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Correlation between amino acid metabolism and self-renewal of cancer stem cells: Perspectives in cancer therapy

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Abstract

Cancer stem cells (CSCs) possess self-renewal and differentiation potential, which may be related to recurrence, metastasis, and radiochemotherapy resistance during tumor treatment. Understanding the mechanisms *via* which CSCs maintain self-renewal may reveal new therapeutic targets for attenuating CSC resistance and extending patient life-span. Recent studies have shown that amino acid metabolism plays an important role in maintaining the self-renewal of CSCs and is involved in regulating their tumorigenicity characteristics. This review summarizes the relationship between CSCs and amino acid metabolism, and discusses the possible mechanisms by which amino acid metabolism regulates CSC characteristics particularly self-renewal, survival and stemness. The ultimate goal is to identify new targets and research directions for elimination of CSCs.

Key Words: Amino acid metabolism; Cancer stem cell; Self-renewal; Resistance

Core Tip: Amino acid metabolism plays an important role in maintaining the stemness of cancer stem cells (CSCs) and is involved in regulating their self-renewal and differentiation potential. This review summarizes the relationship between CSCs and amino acid metabolism and discusses possible mechanisms *via* which amino acid metabolism regulates the self-renewal and differentiation potential of CSCs. The ultimate goal is to identify new targets and research directions for elimination of CSCs.

INTRODUCTION

The concept of cancer stem cells (CSCs) has emerged in recent years. CSCs are a population of self-renewing cell types identified in many types of liquid and solid tumors, and persist predominantly in a low pH, low O_2, and nutrient-deficient tumor microenvironment (TME)[1,2]. CSCs possess the capability to initiate cancer development, recurrence and metastasis[1-4], and play important roles in radio-, chemo- and immunotherapy resistance[5,6]. The TME is a dynamic milieu comprising of cancer cells and stromal cells[7-9] and provides specific conditions favorable to tumor growth such as low pH, hypoxia, ischemia, and limited nutrients[7]. TME regulates the morphology of cancer cells, induces tumor cell activation and CSC production, mediates immunosuppression, and determines tumor response to treatment[2,7,10-12]. The CSC niche is a part of the TME, in which perivascular, invasive, and hypoxic niches are involved in the generation and maintenance of CSCs[13,14]. CSCs also rebuild the microenvironment by transdifferentiation into vascular endothelial cells, fibroblasts, and pericytes[7,14]. CSCs obtain nutrients from TME to support their proliferation[15]. Owing to the increased interest in CSCs, the role of metabolism in the regulation of CSC biology is now being extensively investigated.

Amino acids are indispensable nutrients for the body and play important roles in TME[16]. The human body contains twenty amino acids, which are divided as essential and nonessential. Among these, eight essential amino acids are obtained from food, as they cannot be synthesized by the body or their rate of synthesis cannot meet the body requirements[17]. Although nonessential amino acids can be synthesized by the body, they are equally or more important than essential amino acids for cancer progression[18]. Arginine and histidine are semi-essential amino acids as their organic synthesis is not sufficient for metabolic requirements, and hence they have to be obtained from the environment[19]. However, some researchers consider cysteine and tyrosine as semi-essential amino acids as well because they can be converted from methionine and phenylalanine *in vivo*; thus, conversion and food intake can complement each other[20,21]. In this review we have described these four amino acids as nonessential amino acids.

In addition to being the building blocks of proteins, amino acids participate in many biosynthetic pathways as intermediate metabolites[22]. Previously, researchers have studied the relationship between tumors and amino acid metabolism. In tumor cells, nonessential amino acids may act as essential amino acids to meet the requirement of abnormal proliferation[23]. For example, glutamine is considered to be a "conditional" essential amino acid[24], and therefore, it has been proposed that amino acid metabolism-related enzymes may be used to disrupt amino acid metabolism in targeted therapy[25]. Whether CSCs also harbor similar therapeutic targets warrants detailed investigation, but the exact relationship between CSCs and amino acid metabolism is not completely elucidated. This review tries to summarize the relationship between CSC self-renewal and other characteristics and amino acid metabolism to provide new targets for cancer therapy.

TUMORIGENICITY CHARACTERISTICS OF CSCs AND ESSENTIAL AMINO ACID META-BOLISM

In recent years, researchers have focused on the differences in amino acid metabolism between CSCs and tumor cells, in which essential amino acids play a major role. Several studies have investigated the relationship between methionine and tryptophan metabolism and CSCs; however, studies on phenylalanine metabolism are lacking, and those on the metabolism of the other five essential amino acids are limited. In this review, we have attempted to summarize the role of metabolism of these amino acids in CSC self-renewal.

Methionine metabolism

The methionine cycle maintains the balance of methionine levels *in vivo*. Homocysteine, an intermediate of the methionine cycle, regenerates methionine and tetrahydrofolate (THF) with one-carbon THF (1C-THF) catalyzed by methyltransferase (MTase), while methionine reenters the methionine cycle. THF acts as a carrier in the transfer and utilization of 1C unit, which is crucial for biosynthesis of nucleic acids, DNA stability, and gene expression[26,27]. As conversion of homocysteine to methionine is folic acid-dependent, content of folic acid affects the tumorsphere-forming ability, nucleotide biosynthesis, and DNA methylation in colon cancer cells and glioblastoma cells[27-29]. Cancer cells consume higher amount of methionine than normal cells in some malignant tumors[30,31]; hence, methionine and its derivatives may be labeled with radionuclides in clinics for identification of malignant recurrent glioma, meningioma, as well as prostate cancer and multiple myeloma[28,32-34]. The methionine cycle is enhanced in CSCs of various cancers, such as lung, breast cancer, osteosarcoma, and brain tumor, owing to their disordered proliferation and higher rate of DNA biosynthesis[28,35,36]. As the concentration of methionine increases, the glioblastoma tumorsphere formation ability that supports the self-renewal capacity of CSCs increases; while methionine deprivation not only promotes embryonic stem cell (ESC) differentiation but also weakens clonal formation and tumorigenicity of lung and breast cancer tumorsphere cells, which can be rescued by the re-addition of methionine[28,36]. It was further found

that betaine, synthesized from choline, provides methyl group to homocysteine under the action of betaine homocysteine MTas; this in turn leads to the recycling of methionine[29,37,38]. Stem cell reprogramming requires methionine metabolism and the choline/betaine axis to jointly regulate intracellular homocysteine, abnormality in which causes oxidative stress, mitochondrial toxicity, and inflammation[29].

S-adenosine methionine (SAM) is a crucial intermediate of the methionine cycle, which acts as a direct donor of the methyl group and is involved in genome methylation *in vivo*[28,39,40]. In gastric CSCs, higher methylation of miR-7-5p promoter region reduces its intracellular expression, while in methionine-deficient medium, miR-7-5p expression is up-regulated and inhibits the formation of gastric CSCs by targeting Notch and Hedgehog signaling pathways[41]. Nuclear reprogramming is usually accompanied by an increase in DNA methylation level in ESCs[29]. Methionine adenosyltransferase (MAT) catalyzes the production of SAM and has two isozymes, MATα1 and MATα2[42]. MATα1 is mainly expressed in hepatocytes, while MATα2 is present in extrahepatic tissue[42,43]. MATα2, which maintains the epigenome of CSCs, is a key enzyme involved in the SAM generation in lung, breast, and liver CSCs[36,39,44]. The inhibition of tumorsphere formation and genome methylation by MATα2 inhibitors FIDAS-5 and cycloleucine demonstrates that methionine circulation is necessary to maintain CSC self-renewal and tumorigenicity[36,39]. Another study found that sirtuin 1 (SIRT1), a NAD⁺-dependent protein deacetylase, regulated methionine metabolism and histone methylation by regulating MYC-mediated MAT expression in mouse ESCs (mESC)[45,46]. Nicotinamide N-methyl-transferase (NNMT) catalyzes the transfer of methyl groups from SAM to nicotinamide and is overexpressed in a variety of cancer cells. NNMT promotes cancer cell invasion, migration, and proliferation by inhibiting the methylation potential of cancer cells[47,48]. Overexpression of NNMT in glioblastoma mesenchymal stem-like cells promotes hypomethylation of mesenchymal subtype genes by down-regulating DNA methyltransferase 1 (DNMT1) and DNMT3A[49]. Ras, Stat3, and nuclear factor-kappaB (NF-κB) signaling pathways upregulate NNMT in cancer cells, which may be related to the epithelial-to-mesenchymal transition (EMT)[47]. Several other enzymes that catalyze SAM, such as DNMT1/3L, AMD1, SRM, and MTAP, are downregulated in colon CSCs. The reduction of DNMT1/3L, which catalyzes the transfer of methyl groups from SAM to DNA, leads to the accumulation of SAM in CSCs and thus affects DNA methylation[50].

Overall, maintenance of CSC phenotype mainly requires methionine cycle and folic acid cycle, as they either directly supply CSCs with nutrients or participate in genome methylation as methyl donors. Therefore, reducing the exogenous intake of methionine and folic acid or blocking the methionine cycle may be new therapeutic directions, which are worth investigating[26,51,52].

Tryptophan metabolism

Tryptophan is a source of the 1C unit and high consumption causes changes in TME. Tryptophan 2,3-dioxygenase (TDO2), a rate-limiting enzyme in tryptophan metabolism, was overexpressed in esophageal CSCs and may promote their production by inducing Oct4 and CD44 expression and activating EGFR pathway, which stimulates EMT and invasion of esophageal CSCs[53,54]. TDO2 is involved in the formation of tumorspheres of esophageal CSCs and TDO2 suppression reduces the size and number of spheres[53]. Indoleamine-2,3-dioxygenase-1 (IDO1), one of two IDO isozymes, is another rate-limiting enzyme that catalyzes the production of kynurenine in tryptophan metabolism. Similar to TDO2, the expression of IDO1 is increased in breast and prostate CSCs as well as mesothelioma stem cells[55]. The increased IDO1 promotes immune escape by depleting tryptophan in TME and inducing the binding of tryptophan catabolites to aryl hydrocarbon receptor (AhR) resulting in regulatory T cell activation; this can be reversed by IDO1 inhibitors such as LW106[55-57]. Additionally, IDO also regulates tumor-related immune responses through molecular stress response pathways, mTOR kinase, and NF-κB pathway[56,58,59]. IDO1 and kynurenine pathway metabolites may promote colon cancer cell proliferation and cancer-therapy resistance by altering the PI3K/Akt and β-catenin pathways, which are known to be beneficial for self-renewal of colon CSCs[60-63]. 2-(1'H-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE) is a tryptophan metabolite. ITE reduces the expression of Oct4 in CSCs by activating the AhR transcriptional pathway, thereby inducing CSC differentiation and ultimately reducing CSC tumorigenicity[53]. Tryptophan deprivation in TME decreases endogenous ITE level and increases Oct4 expression in CSCs, which subsequently maintains the stemness of CSCs[53,64]. Recent findings on ITE synthesis and stimulation of the AhR transcriptional pathway have provided crucial targets for the treatment of CSCs[64,65]. Tryptophan derivative, melatonin, may inhibit the proliferation and tumorigenicity of glioma stem cells by inhibiting the zeste homologue 2 and Notch pathways that are important for the survival of glioma stem-like cells[66,67]. In conclusion, tryptophan metabolic enzymes or metabolites, rather than tryptophan itself, are more essential for CSC self-renewal and survival[53,56] and provide new directions for eliminating CSCs.

Threonine, lysine, leucine, isoleucine, and valine metabolism

Threonine is involved in the synthesis of nucleotides and is an important nutrient for mESCs[68]. Threonine content was significantly increased during tumorsphere formation of the colon cancer HCT116 cell line, but it was not explored in-depth[69]. Glycine and acetyl-CoA, produced by threonine dehydrogenase (TDH)-mediated threonine metabolism, are involved in various biosynthetic pathways.

Additionally, glycine produces 1C-THF, which results in SAM synthesis *via* the methionine cycle. SAM ultimately regulates epigenetic modifications in ESCs, which play a significant role in their stemness maintenance and self-renewal[68,70]. TDH is highly expressed in mESCs, and inhibition of TDH or depletion of threonine in the growth medium reduces trimethylation of histone H3 Lysine 4 (H3K4me3) and ESC growth[68,71].

Studies on the relationship between lysine metabolism and CSCs are limited. Only a few studies have suggested that lysine metabolism in CD110+ colorectal CSCs not only reduces the production of reactive oxygen species (ROS), which suppress the proliferation of cancer cells, but also maintains the self-renewal of CSCs by activating the Wnt signaling pathway[72,73]. Instead, researchers have extensively investigated the role of epigenetic modification of histone lysine residues in CSCs[74,75], which may be of greater relevance.

Studies on the relationship between leucine or isoleucine metabolism and CSCs are also limited. Several studies have focused on the leucine-rich repeat of G-protein coupled receptor 5, a gastrointestinal CSC biomarker[71,76-78]. A few studies have found that leucine and isoleucine inhibit the stemness and self-renewal of EpCAM+ hepatocellular carcinoma stem cells by activating the mammalian target of rapamycin pathway complex 1 (mTORC1), in addition to enhanced chemotherapy sensitivity[72,79]. But a recent study suggested that the reduction of leucine caused apoptosis of CD13+ CSCs in hepatocellular carcinoma, but the specific mechanism is not yet clear[80].

Valine is reported to be elevated in canine mammary CSCs[72] and a decrease in valine can cause apoptosis of CD13+ CSCs in hepatocellular carcinoma by unknown mechanisms[80]. 3-Hydroxy-isobutyryl-CoA hydrolase (HIBCH), which catalyzes 3-hydroxyisobutyryl-CoA to 3-hydroxyisobu-tyrate, is a key enzyme in valine metabolism and is highly expressed in a colorectal cancer, prostate cancer, and brain tumor. Elevated HIBCH promotes the initiation and progression of colorectal cancer by increasing the proliferation of tumor cells and the resistance to bevacizumab, while reducing cancer cell autophagy[81,82]. In brain tissue with breast cancer metastasis, HIBCH expression was significantly increased in the areas of reactive gliosis associated with metastatic cells, tumor margins, and hemorrhagic areas, which may provide metabolic substrates[82]. Although studies on metabolism of these five amino acids in CSCs is limited, we confirm their involvement in maintaining self-renewal, survival, and drug resistance of CSCs.

TUMORIGENICITY CHARACTERISTICS OF CSCS AND NONESSENTIAL AMINO ACID METABOLISM

Humans do not have a dietary requirement for nonessential amino acids; however, they have crucial roles to play in CSC survival. To date, only one study illustrates the role of histidine metabolism in the central nervous system of *Drosophila*[83]. This section will further enumerate the roles of other nonessential amino acid metabolism in CSC biology.

Glycine/serine metabolism

Serine and glycine are commonly obtained *via* a branch of glycolysis and subsequent biosynthetic pathways. They can be interconverted by serine hydroxymethyl transferase (SHMT1/2), and participate in the folic acid cycle by providing a carbon unit[84]. Hence, in this review, we have jointly discussed the relationship between serine and glycine metabolism and CSCs. In colon CSCs, canine mammary CSCs, and neuroblastoma stem-like cells, the level of glycine is significantly higher than that in normal cancer cells[69,72,85-87]. If levels of glycine in colon CSC spheres are reduced, EMT suppression and induction of CSC apoptosis will occur[69]. Glycine decarboxylase (GLDC) is highly expressed in several cancers, including lung, ovarian, cervical, prostate, lymphoma, and breast except gastric cancer, catalyzes the conversion of glycine to 1C-THF, and participates in the methionine cycle[36,84,88-90]. The silent GLDC in gastric cancer may be due to hypermethylation of CpG islands in the promoter region of GLDC, which causes invasion and migration of gastric cancer cells[90,91]. GLDC is also related to bone metastases from breast cancer and may increase the aggressiveness of malignant tumors by aiding their metabolic adaptation to hypoxia[89,92]. Overexpressed GLDC in non-small cell lung CSCs alters glycolysis, promotes cellular transformation and synthesis of pyrimidines for cell proliferation that eventually promotes tumorigenesis[88,90]. GLDC knockout suppresses colony formation and CD166 on the surface markers of lung CSCs and reduces tumorigenicity[36,88]. Glycine metabolism *via* glycine and GLDC is a requirement to drive CSCs and promote tumorigenesis[72,88]. Recently, it was found that a new splice variant of GLDC is overexpressed in non-small cell lung CSCs; its tumorigenic ability is similar to that of GLDC and can be exerted by activating MAPK/ERK signaling pathway and regulating cyclin[93]. The binding of c-Myc to GLDC promoter also results in GLDC overexpression in ESCs, which is critical for maintaining their stemness by adjusting H3K4me3 levels; however, whether this is related to c-Myc in CSCs is not yet clear[94-96]. In glioblastoma multiforme, GLDC knockdown results in conversion of excess glycine into toxic aminoacetone and methylglyoxal by glycine C-acetyl-transferase (GCAT), leading to highly expressing SHMT2 cell growth arrest[97]. Importantly, GCAT silencing and preemptive knockdown of SHMT2 can suppress the toxicity due to GLDC knockdown

[97]. Thus, excess glycine is probably toxic to CSCs, and inducing excessive accumulation of glycine in CSC cytoplasm may be a new treatment strategy for glioblastoma[98].

Serine is involved in nucleotide and one-carbon unit biosynthesis. It plays an important role in tumor cell proliferation and is found at a high level in colorectal CSCs, ovarian clear cell adenocarcinoma, cervical squamous cell carcinoma, and neuroblastoma stem-like cells[69,87,99]. In ovarian clear cell adenocarcinoma and cervical squamous cell carcinoma stem-like cells, the high levels of serine are accompanied by elevated levels of aspartate, glutamate, and glutamine, all of which are involved in the tricarboxylic acid cycle (TCA cycle)[99]. In neuroblastoma stem-like cells, upregulated activating transcription factor 4 can activate genes of the glycine/serine pathway to promote formation of tumorspheres[87,100]. Moreover, in melanoma stem-like cells, up-regulated phosphoenolpyruvate carboxykinase, an enzyme in gluconeogenesis, promotes tumorigenesis by promoting glycolysis and serine/glycine pathway[101]. Phosphoglycerate dehydrogenase (PHGDH) is the first key enzyme in the glycolytic serine biosynthetic pathway and is overexpressed in breast cancer[102-104]. The serine synthesized by PHGDH is converted to glycine by SHMT and then forms glutathione (GSH) to maintain intracellular redox balance[105,106]. PHGDH was found to be preferentially expressed in hypoxia-induced breast CSCs and preserved the breast CSC stemness by maintaining the balance of redox reactions and shunting a portion of glucose-derived 3-phosphoglycerate[103,104]. The shunt from glucose metabolism to serine metabolism produces NADPH, which can maintain the reduced state of GSH and forms an antioxidant barrier in breast CSCs[104,107]. Additionally, high intra-tumoral co-expression of PHGDH and Oct4 in NT2/D1 (embryonal carcinoma stem-like cells) is beneficial for the survival of CSCs[103]. PHGDH can interact with kinesin family member 15, which is overexpressed in liver cancer cells and liver CSCs, and increase its stability to promote the liver CSC phenotype[108]. Because CSCs are more dependent on mitochondrial metabolic pathways than glycolytic pathways, mitochondrial inhibitors can limit their growth[107,109,110]. However, increased intracellular PHGDH expression was observed after the use of mitochondrial inhibitors, indicating that PHGDH may play a protective role against mitochondrial inhibitors in CSCs. Additionally, increasing the intake of exogenous serine or synthesis of intracellular serine also countervails the damage to CSCs caused by mitochondrial inhibitors[107]. PHGDH deficiency suppresses tumorsphere formation and reduces expression of stem factors (Oct4, Sox2, Nanog, Bmi-1) in breast CSCs, embryonal carcinoma, and brain tumor stem-like cells, and also impairs metastasis from breast to lung and increases chemotherapy sensitivity[103,104,106]. Mechanistically, the inhibition of PHGDH not only results in redox imbalance but also promotes the differentiation of CSCs through the degradation of Oct4 and the differential ubiquitination of β3-tubulin; it also promotes p-AMPK mediated-Beclin-1 dependent autophagy in a p-mTOR-independent manner. These findings suggest that PHGDH is necessary for maintaining CSC stemness and self-renewal and may be a new metabolic target for eradication of CSCs[103,104,108]. Other enzymes, including SHMT1/2, phosphoserine phosphatase, phosphoserine aminotransferase, and GCAT, required for glycine/serine metabolism, are up-regulated in non-small cell lung CSCs with different amplitudes and promote tumorigenesis by up-regulating glycine/serine metabolism[88].

Glutamate and glutamine metabolism

Glutamate and glutamine, often upregulated in CSCs, have an amine group (-NH2) difference and glutamine is converted to glutamate *via* deamination by glutaminase (GLS), which constitutes the first step of glutaminolysis[111]. Glutaminolysis, a series of reactions in which glutamine is degraded to produce metabolic components and energy, may either replace or complement glucose dependence of cancer cells and CSCs[111-113]. Glutamine and glutamate are structurally similar and their roles *in vivo* are interrelated[114]. Glutamine is used in the biosynthesis of nucleotides, lipids, and amino acids; glutamate forms α-ketoglutarate (α-KG) catalyzed by glutamate dehydrogenase (GDH or GLUD), thereby producing ATP for cellular activities. Interestingly, the biological functions of both these amino acids may be specific to the cancer types[115-118].

Glutamine acts as a "conditionally" essential amino acid in multiple CSCs because the biosynthesis of three major nutrients and nucleic acids requires glutamine to provide the source of carbon and amino nitrogen[99,111,112,117,119]. The glutamine transporter ASCT2 (also known as SLC1A5), encoded by *SLC1A5*, is highly expressed in various CSCs and is associated with tumor progression and poor prognosis[120,121]. CD9-mediated ASCT2 plasma membrane localization increases glutamine uptake and provides energy for CSC growth in pancreatic ductal adenocarcinoma[122]. The up-regulation of MYC-regulated ASCT2 and GLS1 in colorectal CSCs increases glutamine metabolism and metformin resistance[118,123-125]. MYC in CSCs is regulated by the tumor suppressor TP73/p73, and loss of TP73/p73 reduces the expression of MYC and GLS, thereby inhibiting ASCT2 and reducing glutamine-uptake and glutamine metabolism[126,127]. ASCT2 also activates the downstream mTORC1 signaling pathway to promote the growth of prostate cancer cells or melanoma cells by increasing glutamine uptake[128,129]. Of the other SLC1A family members, the upregulation of SLC1A3 (also called glutamate aspartate transporter, GLAST) in CD133+ thyroid CSCs depends on the activation of the NF-κB pathway; SLC1A3 expression in glioblastoma stem-like cells depends on the activation of the STAT3 pathway triggered by glutamate, whereas SLC1A6 that acts as a glutamate exporter is down-regulated in EMT[130-132]. The higher glutamine and glutamate levels in ovarian clear cell adenocarcinoma and cervical squamous cell carcinoma stem-like cells are related to TCA cycle; in glioblastoma stem-like cells

with high GLS expression, GLS inhibition attenuates the influx of glutamine metabolites into the TCA cycle[99,111]. Exogenous glutamine *via* GLS induces tumorsphere formation and expression of ALDH, a stem cell marker of head and neck squamous cell carcinoma, which can be prevented by glutamine deprivation and GLS inhibitors[112]. Glutamine not only promotes the expression of CSC markers and self-renewal potential of pancreatic CSCs, but also increases radiotherapy resistance by maintaining ROS stability[118,133]. Glutamine also promotes clonogenic formation and stemness marker expression in non-small cell lung CSCs and hepatocellular carcinoma CSCs *via* the maintenance of redox balance and activation of the Wnt/β-catenin signaling pathway[119,134]. Additionally, the AMPK-mTOR pathway is involved in the regulation of glutamine metabolism on the metformin sensitivity of colorectal CSCs; in absence of glutamine, the activation of AMPK and inhibition of mTOR will increase the sensitivity of metformin-resistant SW620 colorectal CSCs to metformin; however, as metformin-sensitive HT29 CSCs have an activated AMPK pathway, inhibition of glutamine metabolism will enhance the inhibitory effect of metformin[118,125]. Moreover, mTOR inhibition in ovarian clear cell adenocarcinoma stem-like cells in absence of glutamine confirms that glutamine regulates CSCs through the mTOR pathway[135]. The ammonia molecule released by glutaminolysis also neutralizes the excess acid produced by the Warburg effect in epithelial CSCs, in which stemness and EMT are uncoupled [136]. α-KG, another metabolite produced during glutaminolysis in ESCs, regulates the demethylation of DNA/histone to maintain pluripotency[137]. If the overexpressed GDH1 is suppressed in CSCs, the level of α-KG will be reduced, which not only reduces the production of ATP but also produces a large amount of ROS to damage CSCs[132].

GLS has two isoenzyme forms, GLS1 and GLS2; GLS1 is a tumor promoter in many cancers, while GLS2 appears to be a tumor suppressor[123,134,138]. Recently, studies have found that GLS1 induced by distal-less homeobox-2 promotes the progression of transcription factor Snail-mediated EMT by negatively regulating p53 in colon and breast cancer. However, GLS2 inhibits Snail to prevent EMT in hepatocellular carcinoma independent of glutaminase activity; during breast cancer EMT, GLS2 and glutamine utilization are reduced, which can be rescued by the suppression of transcription factor, FOXC2[117,123,139,140]. In intrahepatic cholangiocarcinoma and lung cancer, the expression of GLS1 is negatively correlated with the expression of E-cadherin but positively correlated with that of vimentin, and cells with low E-cadherin/high vimentin are more sensitive to GLS1 inhibitors[138,141]. Additionally, aspartate aminotransferase (GOT1) is upregulated and system L-type amino acid transporter 1 is down-regulated in ovarian clear cell adenocarcinoma stem-like cells[135,142]. Glutamine depletion and ASCT2/SLC1A3/GLS/GDH/GOT1 inhibition increase CSC apoptosis and sensitivity to therapy, all of which are new ways for CSC therapy[111,119,128,133,134]. For instance, GLS inhibitors CB839 and compound 968 suppress cloning ability of high GLS-expressing glioblastoma stem-like cells and reduce expression of stemness marker CD133. CB839 also selectively leads to cell cycle arrest without inducing apoptosis[111]. Other GLS inhibitors, such as BPTES and Zaprinast, effectively sensitizes pancreatic CSCs to radiotherapy and induces apoptosis through intracellular ROS accumulation[133]. SLC1A3 knockdown reduces intracellular glutamate levels and inhibits the self-renewal activity and tumorigenicity of CD133 + thyroid CSCs; SLC1A3 inhibitor UCPH-101 induces apoptosis of glioblastoma stem-like cells[130,131].

Cysteine metabolism

Cysteine is a special amino acid that can be obtained not only from cystine conversion but also *via* homocysteine transsulfuration[31,143]. The cystathionine produced from homocysteine by cystathionine β-synthase (CBS) is further converted to cysteine by cystathionine γ-lyase (CGL). Cysteine then produces GSH so as to maintain the redox balance[143]. Increased homocysteine to cysteine metabolism is observed in tamoxifen-resistant breast cancer, and both CBS and CGL are significantly upregulated in CD133+ colon CSCs[50,144]. The cystine–glutamate antiporter xCT (SLC7A11) on the cell membrane, which is stabilized by CD44/CD44 variant (CD44v) and overexpressed in breast CSCs, is associated with cystine intake as well as cysteine and GSH production[145-149]. CD133 in liver CSCs, CD44v in lung CSCs, and CD44v8-10 in esophageal squamous cell carcinoma and urothelial cancer stem-like cells upregulate or stabilize xCT against intercellular ROS, and overexpressed CD44v in lung CSCs is not related to stem-like properties[150-153]. Inhibition of xCT leads to changes in redox levels of breast CSCs, decreased survival rate, and reduced self-renewal[146,147]. Sulfasalazine (SSZ), an inhibitor of xCT, selectively inhibits CD44+/CD44v+ CSCs, such as those in gastrointestinal tumors, metastatic bladder cancer, esophageal squamous cell carcinoma, and glioma, decreases intracellular GSH levels, and increases ROS levels[145,152,154-156]. The same SSZ effect also occurs in CD133+ liver CSCs[150, 157]. In CD44v^{high} head and neck squamous cell carcinoma cells, the cytotoxicity of SSZ depends on ASCT2-dependent glutamine uptake and GDH-mediated production of α-KG; GDH depletion and ASCT2 inhibition not only significantly attenuate SSZ-induced intracellular ROS accumulation but also weaken the inhibitory effect of SSZ on cell survival[120]. A phase I study on combined drug therapy in advanced non-small cell lung cancer (UMIN000017854) proposed that SSZ 1.5 g/day can be safely used in combination with standard-dose cisplatin and pemetrexed, but its side effects include intestinal toxicity and limited absorption[158]. Another phase 1 study in patients with refractory cisplatin CD44v+ gastric cancer (UMIN000015595) showed that a combination of 6 g dose of SSZ and cisplatin is feasible, but side effects and disappearance of the inhibitory effect of SSZ on xCT after oral administration were

reported[159]. In addition, vaccines against xCT antigens induce xCT antibody production that mediates antibody-dependent cell cytotoxicity, resulting in redox imbalance, inhibition of breast CSC phenotype and self-renewal, increased chemosensitivity, delay in primary tumor growth, and impaired pulmonary metastasis[146,147,160,161]. Therefore, targeted inhibition and immunotargeting therapy of xCT promotes CSC apoptosis, which may provide new methods for adjuvant anti-cancer therapy[147,150, 154,155,160]. Glutamate cysteine ligase (GCL) catalyzes condensation of cysteine, produced by above mentioned pathways, and glutamate to form γ-glutamyl-cysteine, which reacts with glycine to form GSH by the action of glutathione synthetase[162]. GCL is composed of a catalytic subunit (GCLC) and a modifier subunit (GCLM); upregulated GCLC, regulated by nuclear factor erythroid-derived 2-like 2, in breast CSCs can mediate the production of GSH to upregulate the expression of FoxO3a and Bmi-1, which are essential for maintaining stemness, whereas GCLM is induced in a HIF-1 dependent manner during chemotherapy or hypoxia[162,163]. In short, GSH is the key to elucidating the role of cysteine metabolism in CSCs.

Aspartate and asparagine metabolism

Aspartate and asparagine differ in cellular functions owing to their structural differences. In tumor cells, asparagine is involved in the synthesis of proteins and is a nitrogen source for the synthesis of purines and pyrimidines[164]. Recently, studies have focused on the regulatory role of asparagine in cancers. Asparagine regulates the cellular adaptation to glutamine depletion and inhibits glutamine depletion-mediated apoptosis; in case of sufficient availability of other amino acids, asparagine depletion also causes apoptosis[165]. Asparagine synthetase, which synthesizes asparagine from glutamine, is associated with tumorigenesis in lung cancer and poor prognosis in glioma and neuroblastoma as well as plays a crucial role in glutamine-dependent survival[165,166]. Asparagine can also be used as an exchange factor for cellular uptake of amino acids, such as serine, arginine, and histidine, thereby activating mTORC1 and regulating amino acid metabolism[167]. Aspartate is also involved in the synthesis of nucleotides[168]. Aspartate and asparagine are upregulated in osteosarcoma stem-like cells, and GOT1, an enzyme that converts aspartate to oxaloacetate, is upregulated in ovarian clear cell adenocarcinoma stem-like cells[35,135,169]. The upregulated aspartate in ovarian clear cell adenocarcinoma and cervical squamous cell carcinoma stem-like cells may be involved in TCA cycle reactions [99]. Although research on aspartate and asparagine in CSCs is limited, their established functions in cancer cells provides a basis for further research on CSCs.

Alanine, proline, arginine, and tyrosine metabolism

A recent study revealed that alanine and proline levels are increased in canine mammary CSCs, which may be related to maintenance of stemness[72]. Over-expression of glutamic pyruvate transaminase 2, which catalyzes the reaction between alanine and α-KG to form pyruvate and glutamate, reduces the level of α-KG in cells, thereby leading to proline hydroxylase 2 activity inhibition and HIF-1α stabilization. HIF-1α in turn activates the sonic hedgehog signaling pathway and promotes breast cancer tumorigenesis and CSC growth[170,171]. Arginine and proline metabolism are upregulated in osteosarcoma stem-like cells. The level of arginine and ornithine, converted from arginine *via* arginase, increases significantly and participates in cell proliferation and urea cycle[35]. Addition of proline to Dulbecco's minimum essential media allows ESC to maintain pluripotency. Moreover, proline also induces ESC transformation to mesenchymal-like state and genome-wide reprogramming involving H3K9 and H3K36 methylation[172,173]. TP73/p73 regulates proline metabolism in CSCs; loss of TP73/p73 reduces proline synthesis by inhibiting pyrroline-5-carboxylate reductase 1, which catalyzes proline formation from pyrroline-5-carboxylate[126]. In addition, proline metabolism also plays an important role in the self-renewal of human breast CSCs *via* proline dehydrogenase (PRODH), and inhibiting PRODH damages spheroidal growth and metastasis[72]. CD13+ CSCs are habituated to tyrosine metabolism in hepatocellular carcinoma; acetyl-CoA, produced by tyrosine metabolism, not only enters the TCA cycle to provide energy to CD13+ CSCs, but also promotes the transcription factor Foxd3 acetylation to maintain the CD13+ CSC self-renewal[80]. Due to the lack of phenylalanine hydroxylase in CD13+ CSCs, deprivation of phenylalanine has no effect on cell survival[80]. Thus, the metabolism of arginine, alanine, proline, and tyrosine seem to be necessary in CSCs of specific cancer species.

RELATIONSHIP BETWEEN AMINO ACID METABOLISM AND TME

In addition to regulating CSCs, amino acid metabolism is also interconnected with TME or CSC niches as TME plays an important role in maintaining the self-renewal of CSCs[119,174]. As microenvironments of different cancer types exhibit variable conditions (glucose concentration and oxygen tension), CSCs display diverse metabolic phenotypes to adapt to these microenvironments[113,175-177]. For instance, in the absence of glucose, glutamine compensates for the shortage of glucose[12,113]. However, in the absence of glutamine, extracellular asparagine becomes critical because intracellular asparagine is redirected to glutamine synthesis to avoid apoptosis[165,178,179]. Glutamine metabolism
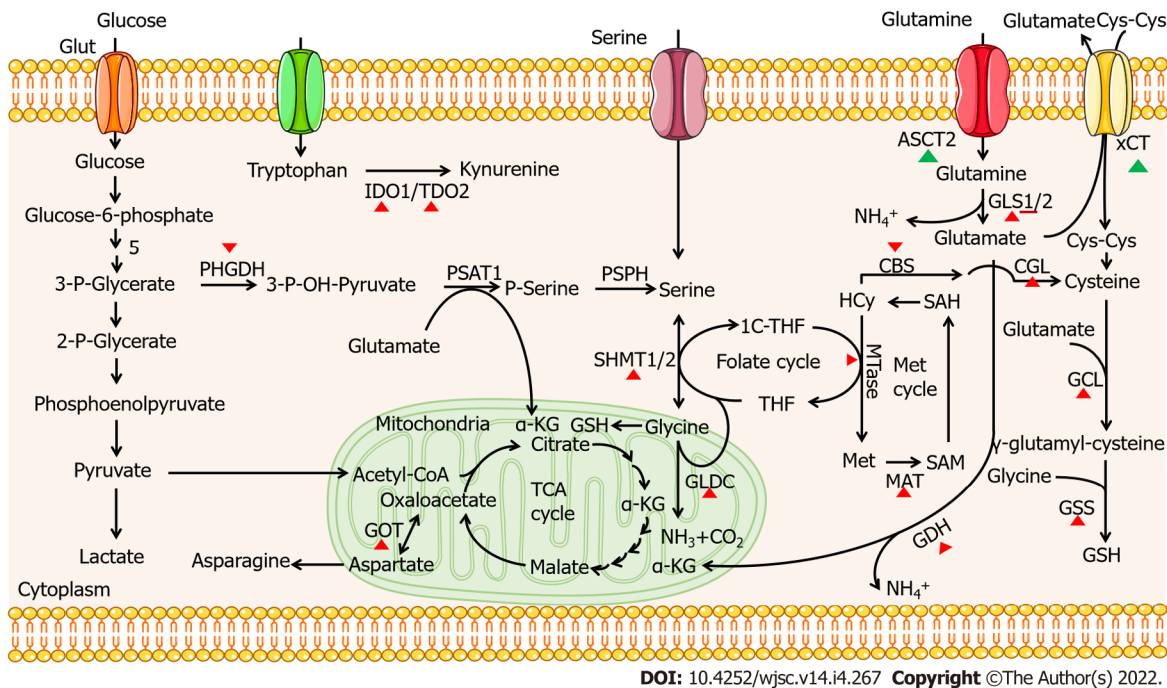
Figure 1 Enzymes and transporters that are potential therapeutic targets in cancer stem cell-based therapy. The red and green triangles indicate the enzyme and transporter, respectively, which may serve as potential targets. IDO1: Indoleamine-2,3-dioxygenase-1; TDO2: Tryptophan 2,3-dioxygenase; PHGDH: Phosphoglycerate dehydrogenase; PSAT1: Phosphoserine aminotransferase; PSPH: P phosphatase; GOT: Aspartate aminotransferase; SHMT: Serine hydroxymethyl transferase; GLDC: Glycine decarboxylase; GLS: Glutaminase; CBS: Cystathionine β synthase; CGL: Cystathionine γ lyase; MTase: Methyltransferase; MAT: Methionine adenosyltransferase; GDH: Glutamate dehydrogenase; GCL: Glutamate cysteine ligase; GSS: Glutathione synthetase; ASCT2: Alanine-serine-cysteine transporter 2; xCT: Cystine-glutamate antiporter; Glut: Glucose transporter; THF: Tetrahydrofolate; 1C-THF: One-canton tetrahydrofolate; HCy: Homocysteine; SAH: S-adenosine homocysteine; Met: Methionine; SAM: S-adenosine methionine; Cys–Cys: Cystine; GSH: Glutathione; α-KG: α-ketoglutarate; TCA cycle: Tricarboxylic acid cycle.

is also affected in TME as interleukin-4 (IL-4), secreted by immune cells, increases ASCT2 expression in breast cancer cells[180,181]. Growth factor IL-3 in TME, through binding to IL-3Rα, up-regulates ASCT2 expression and promotes glutamine uptake *via* the JAK/STAT pathway[180,182]. Hypoxic microenvironment causes the accumulation of lactate, which affects ASCT2 and GLS1 expression by activating c-Myc[180,183]. Glutamine-dependent ovarian cancer cells form a glutamine loop with cancer-associated fibroblasts (CAFs) within TME; tumor cells convert glutamine to glutamate, which is regenerated into glutamine by CAFs to supply to tumor cells[12,184]. CAFs also secrete cysteine and GSH, which are absorbed by ovarian cancer cells to induce resistance to platinum-based chemotherapy. However, drug resistance induced by TME is destroyed by effector T cells, which suppress xCT expression of CAFs through the JAK/STAT1 pathway[180,185].

Cancer cell metabolism produces an acidic, hypoxic, and malnourished TME, which is detrimental to the antitumor immune response[186]. The main amino acids in the tumor immune microenvironment are tryptophan and arginine, whose increased catabolism is a common marker of TME[187]. Cells that decompose tryptophan and arginine, such as myeloid-derived suppressor cells and tumor-associated dendritic cells, induce regulatory T cells and suppress effector T cells to suppress antitumor immunity and promote tumorigenesis[187,188]. Interestingly, tryptophan has a significant effect on T cell survival and function[189]. Tumor cells that overexpress IDO show reduced extracellular tryptophan, which affects the effector function of T cells[189]. Whereas kynurenine, an immunosuppressive product of tryptophan metabolism, induces CD4+ T cells to differentiate into regulatory T cells by activating AhR, which weakens the ability of the immune system to recognize and kill cancer cells[178,190]. However, it does not seem to be a contradiction, as the expression of IDO, extracellularly consumed tryptophan, and synthesized kynurenine all synergistically inhibit T cell proliferation and activation[188]. The increased IDO1 in breast and prostate CSCs also activates regulatory T cells through the kynurenine pathway to promote immune escape[55]. The presence of arginine promotes the effector function and survival of T cells, which indicates that the lack of arginine in TME leads to T cell dysfunction[12,184,188]. Citrulline and ornithine, downstream metabolites of arginine, also affect T cell activation[178]. Glutamine is not only used for cancer cell metabolism, but also provides nitrogen and carbon sources for active T cells in TME[178,191]. Glutamine metabolism is essential to B cell proliferation and differentiation into plasma cells, and macrophage antigen presentation and phagocytosis[188,189]. Amino acids such as serine and alanine are also critical to the tumor immune microenvironment. Serine provides purines for T cell proliferation, but has no effect on T cell function. Conversely, alanine affects T cell effector function and

Table 1 Summary of the role and mechanism of amino acid metabolism in cancer stem cells

Amino acid metabolism	Possible mechanisms in CSCs	Role in CSC properties
Methionine metabolism	Direct nutrients; Genetic modification; DNA biosynthesis	Self-renewal; tumorigenicity
Tryptophan metabolism	Immune escape and resistance; regulates stem genes and signal pathway	Self-renewal; survival
Threonine metabolism	Upregulated in colon CSCs (HCT116), but mechanism is unknown	Self-renewal
Lysine metabolism	Reduces ROS and activates Wnt pathway	Self-renewal
Leucine metabolism	Regulates CD13+ CSCs survival in hepatocellular carcinoma, but mechanism is unknown; Inhibits stemness and growth of EpCAM+ hepatocellular carcinoma stem cells by activating MTORC1	Stemness; survival
Valine metabolism	Regulates CD13+ CSCs survival in hepatocellular carcinoma, but mechanism is unknown	survival
Phenylalanine metabolism	Unknown	Unknown
Isoleucine metabolism	Inhibits stemness and growth of EpCAM+ hepatocellular carcinoma stem cells by activating MTORC1	Stemness
Histidine metabolism	Unknown	Unknown
Glycine metabolism	Direct nutrients within a certain range; epigenetic modification; DNA synthesis; regulates redox homeostasis; carries out TCA cycle	Self-renewal; survival; tumorigenicity; metastasis
Serine metabolism	Regulates redox homeostasis; shunts glucose metabolism; carries out TCA cycle; influences T cell proliferation	Self-renewal; survival; tumorigenicity; stemness; metastasis; resistance
Glutamine metabolism	Direct nutrients; carries out TCA cycle; synthesis of nucleic acids; maintains redox balance; regulates tumor immunity	Self-renewal; survival; tumorigenicity; stemness; resistance
Glutamate metabolism	Carries out TCA cycle; participates serine metabolism; maintains redox balance	Self-renewal; survival; tumorigenicity; stemness
Cysteine metabolism	Mainly maintains redox balance	Self-renewal; survival; tumorigenicity; resistance; metastasis
Aspartate metabolism	Replenishes TCA cycle; synthesis of nucleic acids	Survival
Asparagine metabolism	Replenishes TCA cycle; synthesis of nucleic acids; exchanges amino acids	Survival
Alanine metabolism	Upregulated in breast CSCs, but mechanism is unknown; regulates T cell function	Self-renewal;stemness; tumorigenicity
Arginine metabolism	Participates in cell proliferation and urea cycle; regulates tumor immunity	Self-renewal
Proline metabolism	Maybe epigenetic modification and transform steadily; synthesize collagen	Self-renewal; stemness; metastasis
Tyrosine metabolism	Provides energy; Foxd3 acetylation	Self-renewal

CSC: Cancer stem cell; TCA cycle: Tricarboxylic acid cycle.

proinflammatory cytokine secretion by promoting T cell protein synthesis and initial activation[189, 192]. In addition to tumor metabolism and immune microenvironment, amino acid metabolism also has an influence on the structural microenvironment. Collagen, the main component of the extracellular matrix in TME, is degraded to proline by the action of metalloproteinases and collagenases. The extracellular proline is an energy source for tumor cells and may be resynthesized into collagen to promote the extracellular matrix remodeling, which is responsible for cancer cell reprogramming[174, 193,194]. High-density extracellular collagen matrix also shifts the metabolism of metastatic breast cancer 4T1 cell line from glucose to glutamine[195]. All the above evidence shows that amino acid metabolism, cancer cells or CSCs, and TME form a complex regulatory network that can be efficiently applied in clinical research.

Table 2 Potential enzymes targets for cancer stem cell therapy, role in metabolism, treatment strategy in cancer stem cell-based therapy

Enzyme	Role in amino acid metabolism	CSC therapy
MTase	Translates homocysteine to methionine	Inhibition
MATα2	Induces the production of SAM	Inhibition
IDO1	Catalyzes tryptophan into kynurenine	Inhibition
TDO2	Catalyzes tryptophan into kynurenine	Inhibition
GLDC	Catalyzes glycine into NH_3, CO_2 and CH2-THF	Inhibition (except gastric cancer, better inhibit SHMT and GCAT simultaneously)
PHGDH	Catalyzes 3P-glycerate into 3-P-OH-pyruvate	Inhibition
SHMT1/2	Completes the conversion between serine and glycine	Inhibition
GLS1	Catalyzes glutamine into glutamate	Inhibition
GDH	Catalyzes glutamate into α-KG	Inhibition
CBS	Translates homocysteine to cystathionine	Inhibition
CGL	Catalyzes cystathionine to cysteine	Inhibition
GCL	Catalyzes the production of γ-glutamyl-cysteine	Inhibition
GSS	Catalyzes GSH production	Inhibition
GOT1	Catalyzes the production of oxaloacetate from aspartate	Inhibition
GPT2	Catalyzes transamination between alanine and α-KG to pyruvate and glutamate	Inhibition
PRODH	Oxidize proline to glutamate	Inhibition

CSC: Cancer stem cell; MTase: Methyltransferase; MAT: Methionine adenosyltransferase; SAM: S-adenosine methionine; IDO1: Indoleamine-2,3-dioxygenase-1; TDO2: Tryptophan 2,3-dioxygenase; GLDC: Glycine decarboxylase; 1C-THF: One-canton tetrahydrofolate; PHGDH: Phosphoglycerate dehydrogenase; SHMT: Serine hydroxymethyl transferase; GCAT: Glycine C-acetyltransferase; GLS: Glutaminase; GDH: Glutamate dehydrogenase; α-KG: α-ketoglutarate; CBS: Cystathionine β synthase; CGL: Cystathionine γ lyase; GCL: Glutamate cysteine ligase; GSS: Glutathione synthetase; GSH: Glutathione; GOT: Aspartate aminotransferase; GPT2: Glutamic pyruvate transaminase; PRODH: Proline dehydrogenase.

CONCLUSION

The concept of CSCs, a class of cells with potential for self-renewal and differentiation, was proposed owing to the emergence of recurrence, metastasis, and drug or radiotherapy resistance in tumors, and renders tumor treatment challenging[196]. Currently, researchers are focusing on issues related to the metabolism within CSCs and attempting to identify new research directions and therapeutic targets to eliminate CSC population. In TME, researchers have indicated the involvement of amino acids as a nutrition and energy source, apart from glycolysis, by demonstrating abnormal mitochondrial function in tumor cells[197,198]. Amino acids are not only involved in protein synthesis, but also participate in important biosynthetic pathways as intermediate metabolites. As amino acids are important nutritional components in TME, an increasing number of studies are focusing on the role of amino acids in self-renewal and other biological characteristics of CSCs. This review focuses on the role of amino acid metabolism in CSC biology, particularly self-renewal and their mechanism of action.

The role of 20 amino acids in CSCs is summarized in Table 1. The metabolism of certain amino acid plays an important role in the self-renewal of CSCs, such as methionine, tryptophan, glycine, serine, and glutamine. The effects of amino acids depletion in TME or inhibition of key enzymes on the self-renewal and survival of CSCs further illustrate the influence of amino acid metabolism on the characteristics of CSCs and provide potential targets for cancer therapy. An increasing number of clinical trials focus on targeting key proteins in amino acid metabolism pathways in CSCs. For example, xCT, a cystine-glutamate antiporter, plays an important role in CSC self-renewal; clinical studies involving its inhibitor, SSZ, in advanced non-small cell lung cancer (UMIN000017854) and refractory cisplatin CD44v+ gastric cancer (UMIN000015595) suggest the potential feasibility of targeting amino acid metabolism transporters for tumor therapy[158,159]. In two clinical cases of CD44v9-positive urogenital cancer, SSZ was also used as a new adjuvant treatment approach[199]. Further, parthenolide and piperlongumine targeting aberrant glutathione metabolism in leukemia stem cells[200]; pegcris-antaspase depleting plasma glutamine and asparagine in relapsed/refractory acute myeloid leukemia [201]; and L-asparaginase exhausting asparagine in acute lymphoblastic leukaemia[202] highlight the

extensive prospect of targeting amino acid metabolism in cancer therapy. Additionally, key metabolic enzymes also act as potential targets for CSC-based cancer therapy; they are listed in Table 2 and Figure 1.

In conclusion, the role of amino acid metabolism is varied in different cancer types and metabolism of amino acids are interlinked, which adds to the complexity of TME. Based on these reports, we expect the future research on amino acid metabolism to be based on cancer types, amino acid interrelations, and TME. Only through this research path, can we propose better solutions for CSC clinical therapy and ultimately prolong patient life-expectancy.

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FOOTNOTES

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SYSTEMATIC REVIEWS

Dental stem cell-conditioned medium for tissue regeneration: Optimization of production and storage

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Abstract

BACKGROUND
Mesenchymal stem cells (MSC) effects on tissue regeneration are mainly mediated by their secreted substances (secretome), inducing their paracrine activity. This Conditioned medium (CM), including soluble factors (proteins, nucleic acids, lipids) and extracellular vesicles is emerging as a potential alternative to cell therapy. However, the manufacturing of CM suffers from variable procedures and protocols leading to varying results between studies. Besides, there is no well-defined optimized procedure targeting specific applications in regenerative medicine.

AIM
To focus on conditioned medium produced from dental MSC (DMSC-CM), we reviewed the current parameters and manufacturing protocols, in order to propose a standardization and optimization of these manufacturing procedures.

METHODS
We have selected all publications investigating the effects of dental MSC secretome in *in vitro* and *in vivo* models of tissue regeneration, in accordance with the PRISMA guidelines.

RESULTS
A total of 351 results were identified. And based on the inclusion criteria described above, 118 unique articles were included in the systematic review. DMSC-CM production was considered at three stages: before CM recovery (cell sources for CM), during CM production (culture conditions) and after production (CM treatment).

CONCLUSION
No clear consensus could be recovered as evidence-based methods, but we were able to describe the most commonly used protocols: donors under 30 years of age,

dental pulp stem cells and exfoliated deciduous tooth stem cells with cell passage between 1 and 5, at a confluence of 70% to 80%. CM were often collected during 48 h, and stored at -80 °C. It is important to point out that the preconditioning environment had a significant impact on DMSC-CM content and efficiency.

Key Words: Tissue engineering; Mesenchymal stem cells; Dental; Conditioned medium; Secretome; Regeneration

Core Tip: Dental Mesenchymal stem cells (DMSC) effects on tissue regeneration are highly mediated by their secreted substances [conditioned medium (CM)] such as soluble factors and extracellular vesicles. The manufacturing of CM products suffers from variable procedures and protocols leading to different results between studies. Focusing on CM produced from DMSC (DMSC-CM), we reviewed the current parameters and manufacturing protocols, aiming to facilitate the standardization and optimization of manufacturing procedures, in accordance with PRISMA guideline. No clear consensus could be recovered as evidence-based methods, but it clearly appeared that the preconditioning environment had a significant impact on DMSC-CM content and efficiency.

INTRODUCTION

Although mesenchymal stem cells (MSC) were initially isolated from bone marrow, MSC from dental and periodontal tissue (DMSC) have attracted international attention for future therapies because of their practical and technical advantages[1]. Since 2000, when Gronthos *et al*[2] described a population of pluripotent progenitors in adult dental pulp, studies have shown that dental tissues can be an important resource of MSCs: dental pulp stem cells (DPSC), exfoliated deciduous tooth stem cells (SHED), apical papilla stem cells (SCAP) which are situated at the ends of growing dental roots[3], periodontal ligament stem cells (PDLSC), dental follicle stem cells (DFPC) located around the tooth germ, and responsible for cementum, periodontal ligament and alveolar bone formation during tooth development [4], gingiva-derived mesenchymal stem cells (GMSC) (Figure 1). For teeth at early stage of development (bell stage), multipotent progenitors from dental mesenchyme have been described, named as tooth germ progenitor cells (TGPC)[5].

An important advantage of these sources of MSC is the absence of morbidity and the fact that no additional surgical procedures is required[6]. DMSC are obtained from exfoliated teeth, teeth extracted for orthodontic or medical needs, and supernumerary teeth. While generally discarded as medical waste, teeth could be an abundant source of mesenchymal stem cells.

Numerous studies have indicated that MSC effects on tissue regeneration are mostly mediated by their secreted substances[7] defined as secretome, or MSC-conditioned medium (MSC-CM), which are endowed with paracrine activity. These MSC-CM include soluble factors (proteins, nucleic acids, lipids) and extracellular vesicles (EV)[8]. MSC-CM appears as a potential substitute for cell therapy, with considerable potential to be developed into pharmaceutical products for use in regenerative medicine [9].
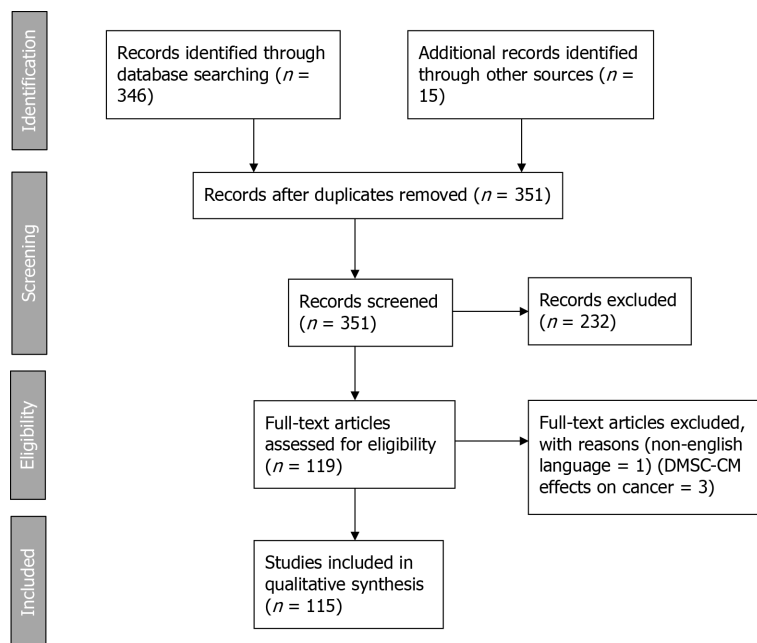
Compared to non-dental MSC-CM, dental mesenchymal stem cell-conditioned medium (DMSC-CM) have greater amounts of transcriptional, metabolic and proliferation-associated proteins, neurotrophins and chemokines. They also present reduced levels of proteins required for extracellular matrix production and adhesion, and superior effects on cell differentiation, maturation and tissue regeneration[10].

Many research works have described the application of DMSC secretome for various diseases treatment and for tissue regeneration[8,10-12]. Dental MSC-CM have been investigated for the repair of neurological disorders[13-18], cardiac lesions[19], diabetic disorders[20], liver diseases[21,22], pulmonary lesions[23], immunity problems[24-26], dental and bone defects[27-30], and growth of hair [31]. These potentials are assigned to positive effects on cell proliferation-migration-protection, to specific effects such as anti-apoptotic, pro-angiogenic, immunomodulation, or to cell differentiation and further tissue regeneration like osteodifferentiation, neuron-like regeneration, dentin-pulp complex formation, periodontal regeneration (Figure 2 and Table 1).

Table 1 Studies investigating the effects of secretomes from dental pulp stem cells, stem cells from human exfoliated deciduous teeth, periodontal ligament stem cells, stem cells from apical papilla, dental follicle stem cells, gingiva-derived mesenchymal stem cells and tooth germ progenitor cells for tissue regeneration

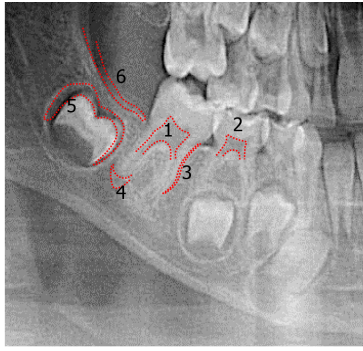| Type of DMSC	Ref.
Dental pulp stem cells	Kumar[33], 2017; Kumar[34], 2018; Kumar[35], 2017; Gervois[36], 2017; Horibe[37], 2014; Zhou[38], 2020; Venugopal[39], 2018; Caseiro [40], 2019; Shen[41], 2015; Mead[42], 2014; Wada[43], 2009; Kolar[44], 2017; Bronckaers[45], 2013; Paschalidis[46], 2014; Piva[47], 2017; Gharaei[48], 2018; Murakami[49], 2013; Li[50], 2019; Yamamoto[51], 2014; Sakai[52], 2012; Hu[53], 2019; Wang[17], 2019; Nakayama [54], 2017; Merckx[55], 2020; Swanson[56], 2020; Gervois[57], 2019; Ivica[58], 2020; Zhang[59], 2020; Yamamoto[60], 2016; Ahmed[13], 2016; Xian[61], 2018; Song[62], 2015; Joo[63], 2018; Shen[64], 2020; Akazawa[65], 2015; De rosa[66], 2011; Ji[67], 2019; Aranha[68], 2010; Huang[69], 2016; Lambricht[70], 2017; Iohara[71], 2008; Ishizaka[72], 2013; Kawamura[73], 2016; Hayashi[74], 2015; Iohara[75], 2014; Iohara[76], 2013; Murakami[77], 2015; Omi[78], 2017; Makino[79], 2019; Omi[80], 2016; Chen[81], 2019
Stem cells from human exfoliated deciduous teeth	Li[82], 2017; Pivoraite[25], 2015; Jarmalaviciute[16], 2015; Kano[83], 2017; Matsubara[84], 2015; Matsushita[21], 2017; Omori[27], 2015; Yamagata[85], 2013; Fujii[86], 2015; Tsuruta[87], 2018; Shimojima[24], 2016; Yamaguchi[19], 2015; Sugimura-Wakayama[88], 2015; Han[89], 2020; Sakai[52], 2012; Yamamoto[51], 2014; Chen[15], 2012; Mussano[90], 2018; Gunawardena[31], 2019; De cara[29], 2019; Wang[91], 2020; Hiraki[28], 2020; Miura-Yura[92], 2020; Ishikawa[26], 2016; Wakayama[23], 2015; Izumoto-Akita[20], 2015; Ogasawara [93], 2020; Sakai[94], 2020; Hirata[22], 2016; Asadi-Golshan[14], 2018; Mita[18], 2015; Inoue[95], 2013; Wei[96], 2020; Li[97], 2019
Periodontal ligament stem cells	Kang[98], 2018; Diomede[99], 2018; Aghamohamadi[100], 2020; Kolar[44], 2017; Nagata[101], 2017; Cianci[102], 2016; Wada[43], 2009; Qiu[30], 2020; Zhang[103], 2020
Stem cells from apical papilla	Kolar[44], 2017; Kumar[35], 2017; Kumar[33], 2017; Kumar[34], 2018; Bakopoulou[104], 2015; Zhuang[105], 2020; Yu[106], 2016; Yu [107], 2020; Yu[108], 2020
Dental follicule stem cells	Kumar[35], 2017; Kumar[33], 2017; Kumar[34], 2018; Chen[109], 2018; Wen[110], 2015; Wen[111], 2011; Liu[112], 2014; Wu[113], 2013
Gingiva-derived mesenchymal stem cells	Jin[114], 2020; Qiu[30], 2020; Mao[115], 2019; Wang[116], 2020; Zhang[117], 2019; Rajan[118], 2017; Rao[119], 2019, Diomede[120], 2018; Silvestro[121], 2020
Tooth germ progenitor cells	Wang[122], 2011; Huo[123], 2010; Ye[124], 2015; Yu[125], 2006; Shan[126], 2015; Yang[127], 2009; Yang[128], 2009

DMSC: Dental mesenchymal stem cells.

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Figure 1 PRISMA 2009 flow diagram. DMSC-CM: Dental mesenchymal stem cell conditioned medium.

However, MSC-CM manufacturing suffers from variable procedures and protocols, leading to different results between studies. Besides, there is no well-defined optimized procedure targeting specific applications in regenerative medicine.

In the present article, we focus on conditioned medium produced from DMSC and their derivative products. We review the current parameters and DMSC culture conditions used in the manufacturing

Figure 2 Orthopantomogram X-ray, with representative sources of various dental mesenchymal stem cells. (1) Dental pulp stem cells; (2) Stem cells from human exfoliated teeth; (3) Periodontal ligament stem cells; (4) Stem cells from apical papilla; (5) Dental follicle stem cells; and (6) Gingiva-derived mesenchymal stem cells.

protocols. The ultimate objective is to facilitate the standardization and optimization of manufacturing procedures needed for the clinical translation of DMSC-CM and their derivative products.

MATERIALS AND METHODS

This systematic review was performed in accordance with the PRISMA guidelines[32].

Data sources and research strategy

We have selected all publications investigating the effects of dental MSC secretome (CM, EV, exosomes) in *in vitro* and *in vivo* models of tissue regeneration by using the PubMed electronic database and the following search terms: ((dental stem cells) AND ((conditioned medium) OR (secretome))) / ((dental stem cells) AND ((extravesicles) OR (exosomes))). The bibliographic search considered articles meeting the inclusion criteria, and published between 2006 and July 2020. Articles taken into account had studied the DMSC secretome as a therapeutic agent, had analyzed the profiles of DMSC-CM, or included experiments based on DMSC-CM or its derivatives (EV, exosomes) to assess the paracrine activity of DMSC. Only articles in English were considered. All results were screened based on titles and abstracts. Full texts of the potentially selected records were obtained for definitive inclusion.

RESULTS

A total of 351 results were identified. And based on the inclusion criteria described above, 118 unique articles were included in the systematic review. Flow diagram is available as Figure 1.

To organize the analysis and interpretation, DMSC-CM production was considered at three stages: Before CM recovery (cell sources for CM), during CM production (culture conditions) and after production (CM treatment). For these three steps, we identified key points that were often taken into account in the published experimental works. A schematic representation of these elements is shown in Figure 3.

Dental mesenchymal cells: Type of cells

Most of studies have investigated the secretome effects of DPSC and SHED. The other secretomes, from PDLSC, SCAP, DFSC, GMSC, or TGPC have been less investigated (Table 1).

Most of these studies analyzed DMSC to verify their stemness. Specific cell surface markers were verified before starting the experimentation: positive expression of CD90, CD73, CD29, CD44, and CD105 (MSC markers), and absence of CD34, CD45, CD11b/c, CD31, CD144, and HLA-DR (endothelial/hematopoietic markers). Cell differentiation capacities were controlled for the adipogenic, chondrogenic, and osteogenic lineage. However, there were several studies in which stemness character was not controlled. And few studies did not considered stemness character for dental cells[58,65,111-113,129] (Supplementary Table 1).

DPSC, SHED, PDLSC, SCAP, DFPC, GMSC and TGPC derive from oral and tooth related structures. However, the specific properties of these different dental stem cell populations are slightly different according to the location from which they are isolated, in term of marker expression and differentiation potencies[130]. Thus, their secretome may also vary. A previously published study compared the neural potential of DMSC-CM obtained from three sources (DPSC, DFSC, and SCAP) with BMSC-CM: the
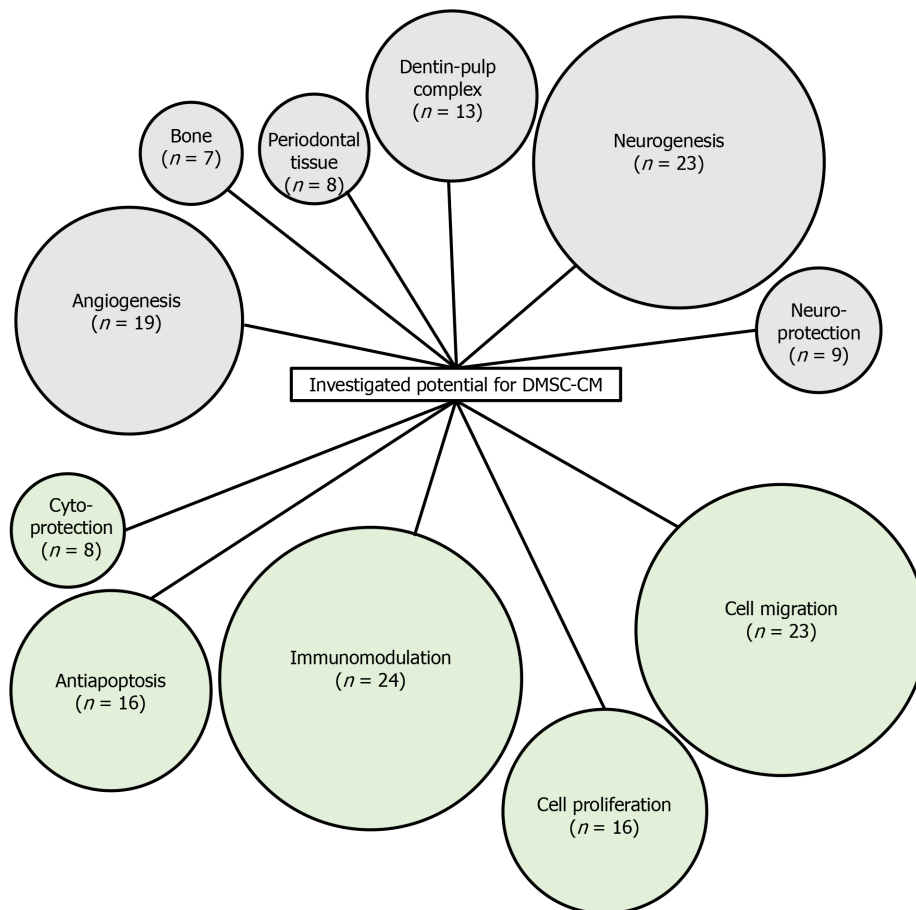
Figure 3 Schematic representation of the characteristics investigated as potential applications of dental mesenchymal stem cell conditioned medium. Number in brackets indicates the number of publications considering the activity. DMSC-CM: Dental mesenchymal stem cell conditioned medium.

results showed that neurite extension in neural cells were significantly lowered when cells were incubated in BMSC-CM, compared to DMSC-CM. However, among these three dental CM, a significant difference was observed, in term of *in vitro* effects and secretome profiles. Thus, neurite length was increased with neural cells incubated with DPSC-CM, compared to DFSC-CM and SCAP-CM. Besides, significantly different levels of neurotrophins and cytokines were observed in the three secretomes[35]. Another study compared also the neural potential of DMSC-CM from three dental sources (DPSC, SCAP, and PDLSC) of the same donor; Although all CM significantly enhanced axon regeneration and showed neuroprotective effects on neurons, results were better with SCAP-CM. By protein quantification, it was revealed that the level of secreted proteins were very similar for DPSC-CM and SCAP-CM, but different from PDLSC-CM[44]. A third study, comparing SHED and DPSC, could not highlight any difference between SHED-CM and DPSC-CM, as both secretome promoted neurite extension[52].

DMSC-CM have been generally compared with ASC-CM and BMSC-CM. Small particles with molecular weights between 30 and 100 nm were more abundant in DPSC-CM, whereas the fraction of intermediate particles (100-300 nm) was larger in BM-MSC[55]. DPSC-CM seemed to be more potent in neurogenesis[35,72], neuroprotection[13,39,42], angiogenesis[72,74], and migration[74] than ADCS-CM and BMSC-CM. Furthermore, the anti-apoptotic potential of DPSC-CM was found to be greater than that of BMSC-CM and ADSC-CM[39,74]. However, endothelial cell chemotaxis and *in ovo* neovascularization were less observed with DPSC-CM than BM-MSC[55]. Compared to umbilical cord mesenchymal stem cells (UMSC), some growth factors dominated in UMSC secretion, while others were more evident in DPSC, despite their overall similar profiles[40].

Similarly, SHED and other MSC differed in their basal expression of several biomolecules. Some relevant factors were abundant in SHED-CM but hardly detectable in ASC-CM[90]. SHED-CM was more anti-inflammatory[19,21] and anti-apoptotic[19] compared to BMSC-CM and ASC-CM. It was more efficient than BMSC-CM for the treatment of arthritis[26] and diabetes[20], for neuroprotection[62] and repair[18].

Concerning SCAP-CM, studies have shown that compared to BMSC, SCAP secrete more proteins associated with metabolic processes and transcription, but less proteins involved in biological adhesion,

immune function, and developmental processes. The amounts of chemokines and neurotrophins secreted by SCAP are significantly higher than those of BMSC, in contrast to extracellular matrix proteins and proangiogenic factors[106].

Cells origin: Donors

Aging has a clear influence on cell proliferation and capacity to differentiate. However, it is difficult to define a barrier discriminating optimal age for cell recovery. Decrease in dental MSC proliferation, migration, and differentiation has been correlated with age in the literature[131]. But among the publications considered in this systematic review, only one investigated the impact of donor age and could not highlight any significant difference between DPSC-CM from aged (44-70 years old) and young donors (19-30 years old), in term of trophic effects[37]. Taking the overall publications, donor age range in most of the studies is between 14 to 30 years old. Some studies considered donors over 30 years old[38,53,66, 67,102], with good results overall. In general, the number of donors, their gender and medical status were not reported. Inter-donor variability has rarely been taken into account, although teeth have been extracted from several donors. In most cases, DPSC were isolated from erupted or impacted third molars, but they were also obtained from premolars, canines and incisors in some studies. SHED were always isolated from deciduous teeth, with donors under 12 years of age. CSDF were obtained mainly from molars and premolars. The developmental stages of the teeth were considered only in few studies, and their clinical status (decayed or not) were not systematically provided.

Some studies used dental cells from rats, dogs, and pigs, making comparison even more complicated, in term of donor. Thus, it is not possible to conclude about optimal donor age range, according to the available data. However, except for SHED, most of the investigations were conducted with donors under 30 years (Figure 4). Complete donors' characteristics of studies investigating DMSC-CM are given in supplementary data (Supplementary Table 2).

Cell expansion

To our knowledge, the impact of cell passage number on their secretions has never been examined, while its effect on the concentration of secreted growth factors has only been noted once by Miura-Yura *et al*[92]. The passage number of cells ranged from 1 to 12 in studies investigating the potential of DMSC secretomes (Supplementary Table 3). Some studies did not even precise cell passage numbers[6,16,20,29, 30,60,66,68,72,82,85,87,88,92,98]. When considering cell expansion (through passage number), we can only observe that the majority of works were conducted with cells before passage 5, to prevent any risk of senescence during cell expansion (Figures 4 and 5).

Cell sorting

Significant variability in proliferative and differentiation capabilities have been observed for individual DPSC populations expanded from human teeth from donors within a similar age range. Inherent differences were even identified between DPSC populations derived from the same patient[132]. In some studies, cell population was selected before investigating DMSC-CM. Side populations were used in some studies, targeting CD31 marker[72,74] or CD31/CD146 markers[71,133]. These sorted populations from dental pulps are enriched in stem/progenitor cells and can significantly stimulate angiogenesis/vasculogenesis[133]. Stem cell mobilization through G-CSF induction was also used in several studies to isolate stem cells[37,49,54,60,73,75-77]. This technique generated a mobilized subpopulation of dental pulp stem cells that are stable and age-independent, with high proliferative, migratory and regenerative potentials. Single cell clones derived from human deciduous tooth pulp cells were used by Akazawa *et al*[65]. Finally, De Rosa *et al*[66] selected DPSC based on their relative positivity profile for markers such as CD34, CD117, CD44, STRO-1, RUNX-2 and OC, before studying the secretome potential of osteodifferentiated DPSC.

No consensus could be described about cell sorting before CM recovery. This is not surprising as mesenchymal/stromal population are heterogeneous populations in which no specific/unique marker is described so far.

Conditioning period

In most studies investigating DMSC-CM effects from secretome, cells were harvested for 48 h, starting with DMSC at 70%-80% of confluency (Histograms in Figure 4 and Supplementary Table 4 in supplementary data). Paschalidis *et al*[46] followed DPSC-CM collections from 4 d to 24 d, and described most pronounced effects within the first collection. The conditioning period was always considered with cells incubated in serum-free media. No clear difference could be enlightened from the various studies about protein concentration or DMSC-CM effects, but 48 h was the most used duration.

Cell culture medium

The principal culture medium used for DMSC-CM processing was DMEM. This medium was used for processing indifferently of the targeted application, or clinical objective: cardiac injury[19], diabetes[20], lung[23] or liver[22] injuries, bone regeneration[27], nerve regeneration[60], dental regeneration[73], and cerebral ischemia[95]. During the processing, in most of the studies, cells were kept in serum-free
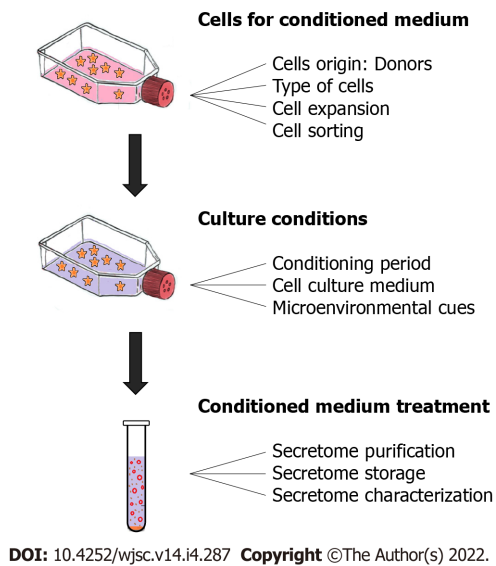
Cells for conditioned medium
- Cells origin: Donors
- Type of cells
- Cell expansion
- Cell sorting

Culture conditions
- Conditioning period
- Cell culture medium
- Microenvironmental cues

Conditioned medium treatment
- Secretome purification
- Secretome storage
- Secretome characterization

Figure 4 Schematic representation of the 3 main steps of evaluation, with corresponding key points.
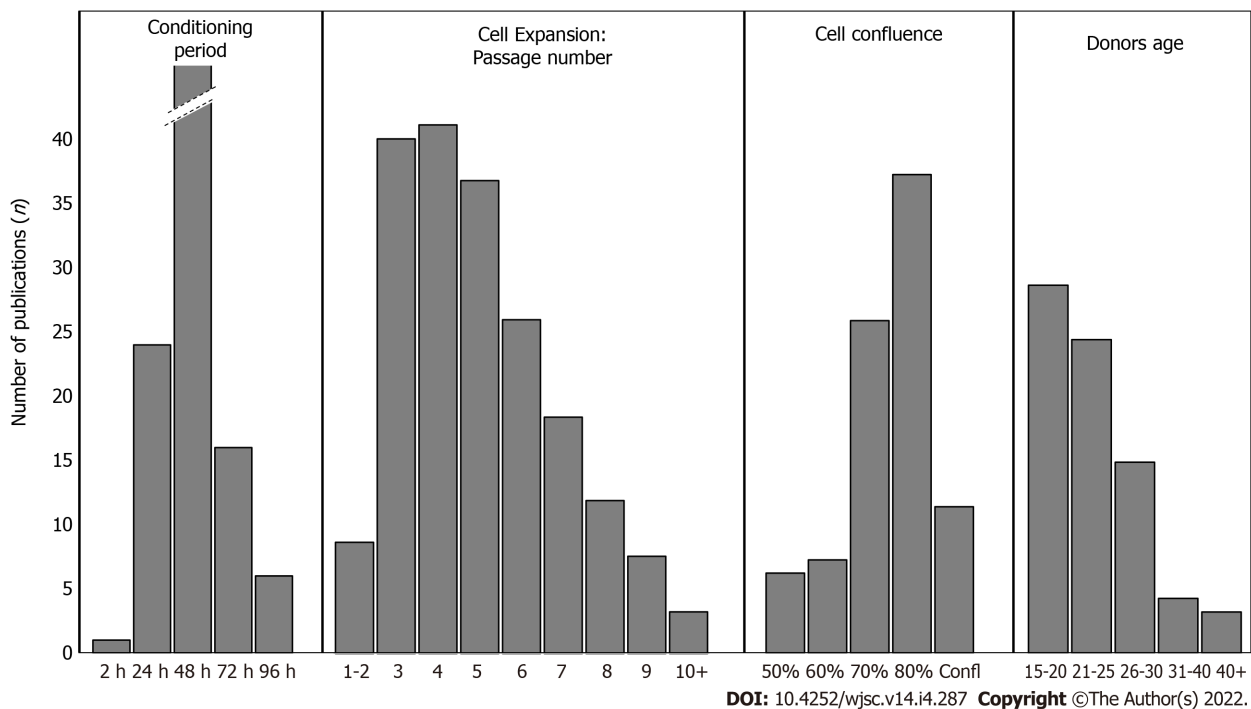
Figure 5 Histograms showing the number of publications for each parameter from the 4 specific key points: Conditioning period; Cell passage number; Cell confluence; Donor age.

conditions, without Phosphate Buffer Saline (PBS) washing step before conditioning period (Supplementary Table 5).

Microenvironmental cues

It has been described that MSC could act as repair cells within the body when stimulated and recruited. This concept lead to MSC implication, not only by natural and constitutive secretion of regenerative factors, but also by producing specific factors in response to stimuli[134]. Thus, the secretion of different potential therapeutic factors by MSC can be modulated according to various physical, chemical, and biological cues.

During DMSC-CM production, the effects of hypoxia, 3D culture, LPS preconditioning, and osteodifferentiation were studied (Supplementary Table 6). Hypoxia is an environmental cue increasing VEGF levels[68,104] in the secretome. A study even described that hypoxia increased angiogenic potential of SCAP conditioned medium[104]. It was shown that 3D culture increased anti-apoptotic effects of SHED

conditioned medium, when used with dopaminergic neurons[16]. LPS preconditioning increased DPSC conditioned medium potential for Schwann cells proliferation, migration, and odontogenic differentiation[50]. Secretome with LPS preconditioning also increased PDLSC anti-inflammatory effects[98]. The cytokine, chemokine and growth factor profiles of SHED-CM were significantly affected after osteoinduction of SHED cells[90], which also strengthened the effect of SHED-Exosomes on the osteogenic differentiation of PDLSC[91]. Osteogenesis of amniotic fluid stem cells was also stimulated by the secretome of osteodifferentiated DPSCs[66]. Kolar *et al*[44] stimulated DMSC using a previously published protocol[135], which increased significantly the secretion of VEGF-A and BDNF proteins and enhanced the effect of DMSC secretome on neurite outgrowth.

Each preconditioning regimen induced an individual expression profile with a wide variety of factors, including several growth factors and cytokines[136].

Secretome purification

DMSC-CM was concentrated before use in some studies and diluted to 50% in others[35,46,109,110]. A 50% diluted DMSC-CM has sometimes been proposed as the most effective[46], probably because of an optimal balance achieved between paracrine stimulatory and metabolic inhibitory products[46,137]. Only protein fractions with a molecular weight between 10 kDa and 3 kDa were able to protect neurons from induced neurodegeneration, indicating a possible responsibility of these microproteins for the neuroprotective character of DPSC-CM[39]. Only the fraction smaller than 6 kDa of SHED-CM promoted neurite growth in dorsal root ganglion neurons[92]. Finally, in most studies, filtration with 0.2µm pore filters was performed, in order to sterilize the CM and/or remove debris. Supplementary Table 7 summarizes the CM purification procedures in studies using DMSC-CM for tissue regeneration.

Numerous studies have been performed to assess if DMSC functions are associated with EV-enriched fractions or not. Merckx *et al*[55] demonstrated that the main angiogenic effect of growth factors secreted by DMSC was not associated with EVs. Nevertheless, Zhou *et al*[38] showed a decrease in the proangiogenic effects of DPSC-CM when EV secretion was blocked. DPSC exosomes in comparison with DPSC-CM showed similar neuroprotective efficacy, with superior anti-necrotic properties and implication in aging processes[39,138]. SHED-exosomes, in contrast to SHED-derived microvesicles, have approved anti-apoptotic effects in dopaminergic neurons[16]. However, they did not show the neuritogenic potential observed in SHED-CM[92]. Supplementary Table 8 in supplementary data summarizes the methods used to purify the DMSC-EV in the literature.

Secretome storage

DMSC-CM was stored at -80 °C in most studies, at -20 °C in three studies[78-80] and at +4 in two studies [87,88]. These different kind of storage did not affect the potentials of CM. Protease inhibitors were added to secretomes just in few studies[13,16,73,74]. Supplementary Table 9 in supplementary data summarizes the storage conditions of DMSC-CM in literature.

Secretome characterization

Secretome characterization is necessary to identify DMSC-CM profiles and to confirm the reproducibility of manufacturing. Different technics were used in the literature (Supplementary Table 10). Protein concentration in the secretome was measured with the BCA and Bradford protein assays. Other techniques were used for qualitative and quantitative identification of CM. With the development of technology, proteomics has become a strong tool for identification, and mass spectrometry has emerged as the main technique applied for the detection of proteins in cell secretomes[139]. A high protein coverage was obtained by mass spectrometry in a study conducted by Tachida *et al*[140] to identify the secretion profile of DPSC-CM. In that study, CM was prepared with DPSC isolated from rat incisors, having a confluence of 80-90%. Three washes with PBS were done before starting the conditioning of serum-free alpha-MEM medium by DPSC for 72 h. A 0.2µm filtration and a 50-fold concentration with a 3 KDa MWCO centrifugal unit were then performed[140]. The CM profile described in this study cannot represent DPSC-CM in general, as the CM preparation protocol is different from those used in other DMSC-CM studies. Comparing protein profiles and concentrations directly between studies is hampered by the variability of all the factors detailed above, and a production standardization may be the key to obtaining clear DMSC-CM profiles and ensure appropriate use of each CM based on its profile.

DISCUSSION

Among all the considered key points, at the 3 Levels of CM production, no consensus could be highlighted as evidence-based methods. We could only describe the most commonly used protocols. However, the various microenviroenmental cues were shown many times to have a significant impact on CM protein content, and improved effect on specifically targeted applications. Hypoxia enhanced the VEGF secretion and proangiogenic; 3D culture increased anti-apoptotic effects on neurons; LPS precon-

ditioning increased cell proliferation and migration, with enhanced anti-inflammatory activity; osteo-induction of DMSC enhanced the CM effect for further cell osteodifferentiation. Taken altogether, each preconditioning manipulation induced a specific protein expression profile.

More recently, we have demonstrated that specific preconditioning protocols could lead to DMSC-CM which significantly increase neurite growth in sensory neurons[141]. This neuroregenerative effect of CM due to preconditioning was linked to a change in secretome composition, with an increase of several factors involved in neurogenesis, neuroprotection, and angiogenesis.

CONCLUSION

We have reviewed here the different conditions and protocols used in the manufacturing of DMSC-CM and their derived products. This literature survey allowed us to describe that donors under 30 years of age are often used to produce CM. DPSC and SHED were the most commonly used cells, with cell passage between 1 and 5, and at a confluence of 70% to 80%. DMEM was the most commonly used culture medium for all applications. CM were often collected during the first 48 h, and frozen at -80 °C. The preconditioning environment (environmental cues) had a significant impact on DMSC-CM content and efficiency. Therefore, further studies should be conducted to confirm which environmental conditions specifically optimize the potentials of DMSC-CM for each application in tissue regeneration, and which parts of DMSC-CM are responsible for these potentials.

ARTICLE HIGHLIGHTS

Research background
Dental Mesenchymal stem cells are progenitor populations recovered from dental and periodontal tissues easily accessible when a tooth is extracted (and usually discarded as medical waste). One of the main effects of Mesenchymal stem cells on tissue regeneration is due to their paracrine activity, mediated by their secreted substances defined as secretome or conditioned medium. Conditioned medium represents a cell-free product with wide potential applications for various diseases treatment and tissue regeneration.

Research motivation
Conditioned medium manufacturing suffers from variable procedures and protocols. Results presented by various studies are difficult to compare, due to the different methods of production. Moreover, there is no well-defined optimized procedure to specifically target specialized tissue in regenerative medicine.

Research objectives
To describe potential consensus for the standardization and optimization of manufacturing procedures, mandatory for further clinical applications. In this systematic review, we focused on conditioned medium produced from Dental Mesenchymal stem cells. We explored the current parameters and culture conditions used in the manufacturing protocols.

Research methods
The bibliographic research was conducted in accordance with the PRISMA guidelines. All articles published between 2006 and 2020 investigating the effects of Dental Mesenchymal stem cells secretome on tissue regeneration were selected. We used the electronic PubMed database with these search terms: ((dental stem cells) AND ((conditioned medium) OR (secretome))) / ((dental stem cells) AND ((extravesicles) OR (exosomes))). Only publications in English were considered.

Research results
Based on the inclusion criteria, 118 articles were included in the systematic review. Conditioned medium production was considered at three levels: before recovery (cell sources), during production (culture conditions) and after production (secretome treatment). We identified key points that were often taken into account in the published experimental works.

Research conclusions
Among all the considered key points, no consensus could be highlighted. However, some tendencies could be described: cells used were mainly from donors under 30 years of age, with cell passage between 1 and 5, at a confluence of 70% to 80%. Conditioned medium was usually collected during the first 48 h, and kept frozen at -80°C. The various microenviroenmental cues were shown many times to have a significant impact on protein content, and improved effects on specifically targeted applications: each preconditioning manipulation induced a specific protein expression profile.

Research perspectives

Standardization of procedures is of prior importance to develop clinical-grade products. However, the protein contents of secretome is more linked to preconditioning than to specific technical methods. The challenge to overcome in the near future is to define specific preconditioning protocols to produce tissue specific conditioned medium (*i.e*, osteogenic environement, neuronal environment, cell incubation with inflammatory proteins…).

FOOTNOTES

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Treatment of syringomyelia using uncultured umbilical cord mesenchymal stem cells: A case report and review of literature

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Abstract

BACKGROUND
Syringomyelia is a disease caused by the formation of a cavity inside the spinal cord and is accompanied by such symptoms as pain, paresthesia, and urination and defecation disorders, and in severe cases causes various paralyses. Currently, there are only surgical methods for the treatment of syringomyelia, but these methods carry the possibility of failure, recurrence, and side effects.

CASE SUMMARY
The patient was a 59-year-old woman who suffered from pain due to syringomyelia. For treatment, the patient received transplant of uncultured umbilical cord-derived mesenchymal stem cells. As intended, the patient's pain was relieved after treatment. Interestingly, an additional benefit was found in that the size of the cavity also decreased. After 2 years from the last treatment, the patient's cavity had almost completely disappeared and her syringomyelia was deemed cured.

CONCLUSION
Using uncultured umbilical cord-derived mesenchymal stem cells may be a new treatment alternative for syringomyelia.

Key Words: Syringomyelia; Umbilical cord mesenchymal stem cells; Cell therapy; Allogenic stem cells; Chiari malformations; Case report

Core Tip: Syringomyelia has no suitable treatment other than surgical methods, but these methods carry the possibility of failure, recurrence, and side effects. In this study, we treated a patient with syringomyelia using uncultured umbilical cord-derived mesenchymal stem cells. This method could be a new treatment alternative for syringomyelia.

INTRODUCTION

Syringomyelia is a disease in which a cavity composed of fluid similar to cerebrospinal fluid or extracellular fluid is formed inside the spinal cord and gradually expands, thereby damaging the spinal nerves[1-4]. Cerebrospinal fluid is a bodily fluid that surrounds the brain and spinal cord and is known to protect the brain and spinal cord from external shocks and to transport nutrients and waste products [5]. Cerebrospinal fluid is produced in the choroid plexus of the ventricles located inside the brain and circulates in the subarachnoid space around the brain and spinal cord[6]. It is known that a spinal cavity is formed when the circulation of the cerebrospinal fluid is blocked in the subarachnoid space[1-4].

Various diseases can cause syringomyelia to include Chiari malformation, arachnoiditis, scoliosis, spinal tumors, spina bifida, and the like[4,7]. It is also known that syringomyelia can occur in patients with spinal cord injury due to trauma[4,8]. The most common sites for syringomyelia are the cervical and thoracic portions of the spinal cord, and sometimes even the medulla oblongata[1-4]. Symptoms caused by syringomyelia vary, depending on the scope. Patients may experience pain, abnormal sensations, and loss of sensation[4,9]. If the autonomic nervous system is invaded, then abnormal body temperature and defecation and urination disorders may occur[10]. If syringomyelia affects the medulla oblongata, then paralysis and atrophy of the tongue, difficulty swallowing, dysarthria, and facial paralysis may occur[4]. If syringomyelia is not treated, the 10-year survival rate is known to be about 50%[4]. In particular, post-traumatic syringomyelia can range from transient neurological deficits due to minor trauma to complete paralysis of the upper and lower limbs[1,4,8].

To date, surgery has been the only treatment method for syringomyelia[4,11,12]. Surgical treatment for syringomyelia focuses on the cause of the cavity and reducing the pressure of the subarachnoid space, and the shunt is mainly used to draw fluid out from the cavity[13,14]. In the case of Chiari malformation, the subarachnoid space is enlarged with foramen magnum decompression and dura mater plastic surgery, and the cerebellar tonsils that obstruct the flow of cerebrospinal fluid are excised. However, these surgical treatments are suboptimal for the treatment of syringomyelia[15-17]. While this treatment prevents further nerve damage and can be expected to improve symptoms, there is a risk of complications such as bleeding, inflammation, and nerve damage. After surgery, symptoms may continue to worsen or the cavity may become enlarged[17]. Therefore, surgery is usually not performed unless symptoms worsen after the observation period of the patient's progress.

In this study, we transplanted uncultured umbilical cord-derived mesenchymal stem cells (UC-MSCs) for the purpose of alleviating pain in a patient with syringomyelia. At the beginning of treatment, it was expected that this treatment would be effective in relieving pain, but it was not expected that the syringomyelia would be cured. As intended, the patient's disease-related pain gradually decreased. After the stem cell treatment, the patient also experienced an unexpected positive change which was observed during subsequent regular check-ups for follow-up after surgery for the Chiari malformation. First detected as a slight decrease in the patient's cavity size, the shrinkage continued until the cavity had almost completely disappeared at 2 years after the stem cell treatment. Although we could not elucidate the underlying mechanism of this beneficial outcome, we expect that the transplantation of uncultured UC-MSCs may have produced a direct or indirect effect on both her syringomyelia and related Chiari malformation.

CASE PRESENTATION

Chief complaints

On May 25, 2016, a 59-year-old woman, suffering from syringomyelia, visited our clinic. The patient had extreme symptoms of pain and dysphonia. The patient reported suffering particularly from pain in the eye, hand, and knee, as well as myalgia and occipital headache.

History of present illness

The patient reported having started experiencing neck and hand pain after a fall in 2009, with those pain symptoms having persisted for more than 1 year. In July 2010, the patient was diagnosed with Chiari malformation with syringomyelia. The patient underwent foramen magnum decompression on November 22, 2010, to treat the Chiari malformation and syringomyelia. After surgery, however, the imaging analysis revealed no changes at the C6-T2 level of the spinal cord in the localized syringomyelia (Figure 1A and B). Subsequent magnetic resonance imaging investigations of the extended cervical spine were performed on August 29, 2011 and August 22, 2012, to check changes in the cavity's size (Figure 1C-F). The data showed no significant changes at the C6-T2 level of the spinal cord in the localized cavity compared to imaging findings from 21 mo prior. The patient did not receive any additional treatment related to this disease other than the treatment received at our clinic after surgery.

History of past illness

The patient had no specific disease, except for Chiari malformation with syringomyelia.

Personal and family history

The patient had no relevant personal and family disease histories.

Physical examination

At the time of the first visit, the patient participated in a brief question-and-answer session to confirm the symptoms. The patient's pronunciation was not clear (but understandable) due to dysphonia. The patient also reported experiencing eye, hand, and knee pain, myalgia, and occipital headache at that time.

Laboratory examinations

Overall, there were no abnormalities on the routine blood and urine tests but total cholesterol (217 mg/dL; normal range: < 200 mg/dL) and low-density lipoprotein cholesterol (157 mg/dL; normal range: < 130 mg/dL) levels were borderline high.

Imaging examinations

Imaging examinations were not performed.

FINAL DIAGNOSIS

The patient was diagnosed with syringomyelia and was suffering from substantial pain and dysphonia.
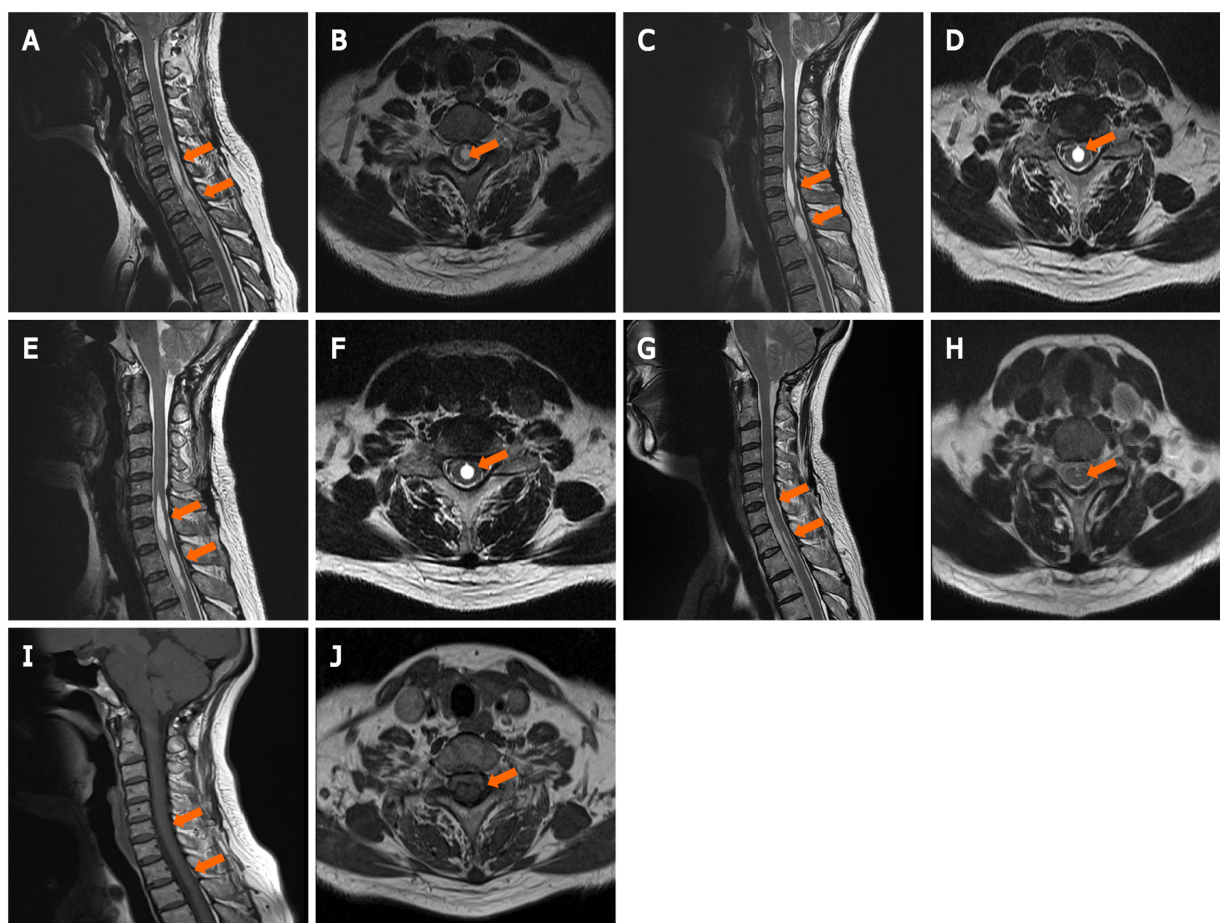
TREATMENT

UC procurement

UCs were donated by the Obstetrics and Gynecology Department at Lynn Woman's Hospital (Seoul, South Korea). The mothers who had donated the UCs had signed informed consent forms. All UCs were confirmed for safety through various tests of the mothers' blood and urine.

Isolation and quality evaluation of UC-MSCs

UC-MSCs were obtained from the donated UCs. The isolation method is as follows[18-21]. The UCs were disinfected with 70% ethanol and washed with 1 × phosphate-buffered saline. Next, three vessels and the amniotic membrane were removed from the UCs. Then, the UC tissues were cut into 2-3 cm pieces and placed into a 50-mL conical tube containing collagenase and hyaluronidase mixture solution. The UC tissues were then cut into smaller pieces using surgical scissors and ground using a disposable tissue grinder. Following, the ground tissues were placed in a 37 °C incubator with 50 mL/L CO_2 for 1 h. After filtration of the solution using a 100-μm filter, the UC-MSCs were harvested by centrifugation of the flow-through. The UC-MSCs were frozen using CryoStor® CS10 (Stemcell Technologies, Cambridge, MA, United States) and then stored at -80 °C for a day. The following day, the frozen cells were stored in liquid nitrogen.

The isolated UC-MSCs were analyzed by the expression level of MSC-specific proteins, such as CD73, CD90, and CD105. A CyFlow® Cube 6 (Sysmex, Lincolnshire, IL, United States) and FCS Express 5 software (De Novo Software, Glendale, CA, United States) were used. We only used cells that met the criteria of CD73 ≥ 70%, CD90 ≥ 90%, and CD105 ≥ 90% as a result of the analysis of the UC-MSCs. The UC-MSCs that were used also passed microbiological tests.

Figure 1 Imaging analyses before and after stem cell treatment. A-F: Magnetic resonance imaging scans of the patient before stem cell treatment, from November 26, 2010 (A: Sagittal; B: Transverse), August 29, 2011 (C: Sagittal; D: Transverse) and August 21, 2012 (E: Sagittal; F: Transverse); G-J: Magnetic resonance imaging of the patient after stem cell treatment, from July 18, 2018 (G: Sagittal; H: Transverse) and August 31, 2020 (I: Sagittal; J: Transverse). Orange arrows indicate where the cavity is or was.

Preparation of injection solution

We prepared 6×10^7 cells *per* treatment to transplant into the patient, calculated for 1×10^6 cells *per* kg body weight. In order to lower the viscosity of the injection solution, we divided the cell content in half, for two solutions with each containing 3×10^7 uncultured UC-MSCs mixed with 10-mL of 0.9% normal saline solution for each.

Treatment

We chose the intravenous injection method instead of local injection into the painful area as the transplantation method because the patient was experiencing pain throughout the whole body. The 10-mL injection solutions (as above, containing 3×10^7 UC-MSCs) were diluted for use by adding to 100 mL of 0.9% normal saline solution. In one treatment, the patient was injected with this 110-mL solution two times. The first 110-mL injection was given over a period of 1-2 h. After a 1 h break, the patient received the second injection. The patient received a total of four treatments over 8 mo. In July 2018, the patient received an additional treatment (Table 1).

OUTCOME AND FOLLOW-UP

During the treatment period, the patient experienced repeated pain reductions and then recurrences. After receiving the first (May 25, 2016) and second (July 26, 2016) treatments, the effectiveness of the treatment did not last long. The effect of the third treatment (August 9, 2016), however, was maintained for about 5 mo, and on January 13, 2017, the patient received the fourth treatment. The patient maintained the pain-reducing effect for a long period.

After syringopleural foramen magnum decompression for the treatment of Chiari malformation, the patient attended regular check-ups for follow-up after the surgery at the hospital where the surgery had

Table 1 Transplantation of uncultured umbilical cord-derived mesenchymal stem cells to the patient					
Treatment number	Date	In an injection		As a treatment	
		Cell amount	Volume in mL	Number of injections	Total cell amount
1	May 25, 2016	3×10^7	110	2	6×10^7
2	July 26, 2016				
3	August 9, 2016				
4	January 13, 2017				
5	July 20, 2018				

been performed. Interestingly, it was confirmed during the regular check-up in July 2018 that the cavity decreased in size (Figure 1G and H). Since the patient did not receive any treatment except for the uncultured UC-MSC transplantation, we considered the possibility that the stem cell treatment induced a reduction of the cavity size. Therefore, in July 2018, the patient received one more treatment. Afterward, the patient's existing symptoms, such as eye, hand, and knee pain, myalgia, occipital headache, and dysphonia, improved significantly. As a result of imaging analysis performed in August 2020, the cavity was almost completely disappeared (Figure 1I and J). Since then, the patient has maintained a significant decrease in pain and dysphonia.

Report of side effects
The patient had temporary nausea and dizziness after the first treatment. However, these symptoms were not present in the second through fourth treatments. The patient did not report any particular side effects during the follow-up period.

DISCUSSION
MSCs have a paracrine effect that helps protect and regenerate neuronal cells and promotes the ability for differentiation into neuronal cells[22-24]. Recently, as MSC transplantation has shown therapeutic potential in patients with spinal cord injury, clinical cases using MSC transplantation are increasing[25, 26]. Meanwhile, a Spanish research team reported the first case of MSC transplantation for a patient with post-traumatic syringomyelia in 2017, and the results of the follow-up study were announced in 2018[27,28]. So far, there have been only these two cases of syringomyelia treatment using cell therapy, and two additional studies using a syringomyelia model were reported in 2018 and 2020[29,30]. Although the mechanism of this benefit is not clear, as there are not many studies on MSCs and the treatment of syringomyelia, previous studies have shown that MSC transplantation has potential as an alternative treatment for syringomyelia.

The patient had undergone syringopleural foramen magnum decompression for the treatment of Chiari malformation with syringomyelia in 2010, but the syringomyelia remained. In the medical records issued by the hospital where the patient had been operated on, there was no significant change detected in the size of the cavity before 2014; however, there was a record of a slight decrease in the size of the cavity detected in March 2014. Unfortunately, since we could not secure magnetic resonance imaging (MRI), C-spine data was obtained in March 2014, but this precluded our ability to directly confirm the change in the size of the cavity. In addition, there was no record of syringomyelia in the medical record from March 2014 until May 25, 2016 (the start-day of UC-MSCs treatment). Therefore, based on the medical records and historic MRI C-spine results, we estimated that the patient's cavity size before UC-MSC therapy was similar or slightly reduced compared to that before surgery. After four UC-MSC treatments had been administered over an 8-mo period, the patient showed cavity size that was significantly reduced according to the magnetic resonance imaging findings in 2018. Confirmed by magnetic resonance imaging, the cavity had almost disappeared in 2020.

Because the initial purpose of the UC-MSC-based treatment of this patient was pain relief, our data are insufficient to support the treatment of syringomyelia by MSCs. In particular, the absence of data for the imaging investigations carried out just before the stem cell treatment and the pressure change that occurred due to the reduction of the cavity's size make it difficult to accurately interpret the study results. However, based on our results and findings from previous studies on the effect of MSC transplantation on syringomyelia, it is possible that MSC transplantation therapy plays a positive role in the treatment of syringomyelia patients[27-30]. We, therefore, expect that a future well-controlled and planned large-scale clinical study will provide support for this therapy as a new alternative for syringomyelia, which is particularly important since an appropriate treatment method is currently not available.

CONCLUSION

In this case study, a patient with syringomyelia was treated by intravenous transplantation of uncultured allogeneic UC-MSCs. Although this study is a case report for a single patient and there is room for interpretation of the data, considering the results of previously reported studies along with it, MSC transplantation may hold promise as a new treatment alternative for syringomyelia.

FOOTNOTES

Author contributions: Ahn H, Lee SY, Jung WJ, and Lee KH designed the report; Ahn H and Jung WJ collected the patient's clinical data; Ahn H, Lee SY, and Jung WJ analyzed the data; Ahn H wrote the manuscript; Lee KH provided professional advice and revised the manuscript; All authors issued final approval for the version to be submitted.

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LETTER TO THE EDITOR

Downregulation of miRNA-21 and cancer stem cells after chemotherapy results in better outcome in breast cancer patients

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Abstract

Epigenetic modifications have been observed as a decline in miRNA-21 expression and breast cancer stem cell (CSC) population after 3 cycles of standard chemotherapy. The epigenetic response (miRNAs expression) and CSCs are also correlated in patients with Breast Cancer. In patients who tolerated chemotherapy well, miRNA-21 (non-coding RNA) expression decreased significantly after three cycles of chemotherapy. The miRNA-21 expression in breast cancer tissue was quantified by quantitative PCR (real-time PCR) using the standard protocol. In addition, breast CSCs (CD44+/CD24-) were also decreased in these patients. The miRNA-21 regulates cell division, proliferation, and autophagy of cancerous cells (as it targets phosphatase and tensin homolog/AKT/transcription factor EB/programmed cell death 4/autophagy-related protein 5 and chemotherapy also produces similar effects), thereby contributing to these benefits. Therefore, when all of the targets on genes have been explored by mimic miRNA, chemotherapy combined with anti-miRNA21 therapy may prove useful in the care of cancer patients.

Key Words: Epigenetic modification; miRNA-21; Breast carcinoma; Autophagy;

Chemotherapy; Breast cancer stem cells

Core Tip: Epigenetic modification by non-coding RNAs (miRNA), along with the discovery of a cancer stem cell (CSC) database for all cancer types, has revolutionized oncology. The hallmarks of cancer include six capabilities acquired during the development of human tumors. These include sustaining proliferative signaling, evading growth suppressors, resisting cell death, facilitating replicative immortality, promoting angiogenesis, and promoting invasion and metastasis. These hallmarks are primarily manifestations of genome instability, which facilitates their acquisition, epigenetic modifications, and CSCs (Heterogenic tissue populations), which play vital roles in nurturing multiple hallmark functions. These alterations can be explored and targeted for better cancer management.

TO THE EDITOR

The article by Mandhair *et al*[1] provides an excellent review of the mechanisms behind autophagy, commonly known during cancer progression for its function as an oncolytic and adaptive signaling pathway. Additionally, the authors discussed various epigenetic modifications such as methylation, acetylation, non-coding RNA (miRNAs), and cancer stem cells (CSCs), which influence genomic diversity and promote carcinogenesis. The relentless work by scientists and clinicians on carcinogenesis has proposed a few hallmarks of cancer, including maintaining proliferative signaling, evading growth suppressors, avoiding cell death, inducing angiogenesis, and activating invasion and metastasis[2].

The author provided an excellent, well-organized description of transcriptional factors expression that are crucial for stemness behavior of CSCs, as well as the contribution of epigenetic modulation, which is involved in regulation of autophagy through various transcriptional factors and signaling pathways. Apoptosis (established hallmark) is regulated by autophagy through the degradation of NOXA (Phorbol-12-myristate-13-acetate-induced protein 1), while inhibiting autophagy increases NOXA protein levels by extending the protein half-life. NOXA accumulation inhibits tumor cell growth by inducing apoptosis, which is further enhanced when p53 is present[3].

The authors have successfully provided an in-depth understanding of autophagy in cancer and its modulation, even though we liked the idea that transcriptional factors, methylations, and non-coding microRNAs have diverse crossroads that affect other indicators of cancer and interfere with them[2]. Similarly, cancer treatment strategies such as chemotherapy and radiation therapy have also been found to interfere with and affect various transcriptional factors and epigenomic milieus of cancers.

Chemotherapy affects autophagy, and the cell cycle in well-tolerated and chemosensitive patients, resulting in better outcomes[4]. We also find, in patients with breast carcinoma (unpublished work), that miR-21 (non-coding RNA) was decreased significantly after three cycles of chemotherapy in those patients who had well tolerated the chemotherapy. Quantitative real-time PCR was used to assess miR-21 expression from breast cancer tissue using the established standard protocol[5].

The breast CSC populations (CD44+/CD24-) also declined in these breast cancer patients. The breast CSC populations (CD44+/CD24) were counted by Flow-cytometer using corresponding antibodies[5]. The miR-21 is one of the most consistently highly expressed onco-miRs in several cancers, where it regulates multiple stemness parameters. Suppression of apoptosis is one of the key roles of miR-21.

Few cancer types appear to be highly affected by epigenetic modifications that modulate miR-21 expression. As a result of treatment with the demethylating agent 5-aza-2'-deoxycytidine, several miRNAs such as miR-21 were strongly induced in the ovarian cell line OVCAR3 (Human ovarian cancer cell lines). In this study, hypomethylation may be responsible for promoting its *in vivo* overexpression. miR-21 inhibits apoptosis by targeting the FASL and by inhibiting an entire network of onco-suppressor genes, including tumor protein p53 (TP53), transforming growth factor β, *PTEN* (phosphatase and tensin homolog), and PDCD4 (programmed cell death 4)[6,7]. In addition, it is also known that tumor suppressor gene PDCD4 inhibits the expression of autophagy-related gene autophagy-related protein 5 (ATG5)[8]. According to the study, miRNA-21 regulates autophagy *via* PDCD4 and the autophagy-related gene ATG5.

We believe that the miRNA21 mimic study should also be conducted prior to planning translational research. The mimic-miRNAs are chemically synthesized duplexes that are designed to activate only one miRNA strand. It is often used to overexpress miRNA transiently and to augment endogenous microRNA activity for investigating gain or loss of function. In conjunction with microRNA and gene expression profiles, miRNA mimics and inhibitors may be tested for their role in identifying specific microRNA-gene relationships.

Several genes can be affected by a single microRNA, which is well established. They may, for instance, influence to the expression of proteins that are essential for normal biochemical reactions and physiological functions, or they may contribute to the development of diseases. In order to achieve therapeutic success, mimic-miRNA-based *in vivo* and *in vitro* studies must be performed to explore all possible target sites. Thus, miRNA-mimics-based studies can provide a complete understanding. It is thus may possible to unravel the whole scenario of gene targets just by increasing the targeted oligonucleotides (miRNA mimics).

Autophagy plays a context-dependent role in the development of cancer. CSCs have been found in almost all types of cancers with mildly altered immunophenotype but almost identical functions. Further, recent findings lend support to the hypothesis that the CSCs microenvironment can intriguingly regulate autophagy. Cells of malignant tumors, for example, induce autophagy in the microenvironment to increase the availability of recycled nutrients to support their own growth. Autophagy inhibition within the tumor has moderate effects on tumor progression through modulation of essential signaling pathways or by promoting resistance to chemotherapy, while autophagy inhibition through chloroquine oral administration reduces tumor growth and invasion more noticeably. Cancer (CSCs) regulates autophagy in vivo, but its exact role in tumor growth remains unclear. Recently, a study in the animal model of *Drosophila melanogaster* malignant tumors confirmed that autophagy is induced within the tumor microenvironment and distant tissues. It also reported that metabolically stressed tumor cells trigger autophagy through *Drosophila* tumor necrosis factor and interleukin-6-like signals[9].

We thus agree with the authors that modulating epigenetic factors (methylation, non-coding RNAs) and CSCs can modulate autophagy, and lead to better cancer treatment. Thus, translational cancer research must be planned in order to facilitate a paradigm shift from laboratory to bedside sites in the future and to pave the way for better cancer management.

FOOTNOTES

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LETTER TO THE EDITOR

Skinny people serum factors promote the differentiation of multipotent stem cells into brown adipose tissue

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Abstract

The original study by Alessio *et al* reported that skinny people (SP) serum can promote the formation of brown adipocytes, but not the differentiation of white adipocytes. This finding may explain why SP do not often become obese, despite consuming more calories than the body needs. More importantly, they demonstrated that circulating factors in SP serum can promote the expression of UCP-1 protein, thereby reducing fat accumulation. In this study, only male serum samples were evaluated to avoid the interference of sex hormones in experiments, but adult males also synthesize estrogen, which is produced by the cells of the testes. At the same time, adult females secrete androgens, and females synthesize androgens that are mainly produced by the adrenal cortex. We believe that the approach of excluding sex hormone interference by sex selection alone may be flawed, so we comment on the article and debate the statistical analysis of the article.

Key Words: Androgen; Cytokine; Estrogen; Gender; Multipotent stem cell; Obesity

Core Tip: Both men and women secrete estrogens and androgens. In females, androgens are mainly derived from the sites of the zona fasciculata and the zona reticularis in the adrenal cortex. In males, estrogens are produced by surrounding tissues, such as the skin, through the conversion of testosterone. Sex hormones in the serum can affect the differentiation and the stereotype of multipotent stem cells.

TO THE EDITOR

We have read with great interest the ingenious article written by Alessio *et al*[1] published in *World Journal of Stem Cells*. Their valuable research explains why skinny people (SP) ingest more calories than the body needs while presenting normal body composition. They reasoned that the existence of certain factors in the serum of SP can promote the differentiation of multipotent stem cells (MSCs) towards brown adipocytes. After reading the article, we agreed that several issues are worthy of discussion. Here, we state our views and contribute to the debate.

Studies have shown that sex hormones, including estrogens and androgens, can affect the differentiation and commitment of MSCs[2]. In a previous study, Alrabadi[3] reported that androgen injections could increase aggressiveness, as well as the body weight of rats, indicative of an association between aggressiveness and body weight. Serum androgen levels in men are usually higher than those in women due to testosterone secretion by the cells of the testes. Androgens can affect body weight by affecting MSCs, and testosterone (a type of androgen) can induce skeletal muscle hypertrophy through a variety of mechanisms. For instance, testosterone can regulate the commitment and differentiation of MSCs. Furthermore, testosterone increases lean body mass and reduces fat mass in young men, and the magnitude of these changes is significantly correlated with testosterone concentration[4]. Therefore, we suggest that the interference of androgen itself on MSC commitment and differentiation should be excluded as much as possible when studying cytokine stimulators of MSCs in the sera of adult males.

In men, estrogen exists in the plasma in a form with high biological activity, estradiol. Approximately 15% of the circulating estrogens are derived directly from the cells of the testes, and the remaining estradiol is derived from aromatase catalysis in peripheral tissues[5,6]. Adult males secrete approximately 30-40 micrograms of estradiol per day, and accumulating evidence supports the key roles of this hormone in the regulation of male metabolism. In men, estradiol may be a stronger influencing factor of obesity than testosterone, and even short-term estradiol deprivation can lead to increased fat mass[4,6]. Therefore, it is not ideal to eliminate the influence of sex hormones through gender selection, and we recommend that the authors expand the study by isolating and eliminating the interfering effects of sex hormones.

In the context of the global pandemic, there are no gender differences in overweight and obesity in most regions, except for a few developing areas with gender or ethnic differences[7,8]. We suggest that the authors expand the female sample size and consider the following factors. Androgen synthesis also occurs in women, and androgens are synthesized by the adrenal cortex and ovary. The adrenal cortex is a major contributor of androgen synthesis in women (Figure 1), and the sites of androgen synthesis are the zona fasciculata and the zona reticularis in the adrenal cortex. The adrenal cortex's ability to secrete androgens is maintained throughout life, with the organ mainly synthesizing dehydroepiandrosterone and androstenedione. Although their biological activity is weak, these hormones are converted into more active forms, where they function in the peripheral blood. More importantly, the active forms of androgens synthesized by the adrenal glands affect MSC commitment and differentiation, which in turn affects body weight and fat metabolism[9-11].

The study stated that serum samples were collected from 12 adult men with a normal body mass index. The author divided these men into two groups, namely, the SP group and the normal people (NP) group, with six samples in each group. Although the author stated that the daily calorie intake of the SP group exceeded 30%-40% of the body calorie requirement, we recommend that the author provide additional details on the individual diets.

Using the author's data, we employed Power and Sample Size Calculation software (HyLown Consulting LLC Atlanta, GA, United States) for sample size estimation. We estimated the sample size of triglycerides to a type II error β of 0.2, that is, a power of 0.8 (1-β) and a first type error (α) of 5%. According to the data in Table 1, which reported the clinical parameters, the mean concentrations of triglycerides in NP and SP groups were 79.2 mmol/L and 98.1 mmol/L, respectively, the sample ratio was 1, and the standard deviation(σ) was 22. According to our calculation, the theoretical conservative estimate of the sample size of each group should be at least 22 cases, while the sample size of each group in this study was only 6 cases. Thus, we believe that the statistical power of this study was low, and we suggest that the author expand the study by increasing the sample size.

Table 1 Normal and skinny patient parameters

	NP	SP
BMI (kg/m^2)	22.10 ± 1.10	20.50 ± 1.30
Glucose (mmol/L)	84.8 ± 6.20	87.0 ± 5.80
Total cholesterol (mmol/L)	190.6 ± 23.18	185.6 ± 20.12
LDL cholesterol (mmol/L)	127.2 ± 21.10	130.6 ± 25.27
HDL cholesterol (mmol/L)	59.3 ± 8.80	62.6 ± 12.14
Triglycerides (mmol/L)	79.2 ± 22.40	98.1 ± 46.42

NP: Normal people; SP: Skinny people; BMI: Body mass index; LDL: Low density lipoprotein; HDL: High density lipoprotein.
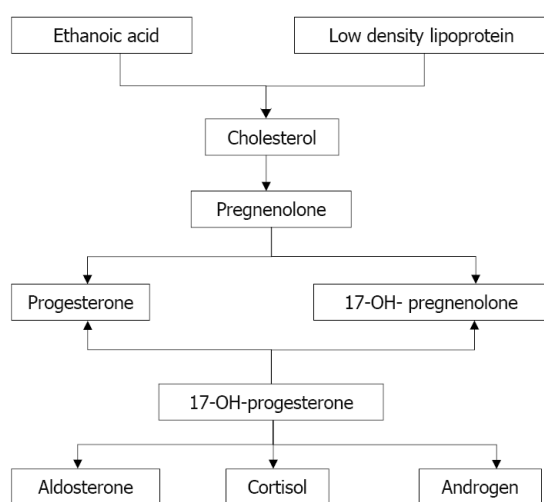
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Figure 1 Schematic diagram of the major steps of corticosteroid synthesis in the adrenal glands. Cholesterol is the synthetic raw material for all steroid hormones, including those produced by the adrenal glands. Approximately 80% of the cholesterol that is synthesized is derived from low density lipoprotein (LDL) in the blood, and a small amount is synthesized from acetic acid in cortical cells. After cholesterol binds to the LDL receptor in the cortical cell membrane, it enters the cell and is stored in the form of cholesterol. Under the actions of cholesterol-associating enzymes, cholesterol is decomposed into free cholesterol, which is transported into mitochondria *via* transporters, converted into pregnenolone by cholesterol side chain lyase, and then further transformed into various cortical stimuli.

FOOTNOTES

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