role in the removal of MSC. Activated NK cells, for example, have been shown to be able to lyse autologous MSC *in vitro*[89]. Also, apoptosis of infused cells may trigger an immunomodulatory response. Lu *et al*[35] demonstrated that macrophages adapt a new immunoregulatory function after the phagocytosis of dead MSC[35,107]. Based on this information, MSC probably interact with resident cells through distinct mechanisms, either by the secretion of paracrine factors or through a direct cell-cell interaction[13-15,108]. Intestinal organoids derived from stem cells also provide a system to mimic *ex vivo* interactions between the lamina propria and epithelium intestinal by cell contact–dependent/independent mechanisms[109].

Thus, the application of MSC *in vivo* requires, in addition to the biological knowledge of the cells, a deep understanding of the mechanisms involved in IBD. For this, the immunological properties of the MSC type, the infusion route, and the inflammatory and immunological conditions of the disease should be considered. Therefore, MSC therapy may exert its therapeutic effects mainly by secreting soluble immunomodulating bioactive factors and/or by cell-cell contact and consequently by interaction with immune cells, establishing a favorable environment for regeneration.

## THERAPY BASED ON THE IMMUNOMODULATORY PROPERTIES OF MSC

Infused MSC have been demonstrated to promote the intestinal repair processes in both humans[42] and animal models[105,110]. Nevertheless, the engraftment and the homing mechanism of MSC are not well elucidated and depend on intricate interactions between signaling pathways. Short survival after infusion and poor biodistribution of MSC have proven to be significant technical challenges to overcome before this therapy can be used for clinical purposes. An alteration in the MSC treatment that avoids these issues, but maintains the immunomodulatory properties of MSC, would enhance this therapy. Also, though positive effects of MSC infusion can last longer than the half-life of the cells themselves, they reduce over time. It is thus plausible that successive MSC administrations are necessary as maintenance therapy[94].

### *Bioactive factors of MSC*

Recent studies have reported that the regenerative potential of MSC therapy has been, at least in part, mediated by paracrine actions[15,111,112]. Thus, studies about MSC-

secreted factors have demonstrated that these factors can induce tissue repair in conditions involving tissue/organ damage[111]. Furthermore, they are referred to in the literature as "secretome" (soluble factors), and extracellular vesicles and can be found in the MSC culture supernatant; thus, the supernatant of MSC is a denominated conditioned medium (CM). CM is commonly prepared by confluent MSC cultures incubated in serum-free media for 24h without any concentration, selection, or purification of cell products. Therefore, the components of CM are in fact both free soluble factors (cytokines, chemokines, and growth factors) and EVs, which may mediate the therapeutic potential of MSC[16,113] (Figure 1).

Few studies have used the MSC-CM as a therapeutic strategy for IBD (Table 1). The paracrine effect of MSC-CM has been observed in experimental models of colitis induced chemically by TNBS and DSS[15,114,115]. Heidari *et al*[116] and Pouya *et al*[117] have shown the effects of MSC-CM in DSS-induced colitis by increasing anti-inflammatory responses associated with an increase of regulatory T cells percentage and IL-10 production. Watanabe *et al*[15] demonstrated that MSC-CM was effective for the inductive stage of TNBS-induced colitis and for the recovery stage of DSS-induced colitis independent of the systemic delivery route. Similarly, Robinson *et al*[115] (2014) and (2015)[118] showed that MSC and CM treatments prevented damage to the enteric nervous system and alleviated gut dysfunction caused by TNBS-induced colitis. In our previous study, we investigated the paracrine role of MSC in DSS-induced colitis of colon organ culture. Our results have shown that colonic inflammation is alleviated by MSC-CM, and this effect is independent of cell contact[119]. Also, CM has been evaluated in several other conditions and diseases, such as myocardial infarction[120], bone defect[121], hepatic insufficiency[122], and spinal cord injury[123]. Liu *et al*[124] suggest that MSC-CM administration has the potential to suppress proliferation of artery smooth muscle cells in an experimental model of pulmonary hypertension. Another study reported that the application of MSC-CM in diabetic rats prevented renal disease, primarily by attenuating the expression of TGF-β1[125]. Therefore, it may be observed that MSC-CM contains several factors that intervene in different pathophysiological manifestations, such as inflammation, proliferation, angiogenesis, and tissue remodeling[15]. Taken together, these results indicate that MSC-secreted factors are able to preserve the intestinal barrier in IBD independent of cell transplantation.

MSC also have been displayed to release EVs, which can be implicated in cell-cell interaction by carrying biologically active lipids, proteins, and nucleic acids in and on their membrane (Table 1)[26,28]. EVs are cell-derived membranous structures released by cells. They can be originated from multivesicular bodies (exosomes) or directly from the cell membrane (microvesicles - MVs). Both exosomes and MVs are comprised of two regions: The membrane and the natural internal cargo. The outer membrane, composed of lipid layer and proteins, packages bioactive molecules and protects the internal cargo, consisting of lipids, proteins, DNA, mRNA, micro-RNA, and other components from the parental cell. The cargo carried by EVs dictated their function[126,127]. The MSC treatment may be mediated by free soluble factors and components contained in EVs, which composition is cell-origin specific[24,25,114]. Kim *et al*[128] profiled MSC-derived MVs proteome and identified 730 proteins engaged in processes associated with self-renewal and/or differentiation of MSC. Also, they pointed out that the micro-RNAs (miRNAs) profile of MVs presents a pattern shared with their cells of origin. Nevertheless, selected miRNAs were evident only in the released MVs but absent in the MSC[128]. Accordingly, another study has extracted RNA from MSC and their MVs for 365 known mature miRNAs, and they observed that 41 miRNAs were co-expressed in MVs and cells, others were cumulated within MVs and absent in the cells after MVs secretion, and still others were maintained within the cells and not liberated in MVs[129].

Thus, several studies have been focused on MSC-derived EVs as a treatment for IBD (Table 1). Wong *et al*[130] identified in circulating exosomes of DSS-induced colitis mouse 56 differentially expressed proteins that are involved in macrophage activation. Wu *et al*[131] demonstrated the role of miR146-a in attenuating experimental colitis since EVs derived from MSC overexpressing miR146-a significantly inhibited TNF receptor–associated factor 6 and IL-1 receptor associated kinase 1. Yang *et al*[132] demonstrated that EVs protect against TNBS-induce colitis by attenuating oxidative stress and apoptosis. Mao *et al*[114] demonstrated that exosomes released from human umbilical cord-derived MSC homed to colon tissue of IBD mice and relieved the severity of the DSS-induced colitis by altering the expression of inflammatory genes and decreasing the infiltration of macrophages. Interestingly, ubiquitination may play an important role in the anti-inflammatory effect observed in IBD animals treated with MSC-derived exosomes. Ubiquitin is upregulated in IBD mice and promotes activation of the nuclear factor kappa B pathway by increasing the ubiquitination and degradation of inhibitor of kappa B alpha and regulating inflammation through

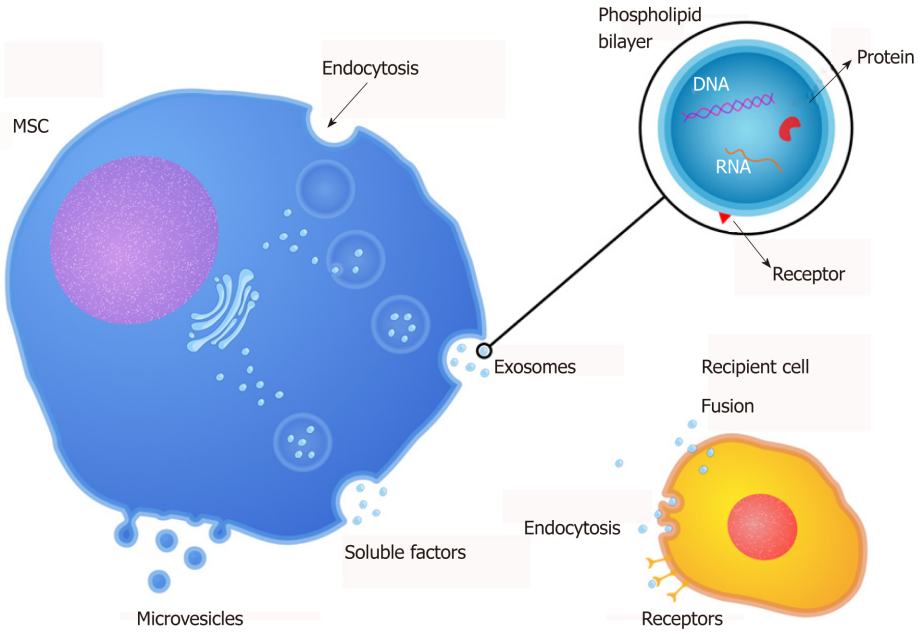


**Figure 1 Release of bioactive factors by MSC.** MSC can secrete bioactive factors including free soluble factors (cytokines, chemokines, and growth factors) and extracellular vesicles (microvesicles and exosomes) that mediate the therapeutic potential of MSC. MSC: Mesenchymal stromal cells.

mTOR signaling. However, the expression of ubiquitin was inhibited in the colonic mucosa and spleen after the MSC-derived exosomes treatment[133].

In this way, the secreted bioactive factors could play an essential role in repairing damaged colon from experimental colitis to regenerative medicine. In relation to cellular therapy, bioactive factors are easier to prepare and store. Then, it is a therapy devoid of cells, and possible risks of rejection between donor and recipient can be minimized. Lastly, pulmonary capillaries are not a barrier for this therapy in IV transplantation, and CM can reach sites beyond the lung[111].

### *MSC membrane*

There is inconclusive evidence that bioactive anti-inflammatory factors alone are responsible for the immunomodulatory effects of transplanted MSC. The short-term survival of MSC in the body after infusion raises the questions of whether MSC have sufficient time to be activated by inflammatory conditions and then secreted factors[9]. Hoogduijn *et al*[106] found that MSC infusion initiates a mild and immediate systemic inflammatory response, which may be the activator of posterior immunosuppression. In this study, the inflammatory response was found in the lung and characterized by an increased expression of pro-inflammatory monocyte and cytokines. Also, MSC infusion provides inflammatory conditions for their activation and that at least part of the immunomodulatory response mediated by MSC is independent of activation by anti-inflammatory soluble factors. Instead, passive interactions with host cells probably mediate these effects[14]. de Witte *et al*[134] demonstrated that infused MSC are internalized by monocytes and induce phenotypical and functional changes in these cells, which subsequently migrate from the lungs to other body sites and modulate immune cells response. Other studies also indicated that MSC induced an immunosuppressive phenotype on macrophages after phagocytosis and that the macrophage depletion reduced the therapeutic effect of MSC[35,135].

Based on this analysis, some studies have shown that MSC exert their effects through intermediary cells by contact with the cell membrane[14,134,136,137]. MSC express immunomodulatory molecules on their membrane, such as CD90 (Thy-1 membrane glycoprotein) that is involved in MSC differentiation pathways, ATPases, and CD73, which dephosphorylate ATP into AMP and AMP into adenosine, respectively[136]. Interestingly, a recent study has determined that heat-inactivated MSC preserve their immunomodulatory capacity after IV infusion in a lipopolysaccharide-induced model, suggesting that cell-membrane-dependent interactions with immune cells are triggering factors of the immunological regulatory effects[14]. Song *et al*[107] systemically administered MSC extract obtained by cell lysis in experimental colitis, and their results demonstrated that the MSC extract polarized the macrophage functional phenotyping from M1 to M2. This new therapeutic approach could overcome the low homing efficiency of MSC in patients with IBD.

**Table 1 Studies using bioactive factors derived from mesenchymal stromal cells as a therapeutic strategy for inflammatory bowel diseases**

| Bioactive factors | MSC origin | IBD model | Pathway | Results | Ref. |
|---|---|---|---|---|---|
| CM | Rat bone-marrow | DSS and TNBS-induced colitis in rats | IP, IV, and enema | Effective for the inductive phase of TNBS-induced colitis and for recovery phase of DSS-induced colitis. | Watanabe *et al*[15], 2014 |
| CM | Human bone-marrow | TNBS-induced colitis in pigs | Enema | Prevent loss of myenteric neurons and damage of nerve process. | Robinson *et al*[115], 2014 |
| EVs | Rat bone-marrow | TNBS-induced colitis in rats | IV | Inhibit NF-κBp65 pathways, modulate anti-oxidant/oxidant balance, and apoptosis. | Yang *et al*[132], 2015 |
| Exosomes | Human umbilical cord | DSS-induced colitis in mice | IV | Increase IL-10 and decrease TNF-α, IL-1β, IL-6, iNOS, and IL-17. | Mao *et al*[114], 2017 |
| Extract | Human umbilical cord | DSS-induced colitis in mice | IP | Inhibit inflammatory cytokines and alter macrophage functional phenotype from M1 to M2 | Song *et al*[107], 2017 |
| CM | Mouse adipose tissue | DSS-induced colitis in mice | IP | Increase Treg, IL-10, and TGF-β, and decreased IL-17. | Heidari *et al*[116], 2018 |
| CM | Mouse adipose tissue | DSS-induced colitis in mice | IP | Increase Treg, IL-10, and TGF-β, and decreased IL-17. | Pouya *et al*[117], 2018 |
| CM | Human umbilical cord | DSS-induced colitis in mice | Culture medium (organ culture) | Decrease IL-6 and increase Ki-67. | DA Costa Gonçalves *et al*[119], 2018 |
| Exosomes | Human umbilical cord | DSS-induced colitis in mice | IV | Downregulated ubiquitin inhibiting NF-κB and mTOR activation. | Wu *et al*[133], 2018 |
| EVs | Mouse bone-marrow overexpressing miR-146a | TNBS-induced colitis in rats | IV | Suppress the activation of NF-kB pathway, decrease TNF-α, IL-6, and IL-1β. | Wu *et al*[131], 2019 |

MSC: Mesenchymal stromal cells; IBD: Inflammatory bowel diseases; DSS: Sodium dextran sulfate; CM: Conditioned medium; EVs: Extracellular vehicles; TNBS: Trinitrobenzene sulfonic acid; IP: Intraperitoneal; IV: Intravenous; TGF: transforming growth factor; Treg: Regulator T cell; NF-κB: Nuclear factor kappa B; IL, Interleukin; TNF-α: Tumor necrosis factor-alpha.

It is known that MSC-mediated immunosuppression is induced by inflammatory cytokines. Treatment with IFN-γ, TNF-α, and IL-1β causes MSC to express immunomodulatory molecules that mediate the suppression *via* cell-contact dependent mechanisms, including TLRs, PD-L1/PD-1 pathway, and FAS-L/FAS interaction[73,138-140] (Figure 2). Duijvestein *et al*[141] showed that MSC stimulated with IFN-γ enhance their immunosuppressive capacities, resulting in diminished mucosal damage in experimental colitis. Kang *et al*[142] found that IFN-γ primed MSC secrete tryptophanyl-tRNA synthetase and alleviate the experimental colitis by inducing apoptosis of immune cells. In our previous study, we have shown that MSC stimulated with IFN-γ decreased lymphocyte population in colonic organ culture of DSS-treated mice, but no effect of CM treatment was observed. That could be explained by the fact that T cell immunosuppression has a partial dependence on the cell-cell contact mechanism[119]. Furthermore, Fan *et al*[143] showed that IL-1β primed MSC have enhanced immunosuppressive capacities and migration ability, and Cheng *et al*[144] demonstrated that IL-25 primed MSC inhibited Th17 immune response and induced regulatory T cell phenotyping in DSS-induced colitis[143,144].

In another previous study conducted by our research group, we developed a cell-free therapy based on small particles from the membranes of MSC stimulated and unstimulated with IFN-γ. These particles contain the membrane-bound proteins of MSC, several of which have an immunomodulatory function and may also overcome the low homing efficiency of MSC. We have demonstrated that membrane particles keep immune regulatory properties of MSC and are potentially capable of passing the

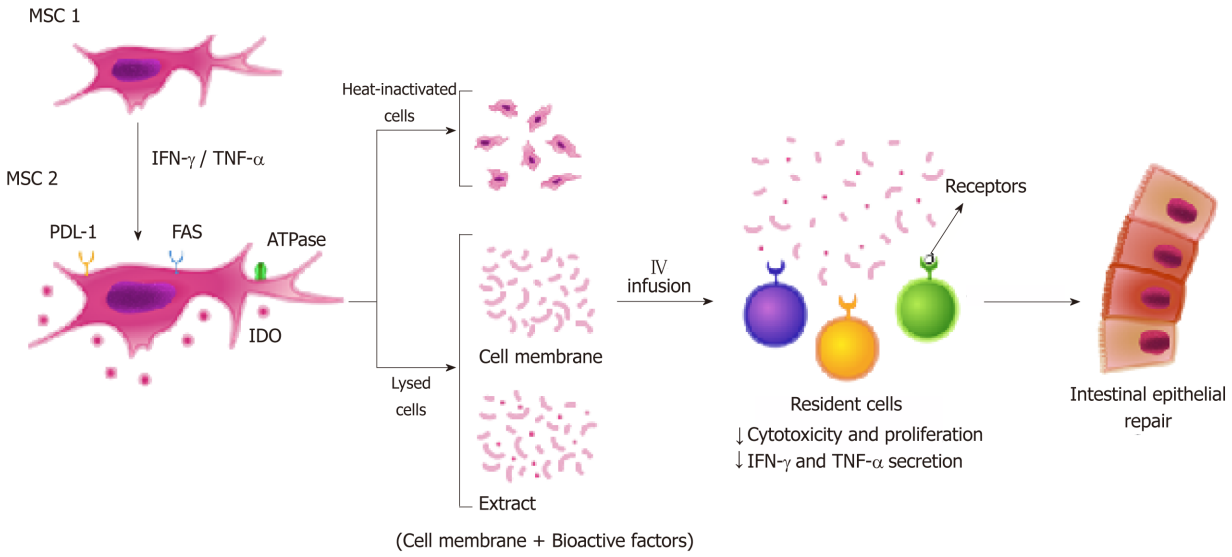


**Figure 2 Enhanced immunosuppressive properties of MSC in cell contact-dependent mechanism.** Treatment with IFN-γ and TNF-α induce MSC1 to express immunomodulatory molecules (MSC2) that mediate the suppression *via* cell contact-dependent mechanisms including PD-L1/PD-1 pathway and FAS-L/FAS interaction. To improve MSC homing, new therapeutic approaches are being developed: Heat-inactivated cells and lysed cells (extract or cell membrane). MSC-based therapy may exert its therapeutic effects mainly by cell-cell contact and consequently by interaction with immune cells, establishing a favorable environment for the regeneration of intestinal tissue. MSC: Mesenchymal stromal cells; IFN: Interferon; TNF: Tumor necrosis factor; PD-L1: Programmed death ligand 1; IDO: Indoleamine 2,3 dioxygenase.

lung barrier and exerting their effects at sites beyond the lungs[136]. Most importantly, we also found that the immunomodulation induced by membrane particles stimulated and unstimulated with IFN- γ is different. According to this information, the surface phenotype of MSC (protein molecules present on their membrane) is determinants for the therapeutic effect of MSC in patients with IBD.

## CONCLUSION

Taken together, these findings highlight that bioactive factors, cell surface proteins, and metabolic pathways derived from MSC offer unique opportunities for the development of promising cell-free therapies for IBD that associate the potential of MSC with the reduction of the practical complexities arising from the use of living cells. Recent experimental studies have suggested that not only the secretion of bioactive factors but also the surface proteins of MSC have great therapeutic potential that can overcome the deficiencies of current therapy for IBD and could open new frontiers in gastrointestinal medicine.

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*REVIEW*

# Using induced pluripotent stem cells for modeling Parkinson's disease

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## Abstract

Parkinson's disease (PD) is an age-related neurodegenerative disease caused by the progressive loss of dopaminergic (DA) neurons in the substantia nigra. As DA neurons degenerate, PD patients gradually lose their ability of movement. To date no effective therapies are available for the treatment of PD and its pathogenesis remains unknown. Experimental models that appropriately mimic the development of PD are certainly needed for gaining mechanistic insights into PD pathogenesis and identifying new therapeutic targets. Human induced pluripotent stem cells (iPSCs) could provide a promising model for fundamental research and drug screening. In this review, we summarize various iPSCs-based PD models either derived from PD patients through reprogramming technology or established by gene-editing technology, and the promising application of iPSC-based PD models for mechanistic studies and drug testing.

**Key words:** Parkinson's disease; Dopaminergic neurons; Induced pluripotent stem cells; Somatic cell reprogramming; Aging



**Core tip:** Human induced pluripotent stem cell (iPSC)-derived dopaminergic neurons hold great promise for studying disease mechanisms underlying Parkinson's disease (PD) and testing drug effects. A number of reviews have previously summarized the potential use of patient iPSCs for modeling PD. However, few of them comprehensively discuss the establishment of gene-editing-based iPSCs for PD and their application in research. Our objective is to consolidate the current literature on various iPSC-based PD models either derived from PD patients through reprogramming technology or established by gene-editing technology, and provide new insights into the application of iPSC PD models.

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## INTRODUCTION

Parkinson's disease (PD) is a progressive neurodegenerative disease caused by the death of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc) and a decrease in dopamine level, which lead to hypokinetic motor symptoms such as shaking, rigidity, slowness of movement, and difficulty in walking[1,2]. Although the standard treatments of PD that focus on boosting dopamine or dopamine receptor signaling can reduce symptoms at an early stage of the disease, none is effective at slowing or preventing progression of PD[3].

The onset of PD typically occurs at age > 60 years and its incidence is a global health concern with the increase in the aged population[4,5]. Aging is considered to be the most important risk factor for PD[6,7], but multiple genetic and environmental factors are also widely recognized to play critical roles in its development[8,9]. PD is generally classified into two forms: sporadic and familial PD. Sporadic PD is usually late onset and accounts for the majority of PD cases[10]. Some genetic backgrounds have been reported to increase the incidence of sporadic PD such as cytochrome P450 2D6 and glutathione S-transferase pi 1. However, these are not defined as genetic diseases because they have not been demonstrated to be associated with the development of PD[11,12]. In addition, long-term exposure to industrial chemicals and pollutants such as pesticides[13], metals[14] and solvents[15] is considered to contribute to development of sporadic PD. Familial PD is caused by gene mutations, which accounts for about 10% of PD cases[16], and shares some clinical features with sporadic PD. Currently, 16 familial PD-related genes have been identified. Their classification depends on chromosomal locus, which is named PARK and numbered in chronological order of their identification[17,18]. Among these mutations, PARK1/4, PARK3, PARK5, PARK8 and PARK11 are identified as autosomal dominant mutations. Except for PARK1/4, the other autosomal dominant mutations are late-onset PD. Autosomal recessive mutations in PARK2, PARK6, PARK7, PARK9, PARK14 and PARK15 are reported in early-onset PD. Other mutations in vacuolar protein sorting 35 and eukaryotic translation initiation factor 4G1 are identified in large families with dominant late-onset PD but not yet assigned with a PARK locus[19].

Previous studies on the pathology of PD have indicated that the deterioration is caused by formation of α-synuclein immunoreactive inclusion bodies that develop into globular Lewy bodies or Lewy neurites[20,21]. α-synuclein aggregation is recognized as not only a key event in familial PD but also the most consistent marker to define Lewy body pathology in sporadic PD[22]. These findings hint that both familial and sporadic PD have similar etiology[23]. In addition to abnormal aggregation of α-synuclein, other pathogenic factors involved in progress of PD have been reported, including mitochondrial dysfunction, oxidative and nitrative stress, neuroinflammation, and impaired autophagy[24-28]. However, up to now, the exact etiology and pathogenesis of PD are still unknown. The major barrier to study PD is the lack of brain tissue samples from patients, and current understanding of PD neuronal dysfunction has been largely derived from postmortem and pathological specimens[29]. Experimentally modeling PD is conventionally based on biochemical abnormalities in the brains of PD patients, such as oxidative stress and mitochondrial dysfunction. Toxins such as 6-hydroxydopamine (6-OHDA), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), paraquat and rotenone are conventionally used in PD modeling[30]. Commonly, 6-OHDA is stereotactically injected into animal brains and the other toxins are injected subcutaneously or intraperitoneally to induce PD models[31-33]. These neurotoxins can be taken up by DA neurons via dopamine transporters and cause neuronal damage[34]. Their toxicities are possibly due to the inhibition of complex I of the mitochondrial electron transport chain, which leads to depletion of ATP and an increase in reactive oxygen species (ROS), and eventually results in neuronal death[35]. Although these toxins can destroy DA neurons in the SNpc, 6-OHDA and MPTP treatment does not yield aggregation of α-synuclein (Lewy bodies), which is a major pathological marker of PD[36]. Typical PD is a type of chronic neurodegeneration. However, 6-OHDA or MPTP causes acute damage, which is not an appropriate model to mimic the pathogenic factors of PD. Chronic exposure to

rotenone in rats leads to aggregation of α-synuclein, DA neurodegeneration and behavior defects. A major concern about rotenone-induced models is that, in addition to degeneration of nigral DA neurons, rotenone causes pronounced degeneration in basal ganglia and brainstem nuclei[37], and leads to high systemic toxicity[38].

Transgenic mice are alternative models for exploring pathogenic mechanisms of PD-linked genetic factors. Currently, duplication or triplication of the α-synuclein locus could cause PD symptoms[39], indicating that increasing expression of PD-related genes could be applied to establishment of PD models[40,41]. Some PD characteristics can be observed in transgenic mice generated by overexpressing wild-type PD genes or PD gene mutation at age ≤ 2 years[42]. However, in mammalian models, knockin or knockout mutation of PD-linked genes can only cause a moderate decrease in striatal DA levels accompanied by low locomotor activity in an age-dependent manner, without evident loss of dopaminergic neurons in SNpc[43]. Transgenic mouse models may illuminate some pathogenic processes of PD, but cannot fully replicate the phenotypes of human PD because they are insufficient to cause significant nigral degeneration within the animal lifespan[40]. The genetic mouse models may not properly represent interaction of genetic factors, aging process, and environmental insults in human PD[19,44]. Thus, establishing suitable models to fully represent the characteristics of PD is urgently needed.

Human induced pluripotent stem cells (iPSCs) are generated from somatic cells by reprogramming. The somatic cells reprogramming technology was pioneered by Shinya Yamanaka in 2006 and showed that introduction of four transcription factors (*OCT4*, *SOX2*, *KLF4* and *c-MYC*) could convert somatic cells into PSCs[45]. The ability of iPSCs to differentiate into DA neurons overcomes the challenges and shortcomings associated with PD modeling. Two types of iPSC-based PD models are widely used: patient-specific and gene-modifying models. Patient-derived specific disease models have been used in discovering novel biomarkers for diagnosis or candidate drug therapy[46]. Genome editing can offset the variation of genetic background among individuals[47,48]. In this review, we summarize various iPSC-based PD models either derived from PD patients through reprogramming technology or established by gene-editing technology, and the promising application of iPSC-based PD models for mechanistic studies and drug testing (Figure 1).

## PATIENT-SPECIFIC iPSCs

It is widely accepted that PD results from a complex interaction of environmental and genetic factors[49]. In the hope of copying traits of PD, DA neurons derived from PD-patient-specific iPSCs display the cellular characteristics of PD *in vitro*. Investigators have established iPSC banks of neurodegenerative disease over the past decade. To date, patient-specific iPSCs have been used in a variety of fields including drug discovery, basic research and cytotoxicity testing.

### *iPSC models for familial PD*

It is well known that sporadic PD occupies > 90% of total PD cases, but genetic factors still play an important role in understanding PD etiology. The use of genetic PD iPSCs offers the promise of addressing the contribution of individual genetic factors and functional relevance of underlying molecular pathways in the development of PD. Currently, 16 familial PD-related genes have been identified. Among them, *SNCA*, *LRRK2*, *Parkin*, *PINK1* and *GBA* mutations have mostly been studied in genetic PD iPSCs[50] (Table 1). Researchers have made efforts to illustrate the pathological features of DA neurons or other neuronal cells derived from these genetic PD iPSCs. It is generally considered that the fate of these mutated genes is often loss of function that induces aberrant accumulation of inactive proteins[51].

Accumulation of α-synuclein is a major feature of PD, which is encoded by *SNCA* gene. *SNCA* A53T mutant and triplication *SNCA* are familial PD *SNCA* mutants. In these patients, α-synuclein level in the midbrain region is three times that in somatic cells, revealing that it is easy to cause accumulation of α-synuclein in DA neurons. After Soldner *et al*[52] successfully generated the first DA neurons from *SNCA* mutant iPSCs, a number of *SNCA* A53T mutant or triplication *SNCA* mutant iPSC lines were generated, and elevated α-synuclein levels were found in these iPSC-derived DA neurons[40,53-57]. Other cellular types such as forebrain cortical neurons, neural precursor cells, and GABAergic neurons were further induced from *SNCA* mutant iPSCs, and endoplasmic reticulum (ER) stress, oxidative stress, or maturation inability but no significant alteration of α-synuclein protein or mRNA level were detected in these iPSC-derived cells[56-59]. These studies show that SNCA gene mutations may have great influence on a variety of neuronal subtypes and not just DA neurons.

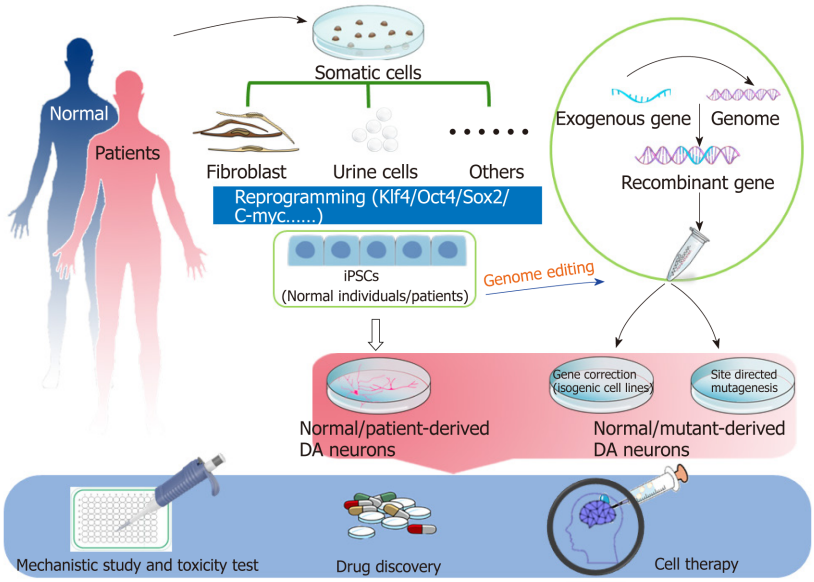


**Figure 1 Potential applications of induced pluripotent stem cells for study of Parkinson's disease.** Somatic cells are extracted from either normal individuals or patients and then reprogrammed to induced pluripotent stem cells (iPSCs). Gene editing technology enables one to generate knockin mutant Parkinson's disease (PD) iPSCs and isogenic control cell lines. Dopamine (DA) neurons can be successfully differentiated from iPSCs. Both patient-derived and gene-editing iPSCs could be powerful tools for modeling PD for mechanistic studies and drug discovery. Healthy or corrected iPSCs could serve as normal controls and cell sources for cell therapy. iPSCs: Induced pluripotent stem cells; DA: Dopaminergic.

N1437H, R1441C and G2019S mutations of *LRRK2* are known to cause PD. Among them, G2019S is the most common familial PD mutation in *LRRK2*[60]. Many studies have pointed out the crucial role of the loss-of-function of *LRRK2* mutant in reducing neurite outgrowth and numbers, and process complexity[61-65]. Oxidative stress, mitophagic dysfunction, and DNA damage have been observed in *LRRK2* G2019S iPSC-derived DA neurons. In addition, self-renewal and neuronal differentiation of *LRRK2* G2019 mutant neural stem cells are reported to be impaired, providing evidence on the crucial role of *LRRK2* in neural development[62].

Synergistic effect of *PINK1* and *Parkin* are important to maintain cellular homeostasis such as mitochondrial quality in DA neurons[66,67]. It is suggested that overexpression of *Parkin* can largely rescue the defects in *PINK1* mutants through mitochondrial translocation[66,68]. Parkin improves the uptake of dopamine through enhancing the expression of DAT as well[69]. *PINK1* or *Parkin* mutant iPSC-derived DA neurons display abnormal phenotypes such as mitophagy and autophagy impairment, vulnerability to various stresses, and accumulation of peroxisome proliferator-activated receptor-γ coactivator 1α (PGC1α), which are consistent with previous studies[63,70-75]. These findings support the synergistic effect of *PINK1* and *Parkin*, providing an inspiration for developing therapeutic strategies for PD.

*GBA1* gene mutations are reported to be associated with an increased risk of sporadic PD[76]. A number of studies on patient-specific iPSC-derived DA neurons harboring *GBA1* mutations have indicated that β-glucocerebrosidase (GBA1) has high correlation with elevated α-synuclein levels as well as autophagic and lysosomal defects[77-79]. Furthermore, calcium homeostasis imbalance, and reduced dopamine storage and uptake are also found in *GBA1* mutant DA neurons[77-79]. However, how these above-mentioned phenotypes are triggered by *GBA1* mutant remains to be explored.

### *iPSC models for sporadic PD*

Sporadic PD-derived iPSCs lack known PD mutations. The first case of generation of sporadic PD patient iPSCs was reported by Soldner *et al*[80]. They generated iPSCs from skin biopsies obtained from sporadic PD patients by application of modified lentiviruses carrying loxP sites flanking the integrated provirus for improving the efficiency of reprogramming. The advantage of this method was the use of inducible excisable lentivirus, rendering the iPSCs free of reprogramming transgenes. However, the phenotypic analysis of these iPSCs was not performed in that study. Sanchez-Danes *et al*[71] generated healthy and sporadic PD iPSCs *via* retroviral delivery of

**Table 1 Patient-derived induced pluripotent stem cell-based modeling of Parkinson's disease**

| Gene mutation | Inheritance type | Differentiated cell types | *In vitro* phenotypes(normalized to normal control / non-isogenic control) | Ref. |
|---|---|---|---|---|
| A53T *SNCA* | Familial | DA neurons | Not demonstrated | [52] |
| Triplication *SNCA* | Familial | DA neurons | Elevated levels of SNCA mRNA Increased cellular and secreted α-synuclein protein | [40] |
| Triplication *SNCA* | Familial | DA neurons | Elevated α-synuclein protein expression Increased expression of oxidative stress-related genes Increased susceptibility to oxidative stress | [53] |
| *SNCA* (A53T)/triplication *SNCA* | Familial | Forebrain cortical neurons | Nitrosative stress Accumulation of ERAD substrates ER stress | [58] |
| Triplication *SNCA* | Familial | Neural precursor cells | High vulnerability to stress Increased ROS production | [59] |
| Triplication *SNCA*<br>*LRRK2* (G2019S) | Familial<br>Familial | Neural precursor cells/DA neurons | Impaired neuronal differentiation and maturation pSer129-aSyn accumulation Increased susceptibility to oxidative stress | [56] |
| *SNCA* (A53T) | Familial | DA, GABAergic,and glutamatergic neurons | Protein aggregation (thioflavin S and pSer129-aSyn) Axonal neuropathology Altered expression of synaptic transcripts | [57] |
| *LRRK2* (G2019S) | Familial | DA neurons | Reduced neurite outgrowth Dysregulated autophagy system Increased cell death in response to neurotoxins Elevated αsynuclein protein level Dysregulation of genes related to DA neurodegeneration | [61] |
| *LRRK2* (G2019S) | Familial | Neural stem cells | Increased sensitivity to stress Progressive impairment in nuclear envelope organization Defective self-renewal and neuronal differentiation | [62] |
| *PINK1 (Q456X)*<br>*LRRK2* (G2019S) | Familial<br>Familial | DA and nonDA neurons, and immature cells | Increased vulnerability to stress Dysfunction of mitochondria | [63]<br>[63] |
| *PINK1* (Q456X or R275W)<br>*PARK2* (V324A) | Familial<br>Familial | DA neurons | Increased neuronal death Degenerated dendrites Impaired AKT signaling | [72]<br>[72] |
| *LRRK2* (G2019S) and Sporadic | Familial and Sporadic | DA neurons | Increased apoptosis Reduced neurite numbers and complexity Increased autophagic vacuoles | [81] |
| *SCNA* (A53T) | Familial | DA neurons | Elevated αsynuclein aggregation and Lewy-body-like deposition Induced nitrosative and oxidative stress Increased vulnerability to mitochondrial toxin-induced cell death | [55] |
| *SCNA* (A53T) | Familial | DA neurons | Decreased αsynuclein tetramers Increased neurotoxicity | [56] |

| | | | | |
|---|---|---|---|---|
| *PARK2* (exon 2–4 deletion or exon 6, 7 deletion) | Familial | DA neurons | Increased oxidative stress, activated NRF2 pathway Abnormal mitochondrial morphology and turnover. Elevated αsynuclein accumulation | [70] |
| *PARK2* (exon 3, 5 deletion or exon 3 deletion) | Familial | DA neurons | Increased oxidative stress Reduced dopamine uptake Enhanced spontaneous dopamine release | [71] |
| *PINK1* (c.1366C>T; p.Q456X or c.509T>G; p.V170G) | Familial | DA neurons | Impaired recruitment of Parkin to mitochondria Increased mitochondria copy number PGC1α upregulation | [74] |
| *PARK2* (exon 3, 5 deletion or exon 3 deletion) | Familial | DA neurons | Reduced neurite complexity Diminished microtubule stability | [73] |
| *PARK2* (R42P, exon 3 deletion, exon 3, 4 deletion, 255A deletion, R275W or R42P) | Familial | DA neurons | Reduced capacity to differentiate into DA neurons Altered mitochondrial volume fraction | [75] |
| *LRRK2* (G2019S) and sporadic type | Familial/sporadic | DA neurons | Elevated oxidative stress response Increased sensitivity to stress-induced cell death | [64] |
| *LRRK2* (G2019S) and Sporadic PD | Familial/sporadic | DA neurons | Hypermethylation in gene regulatory regions Reduced expression of transcription factors related to disease | [65] |
| *GBA1* (RecNcil/+, L444P/+ or N370S/+) and sporadic type | Familial/sporadic | DA neurons | Reduced dopamine storage and uptake Elevated α-synuclein and glucosylceramide levels Defective autophagic and lysosomal machinery Increased basal and induced calcium levels Enhanced vulnerability to ER stress | [77] |
| *GBA* (N370S/+) and sporadic type | Familial/ sporadic | DA neurons | Elevated αsynuclein levels Reduced dopamine levels Induced MAOB expression Disrupted network activity | [78] |
| *GBA1* and sporadic | Familial/ sporadic | DA neurons | Decreased dopamine storage and uptake Elevated αsynuclein levels | [79] |
| *SCNA* SNP | Sporadic | Neurons | Disease-associated risk variant that regulates *SCNA* expression | [49] |
| *SCNA* (A53T) | Familial | DA neurons | Elevated nitrosative stress SNCAA53T or mitochondrial toxins induce S-nitrosylated (SNO)-MEF2C in DA neurons S-nitrosylation of MEF2C reduces PGC1α expression and impairs mitochondrial function | [54] |

ERAD: Endoplasmic-reticulum-associated degradation; MAOB: Monoamine oxidase B; NRF2: Nuclear factor erythroid 2related factor 2; SNP: Single-nucleotide polymorphism; TH: Tyrosine hydroxylase.

OCT4, KLF4 and SOX2, and then differentiated them into DA neurons. By comparing with the healthy control group, they found that DA neurons differentiated from sporadic PD-patient-derived iPSCs after long-term culture displayed increased expression of cleaved caspase 3, shortened neurite length, and defective autophagosome clearance[81]. Fernández-Santiago *et al*[65] reported genome-wide DNA methylation of DA neurons derived from *LRRK2*-mutant and sporadic PD patients to explore the relationship between DNA methylation and alteration of gene expression and enhancer elements. They found that alterations of epigenetic signature significantly affected DNA methylation in sporadic PD patients. This study provides evidence that it is a common phenomenon that aberrant protein turnover and altered morphology and methylation patterns occur in sporadic PD-patient-derived DA neurons. Piwi-interacting RNA (piRNA) seems to have relevance to aberrant protein

turnover and altered morphology and methylation patterns in sporadic PD-patient-derived DA neurons. piRNA is a complex of piwi protein and RNA, and is a large class of small noncoding RNA molecules expressed in animal cells that are involved in the epigenetic and post-transcriptional silencing of transposons. Schulze *et al*[82] have shown that the specific alteration of sin- and line-derived piRNA in DA neurons could be a new mechanism for the causation of PD[82-84].

### *iPSCs-derived astrocytes in PD modeling*

Astrocytes are the major group of cells in the central nervous system, with a range of functions that provide both structural and metabolic support for neurons. Accumulating evidence suggests that astrocyte dysfunction leads to the pathogenesis of PD, especially familial PD[85-88]. As summarized by Booth *et al*[85], mutations in *DJ-1*, *SNCA*, *PLA2G6*, *LRRK2* and *GBA* lead to abnormal glutamate uptake, mitochondrial dysfunction, inflammatory response, water transport defect, and autophagy impairment. Gunhanlar *et al*[86] found that in a coculture system of astrocytes and neurons at a consistent ratio (60:40), neuron maturation was distinctly upregulated according to electrophysiological maturity. Accordingly, astrocytes are widely used in implementing cellular modeling approaches to the study of neurodegenerative disorders[87]. Similarly, astrocytes could help DA neurons defend against the neurotoxins and attenuate the mitochondrial dysfunction, as observed by Feng *et al*[88]. Remarkably, the astrocytes and neuron co-culture system improved the outgrowth of neuron markers, and stabilized the mitochondrial function through downregulation of ROS and increased mitochondrial function[88]. On the contrary, astrocytes were also used in inducing PD degeneration phenotypes by Santos *et al*[89] and di Domenico *et al*[90]. In Santos *et al*'s research, astrocytes were activated and became inflammatory, and were co-cultured with DA neurons[89], while Domenico *et al*[90] co-cultured PD-patient-derived astrocytes with normal DA neurons. Normal DA neurons were induced to display apoptosis and multiple system dysfunction after co-culture with dysfunctional astrocytes[89,90]. These compelling findings emphasized that astrocytes may substantially participate in PD pathogenesis (Table 2).

## GENOME-EDITING-BASED iPSCs and ISOGENIC CONTROLS

Since Yamanaka opened the door for the generation of iPSCs[45], iPSCs differentiation into DA neurons is commonly used for studying PD pathophysiology. Appropriate healthy iPSC-derived cells with higher quality and availability are often used as controls to study the phenotypes of a patient's iPSC-derived cells. However, differences in genetic background are of concern: healthy siblings used as controls share only about 50% of the genome of patients[91]. The differences in genetic background lead to higher variations in phenotypic presentation. This critical issue should only be interpreted *via* comparison with isogenic control cells[92]. A locus mutation or target gene correction is introduced by editing a specific site in the human genome of iPSCs, which has become a routine procedure in many studies of PD iPSCs. Many genome-editing tools, including zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and CRISPR/Cas9 system are commonly used in editing iPSCs[93]. Among them, CRISPR/Cas9 may be the best one in gene editing because of the prospect of flexibility in site selection and affordability, although there are still some concerns about the efficacy and off-target risk[92]. The bottleneck in investigating connections between gene function and disease mechanisms is likely to be overcome by these genome-editing technologies.

Many iPSC PD cell lines have been established with genome editing (Table 3). Soldner *et al*[52] performed genetic correction in A53T (G209A) α-synuclein mutation in PD patient-derived iPSCs via ZFN-based gene editing. The G2019S mutation in *LRRK2* is the most common cause of familial PD. Reinhardt *et al*[94] performed genetic correction of an *LRRK2* mutation in PD-patient iPSCs *via* ZFN-based gene editing and Cre/LoxP systems, and linked parkinsonian neurodegeneration to extracellular signal-regulated kinase (ERK)-dependent changes in gene expression. They further demonstrated that ERK inhibitor reduces multiple PD-associated phenotypes, including lower neurite outgrowth, autophagy defects, synaptic defects, increased apoptosis, accumulation of tau and α-synuclein in *LRRK2* G2019S mature DA neurons. Sanders *et al* reported that *LRRK2* G2019S iPSC-derived DA neurons displayed greater levels of mitochondrial DNA damage, whereas this abnormal mitochondrial damage was no longer detected in corrected iPSC-derived neurons by ZFN-mediated genomic correction[95]. The mutations and genomic multiplications of the *SNCA* gene (α-synuclein) account for up to 15% of cases of familial PD[41,96,97].

**Table 2 Induced pluripotent stem cell-derived astrocytes in Parkinson's disease modeling**

| Cell lines | Differentiated types | Phenotype demonstrated | Ref. |
|---|---|---|---|
| Bone marrow 2-3(BM2-3) hiPSCs | Astrocytes/DA neurons coculture system | Elevated DA neuron identities Stablization of mitochondrial function Downregulation of mitoROS Increased mitochondrial length (normalized to non-co-culture DA neurons) | [88] |
| iPSCs and ESCs | Astrocytes/DA neurons coculture system | Non-activated astrocytes co-culture system improved DA neurons survival Non-activated astrocytes co-culture system increased DA neurons neurite lengths (normalized to inflammatory-activated astrocytes coculture system) | [89] |
| *LRRK2* mutant and normal iPSCs | PD Astrocytes/normal DA neurons coculture system | Non-cell-autonomous damage is triggered by impaired autophagy in PD astrocytes Dysfunctional PD astrocytes accumulate and transfer α-synuclein to healthy DA neurons CMA activator drug prevents α-synuclein accumulation and neurodegeneration (normalized to the single culture system) | [90] |

iPSCs: Induced pluripotent stem cells; ESCs: Embryonic stem cells; hiPSCs: Human induced pluripotent stem cells; PD: Parkinson's disease; DA: Dopaminergic.

Soldner *et al*[52] used CRISPR/Cas9-mediated insertion and exchange of risk-associated enhancer sequences. They identified a common PD-associated risk variant in a noncoding distal enhancer element that regulates expression of *SNCA*. Arias-Fuenzalida *et al*[98] induced heterozygous missense A30P and A53T mutations in the SNCA gene in healthy iPSCs *via* combined use of CRISPR/Cas9 and fluorescence-activated cell sorting analysis. These edited *SNCA* mutant iPSC-derived neuroepithelial stem cells displayed a significant decrease in maximal respiration, proton leak, basal respiration, ATP production, and nonmitochondrial respiration for the extracellular energy flux. Qing *et al*[48] used CRISPR/Cas9 and piggyBac system to establish heterozygous *LRRK2* G2019S mutation in healthy iPSCs. They observed that the number of tyrosine-hydroxylase-positive neurons and their neurite complexity were significantly decreased in *LRRK2* G2019S DA neurons.

## CHALLENGES IN USING iPSCs TO MODEL PD

### *Efficient generation of DA neurons from human iPSCs*

DA neurons modulate several brain functions such as motor control, reward behavior, and cognition[99]. Recapitulation of the *in vivo* developmental profile of a specific cell type provides a powerful strategy for manipulating cell-fate choice during the process of human iPSC differentiation. *In vitro* generation of functional DA neurons is critical in pluripotent cell biology for both experimental and clinical applications. The neural stem/progenitor cell (NSPC) strategy and the floor-plate cell strategy are two useful protocols for generating DA neurons. The NSPC strategy is widely used in neuronal differentiation, in which NSPCs are isolated from rosettes[100,101] or induced by defined medium with many supplements[102]. The floor-plate strategy was proposed by Hynes *et al* and is based on the fact that the floor plate is a critical signaling center during neural development located along the ventral midline of the embryo[103]. Lorens *et al*[100] derived the floor-plate cells from human embryonic stem cells (hESCs) using a modified protocol by dual Smad inhibition. The floor-plate cells are predisposed to differentiate into mature ventral midbrain DA neurons with a higher efficiency than rosette-based neurons[101]. The floor-plate-derived midbrain DA neurons are able to control dopamine release and selective dopamine reuptake, as well as other features such as synaptic transmission. Importantly, PD patient iPSC-derived DA neurons show cellular PD phenotypes such as increased accumulation of mitochondrial ROS and cytoplasmic α-synuclein, mitochondrial DNA damage, shortened neurites, and impaired autophagy[80,104,105].

### *Disease phenotypes in modeling late-onset PD*

**Table 3 Pluripotent stem cell-based genome-editing Parkinson's disease models**

| Gene mutation | Editing system | Cell line | Phenotype demonstrated | Application | Ref. |
|---|---|---|---|---|---|
| *SNCA* A30P | CRISPR/CAS 9 | hiPSC | Not demonstrated | Locus mutation | [48] |
| *LRRK2* G2019S | ZFN | hiPSC | Not demonstrated | Gene correction | [95] |
| *LRRK2* G2019S | CRISPR/CAS 9 | hiPSC | Synaptic defect, fraction of TH+/S129P-αS+ neurons was significantly reduced | Locus mutation | [48] |
| *SNCA* E46K | ZFN | hESC | Not demonstrated | Locus mutation | [53] |
| *SNCA* A53T | ZFN | hiPSC | Not demonstrated | Gene correction | [52] |
| *SNCA* A30P/A53T | CRISPR/CAS 9 | hiPSC | Not demonstrated | Locus mutation | [98] |
| *SNCA* (rs356165 A/G) | CRISPR/CAS 9 | hiPSC | Not demonstrated | Locus mutation | [49] |
| *LRRK2* G2019S | ZFN | hiPSC | Basic phenotypes: autophagy defects, synaptic defects, increased apoptosis, accumulation of τ and α-synuclein. Phenotypes were alleviated after genetic correction | Gene correction | [94] |

hiPSC: Human induced pluripotent stem cell.

Aging is a crucial risk factor for all late-onset neurodegenerative diseases. One important challenge found in iPSC-based PD models is to appropriately reproduce the late-onset characteristics. Reprogramming somatic cells to iPSCs resets their identity back to an embryonic state. The ability of iPSCs to undergo unlimited division while maintaining genomic integrity provides a way to overcome the senescence barrier. Even if these iPSCs differentiate into mature DA neurons, it still needs a lot of time to culture for mimicking aging DA neurons, presenting a significant hurdle for modeling PD. Thus, how to induce aging in iPSC-derived DA neurons is an important issue. Justine *et al*[72] compared young and aged human fibroblasts; they found a predominant difference in progerin level between adolescent and aged cells. They further demonstrated that overexpression of progerin in PD iPSC-derived DA neurons *in vitro* or *in vivo* promoted cell aging for modeling late-onset PD features such as pronounced dendrite degeneration, progressive loss of tyrosine hydroxylase expression, and enlarged mitochondria or Lewy body inclusions[72]. Thus, progerin expression could accelerate aging in iPSC-derived DA neurons for inducing PD pathogenic phenotypes, and introducing progerin expression could be a useful strategy to manifest disease phenotypes in iPSC-based late-onset PD models. However, it should be noted that neurons are fragile for undertaking the delivery of exogenous genes. The low efficiency of transfection in neurons remains a big challenge for widespread and convenient application of progerin expression to model iPSC-based late-onset neurodegenerative diseases.

Previous studies have indicated that chronic treatment with anticancer drug hydroxyurea (HU) could induce cellular senescence in human fibroblasts[106] and mouse neural stem cells[107]*via* induction of genes related to DNA damage and repair, mitochondrial dysfunction, and ROS increase. In our recent study[108], we found that HU induced disease phenotypes of sporadic PD-patient-specific iPSC-derived DA neurons. HU treatment significantly reduced neurite outgrowth, expression of p-Akt and its downstream targets (p-4EBP1 and p-ULK1), as well as increased the level of cleaved caspase 3 in iPSC-derived DA neurons from sporadic PD patients. Transcriptome analysis and Western blotting indicate that HU alters the expression of genes and proteins related to the ER stress pathway in healthy iPSC-derived DA neurons. It reveals that ER stress might contribute to HU-induced aging in iPSC-derived DA neurons from sporadic PD patients. Our study also found that the midbrain characteristics decline after iPSC-derived DA neurons are treated with HU, which is similar to the characterization of PD. Thus, increasing chemically induced ER stress could be an alternative approach to accelerating aging of iPSC-derived DA neurons from PD patients for manifestation of PD cellular phenotypes.

## POTENTIAL APPLICATION OF iPSCs IN PD

### *iPSCs-based mechanistic studies for exploring clinical therapeutic strategies*

Due to the ability of human iPSCs to differentiate into human DA neurons and astrocytes, human iPSCs are a promising model for studying the pathogenesis of PD. Compared with neurotoxin-induced injuries, human iPSC-derived DA neurons from sporadic or familiar PD patients could give help us to understand the progressive changes of PD neuronal phenotypes as culture time increases[50,109]. Through this PD iPSCs model, we can verify the possible mechanisms of pathogenesis suggested in previous studies in other cell or animal models. Most importantly, studying these PD-iPSC-derived DA neurons could explain how the clinical degenerative features of human DA neurons occur[110,111]. At least the changes in some human familial- PD-iPSC-derived neurons can represent the middle or final stage of PD because this iPSC-derived DA neuronal death occurs with α-synuclein accumulation, which is consistent with the observation in PD patients. Compared with familial PD, the etiology of sporadic PD is still a major challenge because of the multifactorial etiopathogenesis of sporadic PD. Since sporadic PD is complicated, the changes in neuronal phenotype cannot reflect the pathogenic alteration in the whole brain or other systems of sporadic PD patients[112,113].

### *iPSCs as a powerful and convincing drug discovery tool in PD*

Current PD therapies help patients relieve motor symptoms, but do not effectively prevent, slow or halt the progression of PD, particularly in the loss of DA neurons[114]. Neurotoxin-based neurons or animal models are commonly used for anti-PD drug screening. Many drugs based on these artificial models have been developed but do not significantly prevent PD progression[115]. One reasonable explanation is that these neurotoxins that usually cause strong injuries in DA neurons cannot mimic the progressive death of human DA neurons in PD. In addition, DA neurons from animals are distinct from human DA neurons. In PD-iPSC-derived DA neurons, the typical PD features such as accumulation of α-synuclein, progressive degeneration, and death could be observed. These robust and reproducible PD phenotypes are amenable to screening potential compounds. Thus, PD-iPSC-derived DA neurons are more suitable for screening anti-PD drugs than artificial models are.

### *iPSC-based research enhanced development of cell therapy*

The inspiring success in PD treatment was achieved through allograft of human fetal midbrain cell suspensions in 1980[116]. In addition, in MPTP-treated monkey brains, monkey ESC-derived neural progenitor cells differentiated into DA neurons and cells integrated well in the striatum, thereby PD-related motor symptoms improved[117]. Compared with ESCs, iPSCs have more potential in cell replacement therapies for PD because they can be generated from patients' own cells and differentiate into DA neural progenitor cells that specifically develop into DA neurons[118]. iPSCs have the advantage of eliminating immune rejection concerns as they are obtained from the host. The generation of iPSCs from a patient's own somatic cells would potentially allow for a plentiful source of cells for autotransplantation. In addition, using iPSCs rather than ESCs means that this treatment would be potentially available in some countries that ban the application of ESCs, including Italy, Ireland, and most African and South American countries[119,120]. For familial PD patients, the corrected iPSCs are also a reasonable source for the transplantation of normal DA neurons to reduce motor symptoms. Recently, scientists from Japan have started a clinical trial[121] (ClinicalTrials.gov NCT02452723) to treat PD with human iPSCs. All these suggest that the transplant of human iPSCs-derived DA neurons will be a promising therapeutic strategy and customized treatment is practical due to individual differences.

## CONCLUSION

In this review, we have summarized iPSC-based PD models from patient-specific as well as genome-editing-based iPSCs. Patient-specific iPSCs that harbor the disease-associated genotype have provided extensive insights into pathogenic mechanisms of PD. However, given inherent genetic heterogeneity between individuals, it is understandable that disease phenotypes could be confounded by use of patient-specific iPSCs with different genetic backgrounds. The use of gene-targeting strategies based on ZFNs, TALENs or CRISPR/Cas9 to induce or correct a particular genetic mutation, has become indispensable in developing isogenic lines with and without a disease genotype. However, although great advances have been made in gene editing, high off-target risk and low efficacy still make it difficult and time-consuming in generating genome-editing-based iPSCs. Thus, improving the efficiency and precision

of gene editing is important for generating more isogenic PD-specific iPSCs and control cell lines. How to reliably establish iPSC-based models for late-onset PD remains to be resolved. Inducing an aged state by long-term culture, overexpression of an aging protein, or small molecules is encouraged in most iPSC-based age-related PD models. Finally, iPSCs cannot mimic motor symptoms and some nonmotor symptoms such as depression, agrypnia, hyposmia and impairment of cognition. Thus, how to bridge the gap between animal and cell studies should be addressed in the future.

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*REVIEW*

# Impelling force and current challenges by chemicals in somatic cell reprogramming and expansion beyond hepatocytes

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## Abstract

In the field of regenerative medicine, generating numerous transplantable functional cells in the laboratory setting on a large scale is a major challenge. However, the *in vitro* maintenance and expansion of terminally differentiated cells are challenging because of the lack of specific environmental and intercellular signal stimulations, markedly hindering their therapeutic application. Remarkably, the generation of stem/progenitor cells or functional cells with effective proliferative potential is markedly in demand for disease modeling, cell-based transplantation, and drug discovery. Despite the potent genetic manipulation of transcription factors, integration-free chemically defined approaches for the conversion of somatic cell fate have garnered considerable attention in recent years. This review aims to summarize the progress thus far and discuss the advantages, limitations, and challenges of the impact of full chemicals on the stepwise reprogramming of pluripotency, direct lineage conversion, and direct lineage expansion on somatic cells. Owing to the current chemical-mediated induction, reprogrammed pluripotent stem cells with reproducibility difficulties, and direct lineage converted cells with marked functional deficiency, it is imperative to generate the desired cell types directly by chemically inducing their potent proliferation ability through a lineage-committed progenitor state, while upholding the maturation and engraftment capacity posttransplantation *in vivo*. Together with the comprehensive understanding of the mechanism of chemical drives, as well as the elucidation of

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specificity and commonalities, the precise manipulation of the expansion for diverse functional cell types could broaden the available cell sources and enhance the cellular function for clinical application in future.

**Key words:** Chemical induction; Pluripotent reprogramming; Direct lineage conversion; Direct lineage expansion; Hepatocyte expansion; Cell fate specificity; Transcriptional memory; *In vivo* induction



**Core tip:** Chemical-mediated reprogramming is a promising strategy for generating desired cells. However, chemical-mediated pluripotent reprogramming has reproducibility difficulties, and direct lineage conversion shows significant deficiency in cell function maturation. On the other hand, direct lineage expansion from target cells not only bypasses pluripotency-related tumorigenesis but also has superior posttransplantation advantages in engraftment and functional maturation. Recent achievements in chemical expansion of human hepatocytes may help solve the cell source limitation in liver disease treatment.

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## INTRODUCTION

The barriers to cell fate conversion between somatic cells and pluripotent cells had a breakthrough with the proposition of the induced pluripotent stem cell (iPSC) reprogramming strategy in 2006, when Takahashi *et al*[1,2] reported a significant discovery that the ectopic expression of four defined transcription factors (TFs; *Oct4*, *Sox2*, *Klf4a*, and *c-Myc*) could force the cell fate conversion. Remarkably, iPSCs can be converted into multiple functional cells under optimal differentiation strategies, not only contributing to the establishment of patient-specific disease models but also benefiting drug discovery and development[3-5]. Later, combinations of lineage-specific TFs were screened and applied to generate various desired cell types, known as direct lineage conversion, including neurons, cardiomyocytes, hepatocytes, and pancreatic β-cells[6-9]; this strategy bypasses the transition pluripotent stage, rendering the process faster and more effective and, meanwhile, evading the risk of pluripotency-related tumorigenesis, holding great promise for biomedical applications[10,11].

However, to date, TF-based reprogramming approaches face numerous challenges in efficiency, especially in safety, regarding the use of oncogenes and likely genetic integration of exogenous factors. Researchers have made substantial efforts to optimize the reprogramming using nonviral and nonintegrating methods, including synthetic RNAs[12,13], cell membrane–permeable proteins[14], episomal plasmids[15], and chemical compounds[16]. Comparatively, chemicals offer several unique advantages such as structural stability; spatiotemporal flexibility; easy for screening, application, and delivery; amenability to manufacturing and scale-up; and the possibility of fine-tuning their effects by altering their concentrations and combinations[17,18]. To date, several small molecules have been proved to facilitate the iPSCs reprogramming and lineage conversion, either by their substitute role for replacing certain TFs, or synergetic effect for augmenting the efficiency[19-22]. Additionally, the progressive attainment of full chemical-induced human pluripotent and lineage-committed cells merits great promise to resolve the cell source limitation for the therapeutic purpose (Figure 1), as well as offer an alternative option for human disease modeling and drug development.

Perhaps, establishing a stable and efficient chemical induction strategy could fundamentally alter the principal concept of the conventional cell reprogramming strategy. Nevertheless, to date, the specificity of chemical targets and the correlative gene network dynamics remain unclear[17,23]. Thus, elucidation of the individual or synergetic chemical force on the cell pathway signaling and epigenetic pattern

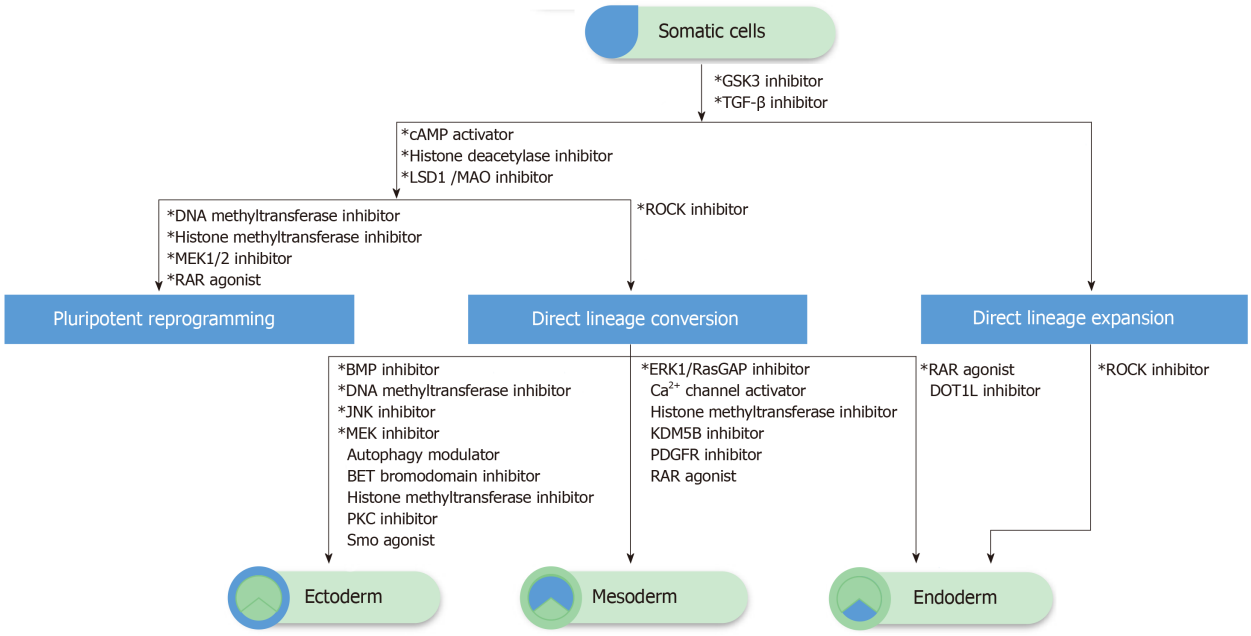


**Figure 1 The schematic of chemical-driven cell fate change and expansion.** The transforming growth factor (TGF)-β and GSK3 pathway inhibitors are commonly required in pluripotency reprogramming, direct lineage conversion, and expansion. Additional epigenetic modulators (histone deacetylase inhibitors and/or deoxyribonucleic acid/histone methyltransferase inhibitors) are applied for pluripotency reprogramming, as well as direct lineage conversion, while different lineage commitments require specific signaling modulations. The combination of TGF-β, GSK3, and ROCK pathway inhibitors could induce the direct lineage expansion of endoderm-committed cells such as hepatocytes. The direct expansion of ectoderm and mesoderm-committed cells remains unclear and could not be listed here. *Necessary and/or commonly used compounds.

modulation would be vital to endorse the induction of desired cell types proficiently. Hence, this review aims to summarize the updated progress and discuss the advantages, limitations, and challenges of the impact of full chemicals on stepwise reprogramming of pluripotency, direct lineage conversion, and direct lineage expansion on somatic cells.

## PLURIPOTENT REPROGRAMMING

The first breakthrough for pluripotency reprogramming by full chemical treatment was attained in 2013[24]. By screening, Hou *et al*[24] first successfully identified the chemical substitutes for Takahashi *et al*[1,2]'s four TFs, including VPA, CHIR99021, 616452, tranylcypromine, Forskolin, and DZNep (VC6TFZ), which could convert mouse fibroblasts into a partially reprogrammed state with high Oct4 expression; embryonic stem cell-like chemically-iPSs (CiPSCs) could be generated after switching to 2i (CHIR99021 and PD0325901) medium. Additionally, it was revealed that an endogenous pluripotency program could be established by chemically manipulating the cell signaling pathways. Although the routes and mechanisms underlying pluripotency reprogramming remain unclear to date, two primary hypotheses reveal how chemicals drive somatic cells stepwise toward pluripotency.

### *Stepwise bridges*

During the attainment of a pluripotent program, unique extraembryonic endoderm (XEN)–like state was first discovered linking fibroblasts to pluripotency, which markedly expressed XEN genes *Sall4*, *Gata4*, *Gata6*, *Sox17*, and *Sox7*[25]. Remarkably, the knockdown of XEN genes during the reprogramming markedly impairs the generation of CiPSCs, whereas their overexpression is adequate to replace essential chemicals (CHIR99021, 616452, and Forskolin) for the Oct4-positive XEN-like colony formation, illustrating that the XEN-like state is a vital intermediate state toward CiPSCs. Of note, despite different cell origins, the similar activation of the XEN-like program was reported during the early stage of reprogramming from neural stem cells (NSCs) and small intestinal epithelial cells (IECs)[26], highlighting that the XEN-like state serves as a vital and exclusive bridge toward pluripotency. Remarkably, the reprogramming kinetics and frequency of XEN-like state were highly distinct between NSCs and IECs. Compared with NSCs, IECs exhibited much rapid and efficient XEN-like colony formation, which could be explained by the fact that the inherent

expression of *Gata4* and *Gata6* in IECs facilitated the conversion of XEN-like program at an early stage.

After 3 years, the same group reported that the 2C (two-cell stage)-like programs were key bridges linking the XEN-like state to pluripotency, and the expression level of the 2C-like program (*Zscan4c*, *Zscan4f*, *Tcstv1*, *Tcstv3*, *Lmx1a*, and *Sp110*) well correlated with the reprogramming potential[27]. The knockdown of 2C genes markedly impaired the reprogramming efficiency of CiPSCs. Additionally, extensive loss of deoxyribonucleic acid (DNA) methylation was detected in this stage, which corroborated the hallmark of global epigenetic reprogramming in early embryogenesis, elucidating further the stepwise establishment of "early embryonic-like programs'' toward the complete pluripotency network. Notably, when the 2C-like program was enhanced by the optimized treatment of VPA (histone deacetylase inhibitor), the reprogramming efficiency was markedly augmented.

### Chromatin accessibility dynamics

BrdU, commonly used in tracing DNA replication, was occasionally reported to facilitate OSKM-induced reprogramming and demonstrated further that it could replace Oct4 in Yamanaka factors–mediated reprogramming and promote full chemical reprogramming[28]. Nevertheless, to date, the precise mechanisms by which BrdU promotes TFs and chemical-mediated reprogramming remain unclear. Based on the benefits of BrdU on closing or opening of chromatin loci, the dynamic chemical reprogramming process was revealed in the view of chromatin accessibility[29]. At stage 1 of chemical induction, the AP1 families–related chromatin loci in fibroblasts were closed gradually, whereas chromatin loci enriched with *GATAs*, *FOXs*, *KLFs*, and *SOXs* were opened, which highly corroborated the XEN-like intermediate state, as reported previously[25]; however, it markedly differed from TF-mediated reprogramming, which does not need to bypass through this particular state[30], illustrating the unique epigenetic dynamics driven by chemicals. Following the treatment of 2iL at stage 2, *GATAs*- and *FOXs*-related loci turned to close gradually, along with the opening for OCT/SOX/KLF families. Notably, BrdU is essential to correctively open and close chromatin loci enriched with the XEN-like and fibroblast program at stage 1. Remarkably, in this study, the overexpression of master XEN genes could not replace BrdU for opening specific loci for the Oct4 expression, which contradicted a previous study[25].

Despite published protocols, currently, the successful induction of CiPSCs is restricted in limited groups, and the core factors and induction efficiency remain debatable[25,27-29,31], which could be partially explained by the nonspecificity of chemicals. Additionally, the targeted signaling pathway and/or epigenetic modulation could change much because of the tiny bias of chemical concentrations and combinations, making it difficult to establish stable intermediate states or correctly open or close the required chromatin loci. Generally, the current low reproducibility raises severe challenges for enhancing the stepwise protocol for generating CiPSCs.

## DIRECT LINEAGE CONVERSION

Recently, the advancements of TF-mediated direct lineage conversion from somatic cells to other cell types have garnered considerable attention; this strategy bypasses the acquisition of pluripotent state and serves a promising approach for generating numerous types of functional cells. The impact of chemicals was first reported to facilitate the TF-mediated lineage conversion. Supplemented with specific chemicals, multiple cells from different germ layers have been successfully generated from fibroblasts or other somatic cells with minimal utilization of lineage-specific TFs, including neuronal cells[32-35], cardiac cells[36,37], hepatocytes[22,38], and pancreatic cells[39,40]. Additionally, chemical cocktails could markedly augment the TF-mediated conversion with relatively high efficiency and purity[22,32]. However, from the perspective potential risks of genetic integration, direct lineage conversion by full chemicals merits much anticipation at present.

### Ectoderm

The first full chemical cocktail reported to generate neural progenitor cells (NPCs) directly from mouse and human somatic cells was a simple and effective VCR (VPA, CHIR99021, and RepSox) combination[41]. The chemical-induced NPCs (CiNPCs) exhibited similar transcription profiles as brain-derived NPCs and could self-renew and further differentiate into different neural lineage cells both *in vitro* and *in vivo*. Later, using alternative chemical cocktails, NSC-like cells were attained by other groups[42,43]. Notably, FGF and Shh signaling pathways recompiled the transcriptional

and epigenetic programs from fibroblast to neural lineage by modulating the binding and activation of immediate downstream TFs Elk1/Gli2 to master neural genes[43].

Bypassing the NPC state, direct chemical conversion of mature neurons (CiNs) from mouse and human fibroblasts has been achieved[44-46]. Compared with fibroblasts, astrocytes are extensively considered as a preferable starting cell source for neurogenesis, as well as direct neuron conversion[47,48]. Under chemical-mediated epigenetic silencing of glial genes, as well as the transcriptional activation of crucial neural transcriptional factors (NEUROD1 and NEUROGENIN2), human astroglial cells were reportedly converted into functional neurons efficiently, with the ability to integrate into local circuits *in vivo*. Additionally, a recent preprint report of *in vivo* direct conversion garnered considerable attention. A study reported successfully converting resident astrocytes to functional neurons *in situ* in adult mouse brain[49]. Remarkably, such *in situ*-generated neurons acquired electrophysiological functions and could functionally interact with resident neurons in the brain.

### Mesoderm

Under the two-step chemical treatment based on CRFVPT (CHIR99021, RepSox, Forskolin, VPA, Parnate, and TTNPB), for the first time, mouse fibroblasts were successfully converted to spontaneously beating cardiomyocyte-like cells (CMs) through a *Sca-1*$^{+}$ cardiac precursor-like stage[50]. Later, another study reported the generation of mouse chemical-induced CMs (CiCMs) by replacing the core chemical RepSox with another TGF-β inhibitor, A83-01[51]. Notably, CiCMs induced by either approach expressed cardiomyocyte-specific markers and displayed typical cardiac calcium flux and electrophysiological features, resembling primary cardiomyocytes. Remarkably, CiCMs could also be induced directly from resident cardiac fibroblasts *in vivo*. Despite the relatively low efficiency, the induced cardiomyocytes could markedly enhance cardiac functions in myocardial infarction mice[52].

In particular, during the conversion of human CiCMs, platelet-derived growth factor receptor inhibitors reportedly facilitated the cardiac conversion by suppressing the fibroblast program[53]. Additionally, the synergistic effects of chemicals for epigenetic modulation were determined, enabling cells responsive to extrinsic cardiogenic signals. Furthermore, the chemicals promoted the chromatin accessibility of core cardiogenesis genes loci, enabling effective binding of cardiogenic signal effectors, such as β-catenin and Smad1, and ultimately facilitating the cell fate conversion.

### Endoderm

During the chemical induction of mouse CiPSCs, some studies reported a unique XEN-like intermediate state[25,26,29]; these extraembryonic endoderm-like cells shared similar global gene expression patterns and *in vivo* developmental potential to the embryo-derived XEN cells[25] and displayed high plasticity for directing endoderm and ectoderm lineage cells. Under favorable induction conditions, both hepatocytes and neurons could be generated[54]. Remarkably, when cultured in a lineage-favorable condition, the multipotential intermediate status appeared prone to incline to a specific direction. Combined with the hepatocyte culture medium and activin A, mouse endoderm progenitor cells (EPCs) were induced with the robust expression of endoderm markers *Sox17*, *Foxa2*, *Gata4/6*, and *Hnf4a*, while lacking ectoderm markers[55]. Additionally, chemically induced EPCs (CiEPCs) displayed marked foregut/liver differentiation potential regarding the markedly elevated expression of *Krt8*, *Krt18*, and *Krt19*. Furthermore, under specific differentiation conditions, both hepatocyte-like cells (HLCs) and pancreatic lineage cells could be generated.

To date, the induction of human EPCs from somatic cells was restricted to the same germ layer. Initiated from gastrointestinal epithelial cells, human CiEPCs were reportedly generated under the support of tissue-specific mesenchymal feeders[56], endowed with bi-potential differentiation capacity toward hepatocytes, pancreatic endocrine cells, and IECs. Nevertheless, despite growing evidence demonstrating that chemicals could facilitate the TF-mediated induction of human endoderm lineage cells from fibroblasts or other distant lineages, such as hepatocytes[22,38] and pancreatic cells[39], there was seldom achievement mediated by a full chemical strategy. Perhaps, gastrointestinal epithelial cells are more amenable for CiEPCs induction than fibroblasts because of their proximity in lineage distance. Regarding the difference in transcriptome and pathway profiles between humans and mice, further chemical cocktail screening is warranted to attain the direct endoderm conversion from other germ layers.

## DIRECT LINEAGE EXPANSION IN HEPATOCYTES

Although direct lineage conversion from fibroblasts or other somatic cells has established predominant advantages for regenerative medicine[41,43], the relative deficient functional maturation and engraftment *in vivo* remain a major obstacle[57]. For decades, human hepatic cell source is in high demand for liver disease treatment because of the shortage of available liver organs[58,59]. The generation of a large number of functional and transplantable hepatic cells merits considerable clinical significance and has garnered substantial attention. In recent years, TF-mediated direct reprogramming of human-induced hepatocytes (hiHeps) has garnered more attention, overwhelming the conventional iPS-derived HLCs, in terms of markedly reduced risk of tumorigenesis. Despite the progressive enhancements in efficiency and purity of hiHeps, the extremely low *in vivo* repopulation capability, as well as deficient functions regarding metabolism, markedly hampered their transplantation applications[60,61]. Most recently, the successive achievements in the chemical induction of primary hepatocytes highlight the acquisition of highly expandable characteristics (Table 1), which could markedly promote the development of hepatic cell-based liver disease therapies.

### Lineage-committed cells and progenitors

To break the blockage for expansion, a simple and effective combination of three core chemicals YAC (Y-27632, A83-01, and CHIR99021) was first reported in 2017, enabling the conversion of terminally differentiated rat and mouse hepatocytes to highly expandable liver progenitor-like state, with bi-potential differentiation capacity toward mature hepatocytes and biliary epithelial cells[62]. Later, using the same core chemical combination, the induction of progenitor-like state was established further by another group with *in vivo* repopulation and maturation capability[63].

However, the induction effect of YAC was only restricted to hepatocytes originated from rodents, until the discovery of HGF, which was highlighted to be essential for establishing a human hepatic progenitor-like state through the ERK-1/2 signaling activation[64]. Remarkably, during the induction of human hepatocytes by modified cocktail HAC (HGF, A83-01, and CHIR99021), not only were hepatic progenitor markers markedly elevated but also endoderm and pluripotency markers were detected[64], suggesting the potential acquisition of multilineage differentiation capacity other than the hepatic fate. Additionally, nicotinamide, commonly used for hepatocyte culture[65,66], inhibited the proliferation and even induced apoptosis through the inactivation of SIRT1, offering a clue for long-term culture optimization. Of note, under the three-dimensional differentiation condition, the expanded progenitor-like cells could regain the expression of hepatitis B virus (HBV) receptor sodium taurocholate cotransporting polypeptide, which could markedly support the HBV infection or reactivation modeling[67].

Besides the markedly elevated expression of progenitor-associated markers, the HAC-based induction approach resulted in the suppressed expression of most mature hepatocyte markers[64,67]. However, when one of the core chemicals, CHIR99021, was replaced with Wnt3a, a unique proliferative state was established, which partly retained mature hepatocyte characteristics while exhibiting progenitor-associated identity[68]. Besides the GSK3-mediated Wnt pathway activation, special insight was provided into the crosstalk between Wnt3a and specific cell signaling regarding hepatic progenitor self-renew and proliferation such as the Hippo–YAP pathway[69]. Moreover, it implied that the CHIR99021-mediated pathway modulation could largely erase the initial memory of hepatocyte identity, while the Wnt3a-mediated modulation partially sustained these signatures. Remarkably, when induced cells were transplanted *in vivo* for over 4 mo, the human albumin secretion and repopulation efficiency could attain a comparable level to primary human hepatocytes, suggesting that these induced cells could serve as the most compatible hepatic cell source that had ever been reported beyond primary hepatocytes.

### Organoid expansion

Using a highly similar chemical and cytokine cocktail, both human and mouse primary hepatocytes have been established to form organoids (Hep-Orgs) in a 3D culture system and stably expand for a long term[70]. Hep-Orgs comprise noncycling mature hepatocytes and cycling hepatocyte progenitor cells, exhibiting comparable gene expression profiles and functions to primary hepatocytes. Remarkably, progenitor markers were not markedly elevated in Hep-Orgs, which was considerably different from that in conventional 2D culture[68]. Despite applying a different Wnt agonist, R-spondin, instead of Wnt3a, the major discrepancy could be contributed by the special circumstance in the organoid culture system, which seemingly recapitulated the regeneration microenvironment of the adult liver *in vivo*. In Hep-Orgs, cell cycle and ribosome synthesis–related gene expression was markedly enhanced, resembling the response of hepatocytes to acute liver damage such as

**Table 1 Expandable hepatic cells induced from primary hepatocytes to date**

| Hepatocyte source(s) | Chemicals | Growth factor(s) | Medium and supplements | Expansion potential | | Yr |
|---|---|---|---|---|---|---|
| | | | | Passage count | Doubling time | |
| Mouse and Rat | A83-01, CHIR99021, Y-27632 | EGF | DMEM/F12, HEPES, L-proline, ITS, dexamethasone, nicotinamide, ascorbic acid-2 phosphate, BSA, antibiotic/ antimycotic | Rat: >10; Mouse: >20 | Rat: 14.7 ± 1.1 h | 2017[62] |
| Mouse | A83-01, CHIR99021, Y-27632 | EGF, HGF | DMEM/F12, N2 or ITS, S1P, LPA | >30 | 15-20 h | 2017[63] |
| Human (resected patient liver tissue, non-lesion) | A83-01, CHIR99021 | EGF, HGF | DMEM/F-12 (high glucose), FBS, nicotinamide, dexamethasone, ITS, penicillin/ streptomycin | >10 | 37.9-39.8 h | 2018[64] |
| Human (resected patient liver tissue, non-lesion) | A83-01, CHIR99021, Y-27632 | EGF, HGF | Advanced DMEM/F-12, N2, B27, sodium pyruvate, ascorbic acid, S1P, LPA | >10 | 24.7 ± 1.4 h | 2018[67] |
| Human (normal, cryopreserved) | A83-01, Y-27632 | EGF, FGF10, HGF, Wnt3a | Advanced DMEM/F-12, FBS, N2, B27 (minus vitamin A), N-acetylcysteine, nicotinamide, [Leu15]-gastrin I, penicillin/ streptomycin, | 4 (normoxia); 8 (hypoxia) | | 2018[68] |
| Human (normal, freshly isolated and cryopreserved) | A83-01, CHIR99021, Y-27632 | EGF, FGF7, FGF10, HGF, TGFa | Advanced DMEM/F-12, HEPES, B27 (minus vitamin A), R-spodin1 conditioned medium, N-acetylcysteine, nicotinamide, gastrin, GlutaMAX, penicillin/ streptomycin, | Fetal hepatocytes: >16 | 5-7 d | 2018[70] |

partial hepatectomy.

### *Limitations*

Notably, human hepatocyte–induced proliferative cell sources displayed large scale expandable potential, as well as superior compatibility and functional maturation, for transplantation compared with iPSCs-derived HLCs or hiHeps, exhibiting a remarkably higher repopulation rate and human albumin secretion[61,71]. As the maturation direction of human hepatocyte-induced proliferative cell sources is lineage-committed, they did not necessarily require an extra differentiation process, severing as ready-to-use transplantable sources, and might merit broader prospects for breaking through the obstacle for liver disease treatment. Nevertheless, currently, the expansion efficiency of human hepatocytes is remarkably lower than that of rodent hepatocytes. Compared with rodent hepatocytes, human hepatocytes required approximately a 10 h longer period for cell doubling, whereas the maximum passage counts for stable expansion declined approximately 10 times[63,67]. Regarding the proliferation kinetics, rodent hepatocytes essentially required three to five passages for establishing an accelerated proliferative state, while human hepatocytes exhibited a gradual loss of proliferation potential after three to four passages, implying that the building of a proliferative intermediate state for human hepatocytes might be relatively insufficient or unstable. Although a hypoxic expansion condition possibly inhibited the senescence and prolonged proliferation[68], an understanding of the different transcriptome and pathway network profiles between humans and mice is required to reveal specific signaling for human hepatocyte expansion.

Telomere shortening during prolonged expansion is another limiting factor[70]. The unlimited self-renewal and genomic stability of pluripotent stem cells were facilitated by sufficiently long telomeres[72]. During pluripotent reprogramming, which was either TF- or chemical-mediated, short telomeres of somatic cells were significantly elongated in a process mediated by telomerase activity upregulation or by recombination-based ALT mechanisms[31,73]. Moreover, reprogramming triggers telomere elongation regardless of donor age status[74,75]. Thus, telomere erosion, characterized in aged and senescent cells, can be efficiently rejuvenated after pluripotent reprogramming. However, direct lineage conversion and expansion from somatic cells bypass the pluripotent intermediate status, thereby preserving age-associated features. Compared with the iPSC-derived counterpart, extensive DNA damage, loss of heterochromatin and nuclear organization, and increased SA-β-Gal activity were observed in lineage-converted functional cells[76]. Therefore, even though the conversion is successfully completed, the converted cells seem to inherit aging signatures from parental cells, including short telomeres, and be prone to senescence after passages. During chemical-mediated expansion of primary human hepatocytes, the inherently short telomeres and insufficient telomerase activation may play a key role in eliciting senescence of induced proliferative cells after limited passages[68], which remains a major obstacle for large-scale expansion *in vitro*. In contrast, we found that when human iPSC-derived HLCs are chemically induced for expansion, they exhibit a superior expansion advantage compared with primary hepatocytes. Induced cells can stably expand for extended passages while sustaining hepatic differentiation potency (unpublished data). The relatively high telomerase activity inherited from iPSCs potentially restores or elongates telomeres, facilitating long-term expansion. The ability to use chemicals to activate telomerase and elongate telomeres in human terminally differentiated cells such as hepatocytes would be of great importance for an extended expansion capability.

Moreover, the advantage of the organoid culture system has been proved to be supportive for the long-term expansion with enhanced functional maturation; however, the current expansion efficiency was much lower than the 2D system, and the culture condition optimization, such as the extracellular matrix, warrants further investigation.

Despite the achievements in hepatocytes, direct expansion of other lineage cells, such as neurons and cardiomyocytes, has been seldom reported. Undoubtedly, the direct conversion from fibroblasts has already proved a feasible option for generating these functional cells. It is expected that when the direct lineage expansion is applied, the *in vivo* repopulation and functional maturation could be enhanced further.

## CHALLENGES

The chemical-mediated direct lineage conversion and expansion, bypassing potential risks using transgenic methods, as well as pluripotency-related tumorigenesis, offer promising options for therapeutic purpose. However, several challenges remain regarding the unrevealed mechanism of chemical effects.

### *Cell fate specificity*

To date, several dozens of chemicals have been identified in various combinations for cell induction[11,16,18,77]. Although the precise mechanism underlying a particular conversion between different cell fates remains unclear, chemical inducers could be classified into two major groups—epigenetic modulators and signaling regulators. Epigenetic modulators, typically HDAC inhibitors and DNA/histone methyltransferase inhibitors, such as VPA and BIX01294, are commonly used for the induction of CiPSCs, CiNSCs, and CiCMs[27,41,53]. Conversely, signaling regulators specify the characteristics of the designated cell identity. Reportedly, ISX9 and AS8351 were specific for the CiNs induction[44,78]. Additionally, SC1 was specific for the CiCMs induction[51,53]. The 2i combination (CHIR99021 and PD0325901) was always required for the late stage of the CiPSCs induction[24,25]. However, RepSox, A83-01, CHIR99021, and Forskolin were extensively used as essential factors for inducing various cell types, including CiPSCs, CiNPCs, CiNs, CiCMs, and CiEPCs[24,43,53,55,78]. The nonspecific feature of a significant portion of the chemicals could be attributed to the fact that same signaling pathways are often shared by the multilineage development such as the Wnt and TGF-β pathways[79]; it may not only extend the induction application for a broad range of cell types but also possibly lead to undesired cell fates. Using the same chemical cocktail for the CiPSCs induction, the generation of an unexpected cardiac fate was uncovered[50]. The possible explanation was that the chemicals might induce a nonspecific multipotential intermediate state, which was highly plastic and

unstable; or a heterogeneous mixture of multiple progenitor cell types. Under favorable conditions, the induction direction could incline further to cardiac or pluripotent fate.

To exclude the unanticipated induction fate, it would be challenging to elucidate further the individual and synergistic effect of chemical cocktails regarding cell signaling and epigenetic regulation, as well as the specificity and commonality for distinct lineages (Table 2), and then develop stepwise reliable chemical cocktails that could precisely direct the desired cell types.

### Cell identity memory

Each cell type has its unique transcriptome, which defines its cell identity. Reprogrammed cells more or less inherit the cell identity memory of their ancestor. An analysis of molecular traces during the induction of iPSC-HLCs and hiHeps identified original fibroblast identity efficiently but not completely erased in both cell types[61]. Additionally, iPSC-HLCs exhibited the expression of endoderm progenitor (*FOXA2* and *GATA6*) and hepatoblast (*AFP* and *EPCAM*) markers, suggesting that the memory of molecular traces during the differentiation path was also sustained in iPSC-HLCs; however, the hiHeps induction bypasses these cell identities transition, exhibiting no expression of these markers. Overall, the sustained memory during reprogramming and differentiation process could elucidate the fact that both c and hiHeps exhibited distinct characteristics from primary hepatocytes in gene expression and related functions[80-82].

Additionally, chemical-mediated reprogramming was also challenged by the initial cell identity modulation. The investigation of the direct induction of neurons identified a bromodomain inhibitor, IBET151, as a core compound needed to erase the initial fibroblast transcriptional network program for cell identity rewriting[78]; the mechanism could be concerned with the blocking effect that IBET151 disrupted the accessibility of bromodomain proteins to acetylated histones related to fibroblast programs, resulting in transcriptional silencing[83]. Additionally, the bromodomain and extra-terminal domain inhibitors remarkably enhanced iPSC and NPC induction *via* switching off a large set of initial somatic transcriptional programs[84]. Besides, platelet-derived growth factor receptor (PDGFR) inhibitors, SU16F and JNJ10198409, reportedly accelerated the downregulation of fibroblast genes during the conversion of human cardiomyocytes, markedly enhancing the efficiency[53]. Perhaps, further investigation of chemicals with the erase effect on specific cell types would booster the efficiency of the cell fate conversion.

Conversely, the initial somatic transcriptional memory could also facilitate the transplantation. In a study, when direct lineage expansion was applied in primary hepatocytes, their partly sustained hepatocyte transcriptional memory could facilitate quick engraftment and functional maturation after transplantation *in vivo*[68]. Of note, based on our study, when cells were chemically induced form iPSC-HLCs, they exhibited relatively low *in vivo* repopulation capability, which corroborated that before induction (unpublished data). The possible explanation was that the induced cells inherited portions of transcriptional memory of iPSC-HLCs, including the deficiency in repopulation capability. This discrepancy not only highlights the hurdles for transplantation of iPSC-derived functional cells but also emphasizes the elucidation of the cell memory dynamics during induction.

### In vivo induction

Conventionally, long-term *in vitro* expansion is critical to obtain sufficient functional cells for transplantation, whereas the potential risk of functional alteration and genetic mutations from the *in vitro* microenvironment raises serious problems[57,85]. Theoretically, *in vivo* reprogramming could produce functional cells followed by inducing resident functional cells with bi-potency or proliferation capability, or directly convert the neighboring cell types proximal in lineage distance and, meanwhile, take advantage of the *in situ* niche to regenerate the damaged tissue or organ efficiently, which remains a major obstacle for *in vitro* circumstances.

In recent years, substantial TF-mediated *in vivo* reprogramming has demonstrated some exciting achievements, including neuroblasts or neurons induced from glial cells[86,87], cardiomyocytes induced from cardiac fibroblasts[88-90], hepatocytes induced from myofibroblasts[91,92], and pancreatic β-like cells induced from exocrine cells[9,93]. Despite great promise for diseases treatment as demonstrated by previous studies, the TF-mediated strategy also poses risks of genome integration, tumorigenesis, as well as manufacturing and delivery problems. Comparatively, chemical-mediated strategy minimized the risk of genetic alteration and is easier for scalable manufacturing, stocking, and delivery[23], eventually preferable for *in vivo* therapeutic applications. Recently, for the first time, *in vivo* chemical reprogramming was reported to successfully convert the resident astrocytes into functional neurons in the adult mouse

**Table 2 The specificity and commonality of chemicals in direct lineage conversion**

| Compounds | Function | Target germ layer and cell types | | | Ref. |
|---|---|---|---|---|---|
| | | Ectoderm: Neural stem/ progenitor cell, neuron | Mesoderm: Cardiomyocyte | Endoderm/ extraembryonic endoderm: Endoderm progenitor cell, XENs | |
| A83-01 | TGF-βRI (ALK4/5/7) inhibitor | + | + | | [42,43,51,53] |
| AM580 | RAR agonist | | | + | [25,54] |
| AS8351 | KDM5B inhibitor | | + | | [53] |
| Bay K 8644 | $Ca^{2+}$ channel activator | | + | | [51] |
| BIX01294 | Histone methyltransferase inhibitor | + | + | | [42,53] |
| CHIR99021 | GSK3 inhibitor | + | + | + | [25,41-46,50,51,53-55,78] |
| DMH1 | BMP inhibitor | + | | | [46] |
| EPZ004777 | DOT1L inhibitor | | | + | [25,54] |
| Forskolin | cAMP activator | + | + | + | [25,44-46,50,51,54,55,78] |
| GO6983 | PKC inhibitor | + | | | [44] |
| Hh-Ag1.5 | Smo agonist | + | | | [43] |
| I-BET151 | BET bromodomain inhibitor | + | | | [78] |
| ISX9 | Neurogenic agent | + | | | [78] |
| JNJ10198409 | PDGF-RTK inhibitor | | + | | [53] |
| LDN193189 | BMP type I receptor (ALK2/3) inhibitor | + | | | [43,45] |
| OAC2 | Oct4 activator | | + | | [53] |
| Parnate (Tranylcypromine) | LSD1/MAO inhibitor | + | + | + | [25,43,50,54] |
| PD0325901 | MEK inhibitor | + | | | [42,45] |
| RepSox (616452) | TGF-βRI (ALK5) inhibitor | + | + | + | [25,41,44,46,50,54,55] |
| RG108 | DNA methyltransferase inhibitor | + | | | [42,43] |
| SB431542 | TGF-βRI (ALK4/7) inhibitor | + | | | [45,78] |
| SC1 | ERK1/RasGAP inhibitor | | + | | [51,53] |
| SMER28 | Autophagy modulator | + | | | [43] |
| SP600125 | JNK inhibitor | + | | | [44,46] |
| SU16F | PDGFRβ inhibitor | | + | | [53] |
| TTNPB | RAR agonist | | + | + | [50,55] |
| VPA | Histone deacetylase inhibitor | + | + | + | [25,41,42,44,46,50,54] |
| Y27632 | ROCK inhibitor | + | + | + | [44,46,53-55] |

XENs: Extraembryonic endoderm-like cells.

brain, resembling endogenous neurons in both neuron-specific marker expression and electrophysiological properties[49]. Meanwhile, cardiomyocytes were induced from adult cardiac fibroblasts by direct full chemical administration *in vivo*, and although the reprogramming efficiency is relatively low, the chemical cocktail treatment markedly decreased the scar formation and enhanced cardiac functions in myocardial infarction mice[52]; these encouraging achievements not only provide a general strategy for *in vivo* reprogramming but also open a novel path to regenerate the diseased organs *in situ*.

However, the off-target or unspecific-target effects of chemicals pose challenges for the *in vivo* induction, as the chemical treatment could target undesired cell types *in situ* or nearby, potentially declining the intrinsic homeostasis of local cell populations. Moreover, exogenous chemicals could also break the homeostasis of local niche and

cause unintended tissue damage. Thus, it will be essential to evaluate the side effects *in vivo* in the long term, and the chemical combinations and concentration should be optimized to adapt physiological homeostasis synergistically. Finally, despite the high permeability advantage of chemicals in tissue, the arrival to unwanted location should be avoided. Advances in the delivery system are warranted to deliver chemicals to designated locations for an expected period precisely. Overall, enhanced targeting and shielding capabilities will be indispensable for attaining effective reprogramming *in vivo*.

## PERSPECTIVES

Generating substantial functional cells with tissue/organ regeneration capability is a major challenge for regenerative medicine. Despite the rapid progress of the conventional strategy regarding the differentiation from pluripotent stem cells[94,95], the direct lineage conversion, bypassing the pluripotent stage, has highlighted a promising alternative strategy in recent years[57]. Additionally, chemical-mediated strategy, targeting the modulation of epigenetic status and signaling pathways without interfering the genome integration, exhibited the unique advantage over transgenic and other approaches for cell reprogramming, especially regarding the expansion potential[23,77]. Remarkably, chemically induced cells, including XEN-like state and lineage-specific stem/progenitors (CiNSCs, CiNPCs, and CiEPCs), exhibited highly expandable characteristics, which could markedly satisfy the predominant requirement for clinical use. Additionally, recently reported direct lineage expansion holds great promise for cell transplantation applications. As these induced cells not only exhibited the robust proliferation capability but also partly sustained the initial cell identity memory, it facilitated direct and rapid revert to mature state after *in vitro* differentiation or been transplanted *in vivo*. However, it remains a major obstacle for direct lineage conversion from fibroblasts or other somatic cells, which exhibited a significant deficiency in functional maturation. Although the achievement to date in direct lineage expansion of human functional cells remains highly limited, the success in human hepatocytes would undoubtedly offer a general idea for extending this strategy to other desired cell types. Moreover, the limited expansion capability of induced human hepatocytes also evokes the importance of exploring chemical candidates to activate telomerase and rejuvenate telomeres, which may potentially extend expansion of induced cells *in vitro* (Table 3).

Holding an excellent promise for direct lineage conversion and expansion, spatiotemporal flexibility and nonintegrative characteristics of chemical strategy could also be favorable for the *in vivo* induction approach, offering superior advantages for therapeutic potential[94]. As safety and efficiency might be the rate-limiting step for *in vivo* reprogramming[96], assistant technology, such as nano-delivery system, might be required to deliver chemicals to specifically targeted sites in a controlled manner.

Along with the advances of chemical screening, discrete combinations of pathway-specific chemicals have progressively been identified to reprogram somatic cells into many lineages. Nevertheless, improved knowledge of the pathway networks, together with the epigenetic pattern that drives the cell fate conversion and proliferation, is warranted to intelligently enhance the induction efficiency and specificity *in vitro* and *in vivo*.

**Table 3 Advantages and disadvantages of different strategies for functional cell induction**

| Strategy | Induction efficiency | Reproducibi-lity/stability | Target specificity | Cellular function | *In vivo* engraftment | Safety | Scaling up | Cost | Ref. |
|---|---|---|---|---|---|---|---|---|---|
| TF-mediated pluripotent reprogramming followed by differentiation | Moderate | Highly reproducible/stable | High | Immature | Low | Genomic integration; tumorigenesis risk | Expandable before differentiation | Very high | [11,97-99] |
| TF-mediated direct lineage conversion | Fast and efficient | Reproducible/stable | High | Deficient | Low | Genomic integration | Expandable in progenitors | High | [100-103] |
| Chemical-mediated pluripotent reprogramming followed by differentiation | Controversial | Poorly reproducible/unstable | Low | Not clear | Not clear | Integration-free | Not clear | Low | [24,25,29,31] |
| Chemical-mediated direct lineage conversion | Low | Reproducible / unstable | Low | Deficient | Low | Integration-free | Expandable in progenitors | Very low | [18,77,104,105] |
| Chemical-mediated direct lineage expansion | Fast and efficient | Reproducible / unstable | Low | Close to primary | High | Integration free | Expandable in rodents/ Limited in humans | Very low | [62,64,67,68] |

TF: Transcriptional factor; XENs: Extraembryonic endoderm-like cells.

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*REVIEW*

# Induction of differentiation of human stem cells *ex vivo*: Toward large-scale platelet production

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## Abstract

Platelet transfusion is one of the most reliable strategies to cure patients suffering from thrombocytopenia or platelet dysfunction. With the increasing demand for transfusion, however, there is an undersupply of donors to provide the platelet source. Thus, scientists have sought to design methods for deriving clinical-scale platelets *ex vivo*. Although there has been considerable success *ex vivo* in the generation of transformative platelets produced by human stem cells (SCs), the platelet yields achieved using these strategies have not been adequate for clinical application. In this review, we provide an overview of the developmental process of megakaryocytes and the production of platelets *in vivo* and *ex vivo*, recapitulate the key advances in the production of SC-derived platelets using several SC sources, and discuss some strategies that apply three-dimensional bioreactor devices and biochemical factors synergistically to improve the generation of large-scale platelets for use in future biomedical and clinical settings.

**Key words:** Megakaryopoiesis; Platelet production; Transfusion; Bioreactors; Stem cells



**Core tip:** Platelets derived from voluntary blood donation pose some challenges, such as susceptibility to pathogen contamination, short preservation time and difficulty in satisfying the increasing number of patients requiring platelet transfusion. Thus, seeking a safe and reliable alternative source of platelets is an effective solution.

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## INTRODUCTION

Platelets are the smallest anucleate cells (2-4 μm in diameter) produced by megakaryo-cytes (MKs) in bone marrow (BM). They play a pivotal role not only in hemostasis and thrombosis but also in neoangiogenesis, innate immunity and inflammation. Until now, platelet transfusion, which may lead to varying degrees of hemorrhage that is sometimes life-threatening, has remained the most effective way to treat patients suffering from thrombocytopenia and/or platelet dysfunction[1,2]. However, platelets derived from voluntary blood donation pose some challenges, including susceptibility to pathogen contamination, short preservation time, and difficulty satisfying the increasing number of patients requiring platelet transfusion[3]. Therefore, seeking a safe and reliable alternative source of platelets is an effective solution.

In recent years, stem cells (SCs) research has developed rapidly in the field of regenerative medicine, and several research groups have focused on cultured platelet production *ex vivo*. In addition to hematopoietic SCs (HSCs), human embryonic SCs (hESCs) and human induced pluripotent SCs (iPSCs) have been considered potential sources for generating human MKs[4-6] and platelets[7,8]. The proper use of biochemical stimuli, including growth factors and cytokines, could augment MK maturation and platelet generation during *ex vivo* stem cell culturing[9,10]. To mimic the physical microenvironment, some bioengineering techniques that promote MK maturation and platelet release have been developed[11,12]. In this review, we will recapitulate the methods that promise to produce sufficient platelets differentiated from SCs *ex vivo* and some obstacles in this field. We believe that the *ex vivo* production of platelets is a state-of-the-art technology integrated with the development of unlimited SCs, newly discovered biochemical reagents and three-dimensional (3D) bioreactor systems.

## MEGAKARYOPOIESIS AND PLATELET FORMATION

Megakaryopoiesis and thrombopoiesis are elaborate processes that can be divided into several successive stages: The commitment of SCs toward the MK lineage, proliferation of MK progenitors, MK maturation, proplatelet formation and terminal platelet production. *In vivo*, HSCs located in the osteoblastic niche on the surface of the bone cavity have the ability for self-renewal and multipotent differentiation. They give rise to common lymphoid progenitors and common myeloid progenitors, which differentiate into granulocyte/macrophage progenitors and MKs-erythrocyte progenitors (MEPs). MEPs subsequently commit to the erythrocytic and megakaryo-cytic lineages, which progressively produce different ages of immature MKs with various proliferative potency. The unique feature of megakaryocytic maturation is endomitosis due to a failure in cytokinesis (not all MKs undergo mitosis). This process is accompanied by an increase in DNA content (up to 128 N) and the following events: Cell enlargement; the emergence of various unique organelles, such as dense bodies and secretory particles; the synthesis and assembly of cytoplasmic proteins; the formation of the demarcation membrane system; and the formation of a membrane reservoir for proplatelets. Upon maturation, MKs migrate to the vascular niche and extend cytoplasmic projections (proplatelets) into the sinusoidal endothelium after cytoskeletal remodeling. Under shear forces produced by the blood stream, proplatelets are released, gradually develop into mature platelets and undergo reassembly and the displacement of microtubules[13-15] (Figure 1).

*In vitro*, SCs (HSCs, hESCs or hiPSCs) are cultured to increase progenitor cell number before differentiation into MKs. After expansion, SCs differentiate into the megakaryocytic lineage as a result of stimulation with growth factors [*e.g.*, thrombopoietin (THPO) and stem cell factor (SCF)] and MK progenitor expansion with a recombinant human THPO mimetic (AMG531)[16]. Immature MKs then undergo the polyploidization process by inducing endomitosis to increase the number of DNA content to 16-128 N. The myosin inhibitor nicotinamide, actin inhibitors and Src inhibitor could be used for MK polyploidization. However, the capacity of MK to undergo polyploidization in culture is significantly lower than in the BM micro-

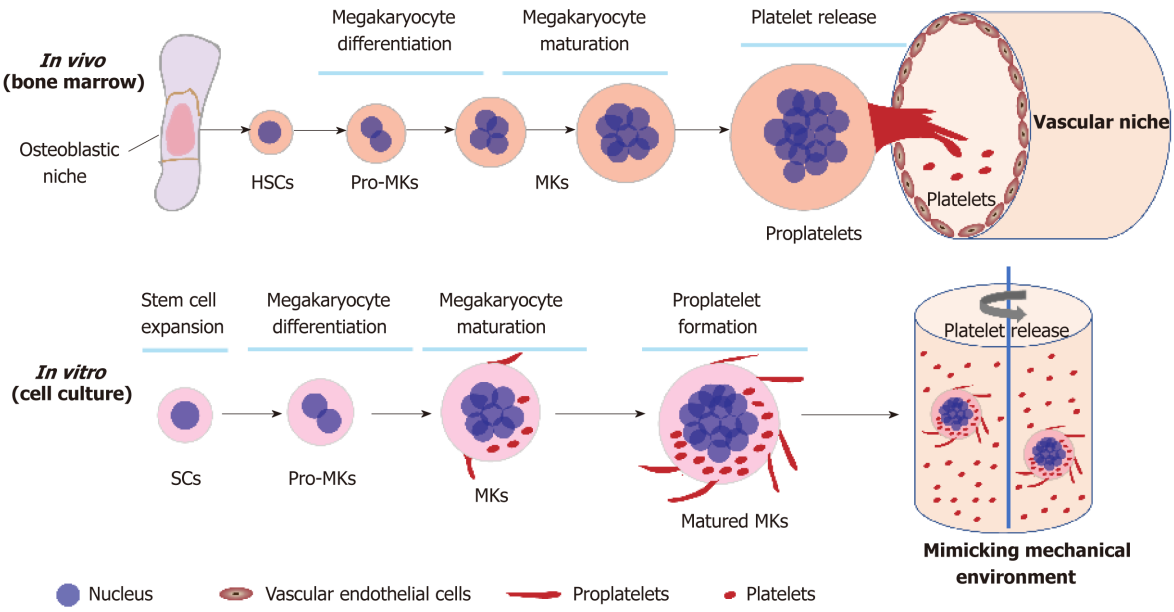


**Figure 1 Overview of megakaryopoiesis, proplatelet formation and platelet release.** *In vivo*, bone marrow is located within the trabecular bones, where the hematopoietic stem cell (HSC) undergo the process of megakaryocyte (MK) differentiation and MK maturation under the control of thrombopoietin. During maturation, MKs migrate to bone marrow sinusoids and form proplatelets. Proplatelets elongate through the vascular endothelium into the vessels, and proplatelet terminal ends are released into the bloodstream by blood shear forces, forming platelets. *In vitro*, HSC-derived $CD34^+$ cells or pluripotent stem cell are first expanded *in vitro* and then differentiate into the megakaryocytic lineage. Once differentiated, immature MKs undergo the polyploidization process *via* inducing endomitosis to increase the number of DNA content to 16-128 N. During the process of polyploidization, some extended proplatelets are formed and released in high ploidy MKs in culture. To increase the efficiency of platelets, MKs extend proplatelets into the bioreactor, with perfused culture medium mimicking blood flow.

environment, with a maximum ploidy of 126 N in hESC-derived MKs and 16 N in iPSC-derived cells[17,18]. During the process of polyploidization, some proplatelets are formed and released in high ploidy MKs in culture. There is a connection between apoptosis and the process of proplatelet formation because the intrinsic apoptosis pathway is necessary for MKs to adequately acquire proplatelets and release platelets[19,20]. To promote the maturation of MKs and accelerate the release of platelets, scientists have altered the culture conditions, including by treating the cells with chemical substances and mimicking the physical microenvironment (Figure 1).

## STEM CELL SOURCES FOR PLATELET PRODUCTION EX VIVO

Pluripotent SCs and hematopoietic progenitors are the main sources that have been used to generate MKs and functional platelets. The former, including hESCs and hiPSCs, is considered an unlimited seeding cell source. The latter involves $CD34^+$ (a surface marker usually expressed by hematopoietic stem/progenitor cells) cells from BM, umbilical cord blood (UCB) and peripheral blood (PB). Because they are primary cells, there is a risk of viral contamination. In total, each of these sources has advantages and disadvantages for clinical platelet transfusion.

### *HSCs*

$CD34^+$ cells extracted from BM, PB and UCB are simple and feasible sources for platelet production *ex vivo*. In 1995, Choi *et al*[21] isolated $CD34^+$ cells from human PB and cocultured them with serum taken from dogs suffering from aplastic anemia. Upon the generation of MKs, human AB serum (AB-HS) was added to the culture system to promote the production of platelet-like particles. When adenosine diphosphate (ADP) was added, aggregation could be observed. This is the first report of human functional platelet production *in vitro*. Since then, the application of THPO has accelerated the development of research on platelet generation *ex vivo*. The scientists found that the addition of different cytokines in the culture medium, except for thrombopoietin, could improve megakaryocytic maturation and platelet production. To some extent, the ultrastructure and physiological functions of derived platelets were similar to those of platelets *in vivo*. However, because of the inconvenience of obtaining BM samples and because the content of $CD34^+$ cells is low in the medullary cavity, the ability to induce $CD34^+$ cells from BM is greatly

restricted[22-25]. In comparison, cord blood has been widely used because of its extensive sources, convenient collection and high amplification efficiency *ex vivo*. Matsunaga *et al*[22] reported that when CB CD34$^+$ cells were cocultured with hTERT stromal cells and interleukin-11 (IL-11), SCF, thrombopoietin and flt-3 ligand (FL) were included in the system, large-scale generation of human platelets could be attained. Using the UCB differentiation system, scientists have investigated various factors that may influence megakaryopoiesis and thrombopoiesis. Employing UCBs depends on donation after childbirth, which makes it more difficult to acquire abundant materials to scale platelet production and increases the risks of bacterial/viral contamination[26].

### hESCs

Since the establishment of the hES cell line for the first time by Thomson *et al*[27] in 1998, scientists have successively attempted to induce hESCs into MKs and platelets. In 2001, Kaufman *et al*[28] cocultured the hES cell line H1 with S17 mouse BM stromal cells to produce CD34$^+$ hematopoietic precursor cells, which can give rise to mixed cell clones containing MKs. This was the first report on the differentiation of hESCs into MKs. Then, Gaur *et al*[4] reported that hESCs could be differentiated into MKs by culturing them with OP-9 stromal cells. After two weeks, CD41a$^+$CD42b$^+$ (surface marker expressed by MKs) cells were detected in the differentiation system. However, these MKs did not exceed a ploidy of 32 N, and proplatelet formation was only occasionally observed. These results suggested that the MKs were immature and that platelets were not produced in the culture system. In 2008, successful generation of platelets was addressed in a study by Takayama's group[29]. Through coculture with OP9 or C3H10T1/2 stromal cells and the use of vascular endothelial growth factor (VEGF), hESCs produced many "sac" structures that could provide a suitable microenvironment for hematopoietic progenitor cells. Thrombopoietin, SCF and heparin were then added to the system. After approximately 10 days, platelets that could be activated by ADP or thrombin were produced in the supernatant. Therefore, the efficiency of platelet production in MKs is far less than that under physiological conditions.

In 2011, Lu *et al*[7] improved the differentiation efficiency of MKs from hESCs by 20-30 times. The breakthrough of the experiment was that the adhesion and aggregation functions of platelets produced *ex vivo* was proven to some extent. More importantly, the cultured platelets were observed to participate in the formation of thrombosis with platelets *in vivo* at the site of damage in arterial blood vessels. However, the application of stromal cells and serum in previous studies reduced the possibility of use in therapies significantly. Subsequently, Pick *et al*[30] committed hESCs to MKs that fragmented into platelet-like particles using a "spin embryoid body" method in serum-free differentiation medium. However, this method also has disadvantages, namely system instability and limitations to the large-scale production of MKs.

### hiPSCs

Compared with hESCs, hiPSCs have several advantages, including the ability to utilize any somatic cell that could develop into MKs and the absence of ethical restrictions. In 2010, Takayama *et al*[18] first reported that when cultured iPSCs derived from human epidermal fibroblasts with a mouse C3H10T1/2 stromal cell line for 22-26 d, platelets could be released from MKs. It is noteworthy that the platelets were observed to form thrombi at the site of damaged vessels in combination with platelets *in vivo*. In addition, hiPSCs from subcutaneous adipose tissues[31], endometrial stromal SCs[32] and adipose-derived mesenchymal stromal/stem cell line[33] could differentiate into functional platelets.

In addition to the above-mentioned stromal cell coculture methods and traditional "embryoid body" differentiation methods, some strategies of altering cell fate by transcription factors have also made progress. Ono *et al*[34] reported that over-expression of the *p45NF-E2*, *Maf* G and *Maf K* genes could convert human and mouse 3T3 fibroblasts into CD41$^+$ MKs, which can produce platelet-like particles with partial coagulation function. Although the method requires much time and the efficiency is low, it showed that exogenous gene manipulation could directly transform other cell types into MKs. The key was to find the appropriate genes. In 2014, Nakamura *et al*[8] showed that overexpression of the *BMI1*, *bcl-xl* and *c-myc* genes in hiPSCs derived from PB cells could generate stable and immortalized megakaryo-cytic progenitor cell lines (imMKCLs). Once the expression of the three genes was stopped, the imMKCLs gave rise to platelets. Similarly, ectopic expression of the three transcription factors GATA1, FLI1, and TAL1 in hiPSCs achieved the same goal, leading to mature MK production with unprecedented efficiency[6]. Both cell lines could be cryopreserved and expanded upon recovery. Compared to MKs derived from iPSCs, the prominent feature of imMKCLs is the generation of platelets with higher efficiency in less time.

Although some characteristics of the imMKCLs deserve recommendation, the cell lines still face some drawbacks, such as relying on serum and feeder cell culture and virus-mediated genetic reprogramming, which may pose potent risks. Recently, important progress has been made by Liu *et al*[35], who developed an efficient system to generate MKs from hiPSCs under feeder-free and xeno-free conditions by adding FDA-approved pharmacological reagents, including romiplostim (Nplate, a THPO analog), oprelvekin (recombinant IL-11), and plas-bumin (human albumin). However, platelet production from iPSCs requires relatively complex and sophisticated methods, and the culture period is long. These results may impede the progress of the industrial-scale generation of cultured platelets and therapeutic applications in regenerative medicine.

## REGULATION FACTORS FOR MEGAKARYOCYTIC MATURATION AND PLATELET RELEASE

Megakaryopoiesis and thrombopoiesis are regulated by various effectors in the BM microenvironment, such as stomal cells, cytokines, extracellular matrix and blood flow. Based on theoretical knowledge, researchers have altered the culture conditions, including chemical substances, and the physical microenvironment, which may promote the differentiation and maturation of MKs and accelerate the release of platelets[14,36,37].

### *Megakaryocytic maturation*

It is generally recognized that the ploidy of MKs is positively correlated with the number of platelets produced. Studies have shown that MKs differentiated from CB, hESCs or hiPSCs have a far lower ploidy level than MKs generated from BM HSCs[38,39], and the reason has not been fully elucidated. Insights into the signaling pathways associated with megakaryocytic polyploidy may contribute to our understanding and result in the production of more platelets *ex vivo* through gene manipulation[40,41].

The Rho/ROCK pathway is a well-known regulator of the actin cytoskeleton. Chang *et al*[42] provided evidence that the pathway acts as a negative regulator of proplatelet formation. Overexpression of a spontaneously active RhoA or dominant-negative mutation led to an increase or a decrease in proplatelet formation. The application of the Rho inhibitor Tat-C3 and the ROCK inhibitor Y27362 promoted the proportion of polyploidy MKs and the formation of proplatelets by decreasing myosin light chain 2 (MLC2) phosphorylation. Another study showed that ROCK inhibition drives polyploidization and proplatelet formation in MKs maturation through downregulation of *NFE2* and *MYC* expression[43].

The tumor suppressor protein P53 plays an important role in regulating the cell cycle and apoptosis[44]. P53 activation inhibits the induction of hematopoietic progenitors and of MKs[45]. Fuhrken *et al*[46] found that in the differentiation of the megakaryocytic cell line CHRF-288-11 induced by PMA, reducing the level of P53 improved the proportion of polyploid MKs. In addition, BM HSCs from P53-/- mice can generate 64 N MKs, while the maximum ploidy of wild-type mice is 32 N. Later, Giammona *et al*[17] showed niacinamide (NIC) can promote polyploidization of MKs by inhibition of SIRT1 and/or SIRT2, which belongs to the histone deacetylase Sirtuins protein family. The function of SIRT1 and SIRT2 is to deacetylate the downstream target protein P53. Therefore, the role of NIC in promoting polyploidization may be related to the enhanced transcriptional activity of P53 after acetylation.

Lannutti *et al*[47] found that Fyn and Lyn, members of the Src family of protein kinases, were highly expressed during the differentiation of BM $CD34^+/CD38^{lo}$ cells to MKs. Lyn-deficient mice produced more mature MKs above 8 N in the presence of the Src kinase inhibitor pyrrolopyrimidine 1. In addition, the Src kinase inhibitor SU6656 increased the proportion of polyploid MKs in the differentiation of cell lines UT-7, HEL, and Meg-01 and in patients with myeloid dysplasia syndrome[48,49].

In addition, some other important molecules, such as DIAPH1 and Gfi1b, also regulate major functions of MK proplatelet formation by controlling the dynamics of the actin and microtubule cytoskeletons[50,51].

### *Platelet release*

Currently, only a few MKs cultured *ex vivo* can release platelets. Therefore, it is essential to improve the ability of MKs to produce platelets *ex vivo*. Factors related to platelet release include the extracellular matrix, blood flow shear force, and MK apoptosis.

There has been evidence that the apoptotic inhibitory proteins Bcl-2 and Bcl-xl are

expressed in the early phase of megakaryopoiesis, but Bcl-2 is absent in neonatal and mature platelets. Moreover, the activity of caspase-3 and caspase-9 are increased in terminally differentiated MKs, suggesting that the maturation of MKs is accompanied by apoptosis[52]. However, Josefsson *et al*[20] found that the platelet numbers in *Bak* and *Bax* knockout mice were normal. It seems that platelet production is independent of intrinsic apoptotic pathway activation. In addition, White *et al*[53] reported that the number and function of platelets in Casp9-/- mice was not affected. Therefore, whether MK apoptosis affects platelet release remains to be investigated.

The extracellular matrix can modulate the production of proplatelets. Type I collagen in the osteoblast niche may inhibit the formation of proplatelets by α5β1α4β1. Fibroblast growth factor-4 (FGF-4) enhances the adhesion between MKs and vascular endothelium, contributing to the survival and maturation of MKs. By chemotaxis of stromal cell-derived factor 1 (SDF1), MKs migrate to the vascular niche and release platelets. In addition, von Willebrand factor (vWF) and fibrinogen play a role in promoting the generation of proplatelets through GPIb-IX-V and αIIbβ3 signaling pathways, respectively[54-57]. On the other hand, the podosome could adhere to extracellular matrix substrates and degrade it, which might play a role in proplatelet arm extension or penetration of the basement membrane[58].

Mechanic stress is an important factor in platelet release from mature MKs [59,60]. Junt *et al*[61] observed the process of MKs interplaying with blood vessels to produce platelets in real time by multiphoton intravital microscopy. In addition, confocal and electron microscopy after fixation were used. They found that MK exposure to high shear rates promotes platelet production *via* GPIb, which depends on microtubule elongation and assembly. The results provided a theoretical foundation for the application of bioreactors. Dunois *et al*[62] suggested that high shear rates from blood flow promote platelet production *via* GPIb, which depends on microtubule assembly and elongation. Recently, Ito *et al*[60] reported that turbulence activates platelet biogenesis and that turbulent flow promotes platelet release from hiPSC-derived MKs, suggesting turbulence as an important physical regulator in thrombopoiesis.

### *Platelet function*

For cultured platelets to be considered for clinical application, they must be very close to donor-derived platelets in terms of quantity and quality. To date, cultured platelets *ex vivo* have fragmented function compared with donors, although ultrastructure and surface markers are similar. The testing of platelet function has mostly relied on the measurement of P-selectin exposure and GPIIb-IIIa activation. In general, a large proportion of cultured platelets are expression of activation markers by agonists stimulating such as ADP or thrombin, while we often observed that a state of pre-activation with P-selection expression in cultured platelets even if it's in the absence of any agonist and thus show poor transfusion properties[63,64]. In addition, platelets generated *ex vivo* can participate in the formation of thrombosis in the site of damage in blood vessels in NOG mice. However, the degree to which these platelets can play an active role in hemostasis remains to be evaluated.

Ideally, platelets cultured *ex vivo* should be evaluated using a standard platelet aggregation assay that requires a platelet count of at least $100 \times 10^9$/L, which has not been reported by the research group. Furthermore, the loss of platelet membrane surface glycoproteins such as GPIb is another important factor that interferes with platelet function. The matrix protein metalloprotease inhibitor GM6001 can prevent GPIba protein hydrolysis by ADAMTS17 and improve platelet function[65,66]. Based on the current situation, platelets collected *ex vivo* have at least some physiological functions, but avoiding self-activation in culture is a difficult challenge.

## PROGRESS AND FUTURE DIRECTIONS IN PLATELET BIOREACTORS

Compared with platelets in PB, the platelets produced *in vitro* were more heterogeneous in size and were produced at a lower output. One explanation is the static quality of cell culture, which is unlike the microenvironment *in vivo*. A reasonable approach for large-scale platelet production is to combine bioreactors with chemical factors. Different bioreactors recapitulate different physiological conditions (Table 1), including gas exchange, media perfusion[67], extracellular matrix proteins, scaffold composition and the effects of blood shear stress[68-71].

In 2003, Li *et al*[72] first discovered that a murine CCE ES cell line could be used to drive hematopoietic cells in a 3D fibrous matrix to direct hematopoietic differentiation using specific cytokines and inhibitors. In 2009, Sullenbarger *et al*[23] reported that a 3D bioreactor with surgical-grade woven polyester fabric or purpose-built hydrogel

**Table 1 Some major advances in bioreactors in platelet formation and platelet release from 2013 to 2018**

| Type of bioreactor | Principles and methods | Designers / users |
|---|---|---|
| Two-directional flow bioreactor | The bioreactor consisted of two-directional flow, in which the angle between the directions of the main and pressure flow is 60 degrees | Nakagawa *et al*[74], 2013 |
| Microfluidic platelet bioreactor | The bioreactor is based on polydimethylsiloxane bonded to glass slides to construct some upper and lower microfluidic channel | Thon *et al*[70], 2014 |
| Spinning-membrane filtration device | Separating *in-vitro*-derived PLTs and recovering immature MKs and the precursor cells of PLTs by use of spinning-membrane filtration device | Schlinker *et al*[77], 2015 |
| RCCS bioreactor | Shear force, simulated microgravity, and better diffusion of nutrients and oxygen from the RCCS | Yang *et al*[67], 2016 |
| Innovative bioreactor | The bioreactor consisted of a membrane, and using flow through the membrane and shear across the membrane to drive the megakaryocytes to release PLTs | Avanzi *et al*[43], 2014 |
| Microfluidic device | This device consists in a wide array of von Willebrand factor-coated micropillars, allowing them to remain trapped and subjected to hydrodynamic shear | Blin *et al*[75], 2016 |
| Turbulent flow-based bioreactor | Stimulation with optimized shear stress and turbulent energy, collaborates with several growth factors for proplatelet formation | Ito *et al*[60], 2018 |

RCCS: Rotary cell culture system.

scaffolds could facilitate platelet output when coated with THPO with/or fibronectin. Subsequently, Lasky *et al*[73] designed the bioreactor by optimizing oxygen concentrations and media perfusion to promote platelet output, but they did not consider blood shear stress. Later, Nakagawa *et al*[74] developed a two-directional flow bioreactor and found that two flows in different directions could promote platelet production by as much as 3.6-fold compared with static cultures. In 2014, Thon *et al*[70] made a microfluidic platelet bioreactor that attempted to use biomimetic BM and blood vessel microenvironments and supported live imaging for platelet generation. Blin *et al*[75] built on previous work, reporting a bioreactor consisting of a wide array of vWF-coated micropillars to act as anchors on MKs. MKs were anchored and subjected to shear stress. Elongation of MK cytoplasm and proplatelet formation were observed. However, these devices have mostly focused on the development of proof-of-concept basic research, low throughput, custom-made tools.

Based on the concept that human MKs are partly regulated by the extracellular matrix, scientists began to design bioreactors with different materials to emulate BM physiology. To increase platelet production, Pallotta *et al*[57,76] applied biocompatible silk microtubes with fibrinogen, type 1 collagen, and SDF1, mimicking the release of platelets in a blood vessel. The MKs extended proplatelets through the micropores of the microtube and released platelets when exposed to low shear stress. Additionally, silk sponges[64], polycarbonate filter membranes[77] or the hyaluronan-based hydrogels[78] were also used to mimic the bioengineered 3D BM environment, while there are major limitations to implementing the material in biomedical devices.

The combination of bioreactors and chemical factors might promote megakaryo/thrombopoiesis. Recently, we used the rotary cell culture system (RCCS), a unique 3D cell culture method, to investigate the potential role in megakaryopoiesis. Our results indicated that RCCS significantly improves the efficiency of platelet generation, which recapitulates some special characteristics, including shear force, simulated microgravity, and better diffusion of nutrients and oxygen. Additionally, we demonstrated that RCCS combined with chemical compounds and growth factors identified *via* small screening can further increase platelet generation efficiency[68], while there is limitation to lacking the function of automatic change of medium. Analogously, a novel bioreactor with a membrane and three ports of input and output was developed and can produce a high number of platelets from UCB-derived $CD34^+$ cells. Based on *in vivo* imaging, Ito *et al*[60] found that turbulence is an important physical regulator of thrombopoiesis. They developed a turbulent flow-based bioreactor (VerMES Bioreactor), which enabled high yield and quality *ex vivo* biogenesis from imMKCLs. In addition, three novel chemical factors (IGFBP2, MIF, and NRDC) that contribute to the remodeling of mature MKs and shedding of

platelets might represent a potential mechanism to promote proplatelet shedding in the VerMES bioreactor[61]. Thus, next-generation platelet bioreactor need to be designed with comprehensive consideration fluid dynamics modeling, automation of flow control and avoid the too highly cost of device design and cell culture.

## CONCLUSION

As the demand for platelet transfusions increases, many countries are competing to improve the efficiency of platelet production *ex vivo*. However, the field of megakaryopoiesis and platelet research still faces several limitations, including (1) The massive number of platelets (100-300 billion platelets) needed for one transfusion; (2) the high economic cost for the entire culture period; and (3) the lower viability of *ex vivo* platelets compared to donor-derived platelets, though they express the proper surface marker for platelet function and participate in the aggregation reaction at the site of damage in a mouse model. Optimizing the key steps of megakaryopoiesis and platelet generation may provide a better understanding of the cellular and molecular mechanisms. Introducing SCs into advanced bioreactors and simultaneous exposure to a subset of chemical compounds may synergistically contribute to the production of a large number of platelets for clinical applications. In addition, before clinical application, the platelet function produced *ex vivo* must be defined in detail and fully verified. Collection, cost-effective and highly controllable strategies and methodologies represent an important step toward large-scale platelet production for future biomedical and clinical applications.

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*REVIEW*

# Tendon stem/progenitor cell ageing: Modulation and rejuvenation

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## Abstract

Tendon ageing is a complicated process caused by multifaceted pathways and ageing plays a critical role in the occurrence and severity of tendon injury. The role of tendon stem/progenitor cells (TSPCs) in tendon maintenance and regeneration has received increasing attention in recent years. The decreased capacity of TSPCs in seniors contributes to impaired tendon functions and raises questions as to what extent these cells either affect, or cause ageing, and whether these age-related cellular alterations are caused by intrinsic factors or the cellular environment. In this review, recent discoveries concerning the biological characteristics of TSPCs and age-related changes in TSPCs, including the effects of cellular epigenetic alterations and the mechanisms involved in the ageing process, are analyzed. During the ageing process, TSPCs ageing might occur as a natural part of the tendon ageing, but could also result from decreased levels of growth factor, hormone deficits and changes in other related factors. Here, we discuss methods that might induce the rejuvenation of TSPC functions that are impaired during ageing, including moderate exercise, cell extracellular matrix condition, growth factors and hormones; these methods aim to rejuvenate the features of youthfulness with the ultimate goal of improving human health

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during ageing.

**Key words:** Tendon stem/progenitor cell; Ageing; Mechanisms; Modulation; Rejuvenation



**Core tip:** Tendon stem/progenitor cells (TSPCs) play an essential role in tendon maintenance, regeneration and repair. Recent studies indicate that an association between the decreased capacities of aged TSPCs and the impaired tendon functions observed with increasing age. In this review, we briefly discuss novel updates in research investigating TSPCs characteristics. Then, we summarize the epigenetic variations in TSPCs that occur with ageing and provide a detailed description of the pathways that play essential roles in the cellular ageing process. Finally, we propose potential methods to rejuvenate ageing TSPCs and provide additional therapeutic targets for the treatment of age-related tendon diseases.

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## INTRODUCTION

Ageing is an intricate physiological progress caused by multiple factors that result in variations in the structure and composition of cells, organs and tissues and a decrease in the capacity and activity of mammals. The global population over the age of 60 years is growing rapidly[1] and the occurrence of tendon-related injuries increases upon ageing[2]. Moreover, the consequences of tendon damage in elderly patients are more severe[3], and older populations also experience a higher occurrence of sport-related tendon injuries and more difficulties in healing process[4], which places a heavy burden on the health systems of individual countries[5]. Epidemiological studies have highlighted the importance of obtaining an in-depth understanding of the pathogenesis of aged-related tendon diseases, with the aim of developing appropriate therapeutic approaches.

Recently, studies focused on stem cells have become emerging areas in regenerative and biomedical medicine because these cells have been confirmed to be remarkably important for tissue maintenance, repair and remodeling; and they have also been used to cure various diseases with satisfactory outcomes[6-9]. Pluripotent stem cells can differentiate into various tissue types under different conditions and serve as an internal repair system, which is also restricted to the embryonic layer of origin[10]. In adults, tendon stem/progenitor cells (TSPCs), as a type of mesenchymal stem cell (MSC), were first confirmed to be present in tendon tissues by Bi *et al*[11] in 2007, and they have been found to possess self-renewal ability, clonogenicity and multidifferentiation potential. Compared with bone marrow stromal cells, TSPCs show express higher level of Oct4, which is known to positively modulate mesodermal lineage differentiation, and have greater ability of proliferative and clonogenicity. Thus, TSPCs potentially represent an more appropriate cell source for the regeneration of musculoskeletal tissue, particularly tendon tissue, which has limited repair and healing abilities with traditional tenocytes[12]. Based on these findings, scholars have a strong interest in identifying the potential role of TSPCs in tendon regenerative medicine and the injury healing process; thus, numerous related studies have been published on this topic in recent years.

However, ageing exerts negative effects on TSPCs functions, which could limit the application of TSPCs in tendon injury repair and the choice of cell sources for regenerative medicine. Ageing also affects cell the genetics of cells, through a series of pathways involved in both accelerating and delaying the ageing process. In the MSC ageing process, the P16/RB pathway and P53/P21 pathway have vital roles in modulating the cellular senescence by regulating telomere length and function. In addition to telomeres, DNA damage, mitochondria dysfunction and reactive oxygen species are involved in suppressing the expression of genes that promote the stem cell cycle progression of stem cells and induce the expression of cell cycle inhibitors[13].

Up-regulation of P53, P16, P14 and P21 genes related to cell cycle arrest and activation of the P53 pathway and P21 pathway have also been observed in aged TSPCs, which are thought to function as accelerators of the cellular ageing process[14]. What's more, stem cell markers expression declines with age in TSPCs, indicating potential causes of the alterations in cell differentiation ability[15]. In this regard, a novel hypothetical model of altered TSPCs fates in the ageing process has been formulated based on the observation of ectopic metaplasia and the decline in the tenogenic differentiation capacity of tendon tissue during the ageing process, which ultimately increases the occurrence of age-related tendon diseases[1]. Recently, the discovery of induced pluripotent stem cells (iPSCs), particularly cells isolated from mature adult, inspired researchers to develop potential therapies to cure clinical diseases and ponder the eternal topic of regaining our youth. Thus, iPSCs provided inspiration to reverse the stem cell fate by modulating the factors that influence cell growth[16-18].

With limited treatment options for tendon diseases and unsatisfactory healing outcomes, studies aimed to explore the biological link between tendon ageing and TSPCs are very meaningful for the development of age-related diseases treatments. In this review, we initially discuss recent studies addressing the characteristics of TSPCs. Then, we summarize the epigenetic variations in TSPCs that occur with age and provide a detailed description of the pathways that play essential roles in the cellular ageing process. Finally, we propose potential pathways to rejuvenate ageing TSPCs, providing further therapeutic targets for the treatment of age-related tendon diseases.

## TENDON STEM/PROGENITOR CELLS

Traditionally, tenocytes were considered the only cell present in tendon tissue and play a critical role in tendons metabolism. This hypothesis did not change until the isolation and identification of TSPCs in many tendon fascicles, including mouse[11], human[19], rat[20], rabbit[21], turkey[22], porcine[23] and fetal bovine tendon fascicles[24] in recent years. Although TSPCs represent a minor percentage of the tendon cell composition, these cells possess features such as self-renewal, clonogenicity and multidifferentiation and TSPCs are distinguished by the presence of stem cell markers[21]. Since these discoveries, substantial interest and progress in the study of the roles of this cell type in tendon maintenance, repair, remodeling and tendon tissue engineering have been reported.

Compared with tenocytes, TSPCs express stem cell markers, proliferate faster, exhibit multidifferentiation potential and express tenogenic markers at higher levels[21,25]. Although Berglund *et al*[26] proposed a different hypothesis that major histocompatibility complex (MHC) mismatched MSCs were not immune privileged because they induced both cell-mediated and humoral immune responses, the majority of studies consistently shown that MSCs display low immunogenicity and immuno-modulatory properties, which avoid immunological rejection. Thus MSCs are a potential allogeneic cell source for transplantation, and TSPCs, a subtype of MSCs, may possess features similar to MSCs[27]. According to Lui *et al*[28], TSPCs expressed lower levels of MHCII, cluster differentiation 86 and cluster differentiation 80 on the cell surface; these proteins are essential for inducing a T-cell response. Additionally, the infiltration of inflammatory cells was not observed in tendon injuries treated with allogeneic TSPCs, revealing the low immunogenicity of TSPCs *in vitro* and *in vivo*[28,29]. Based on these facts, researchers have confirmed that these active TSPCs are immune-privileged and can be used for allogeneic transplantation. Benefiting from the positive aspects, particularly the multi-differentiation capacities and immune-privilege, TSPCs potentially represent an ideal cell source for musculoskeletal tissue regenerative medicine and therapeutic targets for numerous related diseases. Although important research has shown that TSPCs might reside within the tendon fascicles, others researchers have suggested that the epitenon might be another source of TSPCs[30]; subsequent studies have confirmed this hypothesis[30-33]. Although all TSPCs generally exhibit the characteristics of tendon stem cells, they have their own unique features when isolated from different sites in the tendon. These findings reveal the presence of more than one source of distinct TSPCs in tendon tissue, and these populations represent a seed cells source for application in different tendon injuries according to the different cellular characteristics[30,34-36].

Moreover, numerous studies have confirmed that TSPCs play an essential role in the progression of tendon diseases and/or tendon tissue engineering, and the biological features of TSPCs are altered by many factors, which is why many treatment strategies for tendon-related injuries have primarily focused on TSPCs. For example, platelet-rich plasma augments and accelerates the effects of TSPCs on the healing process[37], and the mechanistic basis for the treatment of tendon tear is

attributed to increased DNA synthesis, increased cellular migration velocity and the supplements of TSPCs[38,39]. In several cases, growth and differentiation factor-5 was reported to promote the tenogenic differentiation of TSPCs, and transforming growth factor-β1 and insulin-like growth factor 1 (IGF-1) promotes TSPC proliferation and phenotype maintenance[40]. Additionally, the expression of inflammatory cytokines is dramatically upregulated in injured tendons[41-43], some of which inhibit the proliferation and tenogenic and osteogenic differentiation of TSPCs[43]. Moreover, TSPCs are essential for tendon healing and the regulation of inflammation, and the production of the pro-inflammatory cytokine Interleukin-6 (IL-6) and anti-inflammatory cytokine Interleukin-10 (IL-10), is significantly up-regulated at the late stage of inflammation in injured tendons[7,44]. Based on these findings, IL-6 and IL-10 evidently up-regulate cell proliferation, and IL-10 significantly enhances cell migration. However, both IL-6 and IL-10 inhibit the production of gene and protein functioning as tenocyters markers, including scleraxis and tenomodulin, and dramatically activate the JAK/Stat3 signaling pathway, which has a crucial role in modulating inflammation in TSPCs[45], indicating that IL-6 and IL-10 may exert dual effects on TSPCs *in vitro*[7,44], and connective tissue growth factor plays a role in anti-inflammatory by regulating the IL-6 and IL-10 expression[45]. Decreased annexin A1 (an anti-inflammation protein) expression resulted in elevation of inflammation during the mouse tendon injury process; thus, annexin A1 potentially represents a novel curative target in clinical applications[46]. In addition, many drugs and proteins exert effects on TSPCs that promote tendon healing. Celastrol exerts beneficial effects on human TSPCs stemness and the vital role of HIF1α-Smad7 signaling in the process is elucidated[47]. Celecoxib inhibits the tenogenic differentiation of TSPCs but has no effects on cell proliferation[48], and a high concentration of aspirin induces apoptosis in TSPCs by delaying the activation of Wnt/β-catenin pathway[49]. All these factors might affect the quality of tendon healing by targeting TSPCs, regardless of whether the effects are positive or negative. The recent main factors are summarized in Table 1.

In addition, an altered fate of TSPCs was observed in a collagenase-induced tendon injury model of tendinopathy due to the presence of tenocytes lacking the multidifferentiation capacity[21], consistent with similar results presented in other studies and supporting the hypothesis that TSPCs might play an essential role in the pathogenesis of tendinopathy. A series of recent studies revealed important roles for TSPCs in tendon healing by replacing mature tendon cells that are lost under normal circumstances, which might be the cause of age-related changes in the pathogenesis of tendon disorders[15,50]. Thus, TSPCs are considered to play a crucial role in maintaining tendon homeostasis by affecting tendon repair and regeneration[15,20,51,52]. Recently, Li *et al*[1] proposed that the altered fate of TSPCs contributes to tendon ageing. Other scholars have also observed alterations in TSPCs features during tendon degeneration and the progression of ageing[14,15,50,53,54]. Overall, a range of TSPCs functions are altered, and TSPCs might serve as a potential target due to these alterations. Therefore, a relationship between altered TSPCs features and tendon ageing has been hypothesized, highlighting the importance of TSPCs in the treatment of tendon-related diseases.

## AGEING AND ALTERATIONS IN EPIGENETIC AND THE UNDERLYING MECHANISMS

### *Age-related markers in TSPCs*

TSPCs undergo a series of significant cellular epigenetic alterations with age, which are viewed as age-related markers in TSPCs for that can be used in future studies, and these results are consistent with similar results obtained from other types of stem cells. The main findings are summarized in Table 2.

### *Ageing and cell morphology*

*In vitro* aged-TSPCs (A-TSPCs) exhibit cell shape of star-like flattened, while young-TSPCs (Y- TSPCs) exhibit spindle-shaped morphology[14]. In addition, aged TSPCs are obviously larger in size, have more podia, spread further, and exhibit more robust actin stress fibers and a higher actin content that distorts the balance of the actin cytoskeleton organization[14,55,56], which has also been confirmed by analyses of microarray data in aged TSPCs[14]. Additionally, aged TSPCs display a large, flat and heterogeneous morphology, while younger cells exhibit the morphology of uniform elongated[57]. An increase in the size is often associated with cell senescence[50,55,56]. In addition, the number of heterogeneous and cobblestone-shaped TSPCs is dramatically down-regulated with ageing, and the oldest TSPCs have only a few percent displaying the cobblestone shape[15]. Kohler *et al*[14] reported an important role for

**Table 1 Recent main factors for regulating tendon stem/progenitor cells biological features**

| Factor | Cell source | Interventional details | Results | Ref. |
|---|---|---|---|---|
| PRP | SD rats | 10% PRP | 10% PRP augments and accelerates the effects of TSPCs on the healing process | [37] |
| BMACs and PRP complex | Human | A T75 flask (450 μL of BMACs and PRP) | BMAC–PRP enhances the proliferation and migration of TSPCs | [38] |
| PRP | SD rats | 2% PRGF | PRP can activate TSPCs to improve the quality of Achilles tendon rupture healing | [39] |
| IGF-1, GDF-5 and TGFβ1 | Lewis rats | Each growth factor (1, 10, and 100 ng/mL) | GDF-5 promotes TSPCs tenogenic differentiation, and TGFβ1 and IGF-1 increase TSPCs proliferation and are beneficial for phenotype maintenance | [40] |
| IL-1β | Dogs | - | The expression of inflammatory cytokines is dramatically up-regulated in injured tendon | [41,42] |
| IL-1β | Mouse | IL-1β (1, 5 or 10 ng/mL) | IL-1β strongly and irreversibly impairs tenogenic and osteogenic differentiation potentials of TSPCs | [43] |
| IL-6 | SD rats | IL-6 (0, 0.1, 1, 10, and 100 ng/mL) | IL-6 enhances proliferation and inhibites tenogenic differentiation in TSPCs *via* the JAK/Stat3 pathway | [44] |
| IL-10 | SD rats | IL10 (0, 0.1, 1, 10 or 100 ng/mL) | IL10 enhances cell proliferation and migration, and inhibites tenogenic differentiation in TSPCs | [7] |
| CTGF | SD rats | CTGF (100 ng/mL) | CTGF plays a role in anti-inflammatory, leading to enhanced tendon healing | [45] |
| Annexin A1 | WT and DF508 mice | - | Decreased annexin A1 expression resulted in elevation of inflammation during the mouse tendon injury process | [46] |
| Celastrol | Human | celastrol (0, 1, 2, and 4 μM) | Celastrol exerts beneficial effects on human TSPCs stemness and the vital role of the HIF1α-Smad7 pathway in the process is elucidated | [47] |
| Celecoxib | C57 mouse | celecox (0.1, 1, 10 and 100 ug/mL) | Celecoxib inhibits tenogenic differentiation of TSPCs but has no effects on cell proliferation | [48] |
| Aspirin | SD rats | Aspirin (1, 2, and 5 mM) | A high concentration of aspirin induces apoptosis in TPSCs by delaying the activation of Wnt/β-catenin pathway | [49] |

PRP: Platelet-rich plasma; SD: Sprague–Dawley; TSPCs: Tendon stem/progenitor cells; BMACs: Bone marrow aspirate concentrates; PRGF: Platelet-rich growth factors; CTGF: Connective tissue growth factor; IL-10: Interleukin-10; IL-1β: Interleukin1β; TGFβ1: Transforming growth factor-β1; GDF-5: Growth and differentiation factor-5; IGF-1: Insulin-like growth factor1.

increased Rho associated coiled-coil forming protein kinase (ROCK) activity in accelerating the ageing progress of A-TSPC, and A-TSPCs revert to a morphology similar to Y-TSPCs upon treatment with Y-27632, an common ROCK inhibitor. Similar results have also been detected in aged tenocytes as well as in other types of stem cells[58,59].

### *Ageing and cell proliferation*

**Growth rate:** A-TSPCs showed a proliferation deficit after 120 d of culture and had an

**Table 2 Age-related markers of tendon stem/progenitor cells**

| Object | Species model | Groups | Tendon type | Main findings | Ref. |
|---|---|---|---|---|---|
| Cell morphology | Human | Y-TSPC group: 28 ± 5 yr; A-TSPC group: 63 ± 14 yr | Achilles tendon | A-TSPC exhibit cell shape of star-like flattened, while Y-TSPCs exhibit spindle-shaped | [14] |
| | Human | Y-TSPC: 28 ± 5 yr and A-TSPC: 63 ± 14 yr | Achilles tendons | Aged TSPCs are obviously larger in size, have more podia, spread further, and exhibit more robust actin stress fibers, and exhibit higher actin content | [55] |
| | Rat | old rats: 20 mo and young rats: 8 wk | Achilles tendons | Aged TSPCs display a morphologies of large, flat and heterogeneous morphology, while younger cells exhibit the morphology of uniform elongated | [57] |
| | Mice | young (2.5, and 5 mo) and aging (9 and 24 mo) mice | Patellar tendons | The number of heterogeneous and cobblestone-shaped TSPCs is dramatically down-regulated with ageing, and the oldest TSPCs have only a few percent displaying the cobblestone shape | [15] |
| Growth rate | Human | Y-TSPC group: 28 ± 5 yr; A-TSPC group: 63 ± 14 yr | Achilles tendon | A-TSPCs showed a proliferation deficit after 120 d of culture and had an early plateau phase, while Y-TSPCs didn't exhibit the plateau | [14] |
| | Rat | 3–4 (young) and 24–26 mo (aged) | Patellar tendons | Proliferation rate is decreased and cell cycle progression is delayed with increasing age | [53] |
| | Rat | three different post-natal stages: 1 d, 7 d and 56 d | Achilles tendon | TSPCs-7d displayed that a higher proliferation rate than the groups of TSPCs-1d and TSPCs-56d | [61] |
| | Rat | Early P5, mid P10, and late P20 and P30 passages were used | patellar tendons | TSPCs at late P20 and P30 proliferate more rapidly than those at early P5 and mid P10 | [62] |
| Cell clonogenicity | Human | Y-TSPC group: 28 ± 5 yr; A-TSPC group: 63 ± 14 yr | Achilles tendon | Age-dependent clonogenic deficits in TSPCs are based on a decreased in the colony number and CFU efficiency with ageing | [14] |
| | Human | Group 1: aged 20 (female) and 22 (male); group 2: aged 28 (female) and 31 (male) and Group 3: aged 49 (male) and 50 (female) | Hamstring tendons | The clonogenic potential is dramatically decreased with age; in addition, the size of the colonies was heterogeneous in patients, as the size of colonies produced by cells from aged patients was obviously larger than the colonies composed of cells from younger patients | [50] |

| | | | | | |
|---|---|---|---|---|---|
| | Rat | three different post-natal stages: 1 d, 7 d and 56 d | Achilles tendon | TSPCs-7d have an obviously higher clonogenic ability than TSPCs-1d and TSPCs-56d | [61] |
| | Rat | early P5, mid P10, and late P20 and P30 passages were used | patellar tendons | The colony numbers of TSPCs increase with passaging, | [62] |
| Cell migration | Human | Y-TSPC group: 28 ± 5 yr; A-TSPC group: 63 ± 14 yr | Achilles tendon | The migration of TSPCs exhibits a decreasing trend with advanced age | [14] |
| Cell differentiation | Rat | three different post-natal stages: 1 d, 7 d and 56 d | Achilles tendon | TSPCs from different time groups displays multidifferentiation capability, while the ability of TSPCs-7d is higher than TSPCs-1d and TPSCc-56d, and a similar trend is observed in the tenogenic differentiation capacity | [61] |
| | Human | Y-TSPC: 25 ± 8yr, and A-TSPC: 65 ± 10 yr | Achilles tendon | Tenogenic differentiation capacity of TSPCs significantly decreases with ageing | [54] |
| | Mice | young (2.5, and 5 mo) and aging (9 and 24 mo) mice | Patellar tendons | Aged TSPCs formed adipocytes more readily than younger cells and expressed higher levels of adipogenic markers | [15] |
| | Rat | early P5, mid P10, and late P20 and P30 passages were used | patellar tendons | TSPCs tend to differentiate into osteoblasts, while the adipogenic, chondrogenic and tenogenic differentiation capacities in TSPCs decline during in vitro subculture | [62] |
| | Mice | early P0, and late P5 passages were used | Achilles tendon | The TSPCs experiences a gradual loss of tenogenic differentiation with passaging due to increased expression and activity of Hdac | [65] |
| | Human | Y-TSPC group: 28 ± 5 yr; A-TSPC group: 63 ± 14 yr | Achilles tendon | A-TSPC have been reported to display an evident self-renewal and clonogenic decrease, multipotency is maintained *in vitro* | [14] |
| | Human | Group 1: aged 20 (female) and 22 (male); group 2: aged 28 (female) and 31 (male) and Group 3: aged 49 (male) and 50 (female) | Hamstring tendons | Multi-potency assays were not influenced by advanced ageing, although Y-TSPCs produced higher levels of some osteogenic and adipogenic genes, while chondrogenic genes were expressed at high levels in A-TSPCs | [50] |
| CD marker | Rat | 3–4 (young) and 24–26 mo (aged) | Patellar tendons | Aged TSPCs express lower levels of CD90.1 than young cells, but higher levels of CD44 | [53] |
| | Rat | early P5, mid P10, and late P20 and P30 passages were used | patellar tendons | CD90 and CD73 is down-regulated with increasing numbers of passaging | [62] |

| | | | | | |
|---|---|---|---|---|---|
| Cell stemness marker | Mice | young (2.5, and 5 mo) and aging (9, and 24 mo) mice | Patellar tendons | The expression of the stem cell markers Oct-4, NS, Sca-1 and SSEA-1 in TSPCs decreased in an age-dependent manner | [15] |
| Cell viscoelasticity | Rat | old rats: 20 mo and young rats: 8 wk | Achilles tendons | An overall increase in G′, G″and $h_{TSPC}$ with ageing, revealing an important increase in stiffness of aged TSPCs | [57] |
| | human | Y-TSPC: 28 ± 5 yr and A-TSPC: 63 ± 14 yr | Achilles tendons | Cell stiffness and size increase in A-TSPCs | [55] |
| Cell senescence markers | human | Y-TSPC group: 28 ± 5 yr A-TSPC group: 63 ± 14 yr | Achilles tendon | A-TSPCs undergo an early appearance of cellular senescence, as determined by quantifying the number of β-gal- positive cells at different time points | [14] |
| | rat | Early P5, mid P10, and late P20 and P30 passages were used | patellar tendons | The significant up-regulation of β-gal activity in TSPCs with increasing passaging | [62] |

Y-TSPC: Young-TSPC; A-TSPC: Aged-TSPCs; TSPCs-7d: TSPCs-7days; P5: Passage 5; CFU: Colony-forming unit; Hdac: Histone deacetylase; CD: Cluster differentiation; NS: Nucleostemin.

early plateau phase, while Y-TSPCs didn't exhibit the plateau[14]. Zhou *et al*[53] also observed this decrease in TSPCs proliferation with increasing age, consistent with similar results observed in TSPCs from other aged vertebrate animals[15,50,56,60]. Additionally, TSPCs-7 day (TSPCs-7d) displayed that a higher proliferation rate than the groups of TSPCs-1 day (TSPCs-1d) and TSPCs-56 day (TSPCs-56d)[61]. However, Tan *et al*[62] observed more rapid proliferation of TSPCs at late passage 20 (P20) and P30 than cells at an early P5 and middle P10, revealing a different perspective of the increased proliferation with additional passaging. Moreover, the increased proliferation of aged TSPCs was restored by treatment with ephrin receptor A4-Fc (EphA4-Fc), a moderate treadmill running (MTR) intervention and other factors, revealing that the proliferation rate of TSPCs can be modulated[15,56,60].

**Ageing and cell clonogenicity (colony-forming unit numbers and colony size):** Age-dependent clonogenic deficits in TSPCs are based on a decreased in the colony number and colony-forming unit efficiency with ageing[14,56,63]. Ruzzini *et al*[50] bserved a dramatic decrease in the clonogenic potential with ageing; in addition, the size of the colonies was heterogeneous in patients, as the size of colonies produced by cells from aged patients was obviously larger than the colonies composed of cells from younger patients. Another study reported an obviously higher clonogenic capacity of TSPCs-7d than TSPCs-1d and TSPCs-56d[61]. In summary, the mainstream hypothesis is that ageing exerts negative impact on the clonogenicity of TSPCs. However, Tan *et al*[62] revealed an increase in the numbers of TSPCs colonies with passaging, in contrast to the findings from other studies.

### *Ageing and cell migration*

The migration of TSPCs exhibits a decreasing trend with advanced age in a series of studies[14,56,60]. Popov *et al*[56] observed a significant decrease in the migratory of aged TSPC, and EphA4-Fc and ephrin receptor B2-Fc (ephB2-Fc) restore the decreased migration of A-TSPCs by inducing cell motility. Additionally, young hypoxic conditioned culture medium (HCCM) and inhibition of ROCK, a factor related to accelerate ageing, promote the restoration of cell migration[14,60].

### *Ageing and cell differentiation*

The TSPC pool becomes exhausted considering the size and functional fitness with ageing. However, the maintenance of the multidifferentiation capacities of TSPCs from animals and humans is widely accepted, although a consensus on the direction of alteration has not been reached.

TSPCs from different time groups display multidifferentiation potential, while the ability of TSPCs-7d was greater than TSPCs-1d and TSPCs -56d, and a similar trend was observed in the tenogenic differentiation capacity[61]. The capacity of TSPCs to differentiate into tenocytes is reduced with ageing[61], consistent with the observation that the tenogenic differentiation capacity of TSPCs is profoundly diminished during ageing[54]. Moreover, aged TSPCs are not sensitive to transforming growth factor-β3, a

sublineage of the TGF-β superfamily that regulates cell growth and differentiation[64]. However, A-TSPCs transformed into adipocytes more readily than younger cells and produced higher levels of adipogenic markers that further resulted in the appearance of adipose tissue, which is generally related to aged tendons, while they presented no obvious difference in the capacity to transform into osteoblasts or chondrocytes[15]. Moreover, TSPCs tend to differentiate into osteoblasts as the number of passages *in vitro* increases, while the adipogenic, chondrogenic and tenogenic differentiation capacities in TSPCs decline during *in vitro* subculture[62]. Furthermore, Can Zhang *et al*[65] detected that a gradual loss of the tenogenic differentiation capacity of TSPCs with passaging due to the increased expression and activity of histone deacetylase (Hdac). Additionally, conflicting evidence shows a lack of age-related changes. Although, A-TSPCs have been reported to display an evident decrease in self-renewal and clonogenic capacities, multipotency is maintained *in vitro*[14]. Another research concluded that the multipotency assays were not influenced by advanced ageing, although Y-TSPCs produced higher levels of some osteogenic and adipogenic genes, while chondrogenic genes were expressed at high levels in A-TSPCs[50]. Overall, researchers have concluded that the multidifferentiation capacities of TSPCs are maintained during the ageing process without a conclusive determination of the trends in their variations, but most studies conclude that ageing impairs the tenogenic differentiation capacity of TSPCs.

### Ageing and cell specific cluster differentiation (CD) markers

Greater than 98% of TSPCs are positive for CD73, CD90, CD105, STRO-1, CD146, Musashi-1 and CD44, but are negative for CD19, CD34, CD45 and HLA-DRA[14,50]. Compared with young TSPCs, aged cells exhibit lower CD90.1 level, but higher CD44 expression[53]. CD44 is involved in the healing processes of numerous tissues and its levels are reduced in the process of scar less fetal tendon healing[66]; moreover, an improvement in mouse patellar tendon healing might attributed to a deficiency in CD44[67]. Based on these findings, the up-regulation of CD44 in A-TSPCs might result in a decrease in the self-repair ability of TSPC with ageing. Additionally, the production of CD90 and CD73 decreases with increasing numbers of passage *in vitro*[62].

### Ageing and cell stemness markers

Approximately all TSPCs are positive for stem cell markers, including nucleostemin, Oct-4, and SSEA-4 in different age groups, revealing that the cells still maintained stemness features with age[53]. However, the levels of stem cell markers are dramatically decreased with ageing. Additionally, moderate mechanical stretching (4%) dramatically upregulated the stem marker NS expression of A- TSPCs *in vitro*, but 8% stretching reduced its production; similarly, 4% stretching also upregulated the production of another stem cell marker, Nanog[15].

### Ageing and cell viscoelasticity

One study revealed an overall increase in G′, G″ and $H_{TSPC}$ with ageing, which are valuable indicators of the cellular viscoelasticity that correspond to the storage modulus (G′), loss modulus (G′′) and average thickness ($H_{TSPC}$), respectively. A dense cytoskeletal organization might result in a larger cell size and anomalous cell shape and is the cause of the increase in stiffness and viscosity[57]. Other authors had also detected an increase in the cell stiffness and size of A-TSPCs, as well as a denser and well-structured actin cytoskeleton. Moreover, treatment with a ROCK inhibitor rejuvenated these age-related variations in morphology and stiffness[55]. As it is known, ECM is another critical factor for the viscoelasticity of TSPCs and intervened in the receptor-substrate ligand interactions of cell adhesion[57]. Although, Kostrominova *et al*[68] showed alterations of ECM protein expression in rat tendons with ageing, while composition of ECM related to the cell adhesion was not analyzed. Related experiments can be carried out because ECM proteins and cell niche are likely to highly influence both TSPCs maintenance and turnover in the future.

### Ageing and cell senescence markers

A-TSPCs undergo cellular senescence at an early stage, as determined by quantifying the number of β-gal-positive cells at different time points, and at P4, more A-TSPCs displayed positive staining. In addition, the quantity of β-gal positive A-TSPCs was dramatically increased at later passages. Moreover, the P16 protein was already detected in the P1 A-TSPCs, and its expression was evidently upregulated at P14[14], accompanied by the evident upregulation of β-gal activity in TSPCs with increasing passaging[62]. In addition, an up-trend in the levels of senescence-related markers was observed in A-TSPCs in other studies[53,54,60,63,69,70], and the inhibition of ROCK, up-regulation of Pin1 (peptidyl-prolylcis-transiso merase NIMA-interacting1) or miRNA

(miR)-135a, down-regulation of P16, or modulation of other molecules involved in the ageing process reversed the senescence of TSPCs and effectively delayed the ageing process[14,63,69].

### *Mechanisms involved in the ageing process*

Because TSPCs ageing is an intricate process, its progression is also affected by multiple factors, including hormones, cytokines, enzymes, the oxygen content, mechanical force and exercise. Although the occurrence of epigenetic alterations in TSPCs with ageing has been observed, few scholars have focused on the underlying mechanisms partially because of the ambiguous conclusion regarding changes in aged TSPCs. The following section summarizes recent progress in the discovery of molecules and pathways involved in the TSPC ageing process and their various roles in mediating the ageing process, providing future research directions for TSPCs ageing and potential treatment targets for age-related tendon diseases. The mechanisms involved in the TSPC ageing process are listed in Figure 1.

Compared with Y-TSPCs, cAMP-responsive element-binding protein/p300-interacting transactivator with ED-rich tail 2 (CITED2) was dramatically down-regulated in older–TSPCs (O-TSPCs) at both the mRNA and protein levels and O-TSPCs showed reduced proliferation and elevated senescence. Furthermore, upon induction with TGFβ-2, the nuclear expression of CITED2 and SP1 was significantly decreased, indicating that TGFβ-2 mainly suppresses nuclear expression of CITED2. At the same time, P21 expression was increased, and myelocytomatosis viral oncogene homolog (MYC) was up-regulated following the silencing of CITED2, revealing that the TGFβ2-CITED2-MYC-SP1/P16 pathway medicates TDSC senescence. These findings were further supported by the results of a previous study showing that MYC functions as a transcriptional activator or repressor in regulating cell cycle progression and that the TGFβ receptor kinase inhibitor SB525334 modulates the activity of this pathway[71]. By comparing genome-wide RNA microarray data obtained from human Y-TSPCs and A-TSPCs, an intriguing difference was found: Altered genes were mainly distributed in categories such as cell–cell contact, cell adhesion, motility, migration, cytoskeleton and actin-associated transcripts, which might be the cause of the phenotypic and behavioral variations in A-TSPCs. In addition, the changes in features related to actin in A-TSPCs also significantly disrupted the formation of actin stress fibers and cell-matrix interactions[14]. Moreover, collagen I expression and the corresponding integrins was decreased[14], while Rho-associated coiled-coil protein kinase1/2 (ROCK1/2), a downstream molecule that modulates the stabilization of actin filaments by phosphorylating LIMK, was up-regulated in A-TSPCs[72]. Recently, another study illustrated an apparent increase in cell stiffness in aged TSPCs, which was associated with an increase in the activation of ROCK and a satisfactory rejuvenating effect of ROCK inhibition with Y-27632, because A-TSPCs exhibited similar features to Y-TSPCs after the intervention[55]. Based on these findings, ROCK activity plays an essential role in TSPC ageing, primarily by regulating actin stress fibers and/or cell stiffness. Chen *et al*[63] detected an obvious decrease in miR-135a level in A-TSPCs through direct bind to the 3′-untranslated region of ROCK1 compared with Y-TSPCs. Overexpression of miR-135a inhibits cell senescence, increases proliferation, and enhances migration and tenogenic differentiation of Y-TSPCs, while the inhibition of miR-135a produces the opposite results in A-TSPCs. The effects of miR-135a on TSPCs were attributed to its interaction with the ROCK1 mRNA, which was confirmed by a series of functional studies. Overall, miR-135a-ROCK1 plays a crucial role in TSPC senescence. Han *et al*[54] showed a substantial decrease in the A-TSPC tenogenic differentiation capacity, along with a decrease in the expression of P16 and the senescence-associated β-gal with age. P16 overexpression was responsible for the decrease in the tenogenic differentiation capacity of young TSPCs, and an analysis of the underlying mechanism revealed that this effect was mediated by P16, which enhanced the expression of miR-217 and subsequently inhibited the production of its direct target EGR1. According to these studies, A P16-miR-217-EGR1 pathway modulates TSPC the tenogenic differentiation and senescence of TSPCs. In addition, the EphA4, EphB2 and EphB4 and ephrin ligand B1 (EFNB1) in A-TSPCs is decreased compared with Y-TSPCs, which accelerates the decrease in self-renewal, migration, and actin turnover in A-TSPCs caused by advanced age. Upon stimulation with recombinant EphA4-Fc and EphB2-Fc proteins, significant effects on the key downstream signaling pathways mediated by ephrin-EPN binding were observed, including the activation of the cellular kinases focal adhesion kinase (FAK), extracellular signal-regulated kinase (ERK), Akt, c-Jun N-terminal kinase (JNK), and P38 in A-TSPCs; however, stimulation with EphB4-Fc and EFNB1-Fc did not exert an obvious effect on kinase activity in A-TSPC. Moreover, following stimulation with EphA4-Fc, FAK and JNK activity increased in A-TSPCs and more importantly, ERK phosphorylation was reduced to levels similar to the

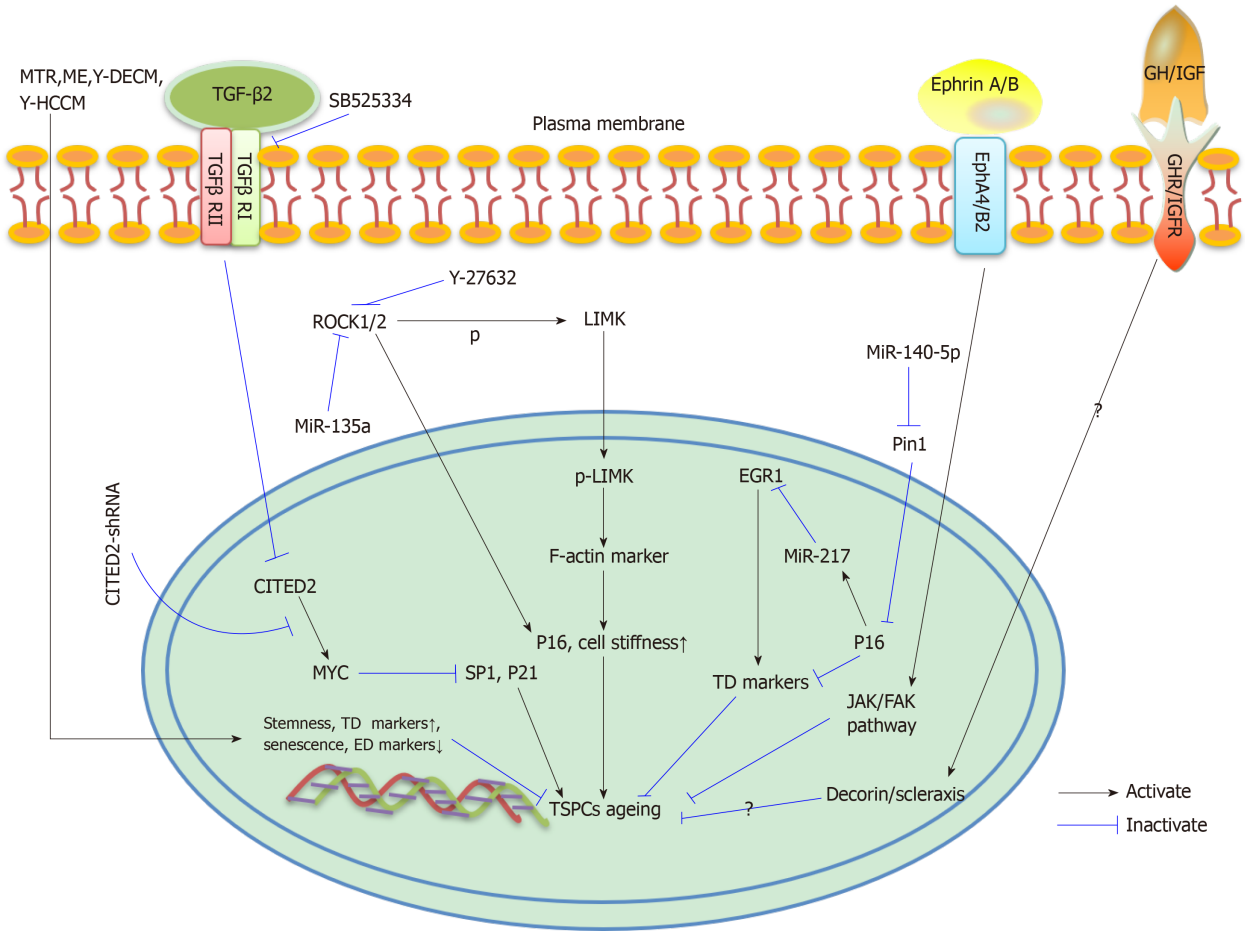


**Figure 1 Mechanisms involved in the tendon stem/progenitor cell ageing process and strategies aimed to rejuvenate the impaired features in aged cells.** TGFβ2 promotes the expression of CITED2. CITED2 up-regulates the expression of MYC, which inhibits the expression of SP1 and P21, revealing TGFβ2-CITED2-MYC-SP1/P21 pathway medicates cell senescence. And this pathway is delayed with the intervention of SB525334, which targets TGF-β2. Moreover, ROCK1/2 plays an important role in accelerating TSPC senescence and stiffness that can be delayed by the inhibition of Y-27632 on ROCK1/2 and miR-135a on ROCK1. MiR-140-5p reduces the expression of pin1 that downregulates the expression of P16 and ultimately delays TSPCs ageing. P16-miR-217-EGR1 pathway negatively modulates the cell tenogenic differentiation and senescence process. JAK/FAK pathways are involved in the modulation of Eprin A/B and EphA4 and EphB2 by affecting cell self-renew, migration and actin dynamics. GH/IGF-I pathway may participate in TSPCs ageing process by increasing the expression of decorin and scleraxis, resulting in delaying TSPCs ageing. Additionally, there are many cell external environment conditions, such as moderate treadmill running, moderate exercise, young decellularized extracellular matrix and young hypoxic-conditioned culture medium, can rejuvenate age-related alterations in aged-TSPCs. TD: Tenogenic differentiation; ED: Erroneous differentiation; MYC: Myelocytomatosis viral oncogene homolog; ROCK: Rho associated coiled-coil forming protein kinase; TGF-β2: Transforming growth factor-β2; TSPCs: Tendon stem/progenitor cells; IGF: Insulin-like growth factor; Pin1: Peptidyl-prolylcis-transiso merase NIMA-interacting1; miR: miRNA; CITED2: cAMP-responsive element-binding protein/p300-interacting transactivator with ED-rich tail 2; FAK: Focal adhesion kinase; P16/21: Passage 16/21.

levels detected in Y-TSPCs. Additionally, EphB2-Fc dramatically up-regulated the levels of phosphorylated JNK and P38 kinases in A-TSPCs, suggesting that EphA4 and EphB2 signaling overlap mainly in the activation of JNK; however, other ephrins showed evident differences in their abilities to activate ERK, FAK, and P38. EphA4, but not EphB2, restores the self-renewal deficit in A-TSPCs, and both EphA4 and EphB2 positively modulate the deficit in migration and increased actin dynamics. Thus, a decrease in EphA4 and EphB2 production with ageing contributes to the limited cell–cell interactions and decreased cell proliferation, motility and actin turnover that play essential roles in the human TSPC ageing process, changes that are potentially mediated by the abovementioned pathways[56]. Other studies revealed a correlation between ageing and a decrease in both plasma growth hormone (GH) and IGF-I levels[73], and the reduced activity of the GH/IGF-I axis in elderly might result in a lower collagen content[74]. Moreover, according to Holladay *et al*[40], IGF-1 affects TSPC proliferation and the maintenance of cellular phenotypes by increasing the levels of decorin and scleraxis; thus, other signaling pathways modulated by the GH/IGF-I pathway might participate in the TSPC ageing process[74].

## REJUVENATION OF AGED TENDON STEM/PROGENITOR CELLS

As a result of in-depth explorations of age-related changes in TSPCs during the cellular ageing process, scholars are now more likely to develop methods to reverse the deficits in TSPC function that result from advanced age. Numerous factors, including macroscopic factors associated with an the uncomfortable exercise intensity and microscopic factors associated with an impaired estrogen balance, deteriorated ECM conditions and inappropriate drug use, alter the features of TSPCs, particularly during ageing, and these alterations are mainly deleterious to TSPC function and the maintenance of tendon homeostasis. Furthermore, repair might be achieved by adjusting these factors, which have potential roles in the rejuvenation of aged TSPCs and are listed in Figure 1.

MTR has also been studied to determine the effects of motion on wound healing in aged tendons[75], resulting in faster healing and a better healing quality through the restoration of the TSPC pool, which is beneficial for delaying TSPC senescence, enhancing the production of collagen fibers and reversing the erroneous differentiation of TSPCs. This approach eventually reverses the histopathological alterations that observed in subjects with age-related tendon diseases[76-78]. Furthermore, the role of moderate exercise in the effects of ageing on TSPCs has been investigated. Moderate exercise ameliorates the depletion of the TSPC pool by up-regulating the expression of cell proliferation and stem cell markers coupled with decreased lipid deposition, proteoglycan accumulation and calcification formation, and it is beneficial for delaying the undesirable effects of age[15]. The impaired capacities of aged TSPCs were rejuvenated in a recent study by culturing cells with young decellularized extracellular matrix (DECM) because the young DECM increased the proliferation and tenogenic differentiation of aged TSPCs. Moreover, the expression of senescence-related marker in aged TSPCs was decreased and that of stem cell markers was increased after culture with young DECM, suggesting that the ECM is an important factor contributing to TSPCs ageing and the modulation of the ECM might be a promising anti-ageing approach[79]. Similar results were also obtained from young TSPCs cultured with HCCM, which restored the impaired function of aged TSPCs[60]. Pin 1 plays an important role in delaying the TSPC senescence process, which was confirmed by the decreased production of senescence markers and P16 and increased telomerase activity coupled with the opposite results following transfection with the Pin1-siRNA. Overexpression of Pin1 also effectively deferred late-stage TSPC senescence progression, but had no evident effect on the progression of early-stage cellular senescence, and miR-140-5p was involved in regulating of Pin1 production, leading to a substantial decrease in Pin1 expression. Thus, Pin1 might be an anti-senescence target in TSPCs, together with miR-140-5p[69]. Numerous studies have reported an important role for ROCK activity in the TSPCs ageing process, and after inhibition of ROCK, A-TSPCs re-established a phenotype and cell stiffness similar to Y-TSPCs[14,55]. Notably, miR-135a also has a crucial role in modulating TSPCs senescence by facilitating the proliferation, migration and tenogenic differentiation of these cells and decreasing the expression of senescence markers inhibiting target downstream molecules of ROCK1 activity[63], revealing that the blockade of ROCK activity is another promising strategy for combating TSPC ageing. A similar process is modulated by CITED2, providing an additional novel direction for fighting TSPC ageing[71]. Culture-expanded TSPCs (an *in vitro* ageing process) tend to exhibit a loss of phenotype, resulting in impaired function of TSPC, and Zhang *et al*[65] found that altered gene expression was related to the increased activity and expression of Hdac subtypes with passaging. Overall, these molecules and their functional states represent potential therapeutic targets for reversing age-related pathological changes in TSPCs.

As shown in the study by Popov *et al*[56], the ephrin receptors EphA4, EphB2 and EphB4 and ligand EFNB1 is decreased in A-TSPCs, and the down-regulation of EphA4 and EphB2 playes crucial roles in the age-associated reductions of the self-renewal, migration, and actin turnover in human TSPCs. Moreover, the activation of EphA4 or EphB2-dependent pathways reverses these harmful consequences, further revealing essential roles in preventing TSPC ageing. According to another study, ageing induces a progressive loss of activity of the GH/IGF-I axis, and the level of IGF-I decreases with age[73,80,81]. At the same time, IGF-1 promotes the proliferation and maintenance of TSPC phenotypes by increasing the expression of decorin and scleraxis[40], indicating that the altered fate of TSPCs is able to be reversed by modulating the relative expression levels of hormones. In addition, rapamycin slows ageing in mice[82,83], and metformin, pentosidine and multiflorum increase the lifespans of animals and humans[84-86]. However, the relationships between these drugs

and the mechanisms underlying the increase in lifespans are unknown due to the limited and insufficient number of studies conducted in this area[84-86]. Additionally, based on most recent development in regenerative medicine, Dale *et al*[87] induced human embryonic stem cells to differentiate into tendon-like cells in the presence of exogenous bone morphogenetic protein (BMP) 12 and BMP 13 and directed parthenogenetic stem cells to differentiate into tenocytes. Moreover, mechanical stretching improved the tenogenic differentiation of pMSCs[88]. Similar results were also obtained using iPSCs[89,90]. Thus, these cells may represent an exogenous supplementation to TSPCs or tenocytes, which is also an ideal way to method for rejuvenating ageing of tendons and provides alternative healing strategies for reversing tendon ageing in the future.

## CONCLUSION

As a result of advanced studies on tendons and the ageing of TSPCs, tendon ageing can be considered to be partially due to the aging of TSPC. TSPCs sustain regeneration at the site of tendon injury, and the loss of their function with advanced age causes aged-related tendon diseases. Although limited studies have been performed and the conclusions regarding the altered differentiation capacities and mechanisms involved are controversial, particularly regarding the erroneous differentiation, researchers generally agree that the cell number and tenogenic differentiation decrease with ageing, providing future directions for studies of TSPCs ageing. In particular, alterations in the ECM environment have been shown to re-establish the regenerative capacity of aged TSPCs, indicating that alterations in stem cell activity may be tractable for intervention, a hypothesis that is supported by the effects of alterations in cell-intrinsic pathways involved in TSPC ageing. Because humans are living longer, improvements in our understanding of the mechanistic networks underlying the age-associated in TSPCs and the tendon repair ability are critical for combating age-related tendon diseases.

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*REVIEW*

# Bioactive lipids in cancer stem cells

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## Abstract

Tumours are known to be a heterogeneous group of cells, which is why they are difficult to eradicate. One possible cause for this is the existence of slow-cycling cancer stem cells (CSCs) endowed with stem cell-like properties of self-renewal, which are responsible for resistance to chemotherapy and radiotherapy. In recent years, the role of lipid metabolism has garnered increasing attention in cancer. Specifically, the key roles of enzymes such as stearoyl-CoA desaturase-1 and 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase in CSCs, have gained particular interest. However, despite accumulating evidence on the role of proteins in controlling lipid metabolism, very little is known about the specific role played by lipid products in CSCs. This review highlights recent findings on the role of lipid metabolism in CSCs, focusing on the specific mechanism by which bioactive lipids regulate the fate of CSCs and their involvement in signal transduction pathways.

**Key words:** Cancer stem cells; Lipid metabolism; Bioactive lipids; ABC transporters



**Core tip:** Cancer stem cells (CSCs) are a minute portion of highly aggressive cells that survive conventional and targeted therapies and ultimately re-populate the tumour. Recent studies have elucidated that stearoyl-CoA desaturase-1 and 3-hydroxy-3-methyl-glutaryl-coenzyme A metabolic pathways involved in lipid metabolism are hyperactive in CSCs. However, the purpose of this enhanced activity is unclear. Here, we review the current literature and discuss the possible pathways and mechanisms that link the enhanced CSC lipid metabolism to bioactivity, specifically, with regard to structural lipids and active bio-molecules involved in cell signalling.

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## INTRODUCTION

Cancer progression is characterised by a continuous changeable state generating a very complex and heterogeneous multitude of cells with different morphology, genotype, and phenotype. This heterogeneity is explained by two main models: The clonal evolution model and the cancer stem cell (CSC) model. According to the CSC model, cancers are a heterogeneous combination of genetically different subclones that are arranged in an organised hierarchy, with CSCs at the apex[1,2]. According to the stem cell theory for cancer, only a subset of cancer cells are accountable for tumour initiation and propagation[3]. The primary functional characteristics of CSCs are similar to those of normal stem cells, such as the capacity to self-renew and the ability to differentiate into different cell types. CSCs present an elevated tumorigenic potential and an increased resistance to conventional and targeted therapy[3-8]. Functional recognition of CSCs from the mass of the tumour population involves the demonstration that they are indeed able to self-renew and differentiate[9-13]. These cells must possess the ability to initiate a novel tumour, often in small numbers. There is much dispute on the specificity of markers to be used to identify CSCs. However, the most reliable are functional markers such as ABC transporter activity, namely ABCG2 and ABCB1, which are able to transport the fluorescent dyes Hoechst 33342 and rhodamine 123, respectively[14]. Aldehyde dehydrogenase activity and the ability to cycle slowly are among other characteristics commonly accepted as defining features of CSCs[5,15-18]. The concept that suggests CSCs rely on oxidative phosphorylation (OXPHOS) is becoming more accepted as the metabolic signature of CSCs, making metabolic targeting a rewarding opportunity within the CSC field[5,6,19-27]. Recent studies have highlighted the link between CSCs and enhanced activity in lipid metabolism, particularly for monounsaturated fatty acids and cholesterol. Recent reviews have brilliantly described the role of lipid metabolism alterations in CSCs[28-30]. However, the purpose behind this enhanced activity is not understood. In this review we discuss the latest advances in CSC lipid metabolism and describe how this enhanced lipid metabolism in CSCs can lead to the production of active biolipids as signalling molecules.

### *CSC metabolism*

Similar to normal cells, CSCs use energy from mitochondrial OXPHOS, which produces more adenosine triphosphate (ATP) compared to glycolysis and produces tricarboxylic acid cycle intermediates utilised for macromolecule synthesis. CSC functions are regulated by a number of specific signalling pathways[31,32]. These pathways change in response to environmental stresses such as fluctuating oxygen and nutrient levels, pH, inflammation, and anticancer therapies[33]. While cancers rely on angiogenesis, the fast proliferation of cancer cells outstrips the blood supply, which is often leaky and lacks a normal hierarchical structure. Consequently, hypoxia and poor perfusion are common in tumours, so that there is a poor supply of nutrients and clearance of waste products. However, mitochondrial respiration is not impaired until the oxygen concentration drops below 1.0 μM[34]. Furthermore, it has been shown that even at oxygen levels of 0.5%, the electron transport chain is still capable of normal functioning[35]. It has also been reported that hypoxia is necessary for the preservation of embryonic stem cells in an undifferentiated state[36] and that it is accountable for the creation and maintenance of the stem cell niche[37-40,41,42]. These studies exemplify that hypoxia is a necessary condition for ensuring a balance between stem cell phenotypes and metabolism. In addition, it has been demonstrated that tumorigenesis is dependent on functioning mitochondria[5,43], since mitochondrial respiration results in the production of metabolites such as citrate, that can be utilised by ATP citrate lyase, to produce oxaloacetate and acetyl-CoA. In conditions where there are high levels of ATP, it has been shown that acetyl-CoA can be utilised for the regulation of protein acetylation and the synthesis of fatty acids[44]. These findings suggest a role for signalling molecules in the maintenance of the stem cell niche. A recent study demonstrated that glycosylation (specifically O-GlcNAc modification) of pluripotency markers sex-determining region Y-box 2 and octamer-binding transcription factor 4 takes place in undifferentiated mouse embryonic stem cells and

this is absent following differentiation[45]. Emerging evidence suggests that the metabolic phenotype of CSCs is dependent on their location, oxygen supply, and metastatic sites. There are studies suggesting that CSCs from lung, breast, glioblastoma, osteosarcoma, ovarian, nasopharyngeal, hepatocellular carcinoma, and colorectal cancers favour glycolysis compared to other differentiated cells *in vitro* and *in vivo*[46-52]. This variation may be due to differential location, availability of nutrients, oxygen, stage of lineage specification, tumour heterogeneity, and isolation techniques. It is possible to speculate that metabolic profiles of CSCs change as they migrate from the original site to the metastatic site and that this change is largely attributed to the tumour microenvironment in which they reside. While both glycolytic and mitochondrial metabolism are utilised by cancer cells, due to the heterogeneity among cancer cells within a tumour, some cells are reliant on glucose[53], while others have a strong dependence on aerobic glycolysis[54,55] due to an impaired TCA cycle or electron transport chain. However, due to the plasticity of cancer, some cells can alter their metabolic profile following therapeutic intervention by undertaking therapy-induced senescence[56]. Another impediment to cancer eradication is that slow-cycling CSCs demonstrate dependence on OXPHOS[5,7,57].

### Aldehyde dehydrogenase metabolism

It was recently found that the prominent CSC marker aldehyde dehydrogenase (ALDH)1A1 modulates energy metabolism in adipocytes from several species[58]. In this study, retinoic acid deficiency in knock-out ALDH1A1 adipocytes inhibited adipogenesis and increased thermogenesis. Functional CSC markers such as ALDH1A1 activity are increasingly highlighted as a reliable marker in the literature. ALDH1A1 activity requires the involvement of metabolic and signalling pathways. Retinoids play an important role in energy metabolism, and their role in maintaining normal embryonic development is well understood. In retinoid metabolism, retinaldehyde can be oxidised to retinoic acid by ALDH1a1-3. Retinoic acid is a potent transcriptional regulator and controls more than 500 genes. The receptors for retinoic acid (RAR-α, RAR-β, and RAR-γ) are members of the nuclear hormone receptor superfamily, which includes receptors for steroid and thyroid hormones. Upon activation, these receptors initiate cell responses related to proliferation, apoptosis, and differentiation. There is also some evidence that retinoic acid can regulate signalling pathways inside the cell and that all-trans-retinoic acid can bind peroxisome proliferator-activated receptor beta-gamma (PPAR β-γ). The enzymes are involved in several biological functions and their functional role is likely related to cellular detoxification and maintenance of low reactive oxygen species[15].

### Lipid metabolism

Lipid dysfunction has been observed as a trait of more aggressive cancers that have adverse survival outcomes. Research is highlighting the specific alterations occurring in pathways involving lipids and cholesterol. An emerging concept is that CSCs are highly dependent on enzymes associated with lipid metabolism, even though the precise reason for this reliance is not completely understood. Hyperactive metabolic routes that produce lipids and cholesterol, together with the increased uptake of exogenous lipids, are required by the tumour to enable proliferation. Lipids are not only substrates but can either provide structural scaffolds for proteins or be incorporated into the protein structure[59], which acts to stabilise signalling proteins to facilitate effective coupling between cellular receptors and signals[59,60]. Lipid metabolism may also be a crucial component in maintaining the cell membrane and protecting against peroxidation by chemotherapeutic agents or the hypoxic niche. It has been shown that the lipid bilayer leaflets have a non-symmetric distribution of lipids[61], and that this is dependent on several factors such as head group, chain length, and degree of saturation, all of which can affect the cell membrane's flexibility and construction[62,63]. Lipids such as steroid hormones or phosphoinositides can leave the cell and act as active signalling biomolecules in the tumour microenvironment. These molecules can act in an autocrine manner to initiate a signalling cascade that induces proliferation in neighbouring cancer cells[64,65].

### De novo lipogenesis

Fatty acid synthesis and oxidation are indispensable components in the maintenance of the adult stem cell and CSC populations from various organs (Figure 1). Both anabolic and catabolic pathways are closely controlled in CSCs and are essential for self-renewal activity. Peroxisome proliferator-activated receptor (PPAR-δ) is crucial for lipid metabolism and is implicated in the control of energy homeostasis. The loss of PPAR-δ results in defects to haematopoietic stem cells but its agonist restores the defect. Similarly, inhibition of mitochondrial fatty acid oxidation generates the disappearance of haematopoietic stem cells[66]. These results suggest that the PPAR

fatty acid oxidation axis may be essential for stem cell conservation. Several investigations have linked lipogenesis to CSCs. *De novo* lipogenesis is more active in glioblastoma multiforme CSCs compared to the bulk tumour population and is needed for stem cell renewal in breast cancer[67,68]. Blockage of fatty acid synthase (FASN) has been shown to diminish breast CSC growth *in vivo* and maintain breast cancer cells through the PPARγpathway by upregulating *de novo* lipogenesis[69]. FASN is overexpressed in patient-derived glioblastoma stem cells, and its inhibition significantly reduces the expression of stemness markers SOX2, NESTIN, CD133, and FABP7, as well as reducing the CSCs' invasiveness and sphere forming ability[67]. Pancreatic CSCs also have higher *de novo* lipogenesis activity where FASN is overexpressed, and the CSCs are more sensitive to inhibition by FASN specific inhibitors[70]. Breast CSCs have shown elevated levels of lipogenic genes compared to non-CSCs, such as ATP citrate lyase, acetyl CoA carboxylase 1 (ACC1), and FASN. Furthermore, ectopic expression of master regulator of lipogenesis sterol-regulatory binding protein-1 upregulates downstream lipogenic genes (ATP citrate lyase, ACC1, and FASN), resulting in enhanced lipogenesis and mammosphere formation[68]. Inhibition of ACC notably impairs mammosphere forming ability and the number of $ALDH1A1^+$ cells in culture[71].

### *Lipid droplets*

The co-culture of adipocytes with bone marrow-derived prostate cancer cells has demonstrated the ability of cancer cells to use lipids from adipocytes in their microenvironment in order to promote cancer growth[72]. When looking at stem cell components, both haematopoietic and leukemic-initiating cells depend on fatty acid oxidation. Elevated levels of lipid droplets have been observed in circulating tumour cells and are associated with more aggressive tumour types and poor survival outcomes. Increased extracellular lipid uptake contributes to lipid droplet accumulation and the tumour-initiating capacity in CSCs[73]. These lipid droplets can act as reservoirs inside the cell since they are filled with energy from various fatty acids, cholesterols, and triacylglycerol. An elevated content of lipid droplets is a distinctive feature of colorectal CSCs. There was a direct correlation between $CD133^+$ cells and lipid droplet amounts, and cells with an elevated level of lipid droplets have enhanced clonogenic potential *in vitro* and *in vivo*[74]. Lipophagy, a process that involves the fusion of lipid droplets with autophagosomes, confers resistance to pancreatic cancer cells through an increase in fatty acid β-oxidation[5]. The latest progresses in proteomics and metabolomics have highlighted the link between fatty acid oxidation and CSC fate[70,75,76]. For example, the homeobox protein NANOG stimulates hepatocellular carcinoma stem-like cells by reprogramming the metabolic state of cells from OXPHOS to fatty acid oxidation[52]. During lipophagy, free fatty acids are mobilised to the mitochondria, which confer survival to cancer cells when metabolic restrictions are induced[77,78]. Although lipid oxidation, lipid synthesis, and glucose metabolism are closely linked, the exact mechanisms underlying these interactions are not well understood. It is plausible to speculate that the lipid content of lipid droplets such as fatty acids, cholesterol, and triacylglycerol can be used to synthesise the cell membrane. These molecules can also be used to synthesise active signalling biomolecules or be exported out of the cell *via* exosomes to prepare the pre-metastatic niche.

### *Monounsaturated fatty acids/stearoyl-CoA desaturase 1 (SCD1)*

Lipid desaturation is important in maintaining stemness, tumour formation, and metastasis in breast, colon, and prostate cancers[79,80]. SCD1 is an enzymatic node central to the conversion of saturated fatty acids to mono-unsaturated fatty acids[81]. Monounsaturated fatty acids are precursors to a number of fundamental plasma membrane lipids such as triglycerides, cholesterol esters, and diacylglycerols[82]. More importantly, they can have signalling properties and act as direct effectors of SCD1 activity. In particular, palmitoleic acid has been found to mediate several processes such as enhanced oxygen consumption, fatty acid oxidation, and ATP content in adipocytes. As previously mentioned, lipids act as essential components of the cell wall, which contributes to signal transduction, migration, and metastatic potential[83,84]. Overexpression of SCDs promotes cancer cell proliferation and inhibits cell death[79,80,85]. Lipid unsaturation has been recognised as a biomarker for ovarian CSCs, and its blockage decreases tumour-forming abilities *in vivo*[76,85]. The same has also been observed in breast CSCs[85]. SCD1 inhibition hindered sphere-forming ability, along with a reduction in markers ALDH1A1, NANOG, and OCT4, and reverted chemoresistance in lung CSCs, while more differentiated cells were unaffected[86]. The presence of carbon-to-carbon single or double bonds can have both physical and chemical properties that are essential in the constitution of cell membranes and signal transduction. As previously mentioned, monounsaturated fatty acids are used as

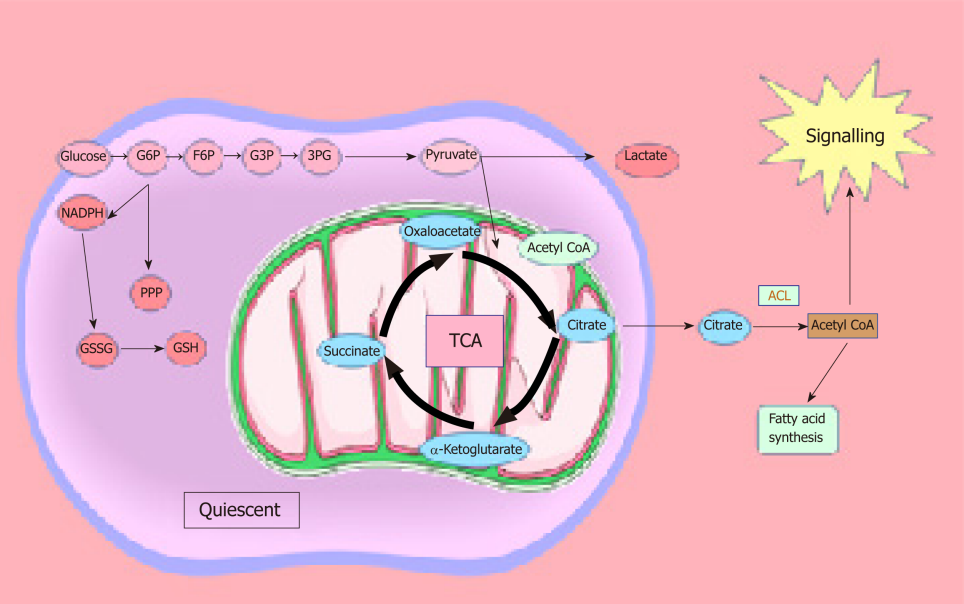


**Figure 1 Cancer cells use glucose-derived metabolites for biosynthesis to support uncontrolled cell proliferation.** Intermediates such as glucose-6-phosphate enter the pentose phosphate pathway and pyruvate is converted to lactate. Cancer stem cells are quiescent by contrast and use glucose-derived pyruvate for mitochondrial metabolism. The reason behind this metabolic shift is unclear. We propose that it is used for the synthesis of bioactive signalling molecules. TCA: Tricarboxylic acid cycle.

progenitors to a number of molecules, which can act as signalling molecules themselves or as substrates for other signalling molecules. For example, cholesterol esters can enter the mevalonate pathway to synthesise steroid hormones. Phosphoinositides can be converted into lysophosphoinositides. Both of these molecules are powerful bioactive lipids. Similarly, the cell membrane and all of its components such as lipid rafts, in which signalling receptors are embedded, cannot function properly without the proper distribution of triacylglycerides and diacylglycerides. Since CSCs are known for their metastatic potential and chemotherapy evasion, it is important to note that these lipid by-products can be involved in signal transduction for both migration and physical protection from peroxidation. These findings suggest that lipid desaturases may be the optimal targets for tumour prevention in a variety of cancers. Interestingly, recent data has shown that SCD-dependent fatty acid desaturation is not the only source of monoun-saturated fatty acids in cancer cells[87]. Indeed, it has identified a novel desaturation pathway, the sapienate biosynthesis, as an alternative source of monounsaturated fatty acids.

### *3-hydroxy-3-methyl-glutaryl-coenzyme A*

The mevalonate pathway is the metabolic pathway responsible for the formation of steroid hormones and cholesterol. This is a highly conserved pathway that involves a series of reactions including the rate-limiting step, catalysed by 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase, which converts HMG-CoA to mevalonate[88]. Mevalonate downstream products comprise cholesterol, geranylgeranyl pyrophosphate, farnesyl diphosphate synthase, and ubiquinone. The mevalonate metabolic route is important in protein prenylation, a post-translational modification that tethers the Ras and Rho family of GTPases to the membrane, which is required for the correct functioning of G protein-coupled receptors, and inhibition of the mevalonate pathway decreased sphere-forming ability in ALDH1A1$^+$ breast CSCs[89]. There is some controversy whether or not increased blood cholesterol is correlated with tumour incidence and mortality. The use of blood cholesterol-lowering statins is correlated with a reduced cancer incidence[90]. However, some reports have shown no correlation[91]. While pre-clinical and mechanistic studies generally support the use of statins for anticancer therapy, conflicting reports may be attributable to compensatory upregulation of HMG-CoA reductase by statins and the resulting dose-limiting toxicities[92]. Nevertheless, total cholesterol is a poor prognostic factor in several different cancers[93], and statin use is associated with reduced cancer-related mortality in cancer patients[94]. Recent studies have found that either blocking cholesterol synthesis or the HMG-CoA pathway exclusively eliminates stem cells of glioblastoma multiforme, colorectal, and lung cancers[95,96]. Further, a high-fat diet enhances *in vivo* tumour growth, which is supressed by statin treatment[97]. These

results strongly suggest that there exists an important and positive role of cholesterol in the biology of CSC functions. Pathways involved in both cholesterol biosynthesis and the synthesis of unsaturated fatty acids have been recently identified as the only selective druggable target in CSCs[98]. Interestingly, a recent study revealed that cholesterol biosynthesis is a key characteristic of breast CSCs and has a clear impact on patient outcome[99]. The findings of the latter study clearly identified the cholesterol biosynthesis pathway as crucial for CSC propagation and a therapeutic target. In addition, this study provides a mechanistic explanation for the beneficial therapeutic effect of the use of statins in breast cancer. Similarly, cholesterol biosynthesis has been found to be a crucial player in the tumorigenicity of human neuroblastoma cell lines and corresponding sphere-forming cells[100].

### *Lipid biomolecules in CSCs*

The majority of studies on lipid metabolism in CSCs have elucidated the enzymes and metabolic pathways involved in lipid synthesis. However, the precise functional role played by the different lipid molecules in CSCs remains unclear. Lipids play a central role in the cell-cell signalling process by maintaining the integrity of the cell membrane and by making lipid rafts, which act as platforms for signal receptors[62,63,101,102]. We can speculate that the hyperactive metabolic activity is used to synthesise lipids, which not only have a structural function by making up the cell membrane, but also have a more active role as bioactive-lipid signalling molecules. These active biomolecules can be released into the extracellular space and activate downstream pathways involved in proliferation, migration/invasion, and differentiation in an autocrine and/or paracrine manner. The latest studies have shown that the metabolism required to produce ATP is tightly regulated in CSCs, and this metabolic profile differs in the bulk of the tumour population[27,103]. CSCs are plastic in nature and change their metabolism as they are migrating from their origin to the metastatic site. They seem to have a preference for OXPHOS and show reduced metabolic plasticity when stressed. As soon as ATP levels reach a certain level, ATP-citrate lyase catalyses the transformation of citrate and CoA to acetyl-CoA and oxaloacetate, respectively. Acetyl-CoA can be converted to malonyl-CoA, which can enter the fatty acid synthesis route. Malonyl-CoA is utilised by AMP-activated kinase in order to regulate the synthesis of fatty acids, which in turn are utilised for the production of phosphoinositides, eicosanoids, lysophospholipids, and sphingolipids[44] (Figure 2).

Lysophospholipids, such as lysophosphatidic acid and sphingosine 1-phosphate, have a key role in stem cell biology[104] and tumour progression[105]. The plasma membrane contains lipid rafts enriched with sphingolipids, which are important participants in signal transmission[106-114]. A recent study of the pancreas highlighted the role of sphingosine-1-phosphate in promoting the survival of progenitor cells and determining acinar and endocrine cell specification[107]. The bioactive lysophospholipid lysophosphatidylinositol can be secreted into the extracellular milieu, initiating a signalling cascade that stimulates the proliferation of surrounding cancer cells[65]. The conversion of acetyl-CoA into acetoacetyl-CoA allows its entry into the mevalonate pathway[44], which is integral for the production of cholesterol esters and steroid hormones that are crucial participants in prostate stem cell maintenance and lineage specification[107-108]. Haematopoietic cells are reliant on phospholipids and essential fatty acids during differentiation[109]. Arachidonic acid is involved in the synthesis of leukotriene, prostacyclin, and thromboxane from phospholipids[109]. Eicosanoids' primary physiological activity is related to inflammation and modulation of cardiovascular function and tone. Leukotrienes and prostaglandins can create a leaky vascular endothelium, which is a requirement for metastatic spread[110]. Interleukin 1B was found to maintain malignant melanoma initiating cells[111,112]. CSCs are known for their increased ABC transporter activity, which requires ATP for its function. We recently proposed that, apart from their role in chemoresistance, ABC transporter hyperactivity is possibly due to their exportation of signalling molecules, including lipids[113]. Several studies have shown that at least one-third of all 48 mammalian ABC transporters are involved in lipid transport[59,63]. Transporters such as ABCA1, ABCG1, ABCG4, ABCG5, and ABCG8 have been identified as sterol transporters[114]. ABC transporters of the C family transport bioactive lysophospholipids such as lysophosphatidylinositol and sphingosine 1-phosphate[64,115,116]. Of particular interest are ABCG2 and ABCB1, the most well studied members in CSCs. We hypothesise that they may play a specific role in CSCs to maintain stemness and sustain cell survival; specifically, by exporting bioactive-lipid signalling molecules such as steroid hormones, cholesterol, and metabolites, which are the result of enhanced lipid uptake and lipid metabolic pathways observed in CSCs[63,64,113,117,118]. Another emerging processthrough which CSCs can also signal is through the release of exosomes. Exosomes are lipid vesicles released from the cell,

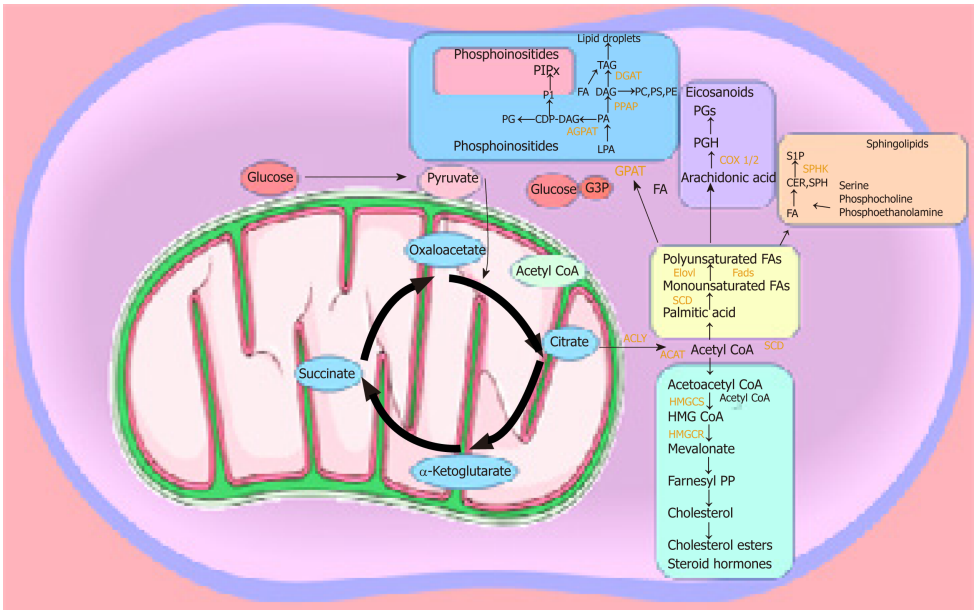


**Figure 2 Citrate produced through mitochondrial metabolism can enter the fatty acid synthesis pathway.** For example, citrate can enter the mevalonate pathway to produce steroid hormones and cholesterol esters, or it can go on to produce phosphoinositides and lysophospholipids. Both of these are powerful examples of signalling molecules. Therefore, the reason behind the enhanced metabolic activity, which was recently observed in cancer stem cells, must be understood.

which carry important messages including bioactive lipids or enzymes and are able to release signalling lipids. Exosomes are thought to be involved in specific cancer functions such as creating the pre-metastatic niche in the specific secondary site[119]. It is likely that enhanced lipid metabolism in CSCs is used to both synthesise exosomes and their content[120-122]. It would be interesting to analyse the lipidomic profile of CSC-derived exosomes to enhance our understanding of the specific role that exosomes play in cancer progression. Exploring these pathways could elucidate a vulnerability that might be beneficial in targeting these highly aggressive cells. However, first an understanding is needed of the mechanisms behind these metabolic pathways and what purpose they fulfil.

## CONCLUSION

In conclusion, lipid metabolism is emerging as a viable target in CSCs. In particular, the enhanced pathways involved in lipid metabolism, such as SCD1 and HMG-CoA activity. However, some questions still need further investigation, such as the purpose for this enhanced activity. We propose that lipid signalling molecules are synthesised as a result of enhanced metabolic activity and that CSCs use those signals for their survival advantage. Lipid metabolism represents an intriguing target for cancer therapy and we further suggest that to target CSCs, these pathways must be understood. The identification of the deregulated pathways is a good starting point to eradicate CSCs. However, increased knowledge of the role played by bioactive lipids will provide a novel opportunity to eliminate these highly aggressive cells.

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ORIGINAL ARTICLE

Basic Study

# Enhanced hepatic differentiation in the subpopulation of human amniotic stem cells under 3D multicellular microenvironment

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## Abstract

### *BACKGROUND*

To solve the problem of liver transplantation donor insufficiency, an alternative cell transplantation therapy was investigated. We focused on amniotic epithelial cells (AECs) as a cell source because, unlike induced pluripotent stem cells, they are cost-effective and non-tumorigenic. The utilization of AECs in regenerative medicine, however, is in its infancy. A general profile for AECs has not been comprehensively analyzed. Moreover, no hepatic differentiation protocol for AECs has yet been established. To this end, we independently compiled human AEC libraries, purified amniotic stem cells (ASCs), and co-cultured them with mesenchymal stem cells (MSCs) and human umbilical vein endothelial cell (HUVECs) in a 3D system which induces functional hepatic organoids.

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**Institutional review board statement:** All specimens and cells from the patients were obtained after their informed consent and ethical permission was obtained for participation in the study.

**Conflict-of-interest statement:** The authors report no relevant conflicts of interest.

**Data sharing statement:** Transcriptome datasets of primary amniotic epithelial cells from the Sequence Read Archive (SRA) of the NCBI are available in the website. SRA number is listed in Table 2. Participants gave informed consent for publication.



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***AIM***
To characterize AECs and generate functional hepatic organoids from ASCs and other somatic stem cells

***METHODS***
AECs, MSCs, and HUVECs were isolated from the placentae and umbilical cords of cesarean section patients. Amnion and primary AEC stemness characteristics and heterogeneity were analyzed by immunocytochemistry, Alkaline phosphatase (AP) staining, and flow cytometry. An adherent AEC subpopulation was selected and evaluated for ASC purification quality by a colony formation assay. AEC transcriptomes were compared with those for other hepatocytes cell sources by bioinformatics. The 2D and 3D culture were compared by relative gene expression using several differentiation protocols. ASCs, MSCs, and HUVECs were combined in a 3D co-culture system to generate hepatic organoids whose structure was compared with a 3D AEC sphere and whose function was elucidated by immunofluorescence imaging, periodic acid Schiff, and an indocyanine green (ICG) test.

***RESULTS***
AECs have certain stemness markers such as EPCAM, SSEA4, and E-cadherin. One AEC subpopulation was also either positive for AP staining or expressed the TRA-1-60 and TRA-1-81 stemness markers. Moreover, it could form colonies and its frequency was enhanced ten-fold in the adherent subpopulation after selective primary passage. Bioinformatics analysis of ribose nucleic acid sequencing revealed that the total AEC gene expression was distant from those of pluripotent stem cells and hepatocytes but some gene expression overlapped among these cells. *TJP1*, associated with epidermal growth factor receptor, and *MET*, associated with hepatocyte growth factor receptor, were upregulated and may be important for hepatic differentiation. In conventional flat culture, the cells turned unviable and did not readily differentiate into hepatocytes. In 3D culture, however, hepatic gene expression of the AEC sphere was elevated even under a two-step differentiation protocol. Furthermore, the organoids derived from the MSC and HUVEC co-culture showed 3D structure with polarity, hepatic-like glycogen storage, and ICG absorption/elimination.

***CONCLUSION***
Human amniotic epithelial cells are heterogeneous and certain subpopulations have high stemness. Under a 3D co-culture system, functional hepatic organoids were generated in a multicellular microenvironment.

**Key words:** 3D micropattern; Amniotic epithelial cells; Amniotic stem cells; Hepatic differentiation; Heterogeneity; Human placental tissue; Human umbilical vein endothelial cells; Mesenchymal stem cells; Multicellular microenvironment; Organoid



**Core tip:** Amniotic stem cells were exploited as a cell source alternative to liver transplantation therapy instead of induced pluripotent stem cells. However, they presented with low hepatic function efficiency. We used 3D co-culture and a combination of supportive somatic stem cells to simulate an *in vivo* microenvironment. Our selected subpopulation of adherent amniotic stem cells self-organized *ex vivo* and generated functional organoids. Cell selection methods and bioinformatics may help refine the differentiation protocol.

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## INTRODUCTION

Liver cirrhosis and liver failure are global problems. They are caused by viral infections, alcoholic- or non-alcoholic steatohepatitis, autoimmune hepatitis, metabolic and hereditary diseases, and others[1]. The only curative treatment is liver transplantation. However, there is a worldwide shortage of liver donors. Moreover, liver transplantation is associated with high mortality and morbidity and high-risk patients with comorbidity do not meet the indication criteria[2,3]. Cell transplantation has been proposed as an alternative therapy to whole organ transplantation. Several cells have been investigated as hepatic cell sources. Human donor-derived hepatocyte transplantation was attempted to cure cirrhosis and it did have some therapeutic benefit[4,5]. However, it required many hepatocytes and failed to solve the problem of donor insufficiency.

Embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) are known to differentiate into hepatocytes[6]. Although they have a high potential for hepatic differentiation, there are ethical, tumorigenicity, and cost issues associated with them. Previous reports indicated that somatic cells such as fibroblasts were induced to differentiate into hepatocyte-like cells by direct reprogramming[7,8]. In this case, virus-mediated overexpression of lineage-specific transcription factors was needed. Other cell types include mesenchymal stem cells (MSCs), hematopoietic stem cells (HSCs), menstrual blood-derived stem cells, and amniotic stem cells (ASCs)[5,9]. Somatic stem cells require only differentiation factors but no gene editing. The latter may cause undesirable and unexpected side effects. Here, we attempted to induce ASCs to differentiate into hepatocytes because they have several beneficial characteristics.

Amniotic epithelial cells (AECs) are easily isolated from amniotic membranes after delivery. This process causes no harm to the donor. Embryologically, the amnion is derived from the epiblast which can differentiate into three germ layers. Even at full term pregnancy, this differentiation potential persists in ASCs which are an immature subpopulation of AECs[10]. AECs also have immune tolerance and are therefore suitable for allogenic transplantation[11,12]. Furthermore, they have certain features, in common with hepatocytes such as the expression of *ALB, AAT*, or *CYP3A4*[13].

Previous studies attempted to differentiate AECs into hepatocyte-like cells in 2D culture[14,15]. However, no differentiation protocol was established. Without systemic and comprehensive analyses in vivo or in vitro, AEC quality could not be verified and it may have been unsatisfactory and inferior to ESCs or iPSCs[5]. Therefore, its functional activity would be insufficient for clinical use when a higher level of hepatic function was required. Moreover, as the general AEC profile had not yet been elucidated, it was difficult to determine which differentiation protocol was appropriate for AECs. Therefore, we endeavored to clarify AEC stemness characteristics, heterogeneity, and general profile by using transcriptomes. We also tried to select an adherent subpopulation to purify ASCs.

A 3D culture system may promote and enhance stem cell differentiation potential in co-culture with endothelial, mesenchymal, and other cells[16,17]. Here, we combined 3D culture conditions and co-culture with other somatic stem cells and supportive stroma cells. We attempted to simulate an *in vivo*-like microenvironment using a selected subpopulation of adherent ASCs for *ex-vivo* self-organization and ultimately obtained functional organoids.

## MATERIALS AND METHODS

### *Isolation of somatic stem cells*

**AECs:** Human placenta was acquired from the University of Tsukuba Hospital with approval from the institutional review board (IRV code: H27-58). All samples were collected from patients who had provided informed consent. Emergent operation cases were excluded. The amniotic membrane was peeled off the placenta in the operating room immediately after birth. After washing in pre-digestion buffer (Hank's balanced salt solution, HBSS; Wako Pure Chemical Industries Ltd., Osaka, Japan) with 0.02% egtazic acid (EGTA; Wako Pure Chemical Industries Ltd., Osaka, Japan), the membrane was incubated in 0.05% trypsin-EDTA (Thermo Fisher Scientific, Waltham, MA, United States) at 37 °C for 20-30 min. HBSS with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Waltham, MA, United States) was added and gentle agitation followed to detach the epithelial cells from the membrane. The cells were incubated with 0.05% trypsin-EDTA and washed again to collect the AECs. A 100-μm strainer (Merck EMD, Darmstadt, Germany) was used to remove a clot from the cell suspension and isolate primary AECs. Viability was checked with 0.4% trypan blue dye (Dojindo Laboratories, Kumamoto, Japan) and found to be > 90%.

**Human umbilical vein endothelial cells (HUVECs):** After the umbilical cords were harvested, the umbilical veins were filled with collagenase buffer (HBSS with 0.24% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; Merck EMD, Darmstadt, Germany), 0.005% trypsin inhibitor (Wako Pure Chemical Industries Ltd., Osaka, Japan), and 0.05% collagenase (Worthington Biochemical Corporation, Lakewood, NJ, United States). The veins were incubated at 37 °C for 15 min. The buffer was centrifuged at 450 × g and 4 °C for 5 min to obtain a cell pellet containing HUVECs. This pellet was seeded to a plate using an EGM Bullet Kit (Lonza, Basel, Switzerland). In most cases, the HUVECs grew to confluence within 4 d.

**MSC:** After HUVEC collection, Wharton's jelly was minced and seeded to a plate with Dulbecco's modified Eagle's medium and 10% FBS. Attached MSCs were isolated and in most cases, they grew to confluence within 2 wk.

### Cell culture

**2D culture:** For the AECs, a custom culture medium was blended and named AEC basal medium (AECBM). The media and protocols are listed in Supplementary Figure 1. Primary AECs were used in most analyses. In the culture experiments, however, AECs were precultured with AECBM for 5-7 d then used for differentiation. In the present study, the multistep hepatic differentiation protocol of Nie *et al*[17] was applied. In that study, iPSCs were differentiated into hepatocytes. The AEC culture medium was changed step by step within 3 wk.

Another simple differentiation protocol from AECs to hepatocytes was reported by Maymó *et al*[14]. In brief, the cells were cultured for 1 wk in Iscove's modified Dulbecco's medium containing epidermal growth factor (EGF) then cultured with EGF and dexamethasone. This method was named the two-step protocol.

**3D culture:** A primary 2D culture was prepared to select a subpopulation of adherent AECs. Unattached cells were removed after 72 h. A 24-well 3D micropattern culture plate (Kuraray, Tokyo, Japan) was used for the next step. After coating the plate with lipidure (NOF Corporation, Tokyo, Japan), the precultured AECs were seeded at a density of $10^6$/well. The AECs formed a sphere within 3-7 d. AECs, HUVECs, and MSCs were used for organoid propagation and they formed spheres within 1 d.

### qRT-PCR

The primers used are listed in Table 1. The TaqMan PCR protocol from Integrated DNA Technologies, Inc. (Coralville, IA, United States) was used. In brief, the RNA was extracted from the cultured cells with Isogen (Nippon Gene, Tokyo, Japan). Then the isolated RNA was converted to cDNA with a Revert Aid RT kit (Thermo Fisher Scientific, Waltham, MA, United States). The cDNA, primers, probes, reference dye, and PrimeTime Gene Expression Master Mix (Integrated DNA Technologies, San Jose, CA, United States) containing hot-start DNA polymerase, dNTPs, enhancers, and stabilizers were combined. A 7500 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, United States) ran the PCR.

### Immunofluorescence analysis

Amnion and organoids were stained with following antibodies: Primary; SSEA4, TRA-1-60, TRA-1-81 (Cell Signaling Technology, Danvers, MA, United States), EPCAM (BD, Franklin Lakes, NJ, United States), E-cadherin (BD, Franklin Lakes, NJ, United States). Frozen sample sections were fixed with 50% acetone (Wako Pure Chemical Industries Ltd., Osaka, Japan) and 50% methanol (Wako Pure Chemical Industries Ltd., Osaka, Japan) for 30 min.

The samples were incubated with primary antibody at 4 °C overnight, washed 3 ×, and incubated with a fluorescence-conjugated secondary antibody for 1 h at 20-25 °C. Before each antibody incubation, blocking was performed with 10% normal goat serum (NGS; Merck EMD, Darmstadt, Germany). In the last step, the samples were mounted with 4',6-diamidino-2-phenylindole (DAPI; Dojindo Laboratories, Kumamoto, Japan) and photographed under an All-in-One Fluorescence Microscope (BZ-X710; Keyence, Osaka, Japan) or a confocal microscope (Leica TCS SP6; Leica Microsystems, Wetzlar, Germany).

For live amnion staining, the amnion was incubated with anti-TRA-1-60 antibody for 30 min at 37 °C and then with Hoechst (Dojindo Laboratories, Kumamoto, Japan) for 15 min at 37 °C. Pictures were obtained with an Inverted System Microscope IX71 (Olympus, Tokyo, Japan).

### Flow cytometry

The following antibodies and DAPI were used: SSEA4-FITC (BioLegend, San Diego, CA, United States), TRA-1-81-PE (Thermo Fisher Scientific, Waltham, MA, United States), TRA-1-60-PE (Thermo Fisher Scientific, Waltham, MA, United States), E-

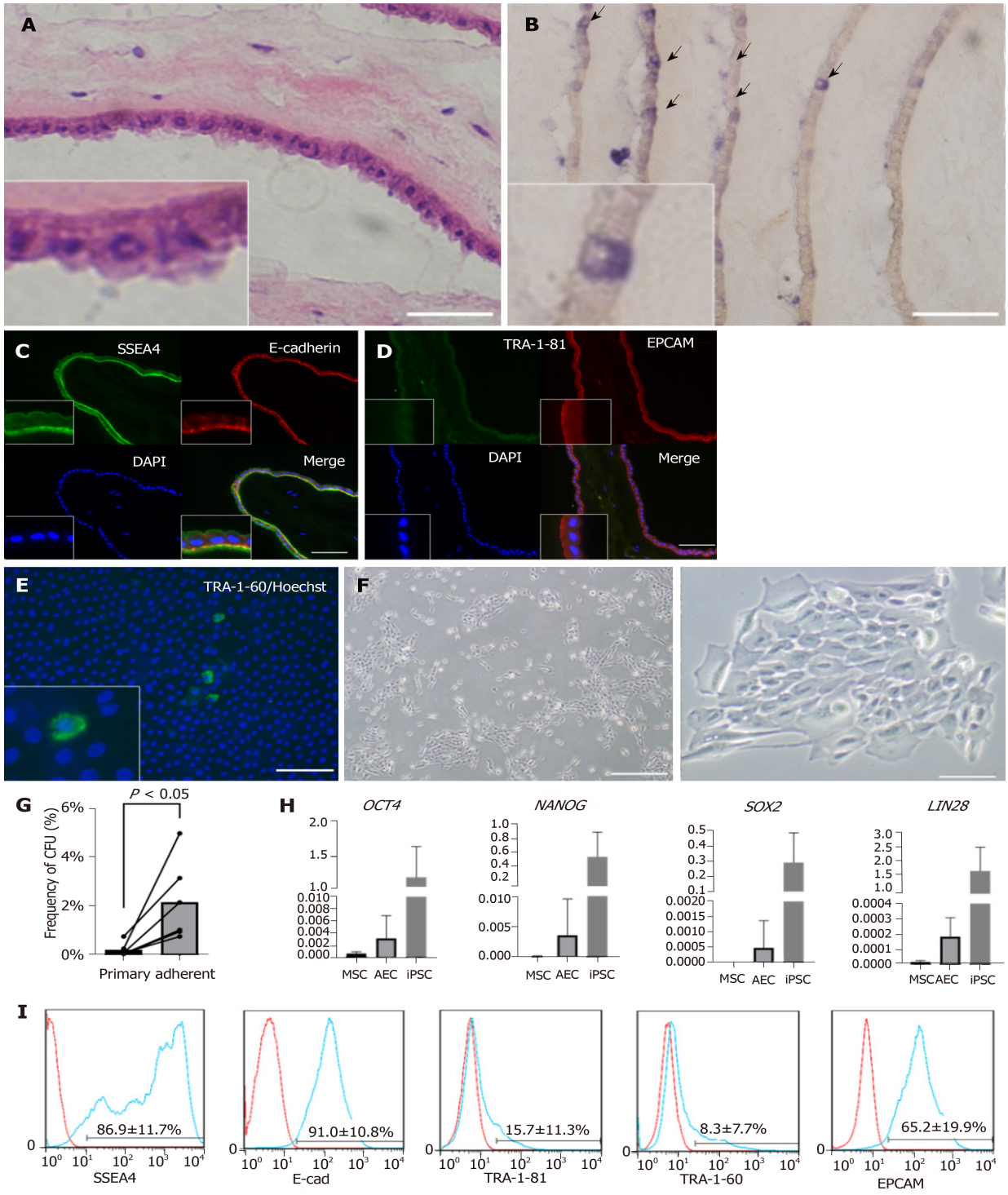


**Figure 1 Characteristics of human amniotic membrane and amniotic epithelial cells.** A: H and E staining of amniotic membrane. Bar, 50 μm; B: AP staining of amniotic membrane. Positive cells are indicated with arrows. Bar, 100 μm. In A and B, the amniotic membrane was rolled before embedding. Therefore, many layers can be seen in one picture; C: Immunofluorescent staining of frozen section of amniotic membrane. Anti-SSEA4 antibody (green), E-cadherin antibody (red), and DAPI were used; D: Same as C. Anti-TRA-1-81 antibody (green), anti-EPCAM antibody (red), and DAPI were used; E: Direct tissue staining of amniotic membrane. Anti-TRA-1-60 antibody (green) and DAPI were used. Bars in C, D, and E represent 100 μm; F: Colonies formed from cultured amniotic epithelial cells (AECs) and observed by phase-contrast microscopy. Bar to left of F represents 200 μm. Bar to right of F represents 500 μm; G: Frequency of colony formation from primary AECs and adherent AECs. Cells which did not attach to the well surface were removed to purify the amniotic stem cells; H: Gene expression of primary AECs, mesenchymal stem cells(MSCs), and induced pluripotent stem cells(iPSCs) detected by qRT-PCR; I: surface markers of primary AECs verified by flow cytometry.

cadherin-Alexa Flour 647 (BD, Franklin Lakes, NJ, United States), and EPCAM-APC (BD, Franklin Lakes, NJ, United States). The cells were incubated with antibody on ice for 30 min then washed twice. The cells were analyzed in FACS Jazz (BD, Franklin Lakes, NJ, United States) and FlowJo v. 7.6.1 (FlowJo LCC, Ashland, CO, United

**Table 1 List of primers**

| Name | | Primer sequence |
|---|---|---|
| *SOX2* | F | TGCACCGCTACGACGTGA |
| | R | GGAGCCAAGAGCCATGCC |
| *NANOG* | F | TGCTGAGATGCCTCACACG |
| | R | TGCAGAAGTGGGTTGTTTGC |
| *OCT4* | F | GAAACCCACACTGCAGCAG |
| | R | GACCCAGCAGCCTCAAAATC |
| *LIN28* | F | GGATGTCTTTGTGCACCAGAGTA |
| | R | TGGATTCCAGACCCTTGGCT |
| *ALB* | F | TGCCTGTTGCCAAAGCTCG |
| | R | GCTACTGCCCATGCTTTGAAAG |
| *AFP* | F | GCAAACGATGAAGCAAGAGTTTC |
| | R | GCAGCATTTCTCCAACAGGC |
| *AAT* | F | ACTGGAACCTATGATCTGAAGAGC |
| | R | GCCTTATGCACGGCCTTGG |
| *CYP3A4* | F | GGATGAAAGAAAGTCGCCTCGA |
| | R | TCCAGATCGGACAGAGCTTTG |
| *ACTB* | F | CCTCGCCTTTGCCGATCC |
| | R | CATGCCGGAGCCGTTGT |

States).

### Colony formation assay

Primary and adherent AEC subpopulations were compared in a colony formation assay. To collect the adherent AEC subpopulation, a selective primary passage was used. This process was the same as the preculture step for the 3D culture, namely, nonattached cells were removed after 72 h culture. Primary and adherent AECs were seeded in a six-well plate at a density of 3000-20000 cells/well with AECBM. After 6 d of incubation, the numbers of colonies containing > 20 cells were counted and traced by scanning under an All-in-One Fluorescence Microscope (BZ-X710). The number of colonies was divided by the seeding number to calculate the colony formation frequency.

### Whole-transcriptome shotgun sequencing and bioinformatics analysis

Total RNA was extracted from primary AECs and RNA sequencing was performed by Eurofins Genomics K.K. (Tokyo, Japan) who used the Illumina HiSeq 2500 (Illumina, San Diego, CA, United States). Gene expression data for the other cell sources were obtained from the National Center for Biotechnology Information Sequence Read Archive (SRA) database (https://www.ncbi.nlm.nih.gov/sra). Data sources are listed in Table 2.

RNA Sequencing. All paired-end reads were trimmed and quality-filtered via CLC Genomics Workbench v. 10.1.1 (Qiagen, Hilden, Germany) which was also used to map the filtered reads against the human reference sequence (hg 19). Mapped reads were counted and transcript abundance was measured in TPM (transcripts per kilobase million) units.

Bioinformatics for RNA Sequencing Data. An original read count matrix was normalized with DESeq2 v. 3.5.2 for unified expression level. All normalized log-transformed expression values were ordered according to the median absolute deviation. The top 3500 genes were selected for a heatmap which was generated with the pheatmap package in R.

### ICG test

AEC spheres or multiple stem cell-derived organoids were incubated with AECGM and 1 mg/mL indocyanine green (ICG) (Daiichi Sankyo, Tokyo, Japan) for 30 min at 37 °C. They were then observed under bright field with an Inverted System Microscope IX71 to confirm ICG absorption. They were then incubated with ICG-free AECGM for 6 h and checked for ICG elimination.

### Statistical analysis

GraphPad Prism v. 8.0.1 (GraphPad Software, La Jolla, CA, United States) was used to

**Table 2 RNAseq data sources used in the bioinformatics analysis**

| Sample name | SRA number | Instrument | Memo | Ref. |
|---|---|---|---|---|
| AEC-1 | SRR9643783 | Illumina HiSeq 2500 | Primary AEC | |
| AEC-2 | SRR9643784 | Illumina HiSeq 2500 | Primary AEC | |
| MSC-1 | SRR6431450 | Illumina HiSeq 2000 | | [44] |
| MSC-2 | SRR6431451 | Illumina HiSeq 2000 | | [44] |
| hESC-1 | SRR4241924 | Illumina HiSeq 4000 | H9 | [45] |
| hESC-2 | SRR4241926 | Illumina HiSeq 4000 | H9 | [45] |
| NiPS-1 | SRR7592168 | Illumina HiSeq 2500 | Normal human iPSC | [46] |
| NiPS-2 | SRR7592169 | Illumina HiSeq 2500 | Normal human iPSC | [46] |
| DE-1 | SRR771468 | Illumina HiSeq 2000 | Definitive endoderm induced from H9 | [47] |
| DE-2 | SRR771469 | Illumina HiSeq 2000 | Definitive endoderm induced from H9 | [47] |
| hiHep-1 | SRR5974291 | Illumina HiSeq 2000 | Umbilical cord fibroblast derived hepatocyte-like like cell | [8] |
| hiHep-2 | SRR5974292 | Illumina HiSeq 2000 | Umbilical cord fibroblast derived hepatocyte-like cell | [8] |
| iPSCHLC-1 | SRR5974295 | Illumina HiSeq 2500 | iPSC-derived Hepatocyte-like cell | [8] |
| iPSCHLC-2 | SRR5974296 | Illumina HiSeq 2500 | iPSC-derived hepatocyte-like cell | [8] |
| Hepa-1 | SRR6176953 | Illumina HiSeq 2500 | Hepatocyte from clinical sample of adult | [48] |
| Hepa-2 | SRR6176948 | Illumina HiSeq 2500 | Hepatocyte from clinical sample of adult | [48] |
| Hepa-3 | SRR5974298 | Illumina HiSeq 2000 | Primary human hepatocyte 2 d | [8] |
| Hepa-4 | SRR5974299 | Illumina HiSeq 2000 | Primary human hepatocyte 4 d | [8] |

process all data. A Wilcoxon signed-rank test compared means between pairs of groups. $P < 0.05$ was considered statistically significant.

## RESULTS

### *Characterization of amnion-derived stem cells*

The amnion consisted of monolayer epithelium and mesenchyme (Figure 1A). We first examined the stemness characteristics of the amnion. Using an AP staining kit (Muto Pure Chemicals, Tokyo, Japan), we found that a small proportion of the epithelial cells were blue (Figure 1B). AP is often used as a multipotency marker. Immunofluorescence analysis of frozen amnion sections (Figures 1C and 1D) indicated that its epithelium was positive for the stemness markers SSEA4, E-cadherin, and EPCAM. However, other stemness markers such as TRA-1-60 (not shown) and TRA-1-81 were not detected. Previous studies[18,19] reported that a small subpopulation of AEC expresses TRA-1-60 or TRA-1-81. We attempted live staining of fresh amnion and found a low frequency of TRA-1-60-positive cells (Figure 1E). We concluded that amniotic epithelium has certain stemness or multipotency markers and only a subpopulation of its cells expresses stemness.

The AEC isolation method yielded 1-6 × $10^7$ AECs with > 90% viability. We then examined AEC morphology, colony formation capability, and stemness markers. We seeded AECs into a plate and observed them by phase contrast microscopy (Figure 1F). Although only a few primary AECs attached to the plate as short, spindle-like cells within 1 d, they proliferated well and formed colonies. After the primary selective passage, the colony formation capacity increased ten-fold relative to that for the heterogenous primary AECs (Figure 1G). We also verified the gene expression of stemness markers by qRT-PCR. *OCT4*, *NANOG*, *SOX2*, and *LIN28*, which are important iPSC markers, were expressed at very low levels in AECs compared with those in iPSCs but at higher levels than those in MSCs (Figure 1H). We also analyzed the stemness surface markers by flow cytometry (Figure 1I). E-cadherin and EPCAM expression was highly positive. Certain SSEA4s were highly positive while others were only weakly positive. Only 10% of the TRA-1-81 and TRA-1-60 were positive. Based on the foregoing information, we concluded that amnion and AECs have stemness and amniotic stem cells widely express stemness markers.

### *Hepatic potentials and profiling by whole-transcriptome shotgun sequencing*

According to previous reports, AECs can differentiate into hepatocytes[20,21]. Here, we performed comprehensive profiling by whole-transcriptome shotgun sequencing to verify this potential in AECs. We also compared the AEC RNA sequences with those

of pluripotent stem cells, hepatocytes or induced hepatocytes, and MSCs. MSCs are also often used as hepatocyte cell sources[9]. First, we normalized the data and checked its repeatability using Pearson's correlation coefficient (Supplementary Figures 2A and 2B). A principal component analysis (PCA) and a general heatmap (Figures 2A and 2B) indicated that AECs and MSCs differ across the spectrum ranging from pluripotent stem cells to hepatocytes. Even human fibroblast-derived hepatocyte-like cells (hiHep) and iPSC-derived hepatocyte-like cells (iPSCHLCs), which had immature hepatic characteristics[8], did not closely resemble hepatocytes. An unexpected finding was that AECs were like hiHep and iPSCHLCs. We gathered 40 stem cell- and 66 hepatic cell markers to make a heatmap (Figure 2C). CD9 and TJP1 were highly expressed in both AECs and ESCs. *IGF2BP2* and *KRT19* were highly expressed in both AECs and the definitive endoderm. The hepatic markers *MET, ABCC2, CYP1A1*, *etc*. were highly expressed in AECs. The markers expressed in AECs are listed in Table 3. The features common to AEC, pluripotent stem cells, and hepatocytes may help elucidate the differentiation mechanism.

### Hepatic differential induction with a 3D culture system

To induce hepatic differentiation, we raised AECs under a hepatic differentiation protocol and a 3D culture system (Figure 3A). First, we tested the hepatic differentiation protocol in 2D culture. We attempted a two-step differentiation protocol using AECBM as a negative control. In the 2D culture, the AECs were short-lived. Nearly all the cells were detached from the culture plate within 3 wk. Moreover, gene expression did not markedly increase during the culture period (Supplementary Figure 3).

We then tried 3D culture with three protocols including the two-step, multistep, and AECBM. With a 3D culture system in a micropattern plate, the seeded cells readily aggregated into a sphere and remained viable for 3 wk. We then checked hepatic gene expression by qRT-PCR. Relative to the multistep and AECBM protocols, hepatic gene expression in the two-step group protocol was higher (Figure 3B and Supplementary Figure 4). *ALB* and *CYP3A4* were upregulated and sustained for 3 wk. Although *AFP* was upregulated in the 2D preculture then downregulated after 7 d, it gradually increased again within the 3-wk differentiation period. *AAT* was also upregulated over 14 d but was downregulated by 21 d. When we seeded the AEC sphere in a culture plate, we observed active AEC proliferation (Figure 3C). Based on the preceding data, we concluded that our 3D cultural system maintains AEC viability for > 3 wk and accelerates hepatic differentiation especially when a two-step protocol is used.

### Hepatic function enhancement with multicellular organoids

We then attempted co-culture by seeding AEC with MSC and HUVEC to form a multicellular sphere organoid. We examined its morphology, cellular distribution, and functional activity. Hematoxylin and eosin (H and E) staining disclosed that the AEC sphere and the organoid were morphologically similar (Figure 4A). However, immunofluorescence with CD31, CD90, SSEA4, and DAPI revealed inner-layer CD31-positive HUVECs (data not shown) and CD90-positive MSCs as well as outer-layer SSEA4-positive AECs (Figure 4B). This architecture resembles normal tissue disposition. Periodic acid Schiff staining showed glycogen accumulation in the center of the organoid (Figure 4C). Both the AEC sphere and the organoid absorbed ICG but only the organoid could excrete it (Figure 4D). From the aforementioned results, we concluded that AECs, MSCs, and HUVECs underwent 3D construction in our 3D co-culture system. Moreover, the organoid resembled a liver bud with complex liver function.

## DISCUSSION

### AEC characteristics and stemness

AECs proliferate well and express stemness markers[20]. They also readily differentiate into hepatocyte-like cells in the presence of certain growth factors[14]. Miki *et al*[20] reported that AECs are derived from epiblasts and some of them remain immature then differentiate into three germ layers[18,20,22]. Amnion immunofluorescence disclosed that only a small proportion of AECs are TRA-1-81- and/or TRA-1-60-positive[19]. Here, it was confirmed by flow cytometry that a small primary AEC subpopulation is TRA-1-60- and/or TRA-1-81-positive and the proportions vary among samples. Izumi *et al*[18] reported that this subpopulation frequency is higher in early-stage than full-term pregnancies. However, all AECs used in the present study were derived from term pregnancies. Moreover, certain AEC lots with comparatively higher proportions

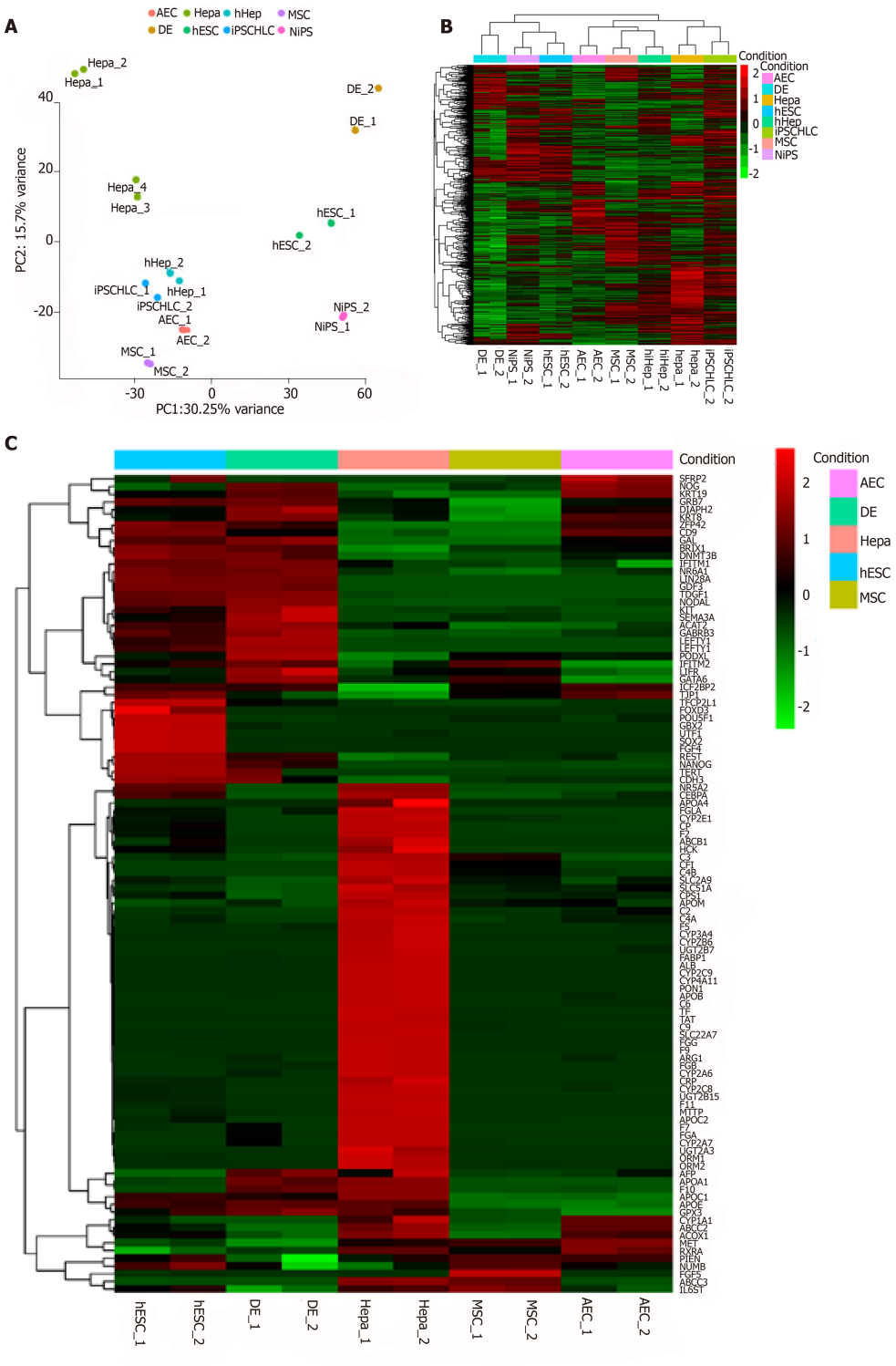


**Figure 2 Characteristics of primary amniotic epithelial cells and other cell sources verified by bioinformatics.** A: Principal component analysis of all cell sources used in this assay; B: General heatmap for each cell source; C: Specific heatmap of stemness and hepatic markers. AEC: Amniotic epithelial cell; Hepa: Hepatocyte; hiHep: Human fibroblast-derived hepatocyte-like cell; MSC: Mesenchymal stem cell; DE: Definitive endoderm; hESC: Human embryonic stem cell; iPSCHLC, iPSC-derived hepatocyte-like cell; NiPS: Normal human iPSC.

of these stem markers had high colony formation frequencies and grew well. For this reason, it is important to select good AEC lots for differentiation.

Previous studies reported that primary AECs could be classified into three different culture layers, namely, floating, attached, or intermediate[20]. In the present study, we found that adherent cells derived from selective passage of primary AECs had a comparatively high colony formation capability. Based on the foregoing information, adherent subpopulations of passaged AECs were used in the 3D culture. Differentiation efficiency could be enhanced by purifying this high-stemness

**Table 3 Stemness (upper)- and hepatic (lower) genes expressed in amniotic epithelial cells.**

| Gene symbol | Explanation | Ref. |
|---|---|---|
| *CD9* | CD9 belongs to the transmembrane 4 superfamily. It is associated with cell proliferation, motility, and adhesion and regulates hematopoietic differentiation | [49] |
| *TJP1* | Tight junction protein 1 is a determinant of plasma cell proteasome and is associated with EGFR, JAK1, and STAT3. It is regulated by TGF-b and involved with cell motility | [30,50] |
| *IGF2BP2* | This member of the insulin–like growth factor 2 mRNA-binding protein family participates in normal embryonic growth and development. It is expressed in the pancreas and associated with type 2 diabetes mellitus | [51] |
| *KRT19* | Keratin 19 is a cytoplasmic intermediate filament protein and belongs to the type 1 keratin family. It is used as a cholangiocyte marker and is not expressed in hepatocytes. It is associated with the progression of several cancers | [31,32] |
| *GRB7* | Growth factor receptor-bound protein 7 mediates signal transduction and cell migration. It is associated with the metastasis of several cancers | [52] |
| *KRT8* | Keratin 8 is a cytoplasmic intermediate filament protein of the type 2 keratin family. It is expressed in single layered epithelial cells. In cancer cells, it is associated with progression in the form of migration and adhesion | [33] |
| **Gene symbol** | **Explanation** | **Ref.** |
| *ApoM* | Apolipoprotein M (apoM) belongs to the lipocalin family. It is expressed in the liver and kidney. Hepatic apoM controls HDL metabolism | [53,54] |
| *CPS1* | Carbamoyl phosphate synthase 1 is a mitochondrial enzyme and participates in the first step of the urea cycle in the liver. | [55] |
| *SLC51A* | Organic solute transporter subunit alpha is a bile acid transporter in the liver, small intestine, and kidney. It prevents the bile acid reflux | [56] |
| *IL6ST* | Interleukin 6 signal transducer controls IL-6 and other cytokines such as IL-11, IL-27, oncostatin M, and leukemia inhibitory factor | [57] |
| *ACOX1* | Acyl-CoA oxidase 1 is a rate-limiting enzyme in fatty acid β-oxidation | [58] |
| *RXRa* | The nuclear hormone receptor retinoid X receptor belongs to the steroid hormone receptor family. It is a key factor of cholesterol synthesis | [59] |
| *MET* | MET encodes the hepatocyte growth factor (HGF) receptor and the key factor of hepatic regeneration. It activates epithelial migration and 3D morphogenesis | [35,36] |
| *ABCC2* | ATP binding cassette subfamily C member 2 is expressed in the hepatocytes and is a biliary transporter. It is also related to drug elimination and multidrug resistance in several cancers | [60] |
| *CYP1A1* | This member of the cytochrome P450 enzyme superfamily participates in fatty acid and steroid metabolism. It is associated with the detoxification of anthropogenic chemicals such as polycyclic aromatic hydrocarbons | [61] |

subpopulation of primary AECs.

### *Hepatic differentiation protocol*

The iPSC-derived organoids have often been considered as models representing organs with highly complex structures and/or functions such as the intestine, lung, neural tissue, *etc*[23-25]. Cell-to-cell interactions[26] and hypoxic reactions were key factors in differentiation[27]. In the case of hepatic differentiation from iPSCs, mesenchymal

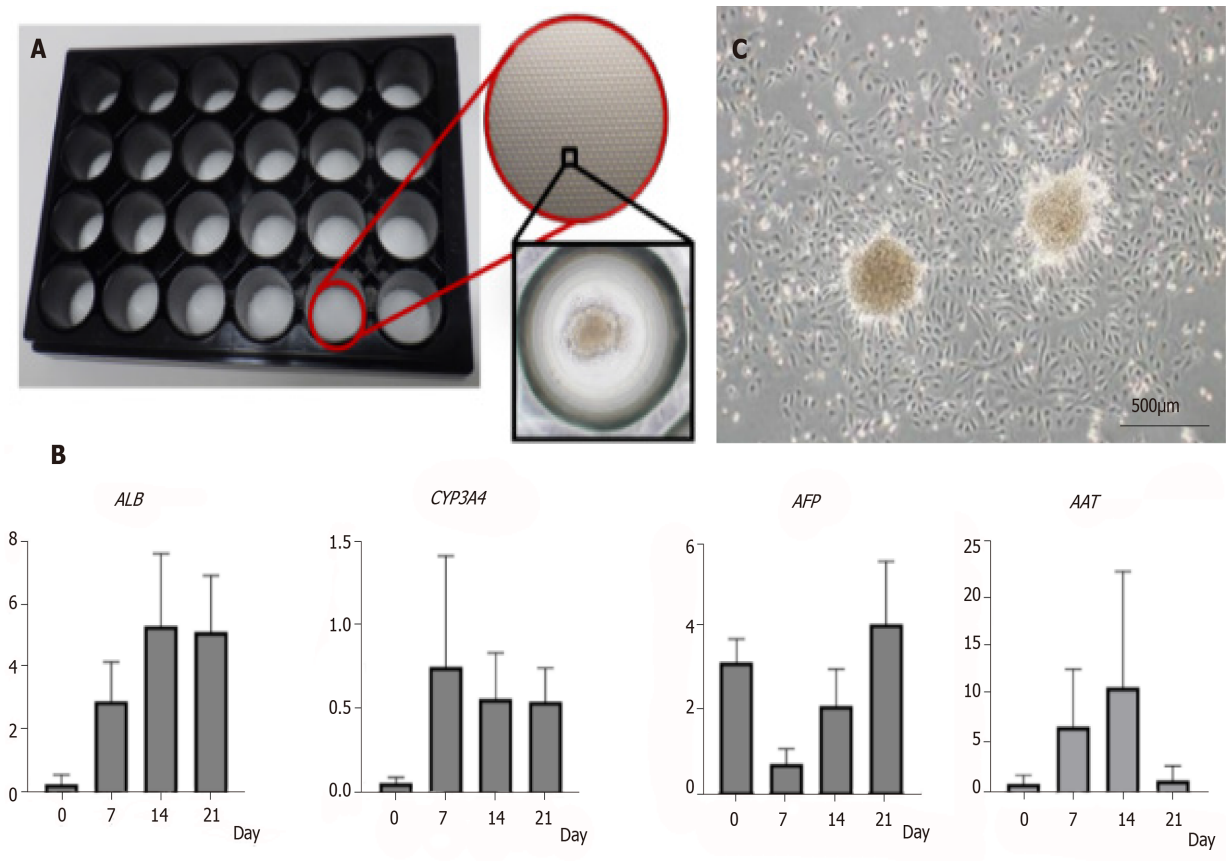


**Figure 3 Characteristics of amniotic epithelial cell spheres formed on 3D-micropattern plate.** A: 3D-micropattern plate used in the present study. Round pits 500 μm in diameter are clustered on the surface. After culture, the amniotic epithelial cells (AECs) formed a sphere; B: Gene expression in the AEC sphere verified by qRT-PCR; C: After reseeding AEC sphere onto 2D culture dish, AEC proliferation was verified by phase-contrast microscopy. Bar, 500 μm.

and endothelial cells were reported as important factors[16]. However, only 2D culture was used in the attempt to differentiate AECs into hepatocytes[14,28] and the protocol used was not comparable to that applied in the present study.

Therefore, we compared 2D and 3D culture with several differentiation protocols. The 2D culture method was unsuitable for AECs because the cells did not remain viable in it. In 3D culture, AECs retain their viability and morphology for 21 d. A previous report stated for an iPSCs' hepatic differentiation model in a 3D culture system that hypoxia suppresses cell proliferation and induces differentiation[26,27]. In the present study, AEC sphere proliferation was temporarily arrested during 3D culture then resumed after plate seeding. Therefore, a similar mechanism was assumed to occur under our AEC 3D culture conditions.

To date, no hepatic differentiation protocol has been established for AECs. In certain studies, an iPSC-to-hepatocyte differentiation protocol was used[15,29]. However, this stepwise protocol is complex and requires numerous growth factors. Since AECs are not pluripotent stem cells, it is uncertain whether every protocol step would be absolutely necessary. One report mentioned that activin A is not required for hepatic differentiation from AECs[15]. Activin A is required for the transformation of pluripotent stem cells to the definitive endodermal state. In contrast, the AEC-to-hepatocyte differentiation protocol is comparatively simple[14]. Our results indicated that this straightforward differentiation protocol upregulates AEC hepatic genes. Nevertheless, it may still be inadequate and must, therefore, be modified. Despite this deficiency, the co-culture method can nonetheless generate hepatic function from AECs. We concluded that a 3D co-culture system or a hepatic microenvironment is needed for hepatic differentiation from AECs.

### *Bioinformatics approach*

Bioinformatics is a strong tool for the evaluation of cellular differentiation. It is often applied to iPSCs but not to AECs. Our PCA and general heatmap revealed that hiHep[8] and iPSCHLCs[8] remotely resemble mature hepatocytes. In fact, they are regarded as immature hepatocytes. AECs and MSCs are distant from iPSCs, ESCs, and DE but more closely resemble hiHep and iPSCHLCs. Therefore, AECs may be

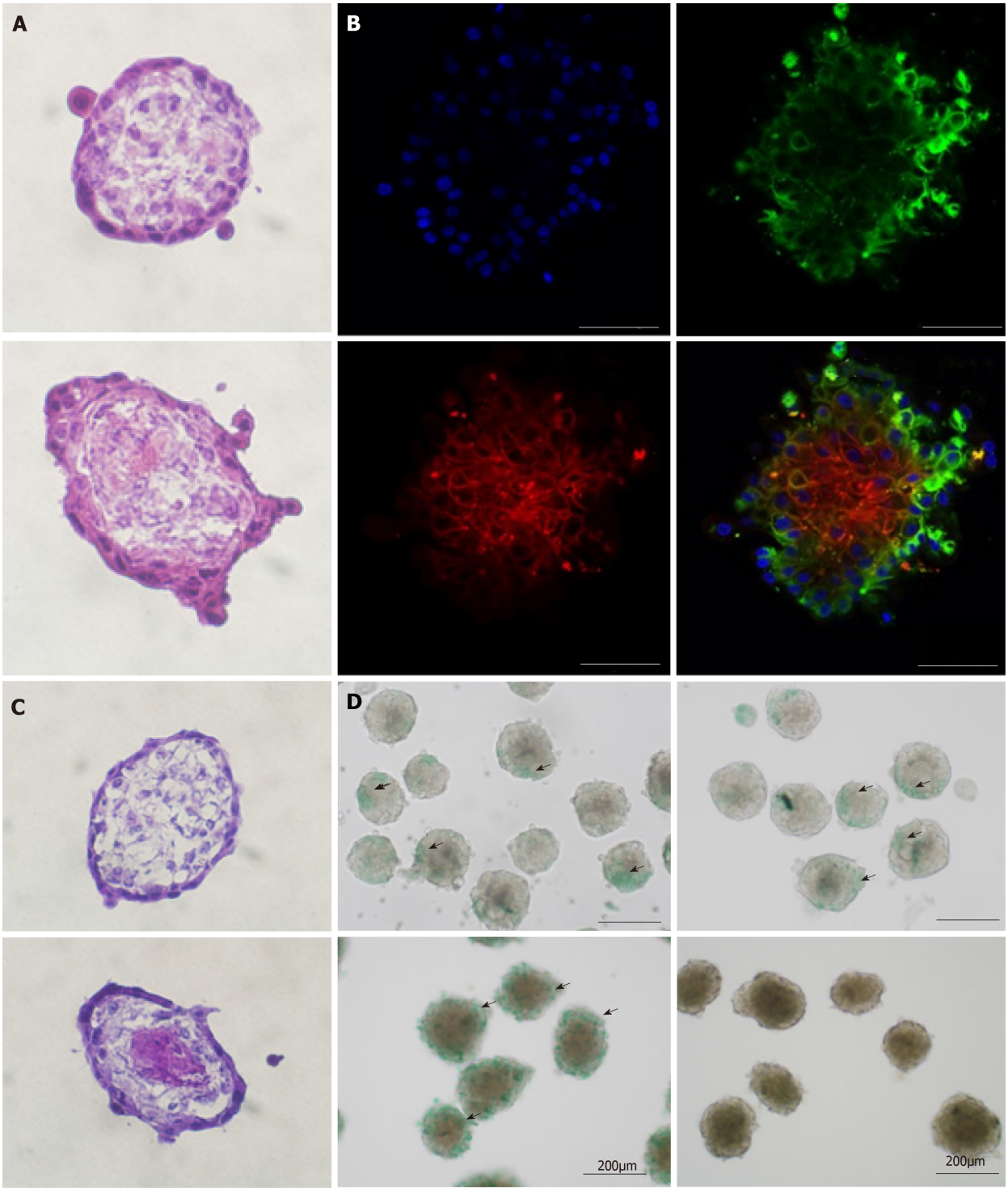


**Figure 4 Organoid morphology and hepatic function.** A and C: Frozen sections of amniotic epithelial cell (AEC) sphere and organoid. The AEC sphere was placed in the upper layer and the organoid was placed in the lower layer. H&E staining is used in A, and Periodic acid Schiff staining is used in C; B: Immunofluorescent organoid staining observed under confocal microscopy. Anti-SSEA4 antibody (green) representing AECs; anti-CD90 antibody (red) representing mesenchymal stem cells and DAPI. Bars in A, B, and C, 50 μm; D: ICG tests on AEC sphere and organoid. The AEC sphere was placed in the upper layer and the organoid was placed in the lower layer.

able to differentiate into hepatocytes in only a few steps.

The stemness and hepatic marker heatmaps disclosed certain genes common to all AECs. *TJP1* is associated with EGFR/JAK1/STAT3 signaling[30]. Every two-step, multistep, and AECBM protocol included EGF and seemed reasonable. *KRT8* and *KRT19* are epidermal markers[31-33] and are also upregulated in AECs. In the hepatic transdifferentiation from fibroblasts, the first step is to transform mesenchymal cells into epidermal cells[34]. In the differentiation of ESC to DE, *KRT8* and *KRT19* are upregulated. Therefore, differentiation of AECs to hepatocytes may require fewer

steps than pluripotent stem cell-to-hepatocyte differentiation.

The hepatic marker *MET* was upregulated in AECs. *MET* encodes the human growth factor (HGF) receptor which is important for hepatocyte proliferation or differentiation[35,36]. HGF may already be sensitive in AECs. In addition, *IL6ST* is upregulated in AECs. It transduces the signals of several chemicals containing oncostatin M. HGF and oncostatin M are normally added to iPSCs in the hepatocyte-like cell protocol but not in our two-step protocol[6]. Therefore, we may be able to improve differentiation by adding HGF or oncostatin M to our two-step protocol. Moreover, other markers of hepatic metabolism may also enhance liver function. A detailed list of the stemness and hepatic genes is presented in Table 3 A bioinformatics approach may help elucidate the differentiation mechanism and improve the protocol used for it.

### Future prospects

An iPSC-derived hepatic organoid has already been created and used in basic research *ex vivo*[17]. However, iPSCs are costly, unstable, and tumorigenic[37,38]. In contrast, our organoid is cost-effective, stable, and non-tumorigenic. AEC organoids with hepatic character could be used both in basic *ex vivo* research and *in vivo* experiments.

The next step of our research is to develop and test a human-to-animal transplantation model with AEC-derived hepatic organoids. According to a previous report, terminal differentiation to a functional hepatocyte would be strongly induced in the recipient liver microenvironment[15]. Even in our organoids simulated the microenvironment, the process would not be as perfect as it would be *in vivo*. Therefore, *in vitro* tests of hepatic organoid function may be imperfect. Nevertheless, we must confirm hepatic function after transplantation. The present study demonstrated that the 3D culture condition temporarily suppressed proliferation but did not entirely cause a loss of this capability. Therefore, proliferation could still occur in vivo with the appropriate signal. Human organoids and animal transplantation models are required for pharmaceutical research and investigations into human-specific infectious disease such as hepatitis B and C[17,39].

A future intended application of our hepatic organoid is as an alternative to organ transplantation as a therapy for hepatic failure. More than one induction step from somatic cells to iPSCs is required and quality and safety must be verified[40,41]. On the other hand, AECs are directly isolated from tissues so they are comparatively safe and reliable.

Safety is of paramount importance in the clinical application of organoids. In this respect, AECs are superior to iPSCs. The safest transplantation model is a private placental and cord blood cell bank. Preserved blood cells can be used as a replacement therapy for hematopoietic disease and the regenerative treatment of congenital heart diseases and other developmental disorders[42,43]. Amnion and umbilical cords also have the same genetic profile as the baby. Therefore, private AEC, MSC, and HUVEC cell banks could be ideal sources of therapeutic materials for infants with developmental conditions.

In conclusion, we selected adherent AECs in which ASCs were purified as a cell source for differentiation into hepatocytes. The 3D- and co-culture systems with MSCs and HUVECs generated multicellular organoids with hepatic function. A bioinformatics approach and cell selection strategy would be useful for further improvement of the protocol and its hepatic function.

## ARTICLE HIGHLIGHTS

### Research background

Cell transplantation is a promising method to solve the problem of organ donor deficiency. Recently, amniotic epithelial cells (AECs) have been studied as a somatic stem cell source for regenerative medicine. It has been reported that AECs possess hepatic differentiation capability and they are more cost-effective and non-tumorigenic compared to other cell types. Therefore, AECs could provide a new cell source for cell transplantation in the future, particularly for liver diseases.

### Research motivation

However, the general profile for AECs has not been comprehensively analyzed and hepatic differentiation protocol for AECs has also not been established. Therefore, by clarifying the comprehensive characteristics of AECs and refining the hepatic differentiation protocol, we could use it effectively as a transplantation cell source in the future.

### Research objectives

This study aimed to elucidate the comprehensive characteristics of human AECs, and to

establish a novel hepatic differentiation protocol. Additionally, 3D multicellular culture condition and purification of stem cells obtained from AECs were studied, with an intention to improve their differentiation capability.

### Research methods

All examined AECs, mesenchymal stem cells (MSCs), and human umbilical vein endothelial cells (HUVECs) were isolated from the placentae and umbilical cords after cesarean section. Stemness characteristics and heterogeneity of amnion and primary AECs were analyzed by immunofluorescence and AP staining, and flow cytometry. In addition, AEC transcriptomes were analyzed and compared with those for other cell sources based on bioinformatics. An adherent AEC subpopulation was selected from primary isolated AECs and their amniotic stem cell (ASC) purification quality was evaluated based on a colony formation assay. Hepatic differentiation capacities of AECs which were cultured in varying 2D or 3D conditions were compared according to their relative gene expression. Finally, ASCs, MSCs and HUVECs were co-cultured in a 3D system to generate hepatic organoids, and the organoid structure and hepatic function were compared with those of 3D AEC spheres using immunofluorescence imaging, Periodic acid Schiff staining, and an indocyanine green (ICG) test.

### Research results

AECs expressed stemness markers such as EPCAM, SSEA4, and E-cadherin, whereas only limited cells in the AEC subpopulation were AP-positive, or expressed TRA-1-60 and TRA-1-81 stemness marker in the flow cytometry. The colony formation assay revealed that primary AECs could form colonies and the frequency was enhanced ten-fold in the adherent subpopulation. According to bioinformatics analysis of RNA sequencing, the primary AEC gene expression was different from those of pluripotent stem cells and hepatocytes; however, some overlapped genes were detected. Compared with the 2D system, AECs could retain their viability for a longer time in 3D culture conditions and the hepatic gene expressions were comparatively elevated using a two-step differentiation protocol. Furthermore, organoids derived from 3D multicellular culture condition using ASCs, MSCs and HUVECs, showed a 3D hepatic structure with polarity, hepatic-like glycogen storage, and ICG absorption/elimination.

### Research conclusions

Human AECs are heterogeneous and certain subpopulations exhibit high stemness. AEC transcriptome analysis suggests some advantages and factors related to hepatic differentiation. 3D multicellular culture conditions improve the differentiation of ASCs into functional hepatic organoids.

### Research perspectives

The results of this comprehensive analysis indicate that AECs have high hepatic differentiation capability which should be optimized. By optimizing the selected high stemness subpopulations of AECs and using a 3D co-culture system, AEC-derived hepatic organoid can be used for performing transplantation experiments to evaluate its *in vivo* function.

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