

World Journal of *Stem Cells*

World J Stem Cells 2014 April 26; 6(2): 69-255





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2010-2015

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INDEXING/ ABSTRACTING *World Journal of Stem Cells* is now indexed in PubMed Central, PubMed, Digital Object Identifier, and Directory of Open Access Journals.

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NAME OF JOURNAL
World Journal of Stem Cells

ISSN
ISSN 1948-0210 (online)

LAUNCH DATE
December 31, 2009

FREQUENCY
Quarterly

EDITOR-IN-CHIEF
Oscar Kuang-Sheng Lee, MD, PhD, Professor,
Medical Research and Education of Veterans General Hospital-Taipei, No. 322, Sec. 2, Shih-pai Road, Shih-pai, Taipei 11217, Taiwan

EDITORIAL OFFICE
Jin-Lei Wang, Director
Xiu-Xia Song, Vice Director

World Journal of Stem Cells
Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
Telephone: +86-10-85381891
Fax: +86-10-85381893
E-mail: bpgoffice@wjgnet.com
<http://www.wjgnet.com>

PUBLISHER
Baishideng Publishing Group Co., Limited
Flat C, 23/F, Lucky Plaza,
315-321 Lockhart Road, Wan Chai,
Hong Kong, China
Fax: +852-65557188
Telephone: +852-31779906
E-mail: bpgoffice@wjgnet.com
<http://www.wjgnet.com>

PUBLICATION DATE
April 26, 2014

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WJSC 6th Anniversary Special Issues (1): Hematopoietic stem cell transplantation

Allogeneic hematopoietic cell transplant for acute myeloid leukemia: Current state in 2013 and future directions

Abraham S Kanate, Marcelo C Pasquini, Parameswaran N Hari, Mehdi Hamadani

Abraham S Kanate, Osborn Hematopoietic Malignancy and Transplantation program, Section of Hematology/Oncology, West Virginia University, Morgantown, WV 26506, United States

Marcelo C Pasquini, Parameswaran N Hari, Mehdi Hamadani, Division of Hematology and Oncology, Medical College of Wisconsin, Milwaukee, WI 53226, United States

Author contributions: All the authors contributed equally to this manuscript.

Correspondence to: Mehdi Hamadani, MD, Associate Professor of Medicine, Division of Hematology and Oncology, Medical College of Wisconsin, 9200 West Wisconsin Avenue, Milwaukee, WI 53226, United States. mhamadani@mcw.edu
Telephone: +1-414-8050643 Fax: +1-414-8050643

Received: November 12, 2013 Revised: January 12, 2014

Accepted: March 13, 2014

Published online: April 26, 2014

Abstract

Acute myeloid leukemia (AML) represents a heterogeneous group of high-grade myeloid neoplasms of the elderly with variable outcomes. Though remission-induction is an important first step in the management of AML, additional treatment strategies are essential to ensure long-term disease-free survival. Recent pivotal advances in understanding the genetics and molecular biology of AML have allowed for a risk-adapted approach in its management based on relapse-risk. Allogeneic hematopoietic cell transplantation (allo-HCT) represents an effective therapeutic strategy in AML providing the possibility of cure with potent graft-versus-leukemia reactions, with a demonstrable survival advantage in younger patients with intermediate- or poor-risk cytogenetics. Herein we review the published data regarding the role of allo-HCT in adults with AML. We searched MEDLINE/PubMed and EMBASE/Ovid. In addition, we searched reference lists of relevant articles, conference proceedings and ongoing trial databases. We discuss the role of allo-HCT in AML patients stratified by cytogenetic- and molecular-risk in first complete remission, as well as allo-HCT as an option in relapsed/refractory

AML. Besides the conventional sibling and unrelated donor allografts, we review the available data and recent advances for alternative donor sources such as haploidentical grafts and umbilical cord blood. We also discuss conditioning regimens, including reduced intensity conditioning which has broadened the applicability of allo-HCT. Finally we explore recent advances and future possibilities and directions of allo-HCT in AML. Practical therapeutic recommendations have been made where possible based on available data and expert opinion.

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Key words: Acute myeloid leukemia; Allogeneic hematopoietic cell transplantation; Reduced intensity conditioning; Myeloablative conditioning; Haploidentical; Umbilical cord blood

Core tip: Acute myeloid leukemia (AML) represents a heterogeneous group of high-grade myeloid neoplasms of the elderly with variable outcomes. We discuss the role of allo-hematopoietic cell transplantation (HCT) in AML patients stratified by cytogenetic- and molecular-risk in first complete remission, as well as allo-HCT as an option in relapsed/refractory AML.

Kanate AS, Pasquini MC, Hari PN, Hamadani M. Allogeneic hematopoietic cell transplant for acute myeloid leukemia: Current state in 2013 and future directions. *World J Stem Cells* 2014; 6(2): 69-81 Available from: URL: <http://www.wjgnet.com/1948-0210/full/v6/i2/69.htm> DOI: <http://dx.doi.org/10.4252/wjsc.v6.i2.69>

INTRODUCTION

Acute myeloid leukemia (AML) comprises a group of high-grade clonal neoplasms of the myeloid progenitor cells. With a median age of 66 years, AML is a disease

of the older age group with an annual incidence of 4.4 per 100000. It is estimated that approximately 15000 new cases of AML will be diagnosed in the United States in 2013^[1]. While the goal of initial therapy in AML is attaining complete remission (CR), without additional post-remission therapy disease relapse is inevitable in vast majority of the cases^[2]. In the past two decades little has changed in AML induction chemotherapy regimens, but our improved understanding of the disease biology in identifying high-risk groups with modern cytogenetics and molecular testing have led to better risk-stratification that facilitates customization of post-remission therapy based on the relapse-risk^[3-5]. While allogeneic hematopoietic cell transplantation (allo-HCT) has been long considered a potentially curative therapy for AML^[6], advances in human leukocyte antigen (HLA)-matching, supportive care, optimal pre-transplant conditioning and advent of alternative donor allografting have broadened the availability and improved transplant outcomes^[7]. Herein we review the role of allo-HCT in adults with AML in first complete remission (CR1), discuss the allograft options in advanced AML (beyond CR1), and review the current state of reduced-intensity and alternative donor allo-HCT in the management of AML.

PROGNOSTIC FACTORS IN AML

Traditionally used prognostic factors in AML include age, leukocyte count at diagnosis, performance status, extra-medullary involvement, antecedent hematologic disorders and initial response to therapy. Cytogenetics by metaphase and interphase analysis are one of the most powerful prognostic factors in AML, providing us the ability to risk-stratify patients at diagnosis. Acute promyelocytic leukemia t(15;17) and core binding factor (CBF) leukemia t(8;21) and inv(16)/t(16;16) are favorable-risk AML, largely retaining their good prognosis even with additional cytogenetic abnormalities^[8-10]. Chromosomal abnormalities conferring poor outcomes include abnormalities of chromosome 3q (abn1 3q), deletions of 5q (-5q), monosomies of chromosome 5 or 7 (-5/-7), and complex karyotype. Large cooperative group studies have confirmed the impact of cytogenetics on survival rates, reporting 55%-65% and 5%-14% 5-year overall survival (OS) for patients with favorable- and poor-risk cytogenetics, respectively^[8,11,12]. Grimawade *et al.*^[10] reported outcomes in 5876 patients treated on Medical Research Council (MRC) trials and identified abn1 3q (excluding t(3;5)(q25;q34)), inv(3)(q21q26)/t(3;3)(q21;q26), add5q/-5q, -5, -7, add(7q)/-7q, t(6;11)(q27;q23), t(10;11)(p11;q23), other t(11q23) (excluding t(9;11)(p21;q23) and t(11;19)(q23;p13)), t(9;22)(q34;q11), -17, abn1(17p) and complex karyotype as poor risk cytogenetic aberrations. Presence of monosomal karyotype (defined as 2 or more autosomal monosomies or combination of 1 monosomy with structural abnormalities) is associated with very poor prognosis with 4-year OS < 5%^[13,14]. Similarly, the presence of subclones within the poor risk cytogenetic category (*i.e.*, clonal heterogeneity) may confer poorer

Table 1 The European LeukemiaNet Standardized Reporting System for risk stratification of acute myeloid leukemia based on cytogenetics and molecular testing¹

| Risk category | Cytogenetic abnormalities | Molecular abnormalities |
|-------------------|--|---|
| Favorable risk | t(15;17) inv(16)/t(16;16) ² t(8;21) ² | CN-AML with biallelic CEBPA mutation CN-AML with NPM1 mutated but FLT3-ITD negative |
| Intermediate risk | CN-AML t(9;11) All others abnormalities not classified as favorable or adverse risk | CN-AML with: NPM1 mutated/FLT3-ITD positive NPM1 wild type/FLT3-ITD negative t(8;21)/inv (16) with c-KIT mutation |
| Adverse risk | inv (3)/t(3;3) t(6;9) t(v;11)/MLL rearranged -5/-5q -7 Monosomal karyotype Abnormal 17p Complex cytogenetics | CN-AML with FLT3-ITD positive |

¹Table modified from Mrózek *et al.*^[24]; ²The good prognosis of inv(16) and t(8;21) is maintained even with additional cytogenetic abnormalities. The presence of concomitant c-KIT mutation may increase relapse risk in t(8;21) and to lesser extent inv(16). CN-AML: Cytogenetically normal acute myeloid leukemia; CEBPA: CCAAT enhancer binding protein alpha; FLT3-ITD: FMS-like tyrosine kinase 3 gene-internal tandem duplication; MLL: Mixed lineage leukemia; NPM: Nucleophosmin.

outcomes^[15]. Recently, Middeke *et al.*^[16] found the presence of abn1(17p) and -5/-5q, within complex and monosomal karyotype AML characterized ultra high-risk disease.

Work done in the last decade has further enhanced our ability to stratify cytogenetically normal AML (CN-AML) based on presence of molecular aberrations into poor-risk [*e.g.*, FMS-like tyrosine kinase 3 gene-internal tandem duplication (FLT3-ITD), mixed-lineage leukemia gene-partial tandem duplication (MLL-PTD), overexpression of Wilms' tumor gene 1 (WT1), brain and acute leukemia, cytoplasmic gene (BAALC), ETS-related gene (ERG), KIT-gene and ecotropic viral integration site 1 gene (EVI1)] and good-risk [nucleophosmin (NPM1), isocitrate dehydrogenase (IDH 1/2) and CCAAT enhancer binding protein alpha (CEBPA)] categories^[15,17-23]. Integrating conventional cytogenetics and the commonly utilized molecular testing markers (FLT3-ITD, CEBPA and NPM1), the European LeukemiaNet validated the effect of prognostic factors on remission rates, disease-free survival (DFS) and OS (Table 1)^[24,25]. The improved understanding of the molecular basis of AML and its ramifications on patient outcomes has important relevance in clinical decision making, heralding the era of "individualized" post-remission therapy (Figure 1).

CONSOLIDATION WITH ALLOGENEIC HCT IN CR1

Remission induction reduces the leukemic burden

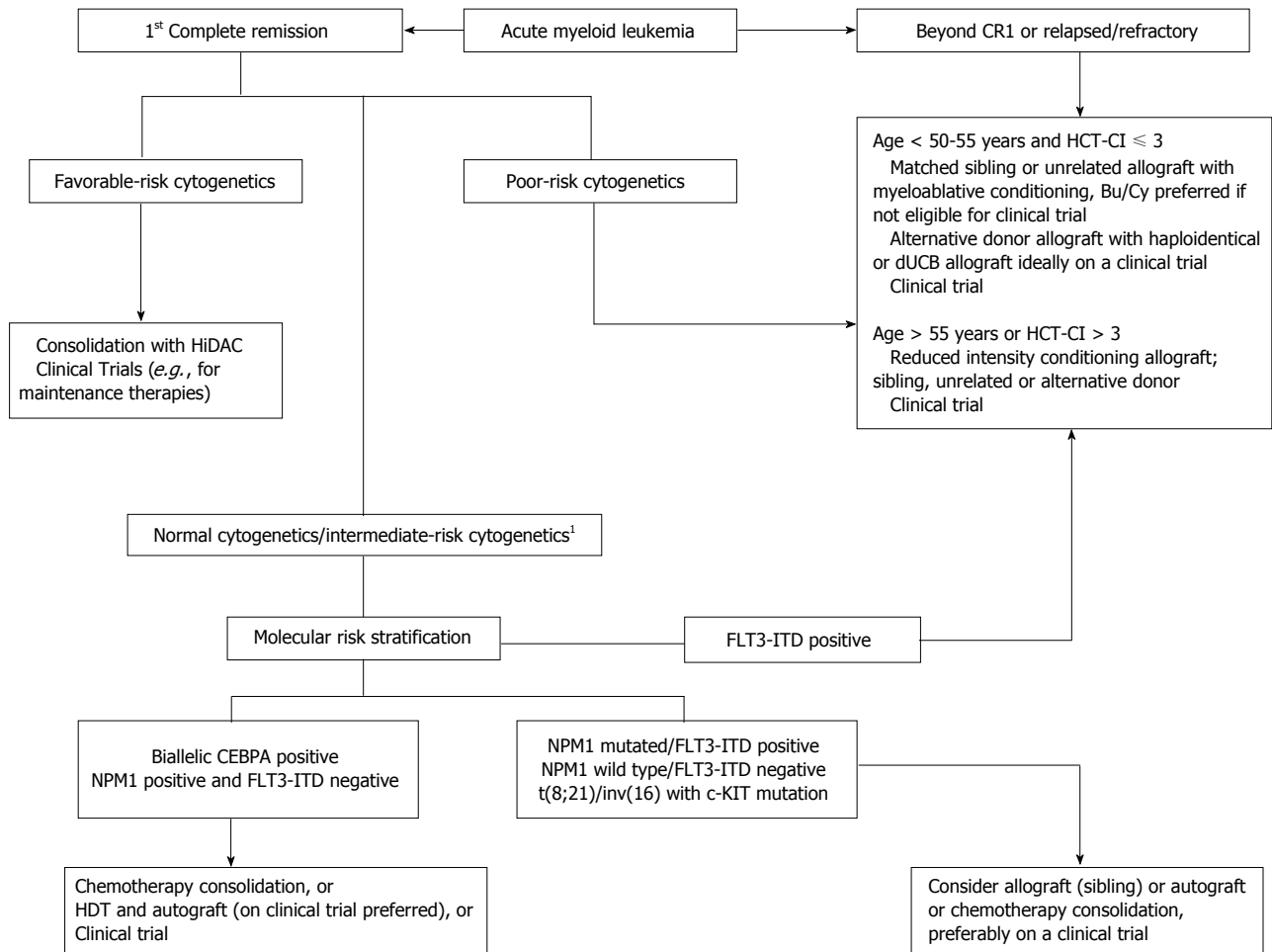


Figure 1 Clinically useful algorithm for optimal consolidation for acute myeloid leukemia patients based on cytogenetic and molecular genetic aberrations, based on available data and practice preference. ¹Allogeneic HCT may be considered in medically fit AML patients with intermediate risk/normal cytogenetics in CR1. Bu/Cy: Busulfan/cyclophosphamide; CEBPA: CCAAT enhancer binding protein alpha; CR: Complete remission; dUCB: Double umbilical cord blood; FLT3-ITD: FMS-like tyrosine kinase 3 gene-internal tandem duplication; HCT-CI: Hematopoietic cell transplantation-comorbidity index; HDT: High dose therapy; HiDAC: High dose cytarabine; NPM: Nucleophosmin.

roughly from 1×10^{12} cells to approximately 1×10^9 cells, if the patient achieves a morphologic CR. Hence additional consolidative therapy is necessary to eradicate a sizeable leukemic clone in patients in morphologic CR to achieve long-term DFS. Generally using chemotherapy-based consolidation approaches alone, the relapse rates in intermediate- and poor-risk cytogenetic groups remain unacceptably high^[26] and represent an area where alternative consolidation approaches are warranted. Allogeneic HCT for patients in CR, not only provides a “tumor-free” graft, but more importantly the donor effector T-cells recognize and mount an effective immune response against the leukemia cells [*i.e.*, the graft-versus leukemia (GVL) effect], to provide patients with durable disease control. While the potent GVL effects of allogeneic HCT provide the most effective post-remission therapy for AML patients in CR1, the associated morbidity and mortality warrants careful selection of high-risk patients, likely to benefit the most from this approach, and sparing the toxicity in lower-risk cohorts.

SIBLING DONOR ALLOGENEIC HCT IN CR1

Prospective single institution studies comparing allo-HCT with consolidation chemotherapy (CC) in the 1980s and early 1990s showed lower relapse rates and improved DFS with allo-HCT for AML patients in CR1, but none conclusively demonstrated an OS advantage^[27,28]. Subsequently, six cooperative group trials (Table 2) have examined the role of allo-HCT in AML in CR1^[28-33]. Those with HLA-matched siblings were offered allo-HCT (“genetic randomization”) while the others were randomized to autologous transplantation or CC on an intention-to-treat analysis. In the European Organization for Research and Treatment of Cancer (EORTC)-Gruppo Italiano Malattie Ematologiche Maligne Ddell’Adulto (GIMEMA) trial^[29], superior 4 year DFS was noted with allo-HCT (55%) and autologous HCT (48%) compared to CC (30%). However, no OS improvement was seen with either transplant modality^[34]. In the Groupe Ouest-

Table 2 Cooperative group trial of allogeneic hematopoietic cell transplantation for acute myeloid leukemia in first complete remission

| Cooperative group | Relapse rate | | | Disease free survival | | | Overall survival | | |
|------------------------------|------------------|------|-----|-----------------------|------------------|-----|------------------|------|------------------|
| | Allo | Auto | CC | Allo | Auto | CC | Allo | Auto | CC |
| EORTC/GIMEMA AML-8 | 24% ¹ | 41% | 57% | 55% ¹ | 48% ¹ | 30% | 59% | 56% | 46% |
| GOELAM | 37% | 45% | 55% | 49% | 48% | 43% | 55% | 52% | 58% |
| ECOG/CALGB/SWOG | 29% ¹ | 48% | 61% | 43% | 34% | 34% | 46% | 43% | 52% ¹ |
| EORTC/GIMEMA AML-10 | 30% ¹ | 52% | - | 52% ¹ | 42% | - | 58% | 50% | - |
| UK MRC AML-10 ^{2,3} | 36% ¹ | 52% | | 50% ¹ | 42% | | 55% | 42% | |
| HOVON-SAKK ³ | 32% ¹ | 59% | | 48% ¹ | 37% | | 54% | 46% | |

¹Represents statistically significant and favorable outcome with the treatment modality; ²The 4-year relapse rate, disease free survival and overall survival shown in all studies, except the UK-MRC AML-10 which reported 7-year outcomes; ³All studies designed to compare outcomes between allograft *vs* autograft *vs* consolidation chemotherapy except the UK MRC AML-10 and HOVON-SAKK trial which did not differentiate between autograft and chemotherapy. Allo: Allogeneic transplantation; auto: Autologous transplantation; CC: Consolidation chemotherapy.

Est Leucémies Aigues Myeloblastiques study, the relapse rates following allo-HCT were unusually high (37% at 4 years) and likely explain the lack of therapeutic benefit with allografting in this study^[30]. The MRC reported improved DFS but not OS in the MRC AML-10 patients randomized to allo-HCT^[31]. Similarly the US intergroup trial showed that the higher treatment related mortality (TRM) in patients randomized to allo-HCT arm negated the benefits of lower relapse rates in this group, resulting in no net OS advantage with transplantation in CR1 over chemotherapy alone^[28]. Although provocative, the data from these cooperative group trials failed to provide any concrete guideline for selecting the optimal post-remission strategy for individual patients with a matched sibling donor available in CR1.

Impact of cytogenetic and molecular markers on allo-HCT in CR1

Integrating information regarding cytogenetic-risk categories in the outcome analysis of aforementioned cooperative group trials was the next logical step. Reanalysis of the EORTC/GIMEMA AML-10 trial by cytogenetic-risk stratification showed superior DFS (43% *vs* 18%) and OS (50% *vs* 29%) with allo-HCT compared to autografting in patients with poor-risk cytogenetics^[32]. However allo-HCT did not benefit patients with good-risk [t(8;21), inv(16)] or intermediate-risk (normal or -Y) cytogenetics. Similar cytogenetic-risk stratification of the US intergroup trial showed a 5-year OS of 44%, 13% and 15% with allo-HCT, autologous-HCT and CC respectively, in patients with poor-risk cytogenetics^[12]. No improvement in OS was observed in patients with good or intermediate-risk disease. Unlike the prior studies, the Dutch-Belgian Haemato-Oncology Co-operative Group (HOVON) and Swiss Group for Clinical Cancer Research (SAKK) trial demonstrated superior DFS with allo-HCT for both intermediate and poor cytogenetic-risk patients^[33]. It may be noted that risk stratification in the HOVON-SAKK trial included additional variables. Patients with intermediate-risk cytogenetics requiring two induction cycles to achieve CR1 were classified as poor-risk, only t(8;21) AML patients with a white blood cell count of $< 20 \times 10^9/L$ were considered favorable and patients with

unknown cytogenetics ($n = 89$) were considered intermediate-risk group. Two separate meta-analyses conducted by the HOVON-SAKK group and Koreth *et al*^[35] have confirmed survival benefit with allo-HCT in patients with intermediate- and poor-risk cytogenetics in CR1. Allogeneic HCT in CR1 also appears to improved DFS and OS in AML with monosomal karyotype, compared to other consolidation strategies.

Recognition of the prognostic value of additional molecular markers is facilitating further risk stratification of the heterogeneous group of patients with CN-AML. The German-Austrian Acute Myeloid Leukemia Study Group showed that transplantation might have an important role in a molecular subset of patients with CN-AML. Patients with normal cytogenetics were randomized based on availability of an HLA-identical sibling donor for allo-HCT in CR1 *vs* chemotherapy alone. No benefit of allogeneic transplantation was seen in patients whose leukemia was *NPM1* mutated without *FLT3*-ITD. Conversely, patients with the *FLT3*-ITD mutation or the genotype consisting of wild-type *NPM1* and *CEBPA* without *FLT3*-ITD, benefited from an allogeneic transplant performed during CR1^[36]. In double mutant *CEBPA* allo-HCT or autografting in CR1 improved DFS without impacting OS compared to CC^[37].

Matched sibling allo-HCT in medically fit AML patients, with poor- and intermediate-risk (at least in the *FLT3*-ITD+ or *NPM1*-/*CEBPA*-/*FLT3*-ITD- subgroups) cytogenetics, who are able to achieve CR1 should be considered a standard option.

UNRELATED DONOR ALLOGENEIC HCT IN CR1

The strength of evidence presented above supports allo-HCT from a sibling donor in intermediate-/poor-risk AML in CR1. Unfortunately only approximately 25%-30% of AML patients have an HLA-identical sibling. No randomized trials have looked at unrelated donor (URD) allo-HCT for AML in CR1. Yakoub-Agha *et al*^[38] reported similar outcomes with respect to acute graft-versus-host disease (GVHD), TRM, and OS in patients with standard-

risk hematologic malignancies who received HLA-A, -B, -C, -DRB1, and -DQ (10/10) allele-matched allografts from either sibling or unrelated donors. Although randomized, prospective trials of URD transplantation for AML in CR1 are lacking, a number of retrospective studies provide evidence in support of the approach. Sierra *et al*^[39] reported outcomes of URD transplantation in 161 AML patients at various stages of disease including 16 patients with poor-cytogenetic risk AML in CR1 with a 5-year DFS of 50%. The corresponding DFS for those undergoing allo-HCT in CR2, relapse, or primary induction failure were 28%, 7%, and 19%, respectively. Bashir *et al*^[40] reported a 3-year OS and TRM of 78% and 15% respectively in a cohort of 44 patients (59% poor risk cytogenetics) who underwent URD allo-HCT in CR1. In a Center for International Blood and Marrow Transplant Registry (CIBMTR) analysis of 476 patients undergoing URD allo-HCT; adjusted 3-year OS, and DFS, in CR1 were 44%, and 43% respectively^[41]. Interestingly, Tallman *et al*^[42] found no difference in survival by cytogenetic-risk stratification for AML patients undergoing URD allo-HCT in CR1. However, the reported 5-year DFS of 30% in cytogenetically poor-risk AML likely represents a better outcome than with other non-HCT treatment strategies^[43]. The presence of complex cytogenetics (> 3), however likely represent a high-risk group with poorer outcomes even with allo-HCT in CR^[44].

European Group for Blood and Marrow Transplantation (EBMT) recently reported outcomes of 206 CN-AML patients in CR1 undergoing HLA-identical sibling or matched URD allo-HCT with reference to their *FLT3-ITD* status (present: $n = 120$, 58%; absent: $n = 86$, 42%)^[45]. *FLT3-ITD*-positive patients, compared with *FLT3-ITD*-negative patients had higher 2-year relapse incidence (30% *vs* 16%, $P = 0.006$) and lower DFS (58% *vs* 71%, $P = 0.04$). More importantly, more than half of the patients harboring this mutation who received matched sibling or URD allo-HCT were alive and leukemia free at 2 years. URD allo-HCT in CR1 however may be associated with a higher TRM as noted in a registry study that reported trends of outcomes over the last two decades, underlining the need to carefully select patients for URD allo-HCT. For poor-risk cytogenetics and *FLT3-ITD*+ CN AML patients in CR1 lacking an HLA-matched sibling donor, it is certainly reasonable to consider matched URD allo-HCT.

OPTIMAL CONDITIONING REGIMENS

Myeloablative conditioning regimens (MAC) utilizing chemotherapy and/or total body irradiation (TBI) have been the basis of most of the studies discussed thus far. The two most commonly utilized MAC regimens are busulfan/cyclophosphamide (Bu/Cy) and cyclophosphamide/TBI (CY/TBI). Although prior studies showed inferior DFS and OS with Bu/Cy conditioning^[46,47], a large meta-analysis did not show any difference between the two regimens with regards to survival and relapse^[48]. It has been widely noted that the erratic bioavailability

of oral busulfan was the likely cause inferior outcomes. Recent EBMT data comparing intravenous Bu/Cy to CY/TBI in AML found increased incidence of GVHD with TBI conditioning, and a trend towards improved TRM with Bu/Cy but no difference in DFS at 2-year^[49]. A larger CIBMTR analysis clearly showed better DFS (RR = 0.70, 95%CI: 0.55-0.88, $P = 0.003$) and OS (RR = 0.68, 95%CI: 0.52-0.88, $P = 0.003$) in AML patients receiving IV, but not oral busulfan compared to TBI^[50]. Similar observations (lower TRM with Bu/Cy and better OS compared to TBI-based regimens) were made in a prospective cohort study of CIBMTR^[50,51]. Collectively these data suggest that in the era of pharmacokinetically driven adjustment of intravenous busulfan dosing, in younger (< 50 -55 year) AML patients Bu/Cy should be considered the preferred MAC regimen for allo-HCT.

The use MAC is limited to medically fit, younger AML patients. The observed lower TRM rates using the so-called non-myeloablative (NMA) or reduced-intensity conditioning (RIC) regimens have broadened the applicability of allo-HCT to elderly patients or younger patients with comorbidities. Unlike MAC regimens; the NMA/RIC allo-HCT relies more heavily on the GVL effects to eradicate disease in the recipient. The decision to use NMA or RIC regimens for AML patients undergoing allo-HCT is not always clearly delineated, and significant variations exist in the selection criteria used by transplant centers across the globe. Sorror *et al*^[52] evaluated the impact of a priori medical comorbidities on transplant outcomes by using the HCT-Comorbidity Index (HCT-CI), and reported significantly higher TRM rates and inferior OS in patients with an HCT-CI score of ≥ 3 . While not validated in prospective clinical trials, it is increasingly becoming common practice to offer RIC allo-HCT to AML patients of advanced age (generally > 50 -55 years), and/or HCT-CI > 3 (regardless of age), or with a prior history of autologous transplantation or less optimal performance status^[53,54].

The acute leukemia working party of the EBMT compared transplantation outcomes for 315 RIC and 407 MAC recipients^[55]. While the incidence of grade II-IV acute GVHD (22% *vs* 31%) and 2-year TRM (18% *vs* 36%) significantly favored the RIC group, more patients with RIC allograft experienced disease relapse compared to MAC regimens (41% *vs* 24%). The DFS and OS did not differ between the two groups. Another report noted grade II-IV acute GVHD rates and 2-year relapse rates of 40% and 39% respectively in 122 AML patients who received a RIC regimen with 2-year DFS of 44%^[56]. A Spanish prospective, multicenter trial of patients with poor-risk AML/myelodysplastic syndrome reported 4-year DFS and OS rates of 43% and 45% with RIC and showed that development of chronic GVHD was strongly associated with reduced risk of relapse and improved OS and DFS, providing proof of concept for clinically relevant GVL effects with RIC allotransplantation^[57].

RIC in AML has generally shown lower TRM with comparable OS and DFS to MAC regimens, but follow up is relatively short thus limiting conclusions. The ongoing

ing prospective randomized BMT-CTN 0901 clinical trial (NCT01339910) comparing RIC regimens against MAC in AML/myelodysplastic syndrome will hopefully clarify the optimal conditioning intensity in AML. The advent of RIC allo-HCT has indeed extended the feasibility and applicability of allogeneic transplantation to include those with advanced age and multiple co-morbidities, thus offering them possibly a better chance for long term DFS.

ALTERNATIVE DONOR TRANSPLANTATION

Umbilical cord blood transplantation

For those high-risk patients who do not have an HLA-identical sibling or unrelated donor available, alternative donor sources may be necessary. Umbilical cord blood transplantation (UCBT) is an attractive alternative donor option due to its rapid and easy availability^[58-62]. UCBT is associated with lower GVHD rates for the degree of HLA-disparity. In a direct comparison of outcomes in adults with hematological malignancies, Laughlin *et al*^[61] reported no difference in TRM or relapse rates between UCBT and mismatched URD bone marrow transplantation, although outcomes were inferior to matched bone marrow allografts. Similarly, Rocha *et al*^[59] in a study that included patients with acute leukemia who received UCB or matched URD marrow ($n = 582$) grafts showed no difference in TRM, relapse rate, DFS, and OS between the two groups.

The low cell dose available from individual cord blood units has been the major limitation against the widespread use of UCBT in adults with AML or other hematologic malignancies. However work done by the group in University of Minnesota has firmly established the feasibility of combining two cord blood units, in the so-called double UCBT (dUCBT), to overcome dose limitation of a single cord unit for adult patients^[63]. A large multicenter collaborative effort comparing dUCBT, matched-sibling allo-HCT, matched URD allo-HCT and mismatched URD allo-HCT showed similar 5-year DFS with all 4 modalities. dUCBT was associated with lower relapse rates but higher TRM^[64]. The preliminary results of Societe Française De Greffe De Moelle Osseuse Et Therapie Cellulaire and Eurocord's multicenter phase II trial for UCBT in patients with AML were presented in abstract form^[65]. At 1 year the rates of OS, DFS, relapse and TRM for the 65 AML patients on the study were 60%, 52%, 30% and 18%, respectively. The wider acceptance of UCBT has markedly extended the application of allogeneic transplantation, particularly to minority populations who are underrepresented in current volunteer donor databases.

Haploidentical transplantation

Almost all AML patients without an HLA-identical donor will find a haploidentical related (parents, sibling or children) donor. Enthusiasm for this modality was subdued early on due to the increased risks of GVHD, TRM, graft

rejection and opportunistic infections. However, renewed interest in haploidentical transplants has been noted with T-cell depleted as well as unmanipulated allografts with novel strategies for GVHD prevention^[66,67]. The Perugia group reported DFS of 30%-45% in AML with rigorous *ex-vivo* T-cell depletion and intense myeloablative conditioning^[68-70]. Although such transplantation has been demonstrated as feasible, it is associated with slow immune reconstitution and high rates of TRM, in smaller centers.

Recently, an alternative approach to haploidentical allo-HCT was developed with the addition of post-transplant cyclophosphamide to prevent GVHD and graft rejection in the setting of a marrow allograft after reduced intensity conditioning^[71,72]. This approach has demonstrated promising results, including acceptable rates of TRM and severe GVHD in single- and multi-institution studies. Variations including myeloablative conditioning and use of peripheral blood grafts with post transplant cyclophosphamide treatment are being studied in prospective trials^[73]. Limited retrospective data suggest comparable outcomes of matched sibling HCT, URD all-HCT and haploidentical transplantation utilizing post-transplant cyclophosphamide administration, in patients with hematological malignancies^[74]. Bone Marrow Transplantation-Clinical Trials Network's (BMT-CTN) two parallel multicenter phase II trials (BMT-CTN 0603 and BMT-CTN 0604) showed comparable 1-year OS and progression-free survival with RIC dUCBT (54% and 46%, respectively) and haploidentical bone marrow transplantation (62% and 48%, respectively) in hematological malignancies^[75]. These trials have paved the way for the ongoing BMT-CTN 1101 trial (NCT01745913) randomizing patients with hematological malignancies to either haploidentical transplantation or dUCBT. This study will hopefully guide us further in choosing the optimal alternative donor source.

Continued research is needed to better define preferred conditioning regimens, methods and degree of T-cell depletion, reduce high relapse rates with haploidentical transplantation and improved delayed immune-reconstitution inherent to all alternative donor HCT. Recently, allelic polymorphism in donor natural killer-cell immunoglobulin like receptor (*KIR*) gene has been shown to impact allograft outcome and may play important role in donor selection, including alternative sources^[76]. In centers with available expertise, alternative donor allo-HCT for carefully selected high- or intermediate-risk AML patients in CR, or those beyond CR1 is reasonable, however enrollment of such patients on any available protocols is preferred.

ALLOGENEIC-HCT FOR AML BEYOND CR1

Second complete remission (CR2)

Relapsed AML patients, who are able to achieve a second CR (CR2), typically do not enjoyed sustained responses

with chemotherapy alone. A retrospective matched-pair analysis that compared the outcomes of autologous HCT versus HLA-identical sibling allo-HCT in AML CR2 ($n = 288$) showed that while allograft recipients had higher TRM it was offset by a much lower relapse rate leading to better OS (39% *vs* 30%) at 4-years^[77]. Burnett *et al*^[78] reported outcomes of 1271 patients aged 16-49 years who entered the MRC AML10, AML12, and AML15 trials and did not receive a transplant in CR1 and then subsequently relapsed. Fifty-five percent of patients who relapsed entered CR2. Sixty-seven percent of remitters received an allotransplant that delivered superior OS compared with patients who did not receive a HCT (42% *vs* 16%). A more-stringent assessment of a transplant by using delayed-entry (Mantel-Byar) analysis confirmed the benefit of transplant overall and within intermediate- and poor-risk groups but not the favorable-risk subgroup. Allo-HCT is the preferred option for most medically fit patients with AML in CR2, including carefully planned alternative donor allografts. For those unable to undergo an allograft (due to comorbidities, personal preference, *etc.*) are best treated in the context of a clinical trial when available.

Beyond CR2

Allogeneic HCT offers the best prospect of long term DFS for patients with relapsed/refractory AML beyond CR2^[79,80]. Sierra *et al*^[39] reported 5-year DFS of 50%, 28%, 27% and 7% with allo-HCT in CR1, CR2, beyond CR2 and in untreated relapse respectively. The corresponding relapse rates were 19%, 23%, 25% and 44%, respectively. A history of prior autologous transplantation adversely affects the success of a subsequent allo-HCT^[79].

The first relapse of AML poses a management dilemma regarding whether to proceed directly with allo-HCT or to administer salvage chemotherapy to attain remission. Retrospective data indicate 3-year DFS rates of approximately 30% for patients transplanted in untreated first relapse^[81,82]. Salvage chemotherapy generally induces subsequent CR in approximately 30% of relapsed AML patients^[83]. Considering that only 35%-45% of these patients may achieve long-term DFS with allo-HCT (approximately 15% of all relapsing patients), theoretically allografting in untreated relapse may cure more patients than additional chemotherapy. However, in clinical practice the logistics of HLA-typing, identifying and evaluating potential donors, and stem cell collection generally necessitate administration of chemotherapy for disease control before transplantation. Moreover, relapse/refractory patients may not be prime candidates for myeloablative conditioning regimes that are likely required for optimal disease control to facilitate graft-versus-leukemia effect. This fact also highlights the importance of initiating the donor search in AML patients at the time of diagnosis^[84,85].

Primary refractory AML

Allo-HCT likely represents the only curative option for patients with primary refractory AML^[83]. Retrospec-

tive analyses have shown long-term survival in a subset of patients receiving allo-HCT for primary refractory AML^[86-89]. Despite the relatively high TRM (30%-50%), the reported 3-year OS and DFS of approximately 20%-30% are encouraging for this otherwise poor prognosis group. CIBMTR reported outcomes of 1673 AML patients undergoing allo-HCT with refractory/active disease^[90]. Five adverse pre-transplantation variables significantly influenced survival: first CR duration < 6 mo, circulating blasts, non-HLA-identical sibling donor, Karnofsky score < 90, and poor-risk cytogenetics. Patients who had 0 adverse factors had 42% OS at 3 years, whereas OS was 6% for a score ≥ 3 . These important results highlight that allo-HCT can salvage a highly select subgroup of AML patients, who are not able to achieve a CR before transplantation. Based on promising phase I / II data, the use of novel clofarabine and busulfan conditioning is being explored in this population (NCT01457885)^[91].

FUTURE DIRECTIONS

Great strides have been made in the field of AML and allo-HCT resulting in a steady increase in the number of allogeneic transplantation done for AML. Risk stratification of AML based on conventional cytogenetics and now molecular profiling has been instrumental in identifying higher-risk groups who may benefit from early allo-HCT. Studies looking at whole-genome and whole-exome sequencing have been reported^[92] and this information will be vital not only in prognostication but is likely to lead to discovery of novel therapeutic targets. The cytogenetic and molecular signature of AML has become expansive and its clinical application ought to be carefully interpreted. The identification of higher-risk cytogenetic groups, novel molecular stratifications incorporating coinciding aberrations and the presence of clonal heterogeneity in poor-risk AML may allow us to better predict relapse risk, recommend allo-HCT and other strategies to improve disease control and survival in an individualized fashion. The presence of minimal residual disease (MRD) is another area of active interest that may help identify those subsets of AML with the highest risk of early relapse and thus may benefit from early interventions such as allo-HCT. This may be especially important in good-risk and intermediate-risk group AML^[93]. Similarly evidence of persistent MRD post allo-HCT is a marker of poor outcomes. Such AML patients with evidence of MRD post allografting could be enrolled in trials designed to eradicate persistent low level disease (*e.g.*, by rapid taper of immune suppression, planned/escalated donor-lymphocyte infusions, low-dose chemotherapies, or novel targeted agents, *etc.*).

Allogeneic HCT itself has indeed undergone tremendous advancement in the last 2 decades. High-resolution allele level HLA-typing, improvements in supportive care, use of alternative donor allograft and RIC has widely broadened the use of allo-HCT in AML. The newest concept of adoptive cellular therapy is the so-

called “microtransplantation” where HLA-mismatched peripheral blood stem cells are infused into the recipient after consolidative chemotherapy with cytarabine, the hypothesis being that the alloreactive HLA-mismatched cells would not engraft, but during their transitory period will destroy AML clone without causing GVHD^[94]. Concerted efforts are needed to devise strategies to prevent relapse post allo-HCT using novel maintenance or consolidation strategies (*e.g.*, FLT3 inhibitors post allo-HCT in FLT3⁺ patients, hypomethylating agent administration to eradicate minimal residual disease). Rigorous research efforts in the development of novel preparative regimens able to provide better early disease control and limiting TRM are need. In this regard total marrow irradiation programs and/or immune-radioisotope-based conditioning appear promising. Additional avenues include using propylene glycol free melphalan (to limited renal toxicity), and pharmacokinetically dose busulfan (to limited organ damage, and prevent underdosing) as safer conditioning drugs. Immunological strategies to modulate patient or donor’s immune system, so that they mount response against tumor specific antigens are ongoing. Various antigens (Wilms Tumor gene, NOTCH, PR1, *etc.*) are being tested to develop vaccine to achieve a lasting immune response in the setting of relapsed leukemia or MRD after transplant. Newer mobilization regimens (*e.g.*, plerixafor for sibling donor mobilization) and more effective methods to prevent GVHD^[95-101] as well as increased availability of alternative-donor approaches, are ongoing and will add to our ability to cure patients with AML in the coming years.

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P- Reviewers: Chen SS, Sharma P, Thomas X **S- Editor:** Gou SX
L- Editor: A **E- Editor:** Zhang DN



WJSC 6th Anniversary Special Issues (2): Mesenchymal stem cells

Enhancing the efficacy of mesenchymal stem cell therapy

Michalis Mastri, Huey Lin, Techung Lee

Michalis Mastri, Huey Lin, Techung Lee, Department of Biochemistry, University at Buffalo, Buffalo, NY 14214, United States

Michalis Mastri, Huey Lin, Techung Lee, Department of Biomedical Engineering, Center for Research in Cardiovascular Medicine, University at Buffalo, Buffalo, NY 14214, United States

Author contributions: Mastri M performed experiments and data analysis; Lin H performed experiments; Lee T contributed to data analysis and manuscript writing.

Supported by NIH, No. R01HL84590; NYSTEM; and University at Buffalo Biomedical Research Service Center

Correspondence to: Techung Lee, Associate Professor, Department of Biomedical Engineering, Center for Research in Cardiovascular Medicine, University at Buffalo, 140 Farber, 3435 Main Street, Buffalo, NY 14214, United States. chunglee@buffalo.edu
Telephone: +1-716-8293106 **Fax:** +1-716-8293106

Received: September 17, 2013 **Revised:** November 29, 2013

Accepted: January 13, 2014

Published online: April 26, 2014

Abstract

Mesenchymal stem cell (MSC) therapy is entering a challenging phase after completion of many preclinical and clinical trials. Among the major hurdles encountered in MSC therapy are inconsistent stem cell potency, poor cell engraftment and survival, and age/disease-related host tissue impairment. The recognition that MSCs primarily mediate therapeutic benefits through paracrine mechanisms independent of cell differentiation provides a promising framework for enhancing stem cell potency and therapeutic benefits. Several MSC priming approaches are highlighted, which will likely allow us to harness the full potential of adult stem cells for their future routine clinical use.

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Key words: Mesenchymal stem cell; Therapy; Clinical trial; Paracrine

Mastri M, Lin H, Lee T. Enhancing the efficacy of mesenchymal stem cell therapy. *World J Stem Cells* 2014; 6(2): 82-93 Available from: URL: <http://www.wjgnet.com/1948-0210/full/v6/i2/82.htm>
DOI: <http://dx.doi.org/10.4252/wjsc.v6.i2.82>

INTRODUCTION

Human bone marrow mesenchymal stem cells (MSCs) are currently being investigated in clinical trials for immune, cardiovascular, neurodegenerative, gastrointestinal, bone/cartilage and blood disorders (<http://clinicaltrials.gov>). The clinical utility of MSCs is in part due to their lack of significant immunogenicity, permitting safe allogeneic cell transplantation without the need for immunosuppression. However, these clinical trials have thus far demonstrated moderate and at times inconsistent benefits, indicating an urgent need to optimize the therapeutic platform and enhance stem cell potency^[1-3]. Along this line, parallel preclinical studies have identified several potentially useful and logistically feasible strategies that may be employed to achieve more robust clinical efficacy of MSC therapy. On the other hand, risk factors associated with MSC therapy cannot be overlooked because long-term safety data remain lacking and unanticipated side effects may appear much later. Potential risks related to disease transmission and activation of latent viruses in allogeneic cell transplantation also highlight the importance of continued surveillance post MSC therapy. Thus, future success of MSC therapy will lie in rational optimization of therapeutic strategies in conjunction with an adequate assessment of benefit and risk factors.

TROPIC ACTION OF MSCS

While early preclinical MSC studies suggested therapeutic mechanisms mediated by MSC trans-differentiation or fusion, these mechanisms do not occur in sufficiently high frequency to account for the observed functional

improvement after stem cell administration. Current evidence indicates that although MSCs exhibit prominent multi-lineage differentiation potential, this cellular feature bears little relevance to their therapeutic effects. Instead, production of multiple paracrine factors by MSCs provides the underlying regenerative mechanism^[4-6]. Therapeutically, the MSC-derived soluble mediators, which include many cytokines and growth factors, are functionally redundant and synergistic, contributing to cytoprotection, angiogenesis, tissue repair, normalization of extracellular matrix (ECM) and alleviation of inflammation. Preclinical studies have indeed highlighted the central role of MSC-derived interleukin (IL)-6-type cytokines, vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) in the treatment for heart failure and multiple sclerosis^[6-8]. MSCs also interact with cells of both the innate and adaptive immune systems, leading to functionally relevant immunomodulation^[9]. Of note, MSCs are widely present *in vivo* and their perivascular origin in multiple organs have been demonstrated^[5,10]. This apparent *in vivo* “drug store” function of MSCs constitutes the primary therapeutic underpinning of MSC therapy.

“COMPETENCE FACTORS” IN MSC THERAPY

Current clinical trial data do not yet support routine use of MSC therapy for the prevention and treatment of organ dysfunction or tissue degeneration. Robust cell therapy is likely dictated by at least two key competence factors affecting both the transplanted stem cells and the treated host tissue. This view necessitates a complete understanding of the cell-tissue crosstalk mechanism and the adoption of an integrative approach in maximizing therapeutic efficacy regardless of the organ system being targeted. Since the mechanisms of action of MSCs in tissue regeneration are likely multifaceted, cell competency can be dictated by the abilities of the injected MSCs to migrate, engraft, survive, differentiate and produce functional paracrine mediators. Tissue competency reflects the ability of the host tissues to favorably respond to the injected MSCs and MSC-derived paracrine factors, resulting in activation of the endogenous regenerative machinery^[11]. While the exogenous repair mechanism is imparted by the implanted MSCs and is often short-lived, the endogenous repair mechanism conferred by the host stem/progenitor cell niches can exert a powerful and long-lasting regenerative benefit. Integration of the exogenous and endogenous repair mechanisms in clinical trial design will prove instrumental in transitioning toward future routine clinical use of adult stem cells. In considering the strategies for boosting the competency factors in MSC therapy, we will focus primarily on non-genetically based methods because genetically modified MSCs will likely pose some concerns and safety issues for clinical application. Given that MSC therapy

is being used to target a wide spectrum of diseases in diverse patient populations, the logistical aspects of MSC therapy will also be considered.

SOURCE OF COMPETENT MSCS

MSCs from different donors may exhibit different degrees of competence due to varying factors such as gender, disease status and age^[12,13]. Limited information indicates that female stem cells may possess a more pronounced regenerative potential than male stem cells^[14], which is in line with the finding that female patients typically exhibit certain cardioprotective phenomenon from acute myocardial infarction and better outcome after the incidence compared to male patients^[15]. Although the gender influence is thought to be mediated through differential sex hormone receptor signaling, a recent study shows that female rodent MSCs produce a higher level of VEGF than male rodent MSC in response to hypoxia^[13]. Given the critical role of paracrine factors in MSC therapy, additional study is warranted to determine whether female MSCs are indeed more robust in production of multiple paracrine factors and should be selected for the use of allogeneic MSCs.

Aside from the gender effect, studies have further revealed disease- and age-associated functional impairment of various types of adult stem cells^[16,17]. While the basal hematopoietic capacity is maintained throughout life, the ability of hematopoietic stem cells (HSCs) to respond to stress and differentiation cues appears to decrease with age^[18,19]. The use of autologous MSCs is not always desirable or feasible because patients can exhibit declined stem cell quality and/or quantity^[20-22]. For instance, diabetes can negatively impact MSCs by reducing angiogenic capacity and therapeutic potential^[23]. Certain disease-causing genotypes may preclude therapeutic use of autologous MSCs due to the inherent genetic defects^[24,25]. Even chemotherapy can induce MSC damage and reduce cell yields in patients with hematological malignancy^[26]. Thus, the use of allogeneic MSCs from healthy donors is gaining acceptance. The use of allogeneic MSCs isolated from healthy donors offers a major advantage because these adult stem cells can be thoroughly tested and formulated into off-the-shelf medicine in advance. MSCs are particularly well suited for this application due to their immune privileged status.

CELL DOSE AND THERAPEUTIC POTENCY

Lessons learned from HSC therapy following myeloablation have revealed that administration of sufficient HSCs promotes faster cell recovery and reduces hospitalizations^[27]. Clinical trials of stem cell therapy for regenerative repair have also demonstrated the importance of administering a sufficient cell dose^[28,29]. Using the hamster heart failure model, we evaluated the relation-

Table 1 Therapeutic benefits in relation to the number of administered mesenchymal stem cells

| Cell number/ animal | Cell number /kg | Cardiac repair | Cell number /70-kg human |
|------------------------|-----------------------|-------------------|-----------------------------|
| 0.01 × 10 ⁶ | 0.1 × 10 ⁶ | - (no) | 7 × 10 ⁶ |
| 0.1 × 10 ⁶ | 1 × 10 ⁶ | + (weak) | 70 × 10 ⁶ |
| 1 × 10 ⁶ | 10 × 10 ⁶ | ++ (moderate) | 700 × 10 ⁶ |
| 4 × 10 ⁶ | 40 × 10 ⁶ | +++ (robust) | 2800 × 10 ⁶ |

ship between the injected MSC doses [(0.1-40) × 10⁶ cells/kg body weight] and cardiac therapeutic benefits as determined by echocardiography, morphometry, gene expression analysis and histochemistry^[30,31]. The series of pharmacodynamic studies established the minimal cell dose, *i.e.*, about 1 × 10⁶ cells/kg (Table 1), which is necessary for achieving quantifiable but weak benefits for the failing hamster heart. The studies also revealed that the most prominent therapeutic benefits were achieved by about 40 × 10⁶ cells/kg, which however approximates 2.8 billion cells per 70-kg human! Notably, published clinical trials of MSC therapy have largely relied on injections of about 1 × 10⁶ cells/kg^[32-36], which appears suboptimal based on our cell dose study. Since the effective treatment dose is influenced by the body size, bio-distribution of the MSC-induced paracrine factors in the human body is likely much less efficient than in the small rodent. Given the large body weight difference between rodents and humans, obtaining sufficient MSCs necessary for mounting a prominent therapeutic response in humans constitutes a daunting challenge. In particular, obtaining sufficient MSCs to achieve maximum clinical benefits may not be economically viable as elaborated in Table 1.

CONUNDRUM OF *EX VIVO* MSC EXPANSION

Normal mitotic somatic cells gradually cease division after continuous expansion in culture and enter a state referred to as replicative aging or senescence, exhibiting a Hayflick limit of 50 population doublings^[37,38]. Embryonic stem cells (ESCs) however proliferate indefinitely in culture, which correlates with their high telomerase activity and long telomeres^[39]. MSCs constitute a minor population of the nucleated cells (0.01%-0.001%) in the adult human bone marrow. Unlike hematopoietic stem cell transplantation, which is a well-established therapeutic regimen for hematological disorders^[40], it is necessary to amplify MSCs in culture to generate sufficient cells required for therapeutic applications. This *ex vivo* cell amplification step unavoidably creates many issues that can confound MSC therapeutics. Long-term *in vitro* passaging alters bone marrow and adipose MSCs^[41]. Prolonged culturing of MSCs from several species causes senescence and prominent changes in gene expression^[42,43]. Down-regulation of genes involved in DNA repair during MSC

senescence^[44] can potentially cause genomic instability. Our study of porcine MSCs shows that late-passage MSCs exhibit significantly reduced expression of many paracrine factors compared to early-passage MSCs (Figure 1). Since cellular aging is a rapid and continuous process in culture, the use of *ex vivo* amplified MSCs, even those derived from early-passages, can generate inconsistent therapeutic effects.

TLR3 SIGNALING AND MSC COMPETENCE

Our MSC and growth factor therapy for hamster heart failure have revealed several major factors critical for tissue repair such as IL-6-type cytokines, VEGF and HGF^[6,7,30,31,45]. We show that MSC therapy increases the levels of paracrine factors present in the serum and multiple organs, suggesting a systemic distribution mode for the soluble mediators at least in the rodent. We further sought to engineer an MSC phenotype exhibiting enhanced expression of paracrine factors, aiming to lower the effective treatment cell dose. We turned our attention to the pattern recognition receptor (PRR) pathway of the innate immune system, which is capable of overproducing many immunomodulatory cytokines, most notably IL-6, upon activation^[46,47]. Distinct immune cell PRRs initiate the cytokine cascade through interacting with a variety of molecular patterns conserved among microbial pathogens. The Toll-like receptor (TLR) pathway is the best characterized PRR system and engagement of TLRs stimulates production of many immunomodulatory cytokines from antigen-presenting cells. TLR3 in particular recognizes double-stranded (ds) RNA, and is activated by the dsRNA mimetic polyinosinic-polycytidylic acid or poly(I:C)^[48,49]. MSCs also express several members of the TLR family^[50], including TLR3, which is an endolysosomal receptor protein.

We initially treated MSCs with three different concentrations of poly(I:C) for 24 h to examine the downstream effect on expression of trophic factors^[31]. Gene expression assays revealed a prominent induction of IL-6 and IL-6-type cytokines by 0.8-20 g/mL poly(I:C). For instance, a 10 fold increase in IL-6 mRNA and 40 fold increase in secreted IL-6 were observed. A less than 2 fold induction of IL-11 mRNA and ~4 fold induction of secreted IL-11 were also observed. Leukemia inhibitory factor (LIF), another member of the IL-6-type cytokines, was also induced. SDF1, VEGF and HGF, all of which are activated by IL-6/JAK/STAT3 signaling, were significantly induced by poly(I:C). Interestingly, the anti-inflammatory cytokine IL-10 was significantly induced. The inflammatory cytokines interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) were only induced by the highest poly(I:C) concentration (20 g/mL). This finding prompted us to adopt an MSC-boosting protocol based on 4 g/mL poly(I:C) for 24 h, which induced IL-6, IL-10, IL-11, LIF, VEGF, SDF1 and HGF without

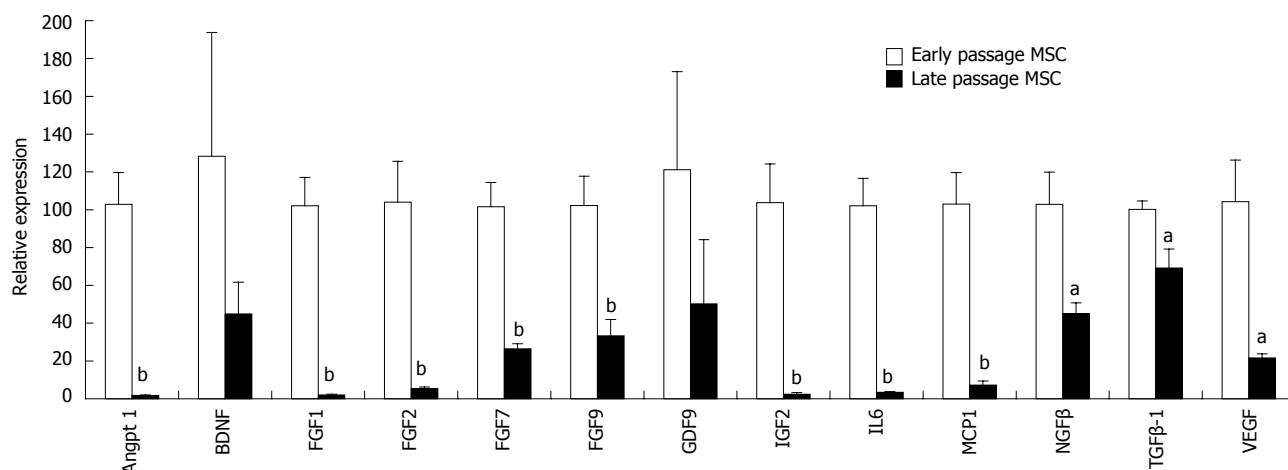


Figure 1 *Ex vivo* expansion of mesenchymal stem cells reduces expression of growth factor/cytokine genes. Porcine mesenchymal stem cells (MSCs) were expanded as described^[42]. Threshold cycle (C_T) for the illustrated genes was determined by real-time reverse transcription polymerase chain reaction. Early and late passage MSCs received less than 5 and more than 10 trypsin passages, respectively. ^a $P < 0.05$, ^b $P < 0.01$ vs early passage MSCs.

induction of the inflammatory cytokines. Longer treatment of MSCs with poly(I:C), *e.g.*, 2 d, was found to be cytotoxic.

Upon testing the potency of the PRR-primed MSCs using the hamster heart failure model, we found that the super MSCs reduced the effective therapeutic cell dose by 40 fold (Table 1) through actively recruiting cardiac progenitor cells and decreasing myocardial inflammation, culminating in a 50% reduction in myocardial fibrosis, a 40% reduction in apoptosis and a 50% increase in ventricular function. This pioneering study of engaging the MSC PRR axis for reducing cell dose requirement in heart failure therapy was recently featured in an AJP editorial^[51]. Although the function of immune cell PRRs has been well established, their role in stem cell function is just beginning to be unraveled. Prolonged TLR activation of the immune system is invariably associated with chronic inflammation. Interestingly, Cole *et al.*^[52] demonstrated an unexpected beneficial role for TLR3 in the arterial wall upon systemic administration of poly(I:C). Further, Packard *et al.*^[53] found poly(I:C) administration to be protective against cerebral ischemia-reperfusion injury. Since MSCs are widely present *in vivo* and their perivascular origin in multiple human organs appears certain^[5,10], it is possible that these prophylactic benefits of poly(I:C) may be mediated through its trophic stimulatory effect on the endogenous MSC niches.

INFLUENCE OF OTHER PRR SYSTEMS

Recognition of various pathogen-associated molecular patterns by immune PRRs leads to transcriptional activation of distinct gene targets, and sets forth a diverse array of pathways that determine the magnitude, duration, and type of the host inflammatory response. Immune cell TLR2 and TLR4 are major PRRs responding to bacterial invasion, and their activation leads to increased

IL-6 and a host of other cytokines similar to the antiviral response mediated by dsRNA-sensing PRRs^[46]. Given the prominent roles of IL-6 in stem cell maintenance and cardiac regeneration^[6,54-56], transient low-dose priming of MSC TLR2/4 may also represent a physiologically significant mechanism for tissue repair. It has indeed been shown recently that TLR2/6-dependent stimulation of MSCs promotes angiogenesis *in vitro* and *in vivo* in bone tissue engineering^[57]. TLR2 forms functional heterodimers with TLR1 and TLR6, and is activated by peptidoglycan. Immune TLR4 upon activation by lipopolysaccharide (LPS) causes elevated levels of IL-6, IL-8, IL-10, IL-12, IL-15, TNF α , IL-1 and TGF β . Potential effects of TLR2 and TLR4 engagement on MSC paracrine profiles can therefore be tested by treating cells with low-dose peptidoglycan and LPS (1-20 g/mL each). However, since TLR2 and TLR4 are also known to be involved in tissue inflammation triggered by ischemia/reperfusion injury^[58], it is unclear whether transient low-dose priming of MSC TLR2/4 may favorably impact the failing heart as demonstrated for MSC TLR3.

Unlike TLR3, TLR2 and TLR4 are present on the plasma membrane, recruiting the adaptor protein MyD88 for signal transduction. Since TLR activation in the absence of MyD88 generally results in delayed kinetics^[59], the difference in the paracrine cascades can be expected to influence MSC therapeutics. Notably, MSCs have been found to be differentially primed by TLR4 and TLR3 ligands to adopt a pro-inflammatory (MSC1) and anti-inflammatory (MSC2) status, respectively^[60]. The MSC1 and MSC2 phenotypes were further found to attenuate and promote tumor growth/metastasis, respectively^[61]. These studies thus indicate that the cytokine secretion profile of MSCs plays a decisive role in dictating the therapeutic potency and treatment outcome, and warrants special consideration in the design of stem cell therapy.

HYPOXIA AND MSC COMPETENCE

Rapid loss of the implanted MSCs has been frequently observed and may be caused in part by hypoxic stress, which triggers apoptosis^[62-64]. The bone marrow environment contains oxygen tensions ranging from 1% to 7%. However, most *in vitro* cell culture work is performed at a pO₂ level of 142 mmHg or 20% O₂, which is much higher than that of the *in vivo* environment^[65]. The implanted MSCs are expected to experience reduced oxygen levels as they attempt to establish contacts with the ECM environment. Preconditioning MSCs by brief hypoxia prior to cell administration may thus allow the cells to better adapt to the lower pO₂ tissue environment and promote cell engraftment. Typically, MSCs are cultured in normoxia (95% air and 5% CO₂) as control and in hypoxia (1% oxygen, 5% CO₂, and 94% nitrogen) for 2 d. Assay of cultured MSCs for cell surface phosphatidylserine, which is a sensitive method for detecting early apoptosis, can be used to determine whether an increase in MSC apoptosis after hypoxic exposure may be induced.

In addition to induction of many angiogenic growth factors, hypoxia is known to induce SDF-1 and its cognate receptor CXCR4^[66,67]. Indeed, low oxygen has been shown to increase expression of CXCR4 and CX3CR1 and promote MSC engraftment^[68,69]. A hypoxia-regulated heme oxygenase-1 vector modification of MSCs was found to enhance the tolerance of engrafted MSCs to hypoxic injury and improves their viability in ischemic hearts^[70]. Note however that although hypoxia promoted MSC proliferation *in vitro*, it unexpectedly attenuated MSC osteogenic potential^[71], suggesting that the utility of hypoxia preconditioning may be application specific. Additional relevant preconditioning strategies intended to enhance MSC survival have been based on the use of unique compounds such as prolyl hydroxylase inhibitor^[62], lysophosphatidic acid^[72], HMG-CoA reductase inhibitor^[73,74], eNOS enhancer^[75] and sphingosine-1-phosphate^[76]. Whether this pharmacological approach may also reduce the effective MSC dose as observed for TLR3-activated MSCs remains to be determined. These pharmacological strategies may also find their application in tackling the issues of host tissue deficiency related to aging and disease (see below). This is because the function and competence of the endogenous host tissue progenitor cell niche also dictates the therapeutic outcome.

CYTOKINE PRECONDITIONING AND MSC COMPETENCE

Rapid loss of the injected cells is perceived as a major hurdle in stem cell therapy^[63,64,77] and may be caused in part by inadequate ECM engagement. Expression of chemokines and their receptors is known to be regulated by cytokines and this phenomenon has been explored to facilitate MSC engraftment after cell implantation^[78,79].

Intervention through the use of growth factors and/or cytokines is appealing because the trophic factor network is typically marked by cross-talk mechanisms enabling mutual induction of gene expression. Priming MSCs with a cocktail of growth factors and cytokines has indeed been found to enhance the cardiac therapeutic efficacy^[80]. In this study, a cocktail of growth factors containing 50 ng/mL FGF-2, 2 ng/mL IGF-1 and 10 ng/mL BMP-2 was used for MSC pretreatment and its effect on the viability under hypoxia and paracrine profiles of MSCs were evaluated. The growth factor pretreatment was found to enhance expression of cardiac transcription factors and promote cell viability under hypoxia. Transplantation of the pretreated MSCs resulted in smaller infarct size and better cardiac function than transplantation of untreated MSCs. This cytokine preconditioning approach is particularly relevant because MSCs are adherent cells and depend on adequate ECM engagement for growth and survival. Anoikis is initiated when trypsinized MSCs are forced into suspension for injections^[81]. Along this line, plasminogen activator inhibitor-1 (PAI-1) has been found to promote anoikis, and PAI-1 null MSCs exhibit enhanced *in vivo* survival after implantation^[64].

Many cytokines are known to exhibit cell adhesion-promoting activities including HGF, IGF-1, SDF-1, TGF- β and VEGF and interestingly these trophic factors are also produced by MSCs, suggesting that MSCs can be regulated by diverse autocrine mechanisms. These cytokines act in part by affecting the integrin and matrix metalloproteinase (MMP) systems. In particular, EGF can promote activation of MMP-2 and cell migration^[82]. TGF-1 can stimulate MMP-9-mediated cell migration^[83]. SDF-1 can increase V 3 integrin expression, cell migration, and therapeutic potentials of EPCs^[84,85]. We also demonstrated that human MSCs overexpressing VEGF exhibited significantly enhanced cardiac repair capacity^[7]. Since no cell retention and survival enhancement strategies have translated to the clinic, strategies aimed at promoting long-term maintenance of the injected cells are worth pursuing, which may ultimately lead to the production of more potent stem cells that can be delivered in lesser quantity.

HOST TISSUE COMPETENCE

Host tissue competence can greatly influence the outcome of MSC therapy because it is increasingly been recognized that aging and disease can adversely affect the tissue milieu into which MSCs are introduced^[86]. The parabiosis study exposing old mice to factors present in young mouse serum^[87] indicates that the age-related decline of muscle satellite cell activity is modulated by systemic factors that change with age. This is because stem cell activity is profoundly influenced by the supporting ECM and cells in the immediate vicinity^[88]. The presence of ECM breakdown products and the extra lamina

caused by the deposition of collagens in aged muscle tissue can potentially interfere with paracrine signaling. Aged muscle for instance exhibits increased Wnt signaling and fibrosis^[89,90], which can impinge unfavorably on the functional paracrine cascade initiated by the implanted MSCs. Importantly, although the intrinsic regenerative potential of aged muscle appears to be largely intact, critical factors such as the Notch ligand Delta required for regeneration appear limiting^[87,91,92].

Increasing age has been found to be associated with adverse prognosis in the setting of ischemic injury, coronary angioplasty, and cardiac surgery^[93-97]. Although the adult heart contains resident cardiac stem cells capable of supporting limited myocardial regeneration^[98], age-associated fibrotic remodeling and senescence of cardiac stem cells lead to contractile dysfunction and gradual loss of cardiomyocytes^[99,100], and the aged heart exhibits significant structural deteriorations including fibrosis and poor angiogenic capacity^[101,102]. Thus, the aged heart is more refractory to regenerative therapy^[103,104]. The harmful host tissue milieu present in the aged tissue may interfere with the trophic actions of MSCs. Several tissue proteases such as elastase, cathepsin and dipeptidylpeptidase (DPP) are known to cleave and inactivate cytokines. Elevated activities of these proteases in the aged tissue may destabilize the trophic factors induced by MSC therapy, rendering the therapy ineffective. Therapeutic efficacy may thus be improved by optimizing tissue retention and stability of the delivered proteins^[105-107]. For instance, administration of Diprotin A, a pharmacologic inhibitor of DPP, enhanced the stability of SDF-1, which increased myocardial homing of CXCR4⁺ progenitor cells and function^[108,109]. Thus, a potential strategy to boost the trophic response of the older tissue is to inject non-toxic protease inhibitor(s) into the host tissue prior to MSC administration. This tissue preconditioning strategy is aimed at promoting trophic factor stability by attenuating abnormally elevated local or systemic protease activities.

The bone marrow compartment harbors many populations of primitive progenitor/stem cells that are mobilized by various chemokines. Of note, a lack of bone marrow support for cardiac repair in aged animals has been documented^[110], indicating that the MSC-initiated healing process may be compromised by the impaired tissue cross-talk mechanism, leading to a greater susceptibility of the old heart to ischemic injury and an inefficient response to protective interventions. IL-6 deficiency, for instance, affects bone marrow stromal precursor cells, resulting in defective hematopoietic support^[54]. This host tissue impairment represents a significant hurdle to regenerative medicine because most preclinical therapeutic studies are based on the use of young animals, but stem cell therapy typically targets the elderly. Development of suitable preconditioning strategies targeting MSCs and aged host tissue is thus expected to lead to more efficacious regenerative treatment regimens.

A RATIONAL DESIGN OF MSC ADMINISTRATION ROUTE

Routes of drug administration are major considerations in pharmacokinetic and pharmacodynamics studies and applications. The choices are however fairly limited for cell-based medicine as cell viability needs to be preserved. Since diseased tissue is often associated with ischemia, inflammation, and fibrosis, which can impair cell survival, therapeutic delivery of stem cells to areas away from the damaged tissue offers an advantage. Intravenously (*iv*) infused MSCs are currently being adopted for clinical trials of neurodegenerative and heart diseases^[36,111], highlighting the significance of formulating a minimally invasive stem cell delivery approach for patient care. Although *iv* MSCs are largely distributed to the lungs, this systemic cell delivery method appears feasible with MSCs because their therapeutic benefits are largely mediated by paracrine mechanisms independent of stemness^[5,6]. Thus, intracoronary infusion of MSCs for heart therapy, which retained only 1%-2% of the infused cells in the porcine myocardium, was found to result in significant functional improvement in the hibernating myocardium^[112,113].

The recognition that IL-6 and IL-6-type cytokines are abundantly produced by MSCs^[6,55] and that skeletal muscle actively induces IL-6 during exercise^[56,114] prompted us to pioneer an intramuscular (*im*) MSC delivery route for cardiac repair^[6,30,115]. This *im* MSC therapeutic strategy is coupled to the inherent ability of skeletal muscle to produce beneficial trophic factors in response to exercise and injury^[116-118], and therefore represents an integrative physiological approach. The skeletal muscle is capable of regeneration after injury, and this ability is coupled to its production of many cardioprotective factors such as VEGF and HGF, which have been used in preclinical or clinical trials for cardiovascular therapy^[119,120]. Although *im* MSCs are trapped in the local musculature, their trophic actions promote increased growth factor levels in the quadriceps, liver, and brain, suggesting a possible global physiological effect^[6,30]. We further demonstrated that blocking JAK/STAT3 signaling abrogated the therapeutic effects of MSCs, indicating the functional relevance of MSC IL-6-type cytokines in initiating the paracrine cascade^[6].

As depicted in Figure 2, MSC-derived IL-6 and IL-6-type cytokines activate the injected muscle through JAK/STAT3 signaling, inducing downstream trophic factor genes such as VEGF, HGF, SDF-1 and IGFs. These factors mediate mobilization of bone marrow progenitor cells, cardioprotective signaling and activation of cardiac progenitor cells, resulting in decreased myocardial fibrosis and inflammation and increased cardiac regeneration and function. Notably, *im* MSCs also induce Suppressor of Cytokine Signaling 3 (SOCS3), which functions in a negative feedback loop to terminate cytokine signaling^[6]. Since excessive and prolonged IL-6 activity can cause tissue inflammation, induction

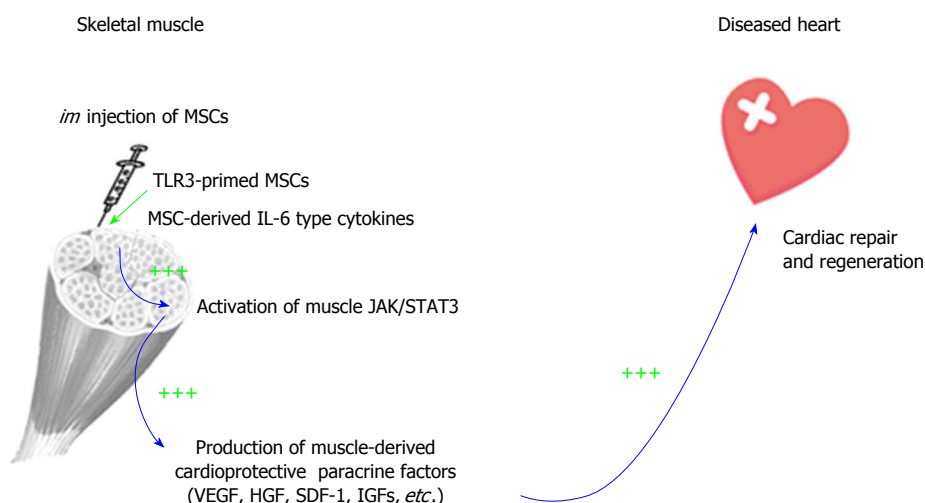


Figure 2 Intramuscular administration of mesenchymal stem cells mediates a paracrine mechanism of distal organ repair. The paracrine cascade initiated by mesenchymal stem cells (MSCs) is illustrated by blue arrows. TLR3 priming by poly (I:C) generates a super MSC phenotype through amplification of paracrine factors, which enhances MSC potency for cardiac repair (indicated by triple green plus signs). Supporting data have been published^[16,30,31,45,126].

of SOCS3 by *im* MSCs reduces the risk of this adverse reaction. The induced paracrine factors further enhance the expression of myocardial growth factors, activating the pro-survival signaling pathways in the diseased heart. Given that exercise is known to increase production of several beneficial trophic factors from the contracting skeletal muscle^[121-123], preventing coronary artery disease and cognitive decline^[124,125], our findings illustrate an *im* MSC-mediated cardioprotective paracrine mechanism mimicking the trophic action of exercise.

CONCLUSION

MSC therapy is entering a new era shifting the focus from initial feasibility study to optimization of therapeutic regimen and enhancement of treatment potency. Since tissue degeneration is often complex in nature and likely entails a therapeutic intervention strategy targeting multiple pathogenic mechanisms, the multiple paracrine factors released by MSCs and the injected host tissue acting in synergy are well suited as a regenerative medicine. Complete identification and understanding of these trophic factors can eventually lead to the development of cell-free trophic factor cocktails ideal for the treatment of tissue injury and degeneration, which may eliminate the concern associated with potential MSC transformation. Major challenges exist, however, regarding suboptimal stem cell potency and age/disease-related host tissue impairment, which may dampen enthusiasm for translational application of stem cells in general. The strategies outlined in this review offer a testable platform to launch innovative clinical trials based on rational design of MSC therapy.

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P- Reviewers: Chakrabarti S, Gazdag G, Grof P
S- Editor: Zhai HH **L- Editor:** A **E- Editor:** Zhang DN



WJSC 6th Anniversary Special Issues (2): Mesenchymal stem cells**"Ins" and "Outs" of mesenchymal stem cell osteogenesis in regenerative medicine**

Dean T Yamaguchi

Dean T Yamaguchi, Research Service, Veteran Administration Greater Los Angeles Healthcare System and David Geffen School of Medicine at University of California at Los Angeles, Los Angeles, CA 90073, United States

Author contributions: Yamaguchi DT solely contributed to this review.

Supported by Veterans Administration Merit Review Award 2 I01 BX000170-05

Correspondence to: Dean T Yamaguchi, MD, PhD, Research Service, Veteran Administration Greater Los Angeles Healthcare System and David Geffen School of Medicine at University of California at Los Angeles, 11301 Wilshire Blvd, Bldg 114, Rm 330, Los Angeles, CA 90073, United States. dean.yamaguchi@va.gov

Telephone: +1-310-2683459 Fax: +1-310-2684856

Received: October 20, 2013 Revised: January 15, 2014

Accepted: January 17, 2014

Published online: April 26, 2014

Abstract

Repair and regeneration of bone requires mesenchymal stem cells that by self-renewal, are able to generate a critical mass of cells with the ability to differentiate into osteoblasts that can produce bone protein matrix (osteoid) and enable its mineralization. The number of human mesenchymal stem cells (hMSCs) diminishes with age and *ex vivo* replication of hMSCs has limited potential. While propagating hMSCs under hypoxic conditions may maintain their ability to self-renew, the strategy of using human telomerase reverse transcriptase (hTERT) to allow for hMSCs to prolong their replicative lifespan is an attractive means of ensuring a critical mass of cells with the potential to differentiate into various mesodermal structural tissues including bone. However, this strategy must be tempered by the oncogenic potential of TERT-transformed cells, or their ability to enhance already established cancers, the unknown differentiating potential of high population doubling hMSCs and the source of hMSCs (*e.g.*, bone marrow, adipose-derived, muscle-derived, umbilical cord blood,

etc.) that may provide peculiarities to self-renewal, differentiation, and physiologic function that may differ from non-transformed native cells. Tissue engineering approaches to use hMSCs to repair bone defects utilize the growth of hMSCs on three-dimensional scaffolds that can either be a base on which hMSCs can attach and grow or as a means of sequestering growth factors to assist in the chemoattraction and differentiation of native hMSCs. The use of whole native extracellular matrix (ECM) produced by hMSCs, rather than individual ECM components, appear to be advantageous in not only being utilized as a three-dimensional attachment base but also in appropriate orientation of cells and their differentiation through the growth factors that native ECM harbor or in simulating growth factor motifs. The origin of native ECM, whether from hMSCs from young or old individuals is a critical factor in "rejuvenating" hMSCs from older individuals grown on ECM from younger individuals.

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Key words: Mesenchymal stem cell; Telomerase reverse transcriptase; Extracellular matrix; Osteogenesis; Regenerative medicine; Tissue engineering; Proliferation; Differentiation

Core tip: When human telomerase reverse transcriptase (hTERT) transformed human mesenchymal stem cells (hMSCs) are used to prolong replicative potential and osteogenic differentiation, consideration should be given to using lower population doubling hTERT-transformed hMSCs to avoid potential oncogenesis. An inducible hTERT system may also avoid oncogenic transformation. Demonstration of *in vivo* bone forming capacity of hTERT-transformed cells should be used as standard in determining osteogenic differentiation of such cells rather than *in vitro* culture mineralization; the CD146 marker may be a suggested surface marker for hTERT-transformed hMSCs that may have the capacity

to form bone *in vivo*. Native ECM from early population doubling hMSCs or hMSCs from a younger source may be best when seeking to extend the proliferative and differentiating potential of hMSCs from either young or older sources.

Yamaguchi DT. “Ins” and “Outs” of mesenchymal stem cell osteogenesis in regenerative medicine. *World J Stem Cells* 2014; 6(2): 94-110 Available from: URL: <http://www.wjg-net.com/1948-0210/full/v6/i2/94.htm> DOI: <http://dx.doi.org/10.4252/wjsc.v6.i2.94>

INTRODUCTION

The regeneration of mesodermal and neural crest-derived structural or connective tissues such as bone, cartilage, muscle and tendon continues to be a widely pursued for the reason that such structural tissues are generally homogeneous with either a predominantly single cell type or limited number of cells that contribute to the make-up of the tissue and that precursors to the mature cell types can be found in adult tissues. These precursor cells are generally multipotent, in that they can differentiate into a variety of connective tissue phenotypes. These precursor cells are generally referred to as adult mesenchymal stem cells (MSCs) or bone marrow stromal cells and can be found in the bone marrow but also as similar multipotent cells in specific tissues as well as circulating cells in blood.

Tissue engineering seeks to replace tissues that are either lost by traumatic events or by disease through the use of specific cell types that can recapitulate the lost or diseased tissue, and generally used in combination with a three-dimensional structural scaffold, and in many instances in combination with various growth factors, cytokines, and hormones or other biological molecules to assist in either the creation of a critical mass of needed cells or to assist in differentiating these cells to the required tissue type.

Because generating a critical mass of cells used in the regenerative process is a key to successful tissue engineering followed by differentiating those cells into the specific cell type comprising the tissue, stem cells have been the preferred starting cell type in many tissue engineering trials. This minireview will focus only on human adult bone marrow MSCs (herein assumed to be synonymous with bone marrow stromal cells) as much as possible and the telomerase strategy of inducing self-renewal of these cells to create a critical cell mass. Secondly, the minireview will examine the strategy of using extracellular matrix as a native scaffold upon which mesenchymal stem cells can self-renew and differentiate into bone.

MESENCHYMAL STEM CELL SELF-RENEWAL

The ability to self-renew is a hallmark of any stem cell^[1].

Self-renewal is simply defined as the ability of the resulting daughter cells, after mitotic division of the original mother cell, to retain the ability to generate a variety of differentiated cell types identical to that of the ability of the mother cell to differentiate in to those same cell types, and for a daughter cell to be able to generate daughter cells that also maintain the ability to differentiate into the same variety of cell types as the original “grandmother” and mother cells^[2]. The maintenance of self-renewal and pluripotency of stem cells occurs in the stem cell niche, where stem cells are able to receive cues from the stroma and other cell types either by direct contact or by secreted soluble factors within this microenvironmental niche^[3,4].

Adult MSCs also share the ability to self-renew. This potential to self-replicate and to differentiate into connective tissue phenotypes has led to the exploration to utilize MSCs in the repair of injured tissues^[5,6]. While the bone marrow has been a common site to harvest MSCs, other cell types similar to bone marrow-derived MSCs can also be found in other sites. Adipose-derived stem cells, satellite cells in muscle, and pericytes around blood vessels and umbilical cord blood cells also may share multipotent characteristics for differentiation into connective tissue phenotypes under specific conditions which include selective differentiation media and growth factors^[7-10]. In a comparison of MSCs from bone marrow, adipose tissue, and cord blood, Rebelatto *et al*^[11] (2008) reported that isolation rate of MSCs from umbilical cord blood was only a third that of bone marrow-derived and adipose-derived MSCs. The initial growth rate of bone marrow-derived and adipose-derived MSCs was much higher than that of umbilical cord blood MSCs. However, others have shown that the proliferation of umbilical cord tissue-derived MSCs show higher population doublings and shorter doubling times compared to adipose-derived MSCs although adipose-derived MSC had higher numbers of colony-forming units compared to MSCs from umbilical cord tissue^[12]. Surface marker expression of CD34 (cluster of differentiation molecule in family of sialomucin proteins) was significantly higher in adipose-derived MSCs compared to that of bone marrow-derived MSCs. Interestingly, CD117 (tyrosine-protein kinase Kit) was found to be positive in about 98% of adipose-derived MSCs but positive in only 52% and 39% of bone marrow-derived and umbilical cord blood-derived MSCs. Additionally, while osteogenic and chondrogenic differentiation was similar in MSCs from all three sources, umbilical cord blood-derived MSCs showed a lesser propensity for adipogenic differentiation. Others have also noted differences in marker expression between bone marrow-derived and adipose-derived MSCs. For instance, CD106 (vascular cell adhesion molecule-1) is expressed in bone marrow-derived MSCs but its expression in adipose-derived MSCs is either low or non-existent while CD49d (integrin $\alpha 4$ subunit) is expressed in adipose-derived MSCs but not in bone-marrow-derived MSCs^[13]. Culture conditions such as the use of fetal bovine serum, human serum, or serum-free medium have been shown to influence

not only the expression of surface markers for adipose-derived MSCs [e.g., CD117, CD166 (activated leucocyte cell adhesion molecule)] and bone marrow-derived MSCs but also in differentiation potential of such MSCs. As an example, fetal bovine serum has a stronger influence on osteogenic differentiation of adipose-derived MSCs than it does on adipogenic differentiation while allogeneic human serum and serum-free conditions have greater propensity to drive adipose-derived MSCs towards adipogenic differentiation than towards either osteogenic or chondrogenic lineages^[14]. Thus while adipose tissue and perhaps umbilical cord tissue sources may provide ample sources for MSCs compared to that of bone marrow and umbilical cord blood, differences in some specific surface markers for MSCs, proliferative potential, and differentiation potential *in vitro* occur based on the source of starting material to isolate MSCs, tissue culture supplements and conditions, and even human individual heterogeneity. Whether non-bone marrow-derived MSCs favor differentiation into specific connective tissue types or even non-mesodermal cell types as in the case of umbilical cord blood MSCs and adipose-derived MSCs in an *in vivo* environment is still a ripe area of investigation^[13-15].

Age of the organism is a determinant of the number of bone marrow MSCs present as well as *in vitro* tissue culture conditions that are critical for MSCs to retain their ability to self-renew yet demonstrate plasticity in their ability to differentiate into various mesodermal tissues^[16]. The number of cells from human bone marrow that are MSCs as determined by colony forming unit-fibroblastic (CFU-f) assay are less than 0.1% of total bone marrow mononuclear cells, thus demonstrating a minimal number of hMSCs that can be used in bone regeneration^[17]. The numbers of CFU-f and the capacity of CFU-fs that can differentiate into osteoblasts further decrease as a function of age of the bone marrow donor up to age 40; after age 40, there does not appear to be any further diminishing of CFU-fs that can differentiate into osteoblasts^[18]. It was suggested that hMSCs have decreased proliferative capacity as a function of age^[19]. Thus hMSCs from young individuals ages 18-29 years achieved an average population doubling level of 41 whereas hMSCs from older individuals ages 66-81 years achieved an average population doubling level of 24 with about a 55% lower population doubling rate than in hMSCs from the younger individuals. However, no difference in *in vivo* bone formation was noted as a function of donor age with early passage cells from either age group. Thus, once placed in primary culture, hMSCs have a limited lifespan (average 20 to 40 population doublings, but the number of population doublings may differ depending on growth medium or any added growth factors)^[19-21] under environmental conditions normally used for *in vitro* cell culture (humidified 5% CO₂ and 95% air (21% O₂) and when grown on tissue culture plastic. hMSCs grown in such conditions attain the Hayflick limit where cell division ceases, and the usual hMSC size becomes larger and the usual spindle shape of normal hMSCs becomes more polygonal or

with a variety of shapes and sizes, at times with multi-nucleation, and overall with less cell density per culture than cells undergoing cell division^[22]. As the number of population doublings for such cells is limited practically in primary culture, slower cell division and finally lack of cell division ensues and the above morphological changes are noted, and the expression of senescence-associated β -galactosidase, and p16, markers of cellular senescence, are increased^[23]. However, it has been shown that if environmental conditions simulate the MSC niche in the bone marrow, specifically low oxygen tension, that self-renewal of hMSCs can be prolonged. D'Ippolito *et al*^[24] (2004) developed a multilineage inducible MSC model from human cadaveric vertebral body marrow (MIAMI cells) and propagated them in 3% O₂/5% CO₂/92% N₂. They reported that more than 50 cell doublings beyond the Hayflick limit for primary cells could be achieved from hMSCs from at least 3 of 12 donors and at least 30 population doublings could be achieved from all of their donors. In a follow-up communication, they reported that MIAMI cells grown in 3% O₂ doubled more quickly than those grown at 21% O₂ and maintained the embryonic transcription factors OCT-4, REX-1, and hTERT and had suppressed osteoblastic differentiation when exposed to osteogenic differentiation medium. At higher O₂ concentrations of 21%, these embryonic transcription factors were lost and osteogenic differentiation was enhanced^[25]. The mechanism by which hypoxia regulates stem cell self-renewal appears to be *via* hypoxia inducible factor-1 α (HIF-1 α). Low oxygen concentrations stabilize HIF-1 α by inhibiting its degradation by the proteasome. Mazumdar *et al*^[26] (2010) reported that hypoxia induced canonical Wnt/ β -catenin signaling and increased transcription of Lef/Tcf genes which have hypoxia response elements in their promoter regions that bind HIF-1 α . Canonical Wnt/ β -catenin signaling thus can induce increased cell proliferation.

HTERT TRANSFORMATION OF HMSCS- THE "IN'S" FOR SELF-RENEWAL

In lieu of special resources needed to grow hMSCs in a hypoxic environment to maintain a proliferative state, a self-renewal strategy, engineering of hMSCs to over express telomerase has been an alternative means to maintain a longer proliferative lifespan of such cells. Telomerase, which is a multi-subunit ribonucleoprotein found in the cell nucleus and perhaps closely associated with nucleoli, allows for the addition of non-coding telomere DNA at the 3' end of linear chromosomes^[27-29]. Maintenance of telomere length by the addition of TTAGGG repeats onto the ends of telomeres allows for cells to continue to divide^[30]. Telomerase is expressed in human embryonic cells and in fetal, newborn, and adult testes and ovaries but not in mature spermatozoa or oocytes. Moreover, expression of telomerase disappears in human somatic cells in the neonatal period and later in life^[31]. Thus lacking telomerase, telomeres shorten with

each cell division leading to replicative senescence once cells reach a critical shortened telomere length. Specifically, with respect to MSCs, a number of laboratories have reported that hMSCs from bone marrow do not express telomerase activity or have activity below detectable levels by telomeric repeat amplification protocol (TRAP) assay when hMSCs are asynchronously dividing^[20,32-34]. However, human telomerase reverse transcriptase (hTERT) expression and telomerase activity could be detected when cells were synchronized to S-phase^[34]. Others have found that telomere length in hMSCs is short upon initial isolation and tend to further shorten with cell passage *in vitro* and appear to correlate with low to undetectable levels of hTERT^[35]. Thus theoretically, maintaining telomerase expression should prevent replicative senescence. Additionally, the decrease in telomere length correlates with CFU-f numbers suggesting that telomere length and telomerase activity could also be related to the ability of hMSCs to differentiate along various cell lineages including the osteogenic lineage^[35]. Gronthos *et al*^[36] (2003) reported that expression of hTERT in human bone marrow-derived MSCs not only increased proliferative capacity by up-regulating G1 to S phase transition cell cycle genes but also increased the expression of osteogenic genes for cbfa-1, osterix, and osteocalcin and induced bone formation earlier and to a much larger degree in an *in vivo* ectopic bone formation assay of hTERT-transformed hMSCs. Saeed *et al*^[37] (2011) demonstrated that in telomerase-deficient mice (*Terc*^{-/-}), there was delayed ossification in occipital bone, sternum, vertebrae, and metatarsals. Overall bone volume was decreased compared to wild type controls, and trabecular bone parameters showed decreased trabecular thickness and increased trabecular spacing^[37]. Additionally, bone formation rate was decreased which correlated with decreased osteoblast surface per bone surface, and osteoclast surface per bone surface was increased. The proliferative ability of bone marrow-derived MSCs from *Terc*^{-/-} mice was diminished compared to wild type mice, and there was increased β -galactosidase staining of *Terc*^{-/-} cells suggesting a more senescent phenotype of MSCs. There was up-regulation of pro-inflammatory genes (*e.g.*, IL-1 receptor type 2, toll-like receptor 6, leukotriene B4 receptor 1, tumor necrosis factor, *etc.*) indicative of osteoclastic activity as well as a decrease of osteoblast-specific bone markers. Thus both decreased bone formation and increased bone resorption as a result of an inflammatory microenvironment were found in this telomerase deficient model.

The critical components of human telomerase include the hTERT catalytic subunit and the RNA subunit, telomerase RNA (hTR), that provides a template for the synthesis of the DNA repeats at the ends of chromosomes. However, generally only hTERT is sufficient to maintain telomere length when transfected into various cell types although integration of ectopic hTERT alone to extend cell replicative ability may be dependent on integration site, availability of other proteins associated

with telomeres, or cell specificity^[32]. Thus a number of studies have demonstrated the feasibility of using hTERT in hMSCs to allow for prolonged replicative lifespan as well as capability of differentiating hTERT-transformed hMSCs towards the osteogenic lineage^[38-42]. The strategy used to transform hMSCs to over express the hTERT gene is generally a retroviral vector approach that uses green fluorescent protein expression as a positive selection marker to enable sorting of positively transformed cells by fluorescence activated cell sorting^[41]. An alternative approach to select transformed cells is an antibiotic resistance strategy^[42]. A technique to control hTERT expression in transfected hMSCs on demand utilizes the tetracycline inducible approach (Tet-On) so that proliferative and differentiation ability can be assessed at selected population doublings although "leakiness" of hTERT even in the Tet-off state could be a limitation^[40]. hTERT-transformed hMSCs have been reported to undergo at least 70 population doubling levels^[42] but upwards of 120 to 400 population doubling levels have been reported depending on the length of time in culture, plating density of cells, and subcultured clonal populations^[32,39-41]. The interesting aspect of hTERT-transformed hMSCs is that they are able to maintain their proliferative ability while being induced to differentiate along osteogenic, but also adipogenic, and chondrogenic lineages. Thus hTERT-transformed cells are different from non-transformed hMSCs and mesenchymal (stromal) cells from other species that are able to differentiate into osteoblasts where it is observed that as osteogenic differentiation proceeds, the proliferative ability of the cells diminishes^[43,44].

Three important criteria must be met when hMSCs are transformed by hTERT expression to achieve a critical mass of cells *via* self-renewal that would be necessary to populate fabricated scaffolds for tissue engineering. Firstly, markers of hMSCs should be maintained after hTERT transformation that would suggest maintenance of multipotency of the cells to undergo differentiation into various mesenchymal cell lineages. Secondly, it is important that hTERT transformation of hMSCs does not lead to malignant transformation either in the pluripotent state or in differentiated cell types. Thirdly, it is critical that hTERT expressing hMSCs will be able to specifically differentiate along the osteogenic lineage and to form bone which is the tissue of interest in this minireview.

Surface markers have been traditionally used to identify hMSCs. The International Society for Cellular Therapy set minimal criteria for positive markers to define hMSCs which are > 95% expression of CD105 (endoglin), CD73 (ecto-5'-nucleotidase), CD90 (Thy-1) and < 2% expression of hematopoietic stem cell markers, CD45 (protein tyrosine phosphatase, receptor type, C), CD34 (sialomucin family adhesion factor), CD14 (monocyte differentiation antigen/lipoglycan receptor) or CD11b (integrin alpha M), CD79 α (immunoglobulin associated alpha) or CD19 (B-lymphocyte antigen), and HLA-DR^[45]. Other markers used to identify hMSCs include STRO-1, CD146 (melanoma cell adhesion molecule/MUC18), CD49a

(integrin alpha subunit), CD271 (low-affinity nerve growth factor receptor), CD63 (lysosome-associated membrane protein-3), found on only on marrow-derived hMSCs and CD166 (activated leucocyte cell adhesion molecule)^[6,16,46-49]. Interestingly, stage-specific embryonic antigen-4 (SSEA-4), found on human embryonic stem cells, was identified as a marker for both mouse and human bone marrow-derived MSCs that had the ability to differentiate into both adipogenic and osteogenic lineages^[50]. Most recently CD44 was identified as a negative marker in freshly isolated although acquisition of the CD44 marker may be a function of *in vitro* cell culture of hMSCs^[51].

Telomerase expression and activity has been found in a majority of human tumors thus suggesting that hTERT expression in human cells could potentially lead to uncontrolled cell proliferation^[52]. However, it has also been suggested that the immortalization induced by hTERT may only in part be due to maintaining telomere length and stabilization and that non-canonical functions of hTERT such as the up-regulation of NF- κ B transcription by TERT binding to the p65 subunit of NF- κ B as well as activating the Wnt/ β -catenin pathway and its target genes, MYC and CCND1 (Cyclin D1), which are regulators of oncogenic targets, and the ability of NF- κ B to inhibit apoptosis, may be more important in promoting tumorigenesis^[53]. The loss of expression of p16^{INK4a}, the protein transcript of the *CDKN2A* gene, in addition to loss of p53 tumor suppressor function, and resistance to growth inhibition by transforming growth factor- β (TGF- β), are among other observations found in the acquisition of oncogenic potential in TERT transformed cells^[54].

Specifically in hMSCs that are transformed with hTERT, there is still the potential of such cells to express tumorigenic properties. Yamaoka *et al*^[55] (2011), constructed hTERT transformed bone marrow hMSCs and found that teratocarcinoma formation could occur when such transformed cells were implanted in immune deficient mice. However, the cells that these investigators transformed with hTERT had first been selected due to their ability to be maintain a proliferative state in the presence of fibroblast growth factor-2 (FGF-2) (> 100 population doubling levels) compared to hMSCs not cultured with FGF-2 that could proliferate to only 20 population doubling levels. As telomerase activity was absent in these FGF-2 maintained clones but had maintained long telomere length, an alternative lengthening of telomeres (ALT) pathway induced by FGF-2 in combination with TERT immortalization could have accounted for the malignant transformation. Serakinci *et al*^[56] (2004) also reported that hMSCs transformed with hTERT could exhibit neoplastic characteristics as shown by loss of contact inhibition and development of mesenchymal tumors after implantation of cells in immunodeficient mice. Loss of p16^{INK4a} and hypermethylation of DBCCR1 (deleted in bladder cancer chromosomal region candidate 1), a cell-cycle associated gene, were observed. Interestingly,

tumors were generated only in high population doubling level hTERT-transformed hMSCs and not in relatively lower population doubling level hTERT-transformed hMSCs. Similarly, Abdallah *et al*^[39] (2005) reported that mesodermal type tumors formed from hTERT transformed hMSCs that had a short population doubling time and accelerated growth, but no tumors developed in hTERT transformed hMSC clones with longer population doubling times that were slower growing. Thus the potential for neoplastic change may be associated with loss of proliferative control as evidenced by cell cycle gene alterations with continued proliferation.

Nevertheless, others have reported that hTERT-transformed hMSCs did not exhibit changes associated with neoplasia even at higher population doubling levels (up to 275)^[32,41,57]. However, whether or not potential oncogenic development occurs in hTERT-transformed hMSCs, functional changes in hMSC parameters need to be considered. Baumer *et al*^[58] (2011) reported that hTERT-transformed human coronary artery endothelial cells demonstrated changes in an *in vitro* co-culture angiogenesis assay where TERT-transformed human coronary artery endothelial cells co-cultured with human fibroblasts and treated with vascular endothelial growth factor (VEGF) did not form tubular networks indicative of angiogenesis; non-TERT-transformed endothelial cells in co-culture with fibroblasts and treated with VEGF were able to form tubular networks. Moreover, hTERT-transformed endothelial cells responded differently to exogenous tumor necrosis factor- α (TNF- α) compared to non-hTERT transformed cells where vascular cell adhesion molecule-1 (VCAM-1) expression was lower, and endothelial barrier function as measured by transepithelial resistance was lost in hTERT-transformed cells. Since hMSCs are immunomodulatory cells that can affect the function of immune hematopoietically derived cells (lymphocytes, monocytes, *etc.*) in an inflammatory environment, there needs to be further investigation if hTERT transformation of hMSCs do not affect these immunomodulating properties of normal hMSCs or have altered function in differentiation or on angiogenesis when interacting with other cell types in a microenvironmental setting.

Perhaps the most prudent approach to ensure that hTERT transformed hMSCs would be useful for bone repair after induction of osteogenic differentiation would be to use inducible vectors for hTERT expression that can then be regulated both temporally and spatially to avert problems with continuous cell proliferation that could result in oncogenic transformation of hTERT-transformed hMSCs^[40].

One other caveat involving the potential enhancement of carcinogenesis may be specific to adipose-derived stem cells (stromal cells) and endothelial cells from white adipose tissue that is independent of hTERT transformation. Zhang *et al*^[59] (2009) reported that the stromal/vascular fraction of white adipose tissue that have proliferative and multipotent differentiative capacity

as well as pericyte-like characteristics can home to human breast and prostate carcinoma cell lines, Kaposi's sarcoma endothelial cell line, and a mouse lung carcinoma cell line implanted in xenograft and allograft mouse models. These stromal/vascular cells engrafted into the tumors and enhanced cancer progression in part through stimulating angiogenesis in the tumors but also perhaps through immunosuppressive effects of the adipose-derived mesenchymal cells found in the stromal/vascular fraction. In follow-up studies, these investigators showed that the increase in the number of adipose-derived stromal (mesenchymal) cells found in obesity could be recruited to mouse and human breast cancer and mouse ovarian cancer models and stimulate tumor growth by increasing tumor vascularity and by differentiating into adipocytes and stimulating proliferation of tumor cells^[60]. In human studies, it was reported that there was increased frequency of mesenchymal stromal (CD34^{bright}CD45⁻CD31⁻) cells (also harboring the pericyte marker, NG2) and CD34^{bright} leucocytes (CD45^{bright}CD34^{bright}) in obese patients (BMI > 30) with colorectal cancer compared to obese non-cancerous subjects^[61,62]. Lean patients with colorectal cancer also had a higher frequency of mesenchymal stromal cells and CD34^{bright} leucocytes compared to lean, non-cancerous controls. However, there was a significant increase in MSCs in obese colorectal cancer patients compared to lean colorectal cancer patients. Thus mobilization of MSCs and CD34^{bright} leucocytes may potentially be markers of colorectal cancer but that there may be a higher frequency of CD34⁺ MSCs (adipose stromal cells) released into circulation even in non-cancerous obese patients suggesting that adipose tissue contributes to MSC mobilization.

OSTEOGENIC DIFFERENTIATION OF HTERT-TRANSFORMED HMSCS

Differentiation of hMSCs along the osteogenic lineage has been demonstrated using both *in vitro* and *in vivo* techniques. Induction of *in vitro* osteogenic differentiation in hMSCs include addition of dexamethasone, ascorbate, and a source of phosphate, mainly β -glycerophosphate to a culture medium base (generally Dulbecco's modified Eagle's medium) containing 10% bovine serum. However, recently it was reported that hMSCs from bone marrow may not require the addition of dexamethasone and ascorbate to form bone *in vivo* although bone marrow-derived hMSCs respond to dexamethasone and ascorbate with increased proliferation *in vitro*^[63]. Osteogenic marker expression by mRNA and protein is usually assessed over the course of *in vitro* cell culture. Early markers of osteogenesis include core binding factor 1 [cbfa1 or runx2 (Runt-related transcription factor 2)] which is found in chondro-osseous precursor cells, osterix which appears in committed osteogenic cells, and collagen type I. Intermediate markers of osteogenesis include alkaline phosphatase and osteopontin and bone sialoprotein and osteocalcin (usually induced in hMSCs by 1.25 dihy-

droxyvitamin D3) are generally used as later markers of terminally differentiated osteoblasts. Determination of mineralization of culture *in vitro* is also critical in assessing terminal differentiation along the osteogenic lineage. This is usually accomplished by staining cell cultures using alizarin red or von Kossa stains which bind to calcium and/or eluting these stains for semi-quantitation of calcium spectrophotometrically. It is also suggested that to distinguish amorphous calcium-phosphate precipitation in cultures from hydroxyapatite [$\text{Ca}_{10}\text{P}_8(\text{OH})_2$], X-ray diffraction, nuclear magnetic resonance, or other technique be used to compare the calcium-phosphate complexes in *in vitro* cell cultures with standard hydroxyapatite patterns by these techniques. Additionally, negative markers for other mesodermal cell types that can be differentiated from hMSCs should be assessed. These are usually markers for the adipogenic lineage [adipsin, peroxisome proliferator-activated receptor gamma (PPAR- γ), adiponectin], the chondrogenic lineage (sox9, collagen type II, collagen type X, aggrecan), tenogenic lineage (scleraxis)^[64], and myogenic lineage Pax3, Pax7 (myogenic precursors), MyoD and myogenin (skeletal muscle), α -smooth muscle actin, vascular endothelial (VE) cadherin (smooth muscle). Essentially, similar techniques to demonstrate osteogenic differentiation have been used for hTERT-transformed hMSCs.

In vivo osteogenesis of hMSCs, whether or not transformed with hTERT, is usually accomplished by ectopic bone ossicle formation assay. In this assay, hMSCs are usually mixed with hydroxyapatite and/or treated with various bone morphogenetic proteins (BMPs) and are implanted into subcutaneous pockets in either immunocompromised rodents (e.g., nude mice; NOD/SCID mice)^[32,39,65,66] or into immune competent rodents^[41]. Assessment for bone formation is done by microCT and/or histology to identify trabecular bone formation and the expression of the above bone marker genes and proteins in tissue sections. hMSCs have been shown to create a locally immunosuppressive microenvironment and are able to avoid allo-recognition^[67] perhaps in rodent species although it is unknown if the same holds true for transplantation of hMSCs into human recipients or if there are any consequences of immunogenicity of hMSCs once they are differentiated into specific lineages in a human recipient^[68].

It is highly important that the both *in vitro* and *in vivo* confirmation of hydroxyapatite or bone formation be done especially in hTERT-transformed hMSCs. It is possible that not all hTERT-transformed hMSCs will be able to form bone *in vivo*. Larsen *et al*^[69] (2010) established subclones from hTERT transformed hMSCs at a relatively early population doubling level (PDL 77) and from a later PDL 233. They found that both subclones retained surface markers for hMSCs (CD63, CD73, CD105, and CD166) as well as expressed osteoblast markers, alkaline phosphatase, collagen type I, and osteocalcin upon induction with osteogenic medium. Both clones also formed mineralized matrix *in vitro* as assessed

by alizarin red staining. However, the PDL 77 clone was able to form bone in an *in vivo* ectopic bone formation assay while the PDL 233 clone did not form bone. Interestingly, these investigators reported that CD146 was highly expressed in the hTERT-transformed hMSC clone that could form bone *in vivo* while CD146 was minimally expressed in the hTERT-transformed clone that did not form bone *in vivo*. Thus the criteria for *in vivo* bone formation and expression of CD146 should be helpful in assessing hTERT-transformed hMSCs that may be useful for potential bone repair or regenerative therapy, and sole dependence on osteogenic markers and *in vitro*, two-dimensional cell culture mineralization assays may be insufficient. Also observed in additional hTERT-transformed hMSC clones that formed bone *in vivo* was the increased number of extracellular matrix genes expressed as well as the increased number of Sp3 binding sites in the promoter regions of these expressed genes compared to that of hTERT-transformed hMSC clones that did not form bone *in vivo*. Sp3 is a transcription factor necessary for bone development and ossification.

In attempts to seed hTERT-transformed hMSCs in areas requiring their presence for tissue repair, strategies such as intracardiac or intravenous injection of hMSCs expressing a fluorescent marker (*e.g.*, green fluorescent protein) have been used to identify sites where such injected hMSCs populate as well as to assess the longevity of transplanted hMSCs in the desired regions. Bentzon *et al*^[70] (2005) reported that hTERT-transformed hMSCs injected intracardiac or intravenously into NOD/SCID mice were trapped mainly in microvasculature of the lungs, kidneys and heart. It was also found that only a small fraction of the injected telomerized hMSCs survived or were retained possibly due to protracted trans-endothelial migration. Thus direct engraftment of hTERT-transformed hMSCs may be a better approach to healing bone.

In addition to cells, such as MSCs, that have the potential to self-replicate and differentiate into the cell type of choice, tissue engineering in regenerative medicine strategies generally combine the cellular component with various growth and differentiation factors that can promote differentiation of undifferentiated precursor cells and with the employment of a structural framework on which either such cells and/or growth and differentiation factors can be assembled. The use of three-dimensional culture platforms may simulate the natural three-dimensional *in vivo* tissue architecture and provide advantages over that of assessing hMSC growth and differentiation on tissue culture plastic in a two-dimensional format^[71,72]. Two dimensional cultures may only yield woven type bone (random orientation of collagen fibrils) and not allow for the formation of lamellar bone, the final desired bone product, and microenvironments that may develop in a three-dimensional framework that could affect cell-cell and cell-matrix interactions cannot fully develop in a two dimensional culture system.

For *in vivo* uses, three-dimensional platforms or scaffold

needs need to be biocompatible, potentially biodegradable, have sufficient porosity to allow great surface area for cell attachment, and in general be non-immunogenic. The more rigid platforms or scaffolds composed of material such as hydroxyapatite or other calcium-phosphate bases which are osteoinductive and can induce ectopic bone formation. Titanium has been used to grow hMSCs that can then be differentiated along the osteogenic lineage with or without BMP stimulation prior to direct surgical implantation into bone defects in translational models of bone repair^[73-75]. Biological scaffolds that are composed of polymer blends such as poly(l-lactide-co-glycolide) (PLGA) are biocompatible and can be degraded by the body have also been used as a base on which hMSCs can be grown and differentiated^[76]. Polymer blends have also been used in combination with inorganic hydroxyapatite crystals or naturally occurring proteins such as collagen to construct composite scaffolds that improve mechanical and osteoinductive properties of the scaffolds have also been designed^[77]. Hydrogels have also been used as scaffold material due to biocompatibility; natural hydrogels are derived from collagen or gelatin, while synthetic hydrogels can be made from poly(ethylene glycol). While natural hydrogels are excellent for cell adhesion and biodegradation, immunogenic reactions may be a concern if the hydrogels are derived from animal-derived extracellular matrix (ECM) protein. Synthetic hydrogels have the advantage of creating scaffolds *in situ* using photopolymerization and also are non-immunogenic^[78]. Hydrogels as well as polymer blends with or without ceramic material (*e.g.*, hydroxyapatite) have also been useful in serving as reservoirs for bioactive molecules such as growth factors^[77-79]. Thus scaffolds impregnated with various growth factors or composed in part of ECM-derived short peptides, modified heparin, chondroitin sulfate or hyaluronic acid to tether growth factors such as the BMPs, epidermal growth factor (EGF), platelet-derived growth factor (PDGF), TGF- β , FGF-2 have been useful in the differentiation of transplanted hMSCs and/or the chemotaxis of native MSCs useful in bone repair^[75,77-82].

Stromal-derived factor 1 (SDF-1), a chemokine, has also been impregnated in scaffolds to serve as a chemotactic factor for bone marrow-derived MSCs^[83-85]. It has also been shown that human cord blood-derived MSCs as well as human adipose tissue-derived MSCs (the stromal/vascular fraction of adipose tissue) express CXC receptor 4 (CXCR4), the receptor for SDF-1, and are induced to migrate in response to SDF-1^[86,87]. Human bone marrow-derived MSCs have also been shown to migrate to bone marrow stroma in a CXCR4-dependent manner^[88]. Bone-marrow-derived MSCs can also express SDF-1 and serve to maintain hematopoietic stem cells in a quiescent state in the bone marrow^[89,90]. However, under conditions of inflammation with the release of pro-inflammatory cytokines such as TNF- α and IL-1 β and hypoxia that can be found in tissue injury and the early phases of wound repair, bone marrow-derived MSCs as well as MSCs from adipose tissue or other local sources could migrate to the

wound location *via* the SDF-1/CXCR4 axis to participate in the repair or regeneration of mesenchymal tissues (*e.g.*, bone)^[91-94]. Potential sources of SDF-1 that could potentially be involved in local MSC migration and homing to disrupted bone may be endothelium, local osteoblasts, platelets involved in initial wound hemostasis, and periosteal cells^[87,95-97]. VEGF has been used to stimulate angiogenesis that would allow for improved blood supply to repairing tissues; use of VEGF incorporated into natural hydrogels or injected directly into scaffolds and in combination with BMPs and MSCs attached to scaffolds have been tested to improve bone healing^[81,98]. In the absence of seeding MSCs onto scaffolds, delivery of SDF-1 *via* implantable infusion pump to poly- ϵ -caprolactone scaffolds preceded by delivery of VEGF to the scaffolds and followed by BMP-6 to induce osteogenic differentiation was able to induce mature mineralized bone formation^[99]. Tasso *et al*^[100] (2009) also reported that in a mouse model of ectopic bone formation, donor murine bone marrow MSCs loaded onto hydroxyapatite scaffolds were needed in the early development of ectopic bone (up to one week after implantation) to recruit host osteoprogenitor cells, but native (host) osteoprogenitor cells actually contributed the most to the bone formation *via* endochondral ossification. Thus native MSCs can be induced to populate scaffolds using SDF-1 and osteogenically differentiate to form vascularized bone. MSCs harboring viral vectors (adeno-associated virus or lentivirus) to over express growth factors and chemoattractants and attached to various types of scaffolds have been used as an alternative strategy to increase local concentrations of bioactive molecules such as BMP-2, BMP-7, VEGF, and CXCR4, the transmembrane G-protein coupled receptor for SDF-1-induced chemoattraction, to enhance osteogenic marker expression^[101-106]. Finally, other chemokines may also play roles in migration of MSCs. Chemokines of the α family (CXC chemokines) as well as the β family (CC chemokines) have been reported to stimulate migration of MSC from both bone marrow and omental adipose tissue^[92,107,108]. Interestingly, under pro-inflammatory conditions as is found in the initial phase of wound healing, priming with TNF- α enhances the expression of these chemokines such as CXCL8 (interleukin 8), CCL5/RANTES (regulated on activation, normal T cell expressed and secreted), CCL22 (macrophage-derived chemokine) which are then able to stimulate MSC migration^[94,95]. Additionally, CXC chemokines with the glu-leu-arg motif in the N-terminus of CXC chemokines are also angiogenic and thus may play a role in new blood vessel formation during wound repair during bone regeneration^[109].

Three dimensional spheroid cultures consisting of high density cell aggregates in agarose or alginate have also been used to traditionally differentiate chondrocytes from hMSCs^[110,111]. Burns *et al*^[112] (2010) used a variation of this method by using caroxymethylcellulose in their high density cell preparation to form spheroids of hTERT transformed hMSC cells. When combined with hydroxyapatite/ β -tricalcium phosphate scaffolds,

induced with osteogenic medium, and implanted into immunodeficient mice in an *in vivo* ectopic bone formation assay, lamellar bone formation was observed in scaffold concavities in addition to the expression of usual osteoblastic markers of cbfa1, alkaline phosphatase, osteonectin, osteopontin, collagen type I and osteocalcin. CD146 expression which had been high in hMSCs was lost as osteogenic differentiation proceeded. Interestingly, transcriptional co-activator with PDZ binding motif (TAZ)^[113], a cbfa1 binding transcription co-activator that allows for commitment to the osteogenic lineage while inhibiting adipogenic differentiation of hMSCs was also induced in the hTERT transformed hMSC spheroids. Stimulated expression of other extracellular matrix proteins such as biglycan, lumican, elastin, periostin, microfibrillar-associated proteins (MFAP2 and MFAP5), tetranectin and decorin also occurred suggesting correlation between these extracellular matrix protein and osteogenesis.

EXTRACELLULAR MATRIX AND HMSCS- THE "OUTS" FOR OSTEOGENESIS

The use of extracellular matrix (ECM) components to enhance either rigid type scaffolds or hydrogel scaffolds or to serve as scaffolds themselves has become more popular in tissue engineering. For instance, collagen type I in the form of gels or sponges or as a protein coating of hydroxyapatite platforms has been useful in providing an attachment for cells in addition to being able to deliver growth factors such as TGF- β , BMPs, or VEGF^[114]. ECM contains proteoglycans which are comprised in part of heparin sulfate that can bind many types of growth factors such as FGFs and VEGF and degradation of ECM by matrix metalloproteases can release these growth factors to subsequently bind to their receptors on specific cells^[115]. Other ECM proteins such as laminin and tenascin have epidermal growth factor (EGF)-like motifs that could potentially bind to EGF receptors on cells and then initiate an EGF signaling cascade through tyrosine kinase activation resulting in cell proliferation and/or differentiation^[116]. The binding of cells to naturally occurring proteins such as collagen occur *via* integrins, comprised of α and β subunits and binding cell membranes to ECM proteins with the arginine-glycine-aspartic acid (RGD) or leucine-valine-aspartic acid (LVD) (consensus sequence L/I (isoleucine)-D/E (glutamic acid)-V (valine)/S (serine)/T (threonine)-P (proline)/S domains^[117,118]. The short cytoplasmic domains of integrins interact with various cytoskeletal elements such as talin and kindlin to initiate inside-out signaling through integrin-linked kinase that is involved in activating integrins to bind to ECM components^[119,120]. Outside-in signaling occurs with the interaction of specific sequences of ECM proteins and activated integrins to activate focal adhesion kinase to allow in part for functions such as cell spreading and migration but also activating other signaling pathways enabling cell proliferation, and survival^[121].

Thus scaffolds composed of native ECM proteins such as collagen have been applied as one strategy to expand hMSCs *ex vivo* and to promote osteogenic differentiation and to enhance bone repair^[17,122,123]. Bone marrow-derived hMSCs, express various integrins such as $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, $\alpha_v\beta_3$, and $\alpha_v\beta_5$; however, the β_1 integrin subunit was found to be most responsible for hMSCs to adhere to collagen, laminin and fibronectin and be involved in proliferation of hMSCs and for their differentiation into osteoblasts^[124]. However, pre-coating scaffolds with a specific protein such as collagen type I or MatrigelTM (BD Biosciences) composed of collagen type IV, entactin, and laminin, may not yield the natural three dimensional environment, nor account for all appropriate ECM proteins that interact with hMSCs *in vivo*, nor retain the natural elasticity or stiffness required for proper self-renewal or tissue-specific differentiation. Degree of stiffness or elasticity of support structures or ECM has been shown to be important in part to be a determinant of stem cell differentiation. In reference to MSCs, softer substrates favor adipocyte or chondrogenic differentiation while stiffer substrates direct osteogenic differentiation. Intermediate stiffness can assist in directing myogenesis from MSCs^[125-128]. ECM or bioengineered support structure stiffness or elasticity can be sensed by cells through the organization of stress fibers composed of actin microfilaments and myosin. Specifically, non-muscle myosin II isoforms, II A, II B, and II C appear to be involved in the MSC's ability to sense matrix stiffness through their interaction with cortical actin that is linked to focal adhesions. Increased matrix stiffness is associated with increased activity of non-muscle myosin II. The increased non-muscle myosin II also correlates with specific lineage determination of MSCs^[129]. Interestingly, ECM stiffness that can set the stage for specific lineage differentiation *via* expression or repression of specific genes is transduced to nuclear chromatin *via* lamin-A^[130]. Cytoskeletal stresses and tension increase with increasing ECM stiffness and the degree of lamin-A expression and phosphorylation is inversely related to ECM stiffness. Thus osteogenic differentiation of MSCs is correlated with increased lamin-A levels and decreased lamin-A phosphorylation when MSCs are grown on a stiff ECM. It would follow that lamin-A would act in a manner to maintain nuclear rigidity or stiffness which could translate into epigenetic regulation of chromatin thus enabling transcription of osteogenic genes and repression of genes specific with other mesenchymal lineages through lamina-associated domains which contain repressive heterochromatin.

Thus the use of cell-free preparations of secreted ECM proteins produced by MSCs or cells of the desired differentiated type (*i.e.*, osteoblasts) may perhaps allow for better osteogenic differentiation of MSCs in a native three dimensional microenvironment similar to the MSC niche found in bone marrow. Chen *et al*^[131] (2007) prepared ECM from mouse MSCs that supported self-renewal of mouse MSCs when cultured on this native

ECM and the proliferative ability of the MSCs grown on native ECM was greater than MSCs grown on fibronectin or collagen type I individually. Differentiation of mouse MSCs into both adipogenic (in response to rosiglitazone) and osteogenic lineages (in the presence of exogenous BMP-2) was also enhanced in cells cultured on native ECM compared to tissue culture plastic or culture plastic coated with fibronectin alone or with collagen type I alone. However, mouse MSCs had a delay in osteogenic differentiation when grown on native ECM in the absence of exogenous BMP-2, and it was suggested that the native ECM components such as collagen and biglycan bind BMP-2, making it less available to MSCs to allow for earlier osteogenic differentiation.

hMSCs can also be used to generate native ECM that supports self-replication of hMSCs, and the degree of enhanced proliferation of hMSCs was found to be greater than that of hMSCs grown on tissue culture plastic, or fibronectin or collagen type I independently^[132]. It was also found that SSEA-4, a marker for bone marrow-derived hMSCs, was maintained at a high level throughout the culture period on native ECM and interestingly, telomerase activity was stable and reactive oxygen species was low on ECM-grown hMSCs compared to hMSCs grown on plastic, fibronectin, or collagen type I. *In vivo* bone formation was also significantly higher in hMSCs grown on native ECM compared to those grown on plastic. Thus native ECM from hMSCs can better support self-renewal and osteogenic differentiation compared to single ECM components or a two dimensional culture platform (plastic).

It has been shown that ECM from human foreskin young fibroblasts (< 20-30 population doublings) supported the proliferation of old fibroblasts (> 68 population doublings) so that the proliferative rate of the old fibroblasts approached that of young cells grown on ECM from young cells^[133]. Additionally, telomere length was restored in old fibroblasts grown on ECM from young cells by a telomerase independent mechanism and reduced reactive oxygen species similar to young cells was also found. Interestingly, SIRT 1, a gene for the NAD-dependent histone deacetylase, sirtuin 1, which was downregulated during senescence was increased when old fibroblasts were grown on ECM from young cells. This suggests that epigenetic mechanism(s) may play a role the mechanism of how young ECM can restore the proliferative ability of old fibroblasts. SIRT 1 can be directly activated by lamin A^[134], which is critically involved in the process of information flow from ECM to the nucleus to perhaps determine chromatin configuration and thus confer epigenetic regulation on gene expression or repression. Thus the potential role of epigenetics in ECM rejuvenation of old fibroblast cells is an area of interesting investigation.

With regards to MSCs, the composition of ECM from young (low passage) adipose-derived MSCs compared to that of old (higher passage) MSCs is different. For instance, while collagen type I is increased in young

MSCs, laminin, fibronectin, vimentin, keratin, and lamin A/C are decreased in old MSCs. When old MSCs are seeded onto ECM from young MSCs, the pluripotency markers of Oct4, Sox2, and Nanog are increased and growth factors such as TGF β are also upregulated^[135]. The ECM component, biglycan, has been shown to increase canonical Wnt/ β -catenin signaling. Wnt signaling is a critical morphogen in osteoprogenitor development. Bone marrow MSCs from mice deficient in biglycan were less proficient in Wnt-induced mineral deposition in culture, did not respond to exogenous Wnt3a, and made significantly less trabecular bone when used in an *in vivo* ectopic bone formation assay^[136]. Thus one could speculate that ECM from young MSCs may have more biglycan than ECM from old MSCs and thus young ECM would be able to enhance Wnt signaling to enhance both proliferation of osteoprogenitors and potentially more bone formation. However, the exact mechanism of how biglycan can regulate either canonical or non-canonical Wnt signaling is unclear.

In another interesting study, Sun *et al*^[137] (2011) reported the differential effect of ECM from mouse bone marrow stromal cells derived from young (3 mo) versus old mice (18 mo). Replicative ability was restored in MSCs from old mice cultured on ECM from young mice, similar to that of the replicative ability of young mice grown on ECM from young mice. However, the replicative ability of MSCs from either young or old mice was significantly less when cultured on ECM from old mice. Telomerase levels were also increased in MSCs from young and old mice cultured on ECM from young mice compared to that of MSCs cultured on tissue culture plastic or on ECM from old animals. Examination of bone forming ability using an *in vivo* assay where MSCs from young or old mice pre-cultured on ECM from young or old mice demonstrated that MSCs from old mice pre-cultured on ECM from young mice had increased cancellous bone formation compared to MSCs from young or old mice pre-cultured on tissue culture plastic. Culture of MSCs from either young or old mice on ECM from old mice demonstrated less bone formation. In trying to dissect the differential effect of ECM from old versus young mice, these investigators found that ECM from old mice contained more mineral phosphate and less collagen although the total amount of ECM produced by young or old cells was the same. Furthermore, reactive oxygen species levels were higher in MSCs grown on ECM from old mice but were reduced in MSCs grown on ECM from young mice; there was also an inverse correlation of the number of colony forming units-osteoblast and the level of reactive oxygen species. How ECM from old mice is incapable of handling reactive oxygen species and how this may relate to changes in ECM composition (lower collagen and proteoglycans) remains unknown.

In a recent communication, Prewitz *et al*^[138] (2013) used early passage bone marrow-derived hMSCs to generate native ECM but used either osteogenic medium to allow the hMSCs to differentiate towards the osteogenic

lineage or ascorbic acid alone in the growth medium to allow the hMSCs under these conditions to generate an “enriched” ECM. These generated ECMs were then tethered to tissue culture plastic using poly(octadecene-*alt*-maleic anhydride). These investigators reported that ascorbic acid-stimulated native ECM contained twice as much collagen and sulfated glycosaminoglycans compared to native ECM generated using osteogenic medium although the spectrum of ECM protein were the same. Release of hepatocyte growth factor, FGF, VEGF, and interleukin-8 was also higher from ascorbic acid-stimulated ECM. Nevertheless, both types of ECM supported higher population doublings of hMSCs grown on these surfaces compared to hMSCs grown on either plasma-treated tissue culture plastic, fibronectin or MatrigelTM. Both ascorbic acid and osteogenic-induced ECM also stimulated more osteogenic differentiation as well as adipogenic differentiation although the ascorbic acid-induced ECM yielded better osteogenic and adipogenic differentiation than osteogenic-induced ECM. Finally, both ascorbic acid-induced and osteogenic-induced ECM were able to support the engraftment of hematopoietic stem and progenitor cells, similar to a hematopoietic stem cell niche. Hence, bolstering native ECM by stimulation its production from hMSCs with either ascorbic acid or osteogenic medium could potentially be a useful strategy in rejuvenating old hMSCs.

Thus whether the total or individual amounts of native ECM, the breadth of composition of native ECM, the geometry of ECM organization, or the ability of ECM to sequester growth factors, retain growth factor-like motifs (*e.g.*, similar to the EGF-like repeats found on laminin and tenascin), or regulate other morphogens such as Wnt signaling that can potentially regulate MSC proliferation and differentiation are important factors in explaining the mechanism(s) of how young ECM can rejuvenate old MSCs are salient areas for future investigation.

CLINICAL UTILITY OF MESENCHYMAL STEM CELLS IN ORTHOPAEDIC CONDITIONS

MSCs from various sources in combination with specific growth factors and/or scaffold material potentially lend themselves to a variety of clinical orthopaedic conditions involving bone and cartilage. There are a number of clinical trials and case reports using MSCs to repair critical sized defects caused by trauma or infection as well as replacing chronically degenerated tissue such as articular cartilage and intervertebral discs. There are a number of excellent and comprehensive published reviews on the subject of orthopaedic applications for MSC therapy^[139-143] and which are listed in Table 1. Two clinical trials and two other case reports using MSCs in human orthopaedic conditions are also included in Table 2. The clinical trial to treat knee osteoarthritis enrolled 25 pa-

Table 1 Reviews of mesenchymal stem cell use in human orthopaedic conditions

| Ref. | Reviewed Orthopaedic conditions treated | MSC source | Additional repair components |
|--|--|---|--|
| Shenaq <i>et al</i> ^[139] , <i>Stem Cell Int</i> , 2010 | Osteonecrosis humerus, femoral head; Fracture non-union; Cartilage defect; Osteogenesis imperfecta; Critical size defect limbs; Calvarial defect | Autologous or allogeneic bone marrow; Fetal liver; Adipose | Ceramic scaffolds; Collagen gels |
| Rastegar <i>et al</i> ^[140] , <i>World J Stem Cells</i> , 2010 | Critical size defect in long bones; Articular cartilage of knee; Osteogenesis imperfecta; Hypophosphatasia | Autologous bone marrow; Allogeneic bone marrow; Fetal liver | |
| Zhang <i>et al</i> ^[141] , <i>Biomaterials</i> , 2012 | Segmental bone defects of limbs; Distraction osteogenesis; Tibial osteotomy; Posterior spinal fusion; Maxilla defects; Sinus augmentation; Osteogenesis imperfecta; Articular cartilage repair; Osteoarthritis | Autologous bone marrow; Allogeneic bone marrow; Fetal liver | Hydroxyapatite scaffolds; autologous platelet rich plasma, allogeneic bone chips or bone grafts; β -tricalcium phosphate scaffolds |
| Veronesi <i>et al</i> ^[143] , <i>Stem Cell Dev</i> , 2013 | Osteoarthritis of knee, hip, elbow, ankle; medial femoral condyle, patellar, patella-femoral joint lesions; osteochondral lesions talar dome and femoral condyle | Autologous bone marrow | Hyaluronate; collagen type 1 sheet; platelet rich plasma; periosteal patch; collagen powder |
| Kim <i>et al</i> ^[142] , <i>Korean J Int Med</i> , 2013 | Osteogenesis imperfecta; Cartilage defects | Allogeneic bone marrow, fetal liver | |

MSC: Mesenchymal stem cell.

Table 2 Clinical trials and case reports using mesenchymal stem cells in human orthopaedic conditions

| Orthopaedic condition | MSC source | Technique | Patients/controls | Study length | Outcome | Ref. |
|----------------------------------|-------------|--|--|--------------|--|-------|
| Osteoarthritis-Knee | Adipose | Autologous MSCs with platelet rich plasma | 25/25 retrospective controls | 12 mo | Study group significantly higher degrees of improvement from pre-treatment levels in pain and activity | [144] |
| Intervertebral Disc Degeneration | Bone marrow | MSC injection into nucleus pulposus | 10/self-controls - pre- and post procedure | 12 mo | Pain, disability, quality of life, disc water content improved | [145] |
| Maxillary Reconstruction | Adipose | Vascular flap with ADCs, β -tricalcium phosphate, BMP-2 | 1 case | 12 mo | Regeneration of normal bone | [146] |
| Mandibular Reconstruction | Bone marrow | BMP-2, collagen sponges + bone marrow MSCs + allogeneic bone chips | 5 cases | 22 mo | Bone regeneration in 2/4 cases using MSCs; failure overall in 2 of 5 cases | [147] |

MSC: Mesenchymal stem cell; BMP: Bone morphogenetic protein.

tients. Infrapatellar fat pad-derived MSCs and platelet rich plasma were injected into knee joints after arthroscopic debridement, excision of degenerative material/osteophytes, or synovectomy^[144]. Comparison was made to retrospective age- sex- and follow-up period matched controls who had received only platelet rich plasma injections with arthroscopic debridement. Various scales used in knee symptoms (visual analog pain scale, Lysholm knee scoring scale, Tegner activity level scale) showed that the initial or pre-treatment scores of the study group were significantly poorer compared to controls but by the last follow-up visit (12 mo) after MSC therapy, the study group showed significantly higher degrees of improvement from pre-treatment levels in all of the assessment scales measured compared to that of the retrospective control group. Orozco *et al*^[145] (2011) injected autologous bone marrow-derived MSCs that were expanded under Good Manufacturing Practice conditions into the nucleus pulposus in 10 patients. These patients apparently served as their own controls and pain (visual analog scale), disability (Oswestry Disability Index), and quality of life (SF-36) were improved over the 12 mo trial. Water content of the diseased discs also improved by 12 mo after

treatment. Two other communications consisting of case reports are also entered into Table 2. One report used autologous adipose-derived stem cells expanded in vitro and combined with β -tricalcium phosphate scaffolding material harboring rhBMP-2 placed in a muscle flap and used to repair a maxillary bone defect^[146]. The other was a series of 5 cases using collagen sponges impregnated with rhBMP-2 with or without autologous bone marrow cells and allogeneic cancellous bone (4 cases) and one case using only rhBMP-2 adsorbed onto collagen sponges to reconstruct mandibular bone defects. Although not stated, it was presumed that bone marrow MSCs were the bone marrow cells referred to in three of the cases, two of which were successful in healing the bone defects^[147].

CLOSING THOUGHTS (A WORKING MODEL)

In summary, MSCs from have promising utility in resolving orthopaedic problems although there is a need for more prospective randomized controlled trials. At this point it is still unclear if MSCs from various sources

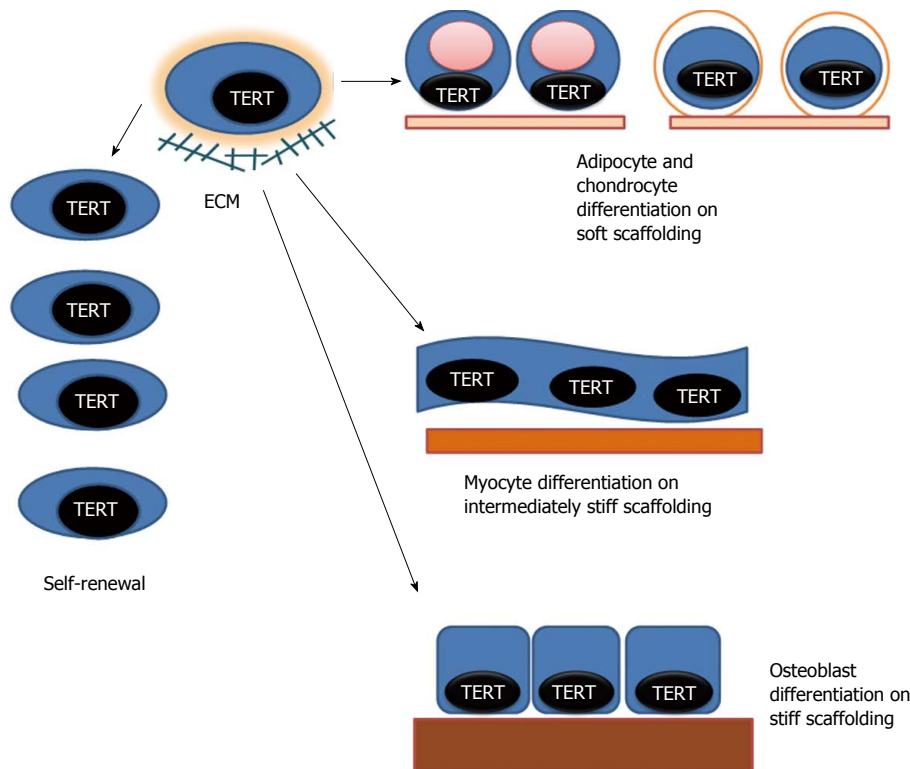


Figure 1 Model of human telomerase reverse transcriptase-transformed mesenchymal stem cell self-renewal and differentiation. Human telomerase reverse transcriptase (hTERT) can be expressed by transfection in human mesenchymal stem cells (hMSCs) from various sources to enhance self-renewal. hTERT transformed cells can be induced to differentiate along multiple mesenchymal lineages. Stiffness of support structures and/or extracellular matrix (ECM) upon which hMSCs are situated is important in differentiated lineage determination. Softer or less stiff support structures/ECM (lightest colored and thinnest bar under the cells) support adipogenic or chondrogenic lineages. Intermediate stiffness (medium colored and thicker bar) can direct myogenesis. Stiffer substrates (darkest colored and thickest bar) can support osteogenic differentiation. Native ECM made from hMSCs from younger hosts may also enhance self-renewal and the differentiative capacity of hMSCs from older sources, and may be superior to singular or limited number of defined ECM components in promoting self-renewal and specific lineage differentiation.

(bone marrow, adipose, cord blood, cord tissue, muscle, etc.) would all be useful in orthopaedic repair and regeneration in general and bone in particular. It does appear that MSCs from either bone marrow or adipose tissue are quite similar in their capacity to serve in bone repair and regeneration. However, work still needs to be done regarding ideal scaffolding material and whether addition of MSCs or growth factors, angiogenic factors, and/or chemotactic factors onto scaffolds alone or in combination with MSCs would be the best strategy for bone repair and regeneration in the human situation.

With specific reference to MSC self-renewal and differentiation into osteogenic tissue, addition of hTERT to MSCs would seem to assist in increasing population doublings and decreasing population doubling times to enhance a critical mass of MSCs (Figure 1). However, there is still debate over initiation of tumorigenesis associated with TERT transformation of MSCs and the potential of MSCs (TERT transformed or not) to enhance the growth of already established tumors. Differentiation of TERT-transformed MSCs into osteogenic cells appear to be kept intact although whether exceeding a certain level of population doublings can lead to a decrease or change in differentiation capacity must still be considered. The use of native ECM from young MSCs appears to enhance the proliferative and differentiative capacity of MSCs

and the stiffness of the ECM appears to steer MSCs to differentiate along specific lineages, with osteogenic differentiation being assisted on a stiffer ECM (Figure 1). Thus TERT expression that can be regulated in a time and stage of differentiation manner may be an ideal strategy to both enhance a critical mass of MSCs necessary for bone repair and regeneration but to try to limit the potential of malignant transformation.

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P- Reviewers: Bonnet D, Karaaz E, Kolonin MG, Scuteri A

S- Editor: Wen LL **L- Editor:** A **E- Editor:** Zhang DN



WJSC 6th Anniversary Special Issues (2): Mesenchymal stem cells

Progress of mesenchymal stem cell therapy for neural and retinal diseases

Tsz Kin Ng, Veronica R Fortino, Daniel Pelaez, Herman S Cheung

Tsz Kin Ng, Daniel Pelaez, Herman S Cheung, Geriatric Research, Education and Clinical Center, Miami Veterans Affairs Medical Center, Miami, FL 33125, United States

Tsz Kin Ng, Department of Ophthalmology and Visual Sciences, The Chinese University of Hong Kong, Hong Kong, China

Veronica R Fortino, Herman S Cheung, Department of Biomedical Engineering, College of Engineering, University of Miami, Coral Gables, FL 33146, United States

Author contributions: Ng TK, Fortino VR, Pelaez D and Cheung HS solely contributed to this paper

Supported by Veterans Affairs (VA) Merit Review Grant; and Senior VA Research Career Scientist Award, Miami

Correspondence to: Herman S Cheung, PhD, Professor, Geriatric Research, Education and Clinical Center, Miami Veterans Affairs Medical Center, 1201 NW 16th Street, Miami, FL 33125, United States. hcheung@med.miami.edu

Telephone: +1-305-5753388 Fax: +1-305-5753365

Received: October 28, 2013 Revised: January 14, 2014

Accepted: March 3, 2014

Published online: April 26, 2014

Abstract

Complex circuitry and limited regenerative power make central nervous system (CNS) disorders the most challenging and difficult for functional repair. With elusive disease mechanisms, traditional surgical and medical interventions merely slow down the progression of the neurodegenerative diseases. However, the number of neurons still diminishes in many patients. Recently, stem cell therapy has been proposed as a viable option. Mesenchymal stem cells (MSCs), a widely-studied human adult stem cell population, have been discovered for more than 20 years. MSCs have been found all over the body and can be conveniently obtained from different accessible tissues: bone marrow, blood, and adipose and dental tissue. MSCs have high proliferative and differentiation abilities, providing an inexhaustible source of neurons and glia for cell replacement therapy. Moreover, MSCs also show neuroprotective effects

without any genetic modification or reprogramming. In addition, the extraordinary immunomodulatory properties of MSCs enable autologous and heterologous transplantation. These qualities heighten the clinical applicability of MSCs when dealing with the pathologies of CNS disorders. Here, we summarize the latest progress of MSC experimental research as well as human clinical trials for neural and retinal diseases. This review article will focus on multiple sclerosis, spinal cord injury, autism, glaucoma, retinitis pigmentosa and age-related macular degeneration.

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Key words: Mesenchymal stem cells; Stem cell therapy; Central nervous system; Retina; Clinical trial

Core tip: Central nervous system (CNS) disorders are the most challenging and difficult for functional repair. Neurons are still diminishing in many patients despite surgical and medical interventions. Stem cell therapy has been proposed as a viable option. Mesenchymal stem cell (MSC) is a widely-studied human adult stem cell population. MSCs can be conveniently obtained from different accessible tissues. MSCs have high proliferative and differentiation abilities, providing an inexhaustible source of neurons and glia. MSCs also show neuroprotective effects and possess extraordinary immunomodulatory properties. These qualities heighten the clinical applicability of MSCs when dealing with the pathologies of CNS disorders.

Ng TK, Fortino VR, Pelaez D, Cheung HS. Progress of mesenchymal stem cell therapy for neural and retinal diseases. *World J Stem Cells* 2014; 6(2): 111-119 Available from: URL: <http://www.wjgnet.com/1948-0210/full/v6/i2/111.htm> DOI: <http://dx.doi.org/10.4252/wjsc.v6.i2.111>

STEM CELL THERAPY AND MESENCHYMAL STEM CELLS

Stem cells are undifferentiated cells defined by their ability to self-renew and differentiate into mature cells. Stem cells are attractive because they are highly proliferative, implying that an inexhaustible number of mature cells can be generated from a given stem cell source. On this basis, cell replacement therapy has been proposed in recent years as a viable alternative for various pathologies. Cell replacement therapy hypothesizes that new retinal cells could be generated from stem cells so as to substitute the damaged cells in the diseased retina. This theory is mainly established from embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs). In addition to cell replacement function, stem cells could have another protective effect, the paracrine effect. The paracrine effects of stem cells are believed to modulate the micro-environment of the diseased tissues so as to protect the injured cells, promote survival and activate any available endogenous repair mechanisms. This latter observation applies mainly to the transplantation of adult stem cells.

Adult stem cells are defined as the stem cells found in fully developed tissues. The function of adult stem cells is the maintenance of adult tissue specificity by homeostatic cell replacement and tissue regeneration^[1]. Adult stem cells are presumed quiescent within adult tissues, but divide infrequently to maintain their own niche by generating a stem cell clone and a transiently-amplifying cell. The transiently-amplifying cells will undergo a limited number of cell divisions before terminal differentiation into mature functional tissue cells. The existence of adult stem cells has been reported in multiple organs; these include: brain, heart, skin, intestine, testis, muscle and blood, among others.

Mesenchymal stem cells (MSCs), also called marrow stromal cells, are an adult stem cell population of stromal progenitor cells of mesodermal origin^[2]. MSCs were originally identified in the bone marrow, representing 0.001%-0.01% of the bone marrow population. MSCs can also be found in other systems all over the body, such as adipose tissue, liver, umbilical cord, central nervous system (CNS) and dental tissues^[3]. According to the International Society of Cellular Therapy^[4], the minimal criteria to define MSCs are: (1) grown in adherence to plastic surface of dishes when maintained in standard culture conditions; (2) positive expression of cytospecific cell surface markers (CD105, CD90 and CD73) and negative expression of other cell surface markers (CD45, CD34, CD14 and CD11b); and (3) capacity to differentiate into mesenchymal lineages, under appropriate *in vitro* conditions. In addition to the expression of the three cell surface markers, MSCs also express CD29, CD44, CD146 and STRO-1^[5].

The function of MSCs is to differentiate into osteocytes, chondrocytes, myoblasts and adipocytes^[6,7]. An increasing number of studies, however, report that MSCs are capable of giving rise to cells of an entirely distinct

lineage, including neuron-like cells. MSCs are not only able to differentiate into neurons for cell replacement therapy, they also exert paracrine effects by modulating the plasticity of damaged host tissues, secreting neurotrophic and survival-promoting growth factors, restoring synaptic transmitter release, integrating into existing neural and synaptic networks, and re-establishing functional afferent and efferent connections^[8]. These paracrine activities have not been reported in ESCs or iPSCs. Moreover, MSCs possess strong immunosuppressive properties and inhibit the release of pro-inflammatory cytokines^[9]. This allows autologous, as well as, allogeneic transplantation of MSCs without the need of pharmacological immunosuppression. Furthermore, MSCs can be transplanted directly without genetic modification or pre-treatments, and are able to migrate to the tissue injury sites^[10]. In addition, there is no teratoma formation concern after transplantation^[11], and no moral objection or ethical controversies involved in their attainment^[12]. These advantageous properties, as well as the expansion potential of MSCs initiate the idea of clinical applications of MSCs to treat different human diseases, especially CNS disorders. Currently, over 100 MSC clinical trials for different diseases have been listed by the United States National Institutes of Health trial database (www.clinicaltrials.gov), indicating that MSC therapy is a popular trend for the field of regenerative medicine in the years to come.

This review article provides an update on the progress of MSC experimental research as well as human clinical trials for neural and retinal diseases with emphasis on multiple sclerosis, spinal cord injury, autism, glaucoma, retinitis pigmentosa and age-related macular degeneration.

MULTIPLE SCLEROSIS

Multiple sclerosis (MS) is an immune-mediated neurodegenerative disorder of the CNS, affecting over 1.3 million people worldwide. The histopathological hallmark of MS is the formation of an inflammatory plaque, which originates from a breach in the integrity of the blood-brain barrier^[13]. The histologic features of lesions in MS include: lymphocyte infiltration, loss of oligodendrocytes, demyelination, and widespread axonal damage^[14]. Myelin-reactive T cells, which secrete interferon- and interleukins, have been suggested to be responsible for the inflammatory demyelination seen in MS^[15]. Currently, there are three treatment options approved by the Food and Drug Administration (FDA) for MS: administration of interferon beta, glatiramer acetate, or mitoxantrone^[16]. However, there is still no medical cure for MS.

Experimental autoimmune encephalomyelitis (EAE), the best known and most commonly used model for MS, mechanistically defines the immune processes responsible for the clinical manifestations and development of MS^[17]. This animal model provides insight for the application of immunotherapy to treat MS^[18]. MSCs have been pro-

posed as a treatment for autoimmune diseases, including MS, because of their immunosuppressive properties and neural repair function^[19]. Transplantation of human MSCs into animals with ongoing EAE results in rapid and sustained functional recovery due to a reduced number of inflammatory myelin-specific Th1 cells and astrocytes as well as an increased number of inflammatory-inhibiting Th2 cells, oligodendrocytes and neurons^[20]. This functional benefit is a critical stepping-stone towards effective MSC therapies in MS patients.

Among all of the CNS disorders, MS has the highest number of registered clinical trials. Altogether there are 14 registered clinical trials for MS (Table 1), and two of them have been published. The study from Israel is a phase-1/2 open safety clinical trial to evaluate the feasibility, safety and immunological effects of intrathecal and intravenous administration of autologous MSCs in 15 MS patients (NCT00781872; <http://clinicaltrials.gov/>)^[21]. No major adverse effects have been reported in this study, and the mean Expanded Disability Status Scale (EDSS) improved from 6.7 to 5.9 (EDSS steps 1.0 to 4.5: MS patients are fully ambulatory, whereas EDSS steps 5.0 to 9.5: MS patients are impaired to ambulation). Moreover, magnetic resonance imaging visualized the MSCs in the occipital horns of the ventricles, indicating migration of the cells. In addition, the proportion of CD4+/CD25+ regulatory T cells increased, whereas the proliferative responses of lymphocytes decreased. The mesenchymal stem cells in the multiple sclerosis trial (MSCIMS) originated in the United Kingdom, is an open-label phase 2a proof-of-concept study of autologous MSCs in secondary progressive MS (NCT00395200; <http://clinicaltrials.gov/>)^[22,23]. In this study, 10 patients received intravenous infusion of autologous bone marrow-derived MSCs (1.6×10^6 cells per kg body weight). The “sentinel lesion approach” assessing the anterior visual pathway was used to measure the efficacy of treatment. Results show that treatment improved visual acuity, visual evoked response latency, and increased the optic nerve area of the recipients. No serious adverse events were identified. For other clinical trials, mainly autologous MSCs have been used, although one study from China uses umbilical cord MSCs (NCT01364246; <http://clinicaltrials.gov/>). Interestingly, an open-label phase I clinical trial from New York was designed to evaluate autologous MSC-derived neural progenitor cells in progressive MS patients (NCT01933802; <http://clinicaltrials.gov/>) even though neural stem cells from EAE animals mainly develop astrocytes rather than oligodendrocytes, or oligodendrocyte precursor cells and neurons^[20].

SPINAL CORD INJURY

Spinal cord injury (SCI) is the most devastating and traumatic disorder among CNS conditions^[24]. The worldwide frequency of SCI is about 40 cases per million individuals^[25]. SCI can be caused by traffic accidents, violent assaults, falls, sport and other traumatic events. Depending on the injury location, extent, phases and time frames,

SCI therapeutic strategies can vary greatly^[26]. Most SCI patients are in the chronic phase, characterized by ongoing demyelination, local inflammation and apoptosis, decreased number of activated macrophages, and formation of glial scar and pseudocysts^[27]. The present standard treatment for SCI patients is surgical intervention, high doses of methylprednisolone, and symptomatic therapy followed by rehabilitation^[28]. New neuroregenerative strategies will be focused on neuroprotection and axonal regeneration in a permissive environment.

Cellular therapy aims to reconstruct the spinal cord through cellular replacement, glial scar remodeling, axonal guidance, and filling of formed syringomyelia^[29]. *In vivo* administration of MSCs in different SCI animal models showed functional recovery including: increased motor activity and sensation in the paralyzed limbs, reduced cavity formation in the spinal cord, and axonal sprouting through the glial scar^[30,31]. The objective of MSC application is to ameliorate the consequences of secondary injury by preserving the host nerve cells, rather than replacing them^[32].

Comparable to MS studies, there are 11 registered clinical trials using MSCs for SCI treatment (Table 1), among which two studies (one from Egypt and one from South Korea) have been completed. The Korean study investigated the safety of single intravenous infusion of autologous adipose tissue-derived MSCs (4×10^8 cells) in 8 male patients with chronic SCI (NCT01274975; <http://clinicaltrials.gov/>)^[33]. No adverse events were observed. Although one patient showed improvement in the American Spinal Injury Association (ASIA) scale from grade A (No sensory or motor function is preserved in sacral segments S4-S5) to grade C (Motor function is preserved below the neurologic level, and most key muscles below the neurologic level have muscle grade less than 3) and three patients showed motor score improvement, this phase I clinical trial might not have the statistical power to conclude on the efficacy of treatment effect with adipose tissue-derived MSCs on SCI. The study conducted in Egypt (NCT00816803; <http://clinicaltrials.gov/>), is a Phase-1/2 clinical trial applying bone marrow-derived MSCs at the injury site of chronic SCI patients. However, no results of this study have been released. Finally, there are two Phase-3 clinical trials taking place in China (NCT01873547; <http://clinicaltrials.gov/>) and Korea (NCT01676441; <http://clinicaltrials.gov/>). The study in China plans to use umbilical cord MSCs to treat 100 chronic SCI patients compared to the rehabilitation-only group and no stem cell and rehabilitation group, whereas the study in Korea was designed to transplant bone marrow-derived MSCs to treat 32 chronic SCI patients. For other ongoing clinical trials in SCI, the approaches are mainly intrathecal transplantation of bone marrow-derived MSCs and adipose tissue-derived MSCs in chronic SCI patients.

AUTISM

Autism belongs to a spectrum of heterogeneous neuro-

Table 1 Registered clinical trials on mesenchymal stem cells for neural diseases

| Identifier | Country | Status | Study | Phase of trial | Estimated number of patients | Estimated trial end | Disease |
|-------------|----------------|-------------------------|---|----------------|------------------------------|---------------------|--------------------|
| NCT01377870 | Iran | Recruiting | Evaluation of autologous mesenchymal stem cell transplantation (effects and side effects) in multiple sclerosis | Phase 1/2 | 30 | 2013 | Multiple sclerosis |
| NCT01895439 | Jordan | Recruiting | Safety and efficacy study of autologous bone marrow mesenchymal stem cells in multiple sclerosis | Phase 1/2 | 30 | 2014 | Multiple sclerosis |
| NCT01883661 | India | Not yet recruiting | Safety and efficacy of MSCs in MS | Phase 1/2 | 15 | 2015 | Multiple sclerosis |
| NCT00395200 | United Kingdom | Completed | MSCIMS | Phase 1/2 | 10 | 2010 | Multiple sclerosis |
| NCT01854957 | Italy | Recruiting | MESEMS | Phase 1/2 | 20 | 2014 | Multiple sclerosis |
| NCT01730547 | Sweden | Recruiting | Mesenchymal stem cells for multiple sclerosis | Phase 1/2 | 15 | 2015 | Multiple sclerosis |
| NCT01364246 | China | Recruiting | Safety and efficacy of umbilical cord mesenchymal stem cell therapy for patients with progressive multiple sclerosis and neuromyelitis optica | Phase 1/2 | 20 | 2014 | Multiple sclerosis |
| NCT01056471 | Spain | Unknown | Autologous mesenchymal stem cells from adipose tissue in patients with secondary progressive multiple sclerosis (CMM/EM/2008) | Phase 1/2 | 30 | 2012 | Multiple sclerosis |
| NCT01228266 | Spain | Active, not recruiting | Mesenchymal stem cell transplantation in MS (CMM-EM) | Phase 2 | 16 | 2013 | Multiple sclerosis |
| NCT00813969 | United States | Active, not recruiting | Autologous MSC transplantation in MS | Phase 1 | 24 | 2014 | Multiple sclerosis |
| NCT01933802 | United States | Not yet recruiting | Intrathecal administration of autologous MSC-NP in patients with multiple sclerosis | Phase 1 | 20 | 2016 | Multiple sclerosis |
| NCT01606215 | United Kingdom | Recruiting | STREAMS | Phase 1/2 | 13 | 2015 | Multiple sclerosis |
| NCT01745783 | Spain | Recruiting | Mesenchymal cells from autologous bone marrow, administered intravenously in patients diagnosed with multiple sclerosis | Phase 1/2 | 30 | 2014 | Multiple sclerosis |
| NCT00781872 | Israel | Unknown | MSCs for the treatment of MS | Phase 1/2 | 20 | 2009 | Multiple sclerosis |
| NCT01694927 | Chile | Enrolling by invitation | Autologous mesenchymal stem cells in spinal cord injury (SCI) patients (MSC-SCI) | Phase 2 | 30 | 2014 | Spinal cord injury |
| NCT01446640 | China | Recruiting | Mesenchymal stem cells transplantation to patients with spinal cord injury (MSC) | Phase 1/2 | 20 | 2014 | Spinal cord injury |
| NCT01676441 | South Korea | Recruiting | Safety and efficacy of autologous mesenchymal stem cells in chronic spinal cord injury | Phase 2/3 | 32 | 2014 | Spinal cord injury |
| NCT01769872 | South Korea | Recruiting | Safety and effect of adipose tissue derived mesenchymal stem cell implantation in patients with spinal cord injury | Phase 1/2 | 15 | 2014 | Spinal cord injury |
| NCT01162915 | United States | Active, not recruiting | Transfer of bone marrow derived stem cells for the treatment of spinal cord injury | Phase 1 | 10 | 2013 | Spinal cord injury |
| NCT01274975 | South Korea | Completed | Autologous adipose derived mscs transplantation in patient with spinal cord injury | Phase 1 | 8 | 2010 | Spinal cord injury |
| NCT01624779 | South Korea | Recruiting | Intrathecal transplantation of autologous adipose tissue derived msc in the patients with spinal cord injury | Phase 1 | 15 | 2013 | Spinal cord injury |
| NCT01393977 | China | Unknown | Difference between rehabilitation therapy and stem cells transplantation in patients with spinal cord injury in China | Phase 2 | 60 | 2012 | Spinal cord injury |
| NCT01873547 | China | Recruiting | Different efficacy between rehabilitation therapy and stem cells transplantation in patients with SCI in China (SCI-III) | Phase 3 | 300 | 2014 | Spinal cord injury |
| NCT01325103 | Brazil | Unknown | Autologous bone marrow stem cell transplantation in patients with spinal cord injury | Phase 1 | 20 | 2013 | Spinal cord injury |
| NCT00816803 | Egypt | Completed | Cell transplant in spinal cord injury patients | Phase 1/2 | 80 | 2008 | Spinal cord injury |
| NCT01343511 | China | Completed | Safety and efficacy of stem cell therapy in patients with autism | Phase 1/2 | 37 | 2011 | Autism |

Information obtained from <http://clinicaltrials.gov/>. MSCs: Mesenchymal stem cells; MS: Multiple sclerosis; MSCIMS: Mesenchymal Stem Cells in Multiple Sclerosis; MESEMS: MEsenchymal StEm Cells for Multiple Sclerosis; MSC-NP: Mesenchymal Stem Cell-derived Neural Progenitors; STREAMS: Stem Cells in Rapidly Evolving Active Multiple Sclerosis.

developmental disorders^[34]. It is characterized by abnormalities in social interaction, impaired verbal and nonverbal communication, and repetitive, obsessive behavior^[35]. According to the Centers for Disease Control, the prevalence of autism hovers around 60 in every 10000 children^[36]. Even though there is no defined gold standard approach, current interventions for autism can be divided into behavioral, nutritional and pharmacological^[37]. Medical interventions aim to ameliorate the neuropsychiatric disorders associated with autism. The medications include selective serotonin reuptake inhibitors (SSRI's), typical and atypical anti-psychotic drugs, psycho-stimulants, α -2 agonists, β blockers, lithium, anti-convulsant mood stabilizers and anti-depressants^[38-40]. Unfortunately, autism is still not treatable.

The pathogenic mechanism of autism is not clearly understood and remains elusive. Nevertheless, two pathologies are commonly found within the autism patients: the first observation is an impaired central nervous system circulation and hypoperfusion to the brain, whereas the second observation is systemic T cell and B cell abnormalities as well as active neuroinflammatory processes in the brain^[41]. Based on the immunomodulatory properties of MSCs, therapies employing MSCs have been proposed to target the immune deregulation observed in autism. Basically, it is believed that MSCs are able to inhibit the release of pro-inflammatory cytokines and have strong immunosuppressive activity^[42]. This not only allows for autologous transplantation, but also heterologous transplantation without the requirement of pharmacological immunosuppression^[43].

Currently, there is only one registered human clinical trial using MSCs to treat autism (NCT01343511; <http://www.clinicaltrials.gov/>; Table 1). This study aimed to test the safety and efficacy of human umbilical cord MSCs and human cord blood mononuclear cell transplantation in Chinese patients with autism^[44]. Outcomes from this study assuaged the safety concerns in using MSCs and mononuclear cells for transplantation in autism patients, and no severe adverse effects were observed. In addition, results also showed that combined transplantation of MSCs and mononuclear cells (combination group) had better therapeutic effects than transplantation of mononuclear cells alone (CBMNC group) in terms of the Childhood Autism Rating Scale (CARS) total score (combination group: 28.00 ± 6.18 ; CBMNC group: 37.14 ± 10.15 ; CARS total score > 30 means the child is considered to be autistic), Clinical Global Impression (CGI) scale (combination group: 88% much improved or higher; CBMNC group: 49% much improved or higher) and the Aberrant Behavior Checklist (ABC) total score (combination group: 36.78 ± 16.95 ; CBMNC group: 58.36 ± 31.73 ; a high score indicates greater severity while a low score indicates a milder degree of difficulty).

GLAUCOMA

Glaucoma is a group of chronic, degenerative optic

neuropathies. It is characterized by a slow progressive degeneration of retinal ganglion cells (RGCs) and their axons, which results in visual field defects^[45]. Glaucoma is the leading cause of irreversible blindness, affecting more than 60 million people worldwide^[46]. Traditional and current treatments for glaucoma are based on surgical or medical interventions to slow disease progression and limit visual loss^[47]. However, in many patients, the numbers of RGCs still diminish, and glaucoma cannot be completely cured.

The molecular basis of glaucoma is complex. The pathophysiological mechanisms leading to RGC degeneration in glaucoma include a complex interaction between primary axonal injury, neurotrophic factor deprivation, ischemia, oxidative stress, mitochondrial dysfunction and inflammation^[48]. New therapies aim to supplement neurotrophic factors, such as brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), glial cell line-derived neurotrophic factor (GDNF) and nerve growth factor (NGF)^[49]. However, repeated injections are needed to achieve an observable effect^[50]. To avoid multiple injections, cell-based delivery of neurotrophic factors was proposed. A phase-I clinical trial for glaucoma (NCT01408472; <http://clinicaltrials.gov/>) using genetically modified CNTF-secreting retinal pigment epithelial cells (NT-501 CNTF implant) has already been launched-the outcomes have not been reported yet. Since MSCs can produce neurotrophic factors, including BDNF, CNTF, GDNF and basic fibroblast growth factor (bFGF), without the requirement of genetic modification, MSC transplantation has been suggested as a potential reservoir for neurotrophic factor secretion^[51]. Bone marrow-derived MSC transplantation increases RGC survival in a model of transient ischemia followed by reperfusion^[52], and reduces RGC loss in ocular hypertension models^[53,54]. Similarly, transplantation of human umbilical cord blood MSCs promotes RGC survival in an optic nerve crush model even after 7 d of injury^[55]. In addition, intracranial human umbilical cord blood MSC transplantation at the site of optic tract transaction also protects RGCs and induces axonal regeneration^[56]. The neuroprotective effect of MSCs on RGC survival has clearly been proven, and the first clinical trial using bone marrow-derived MSCs on glaucoma in Florida (Stem Cell Ophthalmology Treatment Study (SCOTS)) has just started in August 2013 (NCT01920867; <http://clinicaltrials.gov/>; Table 2). This study will be complete in 2017.

RETINITIS PIGMENTOSA AND AGE-RELATED MACULAR DEGENERATION

Retinitis pigmentosa (RP) is characterized by a classic pattern of difficulties in dark adaptation and night blindness in adolescence, loss of mid-peripheral visual field in young adulthood and central vision later in life. These are due to the severe attenuation of rod and cone photoreceptors^[57]. RP is one of the hereditary degenerative diseases, affecting 1 in 4000 individuals. Age-related

Table 2 Registered clinical trials on mesenchymal stem cells for retinal diseases

| Identifier | Country | Status | Study | Phase of trial | Estimated number of patients | Estimated trial end | Disease |
|-------------|---------------|-------------------------|--|----------------|------------------------------|---------------------|--|
| NCT01531348 | Thailand | Enrolling by invitation | Feasibility and safety of adult human bone marrow-derived mesenchymal stem cells by intravitreal injection in patients with retinitis pigmentosa | Phase 1 | 10 | 2014 | Retinitis pigmentosa |
| NCT01914913 | India | Not yet recruiting | Clinical study to evaluate safety and efficacy of stem cell therapy in retinitis pigmentosa | Phase 1/2 | 15 | 2015 | Retinitis pigmentosa |
| NCT01920867 | United States | Recruiting | Stem cell ophthalmology treatment study | | 300 | 2017 | Glaucoma, retinitis pigmentosa, age-related macular degeneration |

Information obtained from <http://clinicaltrials.gov/>.

macular degeneration (AMD) is the leading cause of irreversible blindness in people aged 50 years or above in the developed world^[58]. It influences the central portion of the retina (the macula). Early AMD is characterized by drusen (pale yellowish lesions), or by hyperpigmentation and hypopigmentation of retinal pigment epithelium in the macula. Late AMD is divided into the “non-exudative” and “exudative” forms. The non-exudative form (geographic atrophy) starts with a sharply demarcated round or oval hypopigmented spot in which large choroidal vessels are visible, whereas the exudative form, characterized by choroidal neovascularization, is the detachment of the neuroretina or RPE from Bruch’s membrane by serous or hemorrhagic fluid^[59,60].

Both RP and AMD involve photoreceptor cell death. MSC research studies targeting this common pathology can be divided into two categories: first, cell replacement-based studies aim to generate photoreceptor cells from different sources of MSCs. MSCs from the trabecular meshwork as well as the conjunctiva have been used to produce photoreceptor-like cells *in vitro*^[61,62]. Interestingly, subretinal injection of MSCs has also been reported to induce differentiation into photoreceptor cells in a sodium iodate-induced retinal degeneration rat model^[63]. Second, studies based on paracrine effects hypothesize that MSCs can secrete neurotrophic factors to protect against photoreceptor degeneration in different animal models. Transplantation of bone marrow-derived MSCs can rescue photoreceptor cells of the dystrophic retina in the rhodopsin knockout mouse model^[64]. Moreover, intravenous injection of bone marrow-derived MSCs rescue photoreceptor cells as well as visual function in the Royal College of Surgeons rat model^[65]. For AMD, beside photoreceptor cell loss, retinal pigment epithelial (RPE) cells are also affected. Adipose tissue-derived MSCs can be induced to an RPE phenotype^[66]. In addition, adipose tissue-derived MSCs rescue mitomycin C-treated RPE cell lines (ARPE19) from death in culture^[67]. Furthermore, subretinal injected MSCs adopt RPE morphology and preserve the retinal layer integrity in the sodium iodate-induced retinal degeneration rat model^[68].

To date, there are three ongoing registered clinical trials using MSCs on RP (Table 2). The first clinical trial

aims to determine the feasibility and safety of human adult bone marrow-derived MSCs by intravitreal injection in patients with RP in Thailand (NCT01531348; <http://clinicaltrials.gov/>). The second clinical trial is the Stem Cell Ophthalmology Treatment Study (SCOTS) in Florida (NCT01920867; <http://clinicaltrials.gov/>) proposed to use autologous bone marrow-derived MSCs by different means of injection (retrobulbar, subtenon, intravitreal, intraocular, subretinal and intravenous). The third clinical trial is a Phase-1/2 open labeled study done in India to evaluate the safety and efficacy of bone marrow-derived MSCs in RP (NCT01914913; <http://clinicaltrials.gov/>). For AMD, there is only one registered clinical trial using bone marrow-derived MSCs (Table 2), the Stem Cell Ophthalmology Treatment Study (SCOTS) in Florida (NCT01920867; <http://clinicaltrials.gov/>). Results from these studies have not been reported yet.

CONCLUSION

MSCs have been discovered for more than 20 years^[69], and have been found all over the body. MSCs can be conveniently obtained from different accessible tissues: bone marrow, blood, and adipose and dental tissue. They can also be easily expanded in standard culture conditions. In addition to the above mentioned characteristics, MSCs demonstrate neuroprotective effects, immunomodulatory properties and self-migratory activity, making them an attractive therapeutic tool. In recent years, MSC research has already begun the transition from preclinical experiments to human clinical trials. There are currently more than 60 MSC clinical trials dealing with CNS disorders and three clinical trials on retinal diseases. Although transient rash, self-limiting bacterial infections or fever might occur in some patients after MSC transplantation, serious adverse events have never been observed. This can foresee that MSC transplantation will become routine clinical practice for disease treatment in the near future. However, there are critical challenges still needed to be conquered before MSC therapy can be adopted in daily clinical practice. These include: (1) poor MSC retention *in vivo*; (2) poor MSC engraftment, viability and function *in vivo*; (3) unclear mechanisms of action; and (4) lack

of standardized trials^[70]. Moreover, few studies showed the contradictory results of MSC immunomodulatory properties. This might be explained by the heterogeneous MSC population. TLR4-primed human MSCs (MSC1) mostly secrete pro-inflammatory cytokines (IL-6, IL-8) while TLR3-primed human MSCs (MSC2) express mostly immunosuppressive mediators (IL-10, IDO, TSG-6)^[71]. Addition of fewer MSCs (10-1000) would lead to a less consistent suppression or a marked lymphocyte proliferation in culture, whereas addition of 10000-40000 MSCs have an inhibitory effect^[72]. Besides, there are uncertainties that must be answered. What is the optimal cell number for transplantation? Which MSC types are optimal for regenerative medicine? When is the optimal stage to receive MSC therapy? Which transplantation route is suitable for each individual CNS disorder? Further research is needed to understand the mechanisms elicited by stem cells in regenerating damaged tissues after transplantation.

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P- Reviewers: Peng SM, Phinney DG, Wong J

S- Editor: Song XX **L- Editor:** A **E- Editor:** Zhang DN



WJSC 6th Anniversary Special Issues (2): Mesenchymal stem cells

Mesenchymal stem cells in the treatment of spinal cord injuries: A review

Venkata Ramesh Dasari, Krishna Kumar Veeravalli, Dzung H Dinh

Venkata Ramesh Dasari, Krishna Kumar Veeravalli, Department of Cancer Biology and Pharmacology, University of Illinois College of Medicine at Peoria, Peoria, IL 61656, United States
Dzung H Dinh, Department of Neurosurgery and Illinois Neurological Institute, University of Illinois College of Medicine at Peoria, Peoria, IL 61656, United States

Author contributions: Dasari VR contributed to written and review the manuscript; Veeravalli KK and Dinh DH reviewed the manuscript.

Supported by A grant from Illinois Neurological Institute to DHD

Correspondence to: Dzung H Dinh, MD, Department of Neurosurgery and Illinois Neurological Institute, University of Illinois College of Medicine at Peoria, One Illini Drive, Peoria, IL 61605, United States. ddinh@uic.edu

Telephone: +1-309-6552642 Fax: +1-309-6713442

Received: October 30, 2013 Revised: February 19, 2014

Accepted: March 11, 2014

Published online: April 26, 2014

Abstract

With technological advances in basic research, the intricate mechanism of secondary delayed spinal cord injury (SCI) continues to unravel at a rapid pace. However, despite our deeper understanding of the molecular changes occurring after initial insult to the spinal cord, the cure for paralysis remains elusive. Current treatment of SCI is limited to early administration of high dose steroids to mitigate the harmful effect of cord edema that occurs after SCI and to reduce the cascade of secondary delayed SCI. Recent evident-based clinical studies have cast doubt on the clinical benefit of steroids in SCI and intense focus on stem cell-based therapy has yielded some encouraging results. An array of mesenchymal stem cells (MSCs) from various sources with novel and promising strategies are being developed to improve function after SCI. In this review, we briefly discuss the pathophysiology of spinal cord injuries and char-

acteristics and the potential sources of MSCs that can be used in the treatment of SCI. We will discuss the progress of MSCs application in research, focusing on the neuroprotective properties of MSCs. Finally, we will discuss the results from preclinical and clinical trials involving stem cell-based therapy in SCI.

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Key words: Spinal cord injury; Mesenchymal stem cells; Bone marrow stromal cells; Umbilical cord derived mesenchymal stem cells; Adipose tissue derived mesenchymal stem cells

Core tip: Despite our deeper understanding of the molecular changes that occurs after the spinal cord injury (SCI), the cure for paralysis remains elusive. In this review, the pathophysiology of SCI and characteristics and potential sources of mesenchymal stem cells (MSCs) that can be used in the treatment of SCI were discussed. We also discussed the progress of application of MSCs in research focusing on the neuroprotective properties of MSCs. Finally, we discussed the results from preclinical and clinical trials involving stem cell-based therapy in SCI.

Dasari VR, Veeravalli KK, Dinh DH. Mesenchymal stem cells in the treatment of spinal cord injuries: A review. *World J Stem Cells* 2014; 6(2): 120-133 Available from: URL: <http://www.wjgnet.com/1948-0210/full/v6/i2/120.htm> DOI: <http://dx.doi.org/10.4252/wjsc.v6.i2.120>

INTRODUCTION

Traumatic spinal cord injury (SCI) continues to be a devastating injury to affected individuals and their families and exacts an enormous financial, psychologi-

cal and emotional cost to them and to society. Despite years of research, the cure for paralysis remains elusive and current treatment is limited to early administration of high dose steroids and acute surgical intervention to minimize cord edema and the subsequent cascade of secondary delayed injury^[1-3]. Recent advances in neurosciences and regenerative medicine have drawn attention to novel research methodologies for the treatment of SCI. In this review, we present our current understanding of spinal cord injury pathophysiology and the application of mesenchymal stem cells (MSCs) in the treatment of SCI. This review will be more useful for basic and clinical investigators in academia, industry and regulatory agencies as well as allied health professionals who are involved in stem cell research.

Direct mechanical damage to the spinal cord usually results in either partial or total loss of neural functions such as sensory perception and mobility^[4]. The prevalence of people with SCI who are alive in the United States in 2013 is estimated to be approximately 273000^[5]. According to census data, motor vehicle accidents (36.5%), falls (28.5%), and acts of violence (14.3%) are the most frequent causes of SCI since 2010. The average age at injury is 42.6 years and 80.7% of spinal cord injuries occur in males. Among those injured since 2010, 67.0% are Caucasian, 24.4% African American, 0.8% Native American and 2.1% Asian. The most frequent neurologic category at discharge of persons reported to the database since 2010 is incomplete tetraplegia (40.6%), followed by incomplete paraplegia (18.7%), complete paraplegia (18.0%) and complete tetraplegia (11.6%). Less than 1% of SCI patients experienced complete neurologic recovery by the time of hospital discharge. Over the last 20 years, the percentage of SCI patients with incomplete tetraplegia spinal cord injury has increased while the more devastating complete paraplegia and complete tetraplegia numbers have decreased^[5]. Whether complete or incomplete injury, SCI is a devastating condition that not only paralyzes the affected individuals but also exacts tremendous emotional, social and financial burdens^[6]. These patients also face increased risks of cardiovascular complications, deep vein thrombosis, osteoporosis, pressure ulcers, autonomic dysreflexia and neuropathic pain^[3]. The limitation of any clinical treatment success is most likely due to the complex mechanisms of SCI and the relative inability of the human body to repair or regenerate neurons in the spinal cord^[7].

PATHOPHYSIOLOGICAL FEATURES AFTER SCI

The pathophysiological processes that underlie SCI comprise the primary and secondary phases of injury^[1,8]. Initial physical trauma to the spinal cord includes traction injury, compression forces and direct mechanical disruption of neural elements. Immediate microvascular

injuries with central gray hemorrhage and disruption of cellular membrane and blood-spinal cord barrier are followed by edema, ischemia, release of cytotoxic chemicals from inflammatory pathways and electrolyte shifts. Subsequently, a secondary injury cascade is triggered that compounds the initial mechanical injury with necrosis and apoptosis that are injurious to surviving neighboring neurons, further reducing the chance of recovery of penumbra neurons and rendering any functional recovery almost hopeless^[3,8]. Pathophysiological processes that occur in the secondary injury phase are responsible for exacerbating the initial damage and creating an inhibitory milieu that is hostile to endogenous efforts of repair, regeneration and remyelination. These secondary processes include inflammation, ischemia, lipid peroxidation, production of free radicals, disruption of ion channels, axonal demyelination, glial scar formation, necrosis and programmed cell death^[3]. The post-trauma inflammatory response plays a critical role in the secondary phase after SCI through modulation of a series of complex cellular and molecular interactions^[9]. After SCI, the blood-spinal cord barrier is disrupted due to hemorrhage and local inflammation^[10]. The activation and recruitment of peripheral and resident inflammatory cells including microglial cells, astrocytes, monocytes, T-lymphocytes, and neutrophils promotes the development of secondary damage following SCI^[11]. This secondary injury can be subdivided into the acute-phase (2 h-2 d), the sub-acute phase (days-weeks), and the chronic phase (months-years), each with distinct different pathophysiological processes^[12-14]. These changes include edema, ischemia, hemorrhage, reactive oxygen species (ROS) production and lipid peroxidation, glutamate-mediated excitotoxicity, ionic dysregulation, blood-spinal-cord barrier permeability, inflammation, demyelination, neuronal cell death, neurogenic shock, macrophage infiltration, microglial activity, astrocyte activity and scar formation, initiation of neovascularization, Wallerian degeneration, glial scar maturation, cyst and syrinx formation, cavity formation and schwannosis. The end of spontaneous post-SCI changes is identified as a pathophysiological phenomenon with solid glial scar formation, syrinx formation, and neuronal apoptosis^[15]. However, endogenous repair and regenerative mechanisms do occur during the secondary phase of injury to minimize the extent of the lesion (through astrogliosis), reorganize blood supply through angiogenesis, clear cellular debris, and reunite and remodel damaged neural circuits, and as such, offer exploitable targets for therapeutic intervention^[3], the most promising of which is stem cell-based therapy^[16].

MSC THERAPY AFTER SCI

An array of new and promising strategies is being developed to improve function after SCI. At present, two main therapeutic strategies, cell-based and gene-based therapies

are being investigated to repair the injured mammalian spinal cord. At this time it appears that neither strategy by itself is efficacious, whereas a combinatory strategy appears to be more promising. The targeting of an array of deleterious processes within the tissue after SCI will require a multi-factorial intervention, multi-phasic polytherapy such as the combination of cell- and gene-based approaches^[17]. This review focuses only on stem cell-based therapy. Cell-based therapy faces numerous challenges including selection of a SCI model, timing and mode of cell implantation, location of cellular injection and their subsequent migration, survival, transdifferentiation, immune incompatibility and rejection, and tracking of implanted cells^[17]. Cellular therapies for SCI repair may involve modification or recruitment of endogenous cells *in vivo*, harvest and/or alteration *ex vivo* of endogenous cells that are subsequently implanted as autogeneic graft or transplanted into the injured organism as allogeneic or xenogeneic grafts. Transplanted stem cells promote neural regeneration and rescue impaired neural function after SCI by paracrine/permissive neurotrophic molecules at the lesion site to enhance the regenerative capacity thereby providing a scaffold for the regeneration of axons and replacing lost neurons and neural cells^[17]. Mesenchymal stem cells have recently been advocated as a promising source for cellular repair after central nervous system (CNS) injury^[15]. MSCs, also known as marrow stromal cells^[18] or mesenchymal progenitor cells^[19] are self-renewing, multipotent progenitor cells with the capacity to differentiate into several distinct mesenchymal lineages^[20]. These cells are multipotent adult stem cells present in all tissues as part of the perivascular population. As multipotent cells, MSCs can differentiate into different mesodermal tissues ranging from bone and cartilage to cardiac muscle^[21]. Several small clinical trials have investigated the efficacy and safety of MSCs in diseases including chronic heart failure, acute myocardial infarction, hematological malignancies and graft *vs* host disease. Pre-clinical evidence suggests that MSCs exert their beneficial effects largely through immunomodulatory and paracrine mechanisms^[22].

MSCs are favored in stem cell therapy for SCI for the following reasons: (1) ease of isolation and cryopreservation^[23], (2) maintenance of viability and regenerative capacity after cryopreservation at -80 °C^[24], (3) rapid replication with high quality progenitor cells and high potential of multilineage differentiation^[25], and (4) minimal or no immunoreactivity and graft-versus-host reaction of transplanted allogeneic MSCs^[26]. MSCs were initially identified in bone marrow and later in muscle, adipose and connective tissue of human adults^[21]. Bone marrow and umbilical cord blood are rich sources of these cells, but MSC have also been isolated from fat^[27], skeletal muscle^[28], human deciduous teeth^[29], and trabecular bone^[30]. Mesenchymal stem cells are ideally suited to address many pathophysiological consequences of SCI^[3]. The major goals for the therapeutic use of stem cells is regeneration of axons, prevention of apoptosis and re-

placement of lost cells, particularly oligodendrocytes, in order to facilitate the remyelination of spared axons^[31]. In this review, we touch upon the therapeutic applications of MSCs after SCI.

BONE MARROW STROMAL CELLS

Bone marrow-derived mesenchymal stem cells (BMSC) differentiate into cells of the mesodermal lineage but also, under certain experimental conditions, into cells of the neuronal and glial lineage. Their therapeutic translation has been significantly boosted by the demonstration that MSCs display significant anti-proliferative, anti-inflammatory and anti-apoptotic features. These properties have been exploited in the effective treatment of experimental autoimmune encephalomyelitis (EAE), experimental brain ischemia and in animals undergoing brain or spinal cord injury^[32]. Several investigators have reported that MSCs possess immunosuppressive features^[33-36]. These immunosuppressive properties, in combination with the restorative functions of BMSC reduce the acute inflammatory response to SCI, minimize cavity formation, as well as diminish astrocyte and microglia/macrophage reactivity^[37-39]. BMSC transplantation in an experimental SCI model has been shown to enhance neuronal protection and cellular preservation *via* reduction in injury-induced sensitivity to mechanical trauma^[39]. It was suggested that the beneficial effects of MSCs on hindlimb sensorimotor function may, in part, be explained by their ability to attenuate astrocyte reactivity and chronic microglia/macrophage activation^[39]. These significant results demonstrated the potential of MSCs to serve as attenuators of the immune response. It was proposed that as attenuators, MSCs could potentially serve in an immunoregulatory capacity in disorders in which chronic activation of immune cells, such as reactive astrocytes and activated microglia/macrophages play a role. Studies by Hofstetter *et al.*^[40], indicated that transplanted MSC attenuates acute inflammation and promotes functional recovery following SCI. Ohta *et al.*^[41], suggested that BMSCs reduced post-SCI cavity formation and improved behavioral function by releasing trophic factors into the cerebrospinal fluid (CSF) or by direct interaction with host spinal tissues. Infusion of transplants through CSF provides no additional traumatic injury to the damaged spinal cord and BMSCs might be administered by lumbar puncture to the patients. Lumbar puncture can be done without severe invasion, so BMSCs can be repeatedly administered to maintain their effects. This study has demonstrated for the first time that the transplantation of BMSCs through CSF can promote the behavioral recovery and tissue repair of the injured spinal cord in rats, thus providing a road map for the clinical autograft of BMSCs without severe surgical infliction to human patients^[41]. In another study, human mesenchymal stem cells (hMSCs) isolated from adult bone marrow were found to infiltrate primarily into the ventrolateral white matter tracts, spreading to adjacent segments

rosto-caudal to the injury epicenter, and facilitate recovery from SCI by remyelinating spared white matter tracts and/or by enhancing axonal growth^[42]. In our laboratory, we used mesenchymal stem cells from rat bone marrow to evaluate the therapeutic potential after SCI in rats^[43]. We observed that caspase-3 mediated apoptosis after SCI on both neurons and oligodendrocytes was significantly downregulated by BMSC. Treatment with BMSC had a positive effect on behavioral outcome and better structural integrity preservation as seen in histopathological analysis. BMSC secrete protective factors that prevent neuronal apoptosis through stimulation of endogenous survival signaling pathways, namely PI3K/Akt and the MAPK/ERK1/2-cascade. Overall, these findings demonstrate that BMSC trigger endogenous survival signaling pathways in neurons that mediate protection against apoptotic insults. Moreover, the interaction between stressed neurons and BMSC further amplifies the observed neuroprotective effect^[44].

Lu *et al.*^[45], investigated the nature of the chronic scar and its ability to block axon growth by testing the hypothesis that chronically injured spinal cord axons can regenerate through the gliotic scar in the presence of local growth-stimulating factors. BMSC, genetically modified to secrete neurotrophin-3 (NT-3) were injected into the lesion site of rats with cervical SCI^[45]. It was observed that a modest number of axons penetrated through the chronic scar that contained a mixture of inhibitory and growth stimulating factors. Furthermore, robust axonal growth can be induced by the local provision of neurotrophic factors without resecting the chronic scar. In another study, Urdžíková *et al.*^[46], have shown that treatment with different cell populations obtained from bone marrow (MSCs, BMCs and the endogenous mobilization of bone marrow cells) has a beneficial effect on behavioral and histological outcomes after SCI. However, it is not clear whether the injection of MSCs, BMCs or granulocyte-colony stimulating factor (G-CSF) treatment induces functional and morphological improvement through the same mechanisms of action. Transplanted MSCs mollify the inflammatory response in the acute setting and reduce the inhibitory effects of scar tissue in the subacute/chronic setting to provide a permissive environment for axonal extension. In addition, grafted cells may provide a source of growth factors to enhance axonal elongation across spinal cord lesions^[47]. Down-regulation of TNF- α expression in macrophages/microglia was observed at an early stage after SCI in rats transplanted with a gelatin sponge (GS) scaffold impregnated with rat bone marrow-derived mesenchymal stem cells at the site of injury^[48]. It was also shown that 3D gelatin sponge scaffolds allowed MSCs to adhere, survive and proliferate and also for fibronectin to deposit. *In vivo* transplantation experiments demonstrated that these scaffolds were biocompatible and MSCs seeded to the scaffolds played an important role in attenuating inflammation, promoting angiogenesis, and reducing cavity formation. Novikova *et al.*^[49], observed that differentiated BMSC provided neuroprotection for

axotomized rubrospinal neurons and increased the density of rubrospinal axons in the dorsolateral funiculus rostral to the injury site. They suggested that BMSC induced along the Schwann cell lineage increased expression of trophic factors and have neuroprotective and growth-promoting effects after SCI^[49]. Cizkova *et al.*^[50], standardized the intrathecal (IT) catheter delivery of rat MSCs after SCI in adult rats. Based on these results, it was suggested that repetitive IT transplantation, which imposes a minimal burden on the animals, may improve behavioral function when the dose, timing, and targeted IT delivery of MSCs towards the lesion cavity was optimized. Kang *et al.*^[51], indicated that therapeutic rat BMSCs in a poly (D,L-lactide-co-glycolide)/small intestinal submucosa scaffold induced nerve regeneration in a complete spinal cord transection model and demonstrated that functional recovery further depended on defect length.

Park *et al.*^[52] evaluated the therapeutic efficacy of combining autologous BMSC transplantation with granulocyte macrophage-colony stimulating factor (GM-CSF) by subcutaneous administration directly into the spinal cord lesion site of six patients with complete SCI. At the 6-mo and 18-mo follow-up periods, four of the six patients showed neurological improvements by two ASIA (American Spinal Injury Association) grade (from ASIA A to ASIA C), while another improved from ASIA A to ASIA B^[52]. Moreover, BMSC transplantation together with GM-CSF was not associated with increased morbidity or mortality. In another clinical trial, the safety of autologous bone marrow cell implantation was tested in twenty patients^[53]. Motor-evoked potential, somatosensory-evoked potential, magnetic resonance imaging, and ASIA scores were measured in a clinical follow-up. This study demonstrated that BMSC transplantation is a relatively safe procedure, and BMSC-mediated repair can lead to modest improvements in some injured patients. It is also anticipated that a Phase II clinical trial designed to test the efficacy will be initiated in the near future. In a study conducted by Deng *et al.*^[54], implantation of BMSC elicited *de novo* neurogenesis, and functional recovery in a non-human primate SCI model with rhesus monkeys achieved Tarlov grades 2-3 and nearly normal sensory responses three months after transplantation. Zurita *et al.*^[55], observed progressive functional recovery three months after SCI in paraplegic pigs injected with autologous BMSC in autologous plasma into lesion zone and adjacent subarachnoid space. Intramedullary post-traumatic cavities were filled by a neoformed tissue containing several axons, together with BMSC, that expressed neuronal or glial markers. Furthermore, in the treated animals, electrophysiological studies showed recovery of the previously abolished somatosensory-evoked potentials. Despite promising data, further research is needed to establish whether bone marrow cell treatments can serve as a safe and efficacious autologous source for the treatment of SCI^[47]. However, the use of BMSC in SCI does present certain issues-migration beyond the injection site (for intraspinally delivered cells) is limited and

Table 1 Overview of effects of bone marrow stromal cells after spinal cord injury

| Source of MSC | Main pathological features improved/repared | Limitations/recommendations/conclusions | Ref. |
|---------------|---|--|--|
| Human | Axonal growth, partial recovery of function | Differences in donor or lot-lot efficacy of MSC | Neuhuber <i>et al</i> ^[37] , 2005 |
| Human | Axonal growth, significant behavioral recovery | Survival of BMSC grafts for longer duration | Himes <i>et al</i> ^[38] , 2006 |
| Human | Significant motor improvements in human patients | Autologous bone marrow cell transplantation with GM-CSF administration has no serious complications. More comprehensive multicenter clinical studies are recommended | Park <i>et al</i> ^[52] , 2005 |
| Human | Homing of MSC, functional recovery | Mechanisms of engraftment, homing, long-term safety | Cizkova <i>et al</i> ^[42] , 2006 |
| Rhesus monkey | <i>De novo</i> neurogenesis and functional recovery in rhesus monkeys | Synergetic effects of MSC implantation and locally delivered neurotrophic factors in rhesus SCI models | Deng <i>et al</i> ^[54] , 2006 |
| Pig | Improvement in somatosensory-evoked potentials, functional recovery in pigs | Possible utility of BMSC transplantation in humans suffering from chronic paraplegia | Zurita <i>et al</i> ^[55] , 2008 |
| Rat | No allodynia, anti-inflammatory, increase in white matter volume and decrease in cyst size, sensorimotor enhancements | Survival of MSC | Abrams <i>et al</i> ^[39] , 2009 |
| Rat | MSC form bundles bridging the lesion epicenter, functional recovery | Neuron-like MSC lacked voltage-gated ion channels for generation of action potentials | Hofstetter <i>et al</i> ^[40] , 2002 |
| Rat | Cavity reduction, functional recovery | Unknown trophic factors secreted by BMSC | Ohta <i>et al</i> ^[41] , 2004 |
| Rat | Downregulation of apoptosis, functional recovery | Intrinsic properties of MSC, microenvironment of the injured spinal cord, host-graft interactions | Dasari <i>et al</i> ^[43] , 2007 |
| Rat/gerbil | Activation of survival signaling pathways, neuroprotection | Neuroprotective factors released by BMSC, interactions between neurons and BMSC | Isele <i>et al</i> ^[44] , 2007 |
| Rat | Axonal regeneration, myelination of axons | Resection of the chronic scar | Urdzíkova <i>et al</i> ^[46] , 2006 |
| Rat | Increase in spared white matter, functional recovery | Differences in mechanism of action of MSCs or BMCs (bone marrow cells) or G-CSF in inducing functional and morphological improvement | |
| Rat | Reduction in inflammation, promoting angiogenesis, and reducing cavity formation | GS scaffolds may serve as a potential supporting biomaterial for wound healing after SCI | Zeng <i>et al</i> ^[48] , 2011 |
| Rat | Extensive in-growth of serotonin-positive raphespinal axons and calcitonin gene-related peptide-positive dorsal root sensory axons, attenuation of astroglial and microglial activity | Production of trophic factors support neuronal survival and axonal regeneration | Novikova <i>et al</i> ^[49] , 2011 |
| Rat | Functional recovery | Repetitive IT transplantation may improve behavioral function depending on optimization of dose, timing, and targeted IT delivery of MSCs | Cizkova <i>et al</i> ^[50] , 2011 |
| Rat | Axonal regeneration, functional recovery | Feasibility of therapeutic cell delivery using 3D scaffolds, especially in complete spinal cord transection | Kang <i>et al</i> ^[51] , 2011 |
| Rat | Partial improvement in ASIA score in human patients | Polymer hydrogels may become suitable materials for bridging cavities after SCI | Sykova <i>et al</i> ^[53] , 2006 |

SCI: Spinal cord injury; MSC: Marrow stromal cell; IT: Intrathecal; CSF: Cerebrospinal fluid; GS: Gelatin sponge; BMSC: Bone marrow-derived mesenchymal stem cell.

inter-donor variability in efficacy and immunomodulatory potency might be reflected in variable clinical outcome^[37], making BMSC evaluation as a therapy for SCI difficult^[3]. The pathological improvements of BMSC after SCI are summarized in Table 1.

ADIPOSE TISSUE-DERIVED MESENCHYMAL CELLS

Adipose tissue is abundant in the body and contains a stromal fraction rich in stem- progenitor cells capable of undergoing differentiation into osteogenic, chondrogenic, and adipogenic lineages^[56]. The *in vitro* as well as *in vivo* properties of adipose tissue-derived stromal cells (ADSCs) resemble those of MSCs obtained from bone marrow, and the liposuction procedure employed to harvest ADSCs is minimally invasive for the patient^[57]. Kang *et al*^[58], reported that intravenous infusion of oligodendrocyte precursor cells (OPCs) derived from rATSC autograft cells improved motor function in rat models of

SCI. Moreover, cytoplasmic extracts prepared from adipose tissue stromal cells (ATSCs) inhibit H₂O₂-mediated apoptosis of cultured spinal cord-derived neural progenitor cells (NPCs) and improved cell survival^[59]. ATSCs extracts mediated this effect by decreasing caspase-3 and c-Jun-NH₂-terminal kinase (SAPK/JNK) activity, inhibiting cytochrome c release from mitochondria and reducing Bax expression levels in cells. Direct injection of ATSCs extracts mixed with matrigel into the spinal cord immediately after SCI also resulted in less apoptotic cell death, astrogliosis and hypo-myelination and showed significant functional improvement. Zhang *et al*^[60], showed that ADSCs can differentiate into neural-like cells *in vitro* and *in vivo*. However, neural differentiated ADSCs did not result in any better functional recovery than did undifferentiated ones following SCI. Ryu *et al*^[61], evaluated the implantation of allogenic adipose-derived stem cells (ASCs) for the improvement of neurological function in a canine SCI model. Using both *in vitro* and *in vivo* injury models, Oh *et al*^[62], confirmed that hypoxic

Table 2 Overview of effects of Adipose tissue-derived mesenchymal cells after spinal cord injury

| Source of MSC | Main pathological features improved/repared | Limitations/recommendations/conclusions | Ref. |
|---------------|---|---|--|
| Human | Functional recovery | Interaction between engrafted rATSC-OPCs and endogenous spinal cord-derived NPCs promotes host injury repair | Kang <i>et al</i> ^[58] , 2006 |
| Human | Improvement in both the cell survival and the gene expression of the engineered NSC observed in SCI rats | Hypoxia preconditioning strategy and combined stem cell/ gene therapies can be used to augment the therapeutic efficacy at target injury sites | Oh <i>et al</i> ^[62] , 2010 |
| Human | mNSCs transplanted into rat spinal cords with AT-MSCs showed better survival rates than mNSCs transplanted alone | Co-transplantation of mNSCs with AT-MSCs may be a more effective transplantation protocol to improve the survival of cells in the injured cord | Oh <i>et al</i> ^[63] , 2011 |
| Human | Transplantation of 3DCM-ASCs into the injured spinal cord significantly elevated the density of vascular formations and enhanced axonal outgrowth at the lesion site, functional recovery | Transplantation of 3DCM-ASCs may be an effective stem cell therapy | Oh <i>et al</i> ^[64] , 2012 |
| Human | No toxicity of hAdMSCs in immunodeficient mice, none of 8 male patients developed any serious adverse events related to hAdMSC transplantation in phase I clinical trial | Systemic transplantation of hAdMSCs appears to be safe and does not induce tumor development. Slow intravenous infusion of autologous hAdMSCs may be safe in SCI patients | Ra <i>et al</i> ^[66] , 2011 |
| Human | Increase in BDNF levels, increased angiogenesis, preserved axons, decreased numbers of ED1-positive macrophages, reduced lesion cavity formation, functional recovery in rats | Compared with hBMSCs, hADSCs may be a better source of MSCs for cell therapy for acute SCI because of their relative abundance and accessibility | Zhou <i>et al</i> ^[67] , 2013 |
| Dog | Significant improvement in nerve conduction velocity based on SEP, partial improvement in neurological functions of dogs | ASCs in spinal cord injuries might be partially due to neural differentiation of implanted stem cells | Ryu <i>et al</i> ^[61] , 2009 |
| Dog | Anti-inflammation, anti-astrogliosis, neuronal extension, neuronal regeneration, functional recovery | The combination of Matrigel and NMSC produced beneficial effects | Park <i>et al</i> ^[65] , 2012 |
| Rat | Reduced apoptotic cell death, astrogliosis and hypo-myelination, functional recovery | ATSC extracts may provide a powerful autoplasmic therapy for neurodegenerative conditions in humans | Kang <i>et al</i> ^[59] , 2007 |
| Rat | Neural differentiated ADSCs did not result in better functional recovery than undifferentiated ones following SCI | <i>In vitro</i> neural transdifferentiation of ADSCs might therefore not be a necessary pre-transplantation step | Zhang <i>et al</i> ^[60] , 2009 |
| Rat | Functional recovery | Predifferentiation of ASCs plays a beneficial role in SCI repair | Arboleda <i>et al</i> ^[57] , 2011 |
| Rat | Axonal regeneration, remyelination, functional recovery | Adipose tissue-derived Schwann cells can support axon regeneration and enhance functional recovery | Zaminy <i>et al</i> ^[68] , 2013 |

OPCs: Oligodendrocyte precursor cells; NPCs: Neural progenitor cells; NSC: Neural stem cell; SCI: Spinal cord injury; MSC: Marrow stromal cell; AT: Adipose tissue; 3DCM-ASCs: Three-dimensional cell mass transplantation of adipose-derived stem cells; hAdMSCs: Human Adipose tissue-derived mesenchymal stem cells; NMSC: Neural-induced mesenchymal stem cells; ATSC: Adipose tissue stromal cell; ADSCs: Adipose tissue-derived stromal cells; BMSC: Bone marrow-derived mesenchymal stem cell.

preconditioning (HP)-treated adipose tissue-derived mesenchymal stem cells (HP-AT-MSCs) increased cell survival and enhanced the expression of marker genes in DsRed-engineered neural stem cells (NSCs-DsRed). Based on their findings, it was suggested that the co-transplantation of HP-AT-MSCs with engineered neural stem cells (NSCs) can improve both cell survival and gene expression of the engineered NSCs. This novel strategy can be used to augment the therapeutic efficacy of combined stem cell and gene modulation therapy for SCI. In another study, Oh *et al*^[63], examined the effects of co-transplanting mouse neural stem cells (mNSCs) and adipose tissue-derived mesenchymal stem cells (AT-MSCs) on mNSC viability. It was observed that mNSCs transplanted into rat spinal cords with AT-MSCs showed better survival rates than mNSCs transplanted alone, thereby suggesting that co-transplantation of mNSCs with AT-MSCs is a more effective strategy to improve the survival of transplanted stem cells into the injured spinal cord. In a more recent study, the same group investigated the effectiveness of a three-dimensional cell mass trans-

plantation of adipose-derived stem cells (3DCM-ASCs) in hind limb functional recovery by the stimulation of angiogenesis and neurogenesis^[64]. These results revealed a significantly elevated density of neovascular formations through angiogenic factors released by the 3DCM-ASCs at the lesion site, enhanced axonal outgrowth, and significant functional recovery. These findings suggest that transplantation of 3DCM-ASCs may be an effective stem cell transplantation modality for the treatment of spinal cord injuries and neural ischemia. In a similar study, Park *et al*^[65], observed that a combination of matrigel and neural-induced mesenchymal stem cells (NMSC) reduced the expression of inflammation and/or astrogliosis markers and improved hind limb function in dogs with SCI. The predifferentiation of ASCs plays a beneficial role in SCI repair by promoting the protection of denuded axons and cellular repair that was induced mainly through paracrine mechanisms^[57]. The propensity of proliferation and the potential of unchecked differentiation of stem cells raised the concern of inherent tumorigenicity and toxicity. Ra *et al*^[66], observed that systemic transplanta-

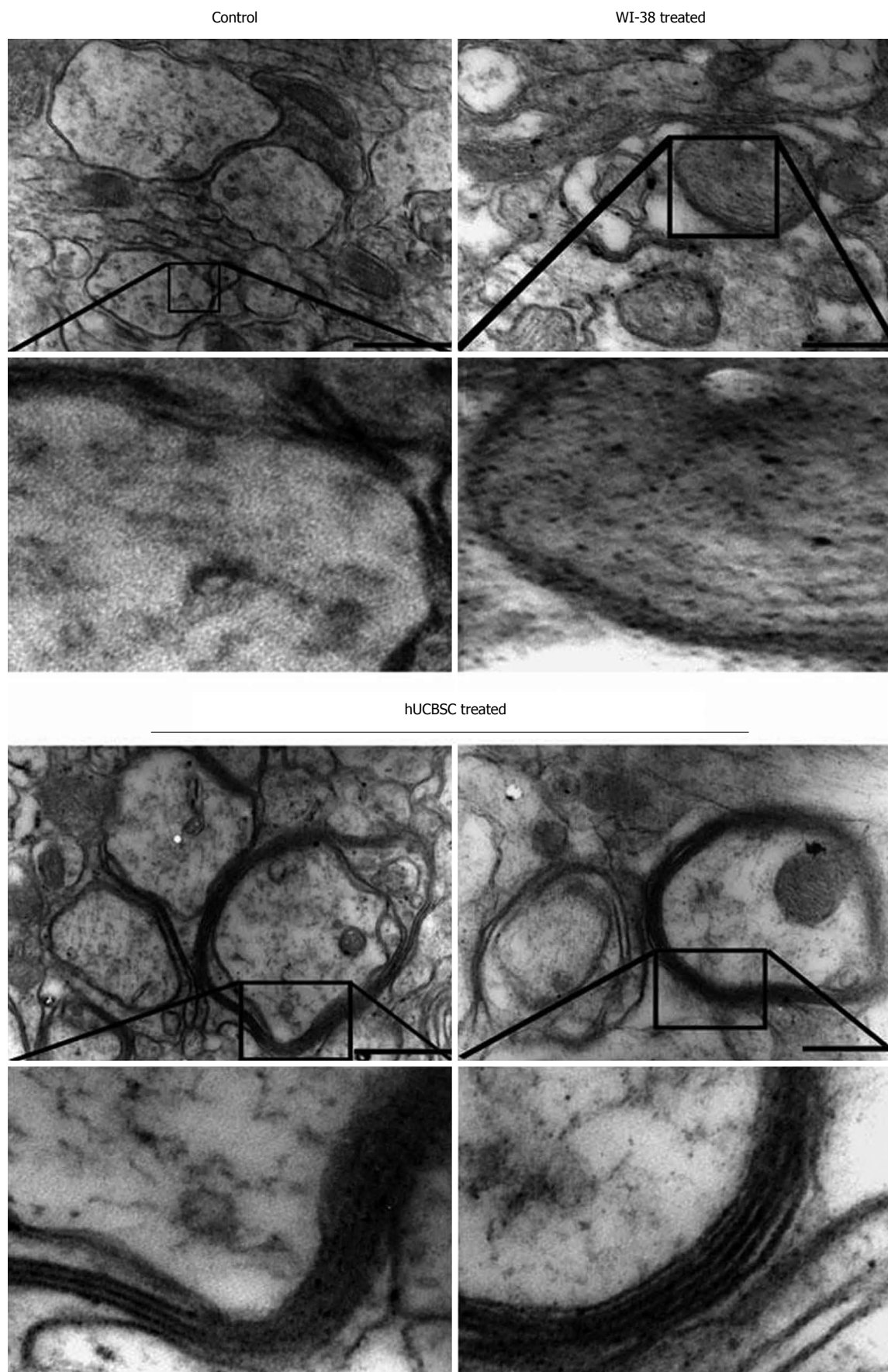


Figure 1 Transmission electron micrographs of shiverer mice brain showing thin and fragmented myelin around the axons in control and WI-38- implanted mice. In contrast, human umbilical cord blood-derived mesenchymal stem cells-treated shiverer brains showing myelin with several layers. Images are representatives of the several sections obtained from 3 different animals ($n = 3$). Scale bar = 33000. *Stem Cells Dev* 2011; **20**: 881-891.

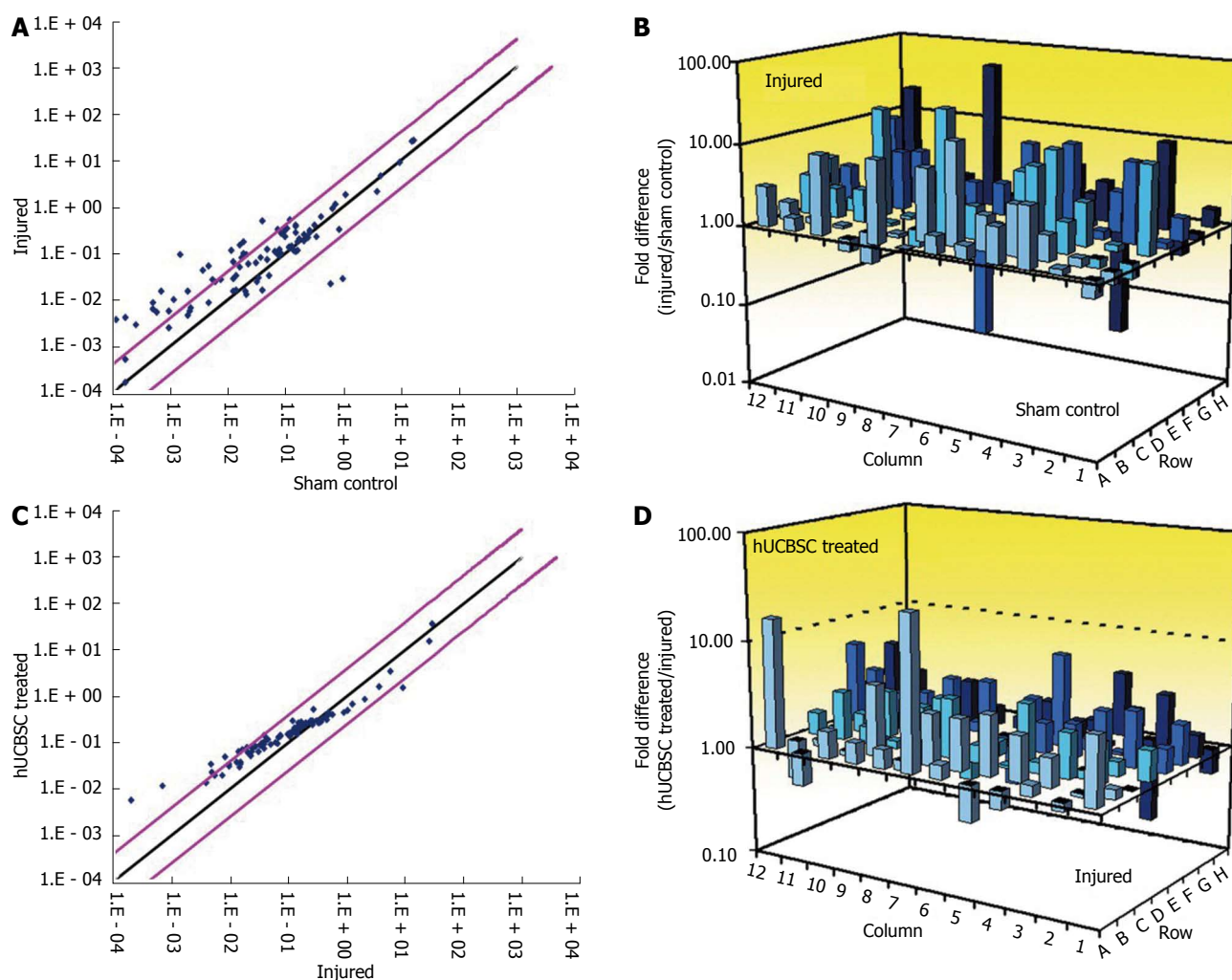


Figure 2 Microarray analysis of apoptotic genes after spinal cord injury. Total RNA was extracted from sham control, 3-wk post-spinal cord injury (SCI), and 3-wk post-SCI plus human umbilical cord blood-derived mesenchymal stem cells (hUCBSC)-treated tissues, reverse-transcribed, and the corresponding cDNA was loaded into a 96-well plate. In each group, RNA from at least three different animals was pooled together. A and C: Representative scatter plots show the validity of the experiment and the expression level of each gene in the control vs injured and injured vs hUCBSC-treated samples; B and D: These 3D profile graphs show the fold difference in expression of each gene between sham control vs injured and injured vs hUCBSC-treated samples. These experiments were performed in duplicate (hUCBSC, human umbilical cord blood-derived mesenchymal stem cells; SCI, spinal cord injury). *J Neurotrauma* 2009; **26**: 2057-2069.

tion of human Adipose tissue-derived mesenchymal stem cells (hAdMSCs) appeared to be safe and did not induce tumor development as none of the patients developed any serious adverse events related to hAdMSC transplantation during the three-month follow-up. Zhou *et al.*^[67], compared mesenchymal stromal cells from human bone marrow and adipose tissue for the treatment of spinal cord injury and suggested that hAdMSCs would be more appropriate than hBMSCs for transplantation to treat SCI. Recently, Zaminy *et al.*^[68], proved that adipose tissue-derived Schwann cells can modulate the hostile environment of the damaged spinal cord and generate a more stimulating environment to support axon regeneration and enhance functional recovery (Table 2).

HUMAN UMBILICAL CORD BLOOD-DERIVED MSCS

Human umbilical cord blood-derived mesenchymal stem

cells (hUCBSC) offer great potential for novel therapeutic approaches targeted against many CNS diseases. Previous studies have reported that hUCBSC are beneficial in reversing the deleterious effects of SCI, even when infused five days after injury^[69]. Transplanted hUCBSC differentiate into various neural cells and induce motor function improvement in SCI rat models^[70]. In our laboratory, hUCBSC transplanted in rats one week after SCI were shown to transdifferentiate into neurons and oligodendrocytes and also to downregulate Fas-mediated apoptosis^[71,72]. These transdifferentiated oligodendrocytes facilitated the secretion of neurotrophic hormones NT3 and BDNF and synthesized MBP and PLP, thereby promoting the remyelination of demyelinated axons in the injured spinal cord^[71]. We observed that hUCBSC treatment increased myelin basic protein *in vitro* in PC-12 cells, which are normally not myelinated. To further confirm the ability of transplanted hUCBSC in remyelination, we injected hUCBSC into shiverer mice brains. This study

Table 3 Changes in the expression of apoptotic genes and inhibitors after spinal cord injury and human umbilical cord blood stem cells treatment

| UniGene | GenBank | Gene name | Fold change after SCI | Fold change after hUCBSC treatment |
|------------|-----------|--------------|-----------------------|------------------------------------|
| Rn. 36696 | NM_022698 | Bad | 3.12 ± 1.34 | -1.47 ± 0.14 |
| Rn. 14598 | NM_053812 | Bak1 | 2.28 ± 0.99 | 1.36 ± 0.79 |
| Rn. 13007 | NM_031328 | Bcl10 | 8.83 ± 1.91 | 1.51 ± 1.45 |
| Rn. 19770 | NM_133416 | Bcl2a1 | 7.95 ± 1.98 | 1.79 ± 0.75 |
| Rn. 10323 | NM_031535 | Bcl2l1 | 2.13 ± 0.85 | -2.01 ± 0.89 |
| Rn. 162782 | NM_022684 | Bid | 2.45 ± 1.27 | 1.86 ± 0.99 |
| Rn. 89639 | NM_057130 | Bid3 | 5.43 ± 1.06 | 2.62 ± 0.75 |
| Rn. 38487 | NM_053704 | Bik | 4.41 ± 0.64 | 3.58 ± 0.14 |
| Rn. 92423 | XM_226742 | Birc1b | 25.84 ± 0.85 | 3.01 ± 0.67 |
| Rn. 64578 | NM_023987 | Birc3 | 10.14 ± 1.06 | 3.01 ± 0.78 |
| Rn. 54471 | NM_022274 | Birc5 | -2.84 ± 1.98 | 4.57 ± 1.14 |
| Rn. 55946 | NM_057138 | Cflar (Flip) | 3.12 ± 1.77 | -1.20 ± 0.86 |

Results are expressed as mean ± SD. hUCBSC: Human umbilical cord blood stem cells; SCI: Spinal cord injury. Refer Dasari *et al*^[73].

clearly demonstrated that transplanted hUCBSC survived, migrated *in vivo* and myelinated genetically denuded axons in shiverer mice brains. The expression level of myelin basic protein, a major component of the myelin sheath, was significantly elevated *in vivo* and *in vitro* as revealed by Western blotting, reverse transcription polymerase chain reaction, immunohistochemistry, immunocytochemistry, and fluorescent *in situ* hybridization results. Further, transmission electron microscopic images of hUCBSC-treated shiverer mice brains showed several layers of myelin around the axons compared with a thin and fragmented layer of myelin in untreated animals (Figure 1). Moreover, the frequency of shivering was diminished one month after hUCBSC treatment. Our results strongly indicated that hUCBSC transplantation played an important role in re-myelination and could be an effective therapeutic approach for demyelinating or hypomyelinating disorders^[73]. Furthermore, apoptotic pathways mediated by caspase-3, Fas and TNF- α were downregulated by hUCBSC^[72,74]. The locomotor scale scores in hUCBSC-treated rats were significantly improved as compared to those of the control injured group. To further extend our studies, we utilized RT-PCR microarray and analyzed 84 apoptotic genes to identify the genetic modulation that occurred after traumatic SCI and after hUCBSC transplantation^[75]. We observed that the genes involved in inflammation and apoptosis were up-regulated (TNF- α , TNFR1, TNFR2, Fas, Bad, Bid, Bid3, Bik, and Bak1) in the injured rat spinal cords, whereas genes such as XIAP, which are involved in neuroprotection, were up-regulated in the hUCBSC-treated rats (Figure 2, Tables 3 and 4). Our findings from co-cultures of cortical neurons with hUCBSC and blocking of the Akt pathway by a dominant-negative Akt and Akt-inhibitor IV confirmed that the mechanism underlying hUCBSC neuroprotection involved activation of the Akt signaling pathway. These results suggested the neuroprotective potential of hUCBSC against glutamate-induced apoptosis of cultured cortical neurons^[74]. Both the *in vivo* and *in vitro* studies supported

Table 4 Changes in the expression of caspase-related and nuclear factor- κ B-related apoptotic genes after spinal cord injury

| UniGene | GenBank | Gene name | Fold change after SCI | Fold change after hUCBSC treatment |
|------------|-----------|------------------|-----------------------|------------------------------------|
| Rn. 37508 | NM_012762 | Casp1 | 9.14 ± 1.70 | 1.27 ± 0.78 |
| Rn. 81078 | NM_130422 | Casp12 | 2.91 ± 1.34 | 1.46 ± 0.68 |
| Rn. 10562 | NM_012922 | Casp3 | 3.56 ± 0.92 | 1.18 ± 0.84 |
| Rn. 88160 | NM_031775 | Casp6 | 3.34 ± 1.06 | 1.46 ± 0.79 |
| Rn. 53995 | NM_022260 | Casp7 | 2.81 ± 1.27 | 2.81 ± 1.21 |
| Rn. 54474 | NM_022277 | Casp8 | 3.84 ± 1.20 | 1.62 ± 0.89 |
| Rn. 32199 | NM_031632 | Casp9 | 2.86 ± 0.71 | 1.36 ± 0.62 |
| Rn. 67077 | NM_053362 | Dffb (Cad) | 32.94 ± 0.78 | 2.72 ± 0.84 |
| Rn. 16183 | NM_152937 | Fadd | 2.21 ± 0.78 | 1.51 ± 0.73 |
| Rn. 162521 | NM_139194 | Tnfrsf6 (Fas) | 10.87 ± 1.77 | 1.79 ± 0.67 |
| Rn. 44218 | NM_053353 | CD40lg | 15.91 ± 0.99 | 3.46 ± 0.78 |
| Rn. 160577 | NM_080769 | Lta (Tnfb) | 28.67 ± 0.07 | 2.06 ± 0.68 |
| Rn. 2275 | NM_012675 | TNF- α | 7.17 ± 1.63 | 2.36 ± 1.03 |
| Rn. 11119 | NM_013091 | Tnfrsf1a (TNFR1) | 2.53 ± 1.48 | 1.22 ± 0.78 |
| Rn. 83633 | NM_130426 | Tnfrsf1b (TNFR2) | 5.25 ± 1.56 | 3.01 ± 0.99 |
| Rn. 25180 | NM_134360 | Tnfrsf5 (CD40) | 4.26 ± 1.84 | 1.99 ± 0.78 |
| Rn. 54443 | NM_030989 | Tp53 (P53) | 3.46 ± 1.41 | -1.12 ± 0.61 |
| Rn. 18545 | XM_341671 | Tradd | 5.62 ± 1.13 | 1.46 ± 0.59 |
| Rn. 136874 | AI406530 | Traf1 | 4.12 ± 1.34 | 2.06 ± 0.84 |

Results are expressed as mean ± SD. hUCBSC: Human umbilical cord blood stem cells; NF- κ B: Nuclear factor- κ B; SCI: Spinal cord injury. Refer Dasari *et al*^[75].

our hypothesis that the therapeutic mechanism of hUCBSC was remyelination of demyelinated axons and inhibition of the neuronal apoptosis during the repair phase of the injured spinal cord. Veeravalli *et al*^[76] reported the involvement of tissue plasminogen activator (tPA) after SCI in rats and the role of hUCBSC. The tPA expression and activity were studied *in vivo* in rats after SCI and *in vitro* in rat embryonic spinal neurons in response to injury with staurosporine, hydrogen peroxide and glutamate. Infusion of hUCBSC downregulated tPA activity *in vivo* in rats as well as *in vitro* in the spinal neurons. Furthermore, MMP-2 is upregulated after hUCBSC treatment in spinal cord injured rats and in spinal neurons injured either with staurosporine or hydrogen peroxide. Also, hUCBSC-induced upregulation of MMP-2 diminished the formation of the glial scar at the site of injury along with reduced immunoreactivity to chondroitin sulfate proteoglycans. This upregulation of MMP-2 levels and reduction of glial scar formation by hUCBSC treatment after SCI created an environment more favorable for endogenous repair mechanisms^[77] (Figure 3). Kao *et al*^[78], suggested that hUCB derived-CD34⁺ cells can induce angiogenesis and endo/exogenous neurogenesis in SCI. In addition, Chen *et al*^[79] recently showed that hUCB stem cells have the ability to secrete multiple neurotrophic factors. Their study demonstrated an elevation of neuroprotective cytokine serum IL-10 levels and a decrease in TNF- α levels after hUCB stem cells infusion. Moreover, both GDNF and VEGF could be detected in the injured spinal cord

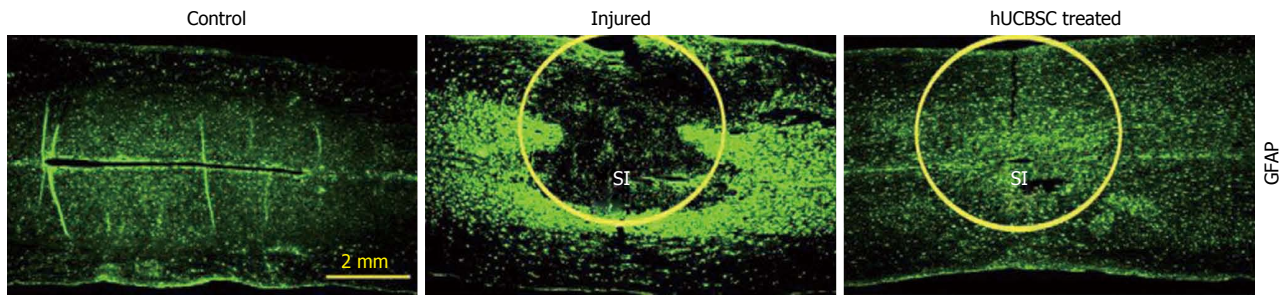


Figure 3 Reduction of inflammation in human umbilical cord blood-derived mesenchymal stem cell-treated spinal cords of rats. Immunohistochemical comparison of control, injured (21 d after spinal cord injury) and human umbilical cord blood-derived mesenchymal stem cells-treated spinal cord sections was performed to analyze the expression of reactive astrocytes at the site of injury. GFAP immunoreactivity is more evident and is localized at the lesion epicenter in the injured spinal cords. Astrogliosis is reduced in human umbilical cord blood-derived mesenchymal stem cells-treated sections. SI: Site of injury. *Neurobiol Dis* 2009; **36**: 200-212.

Table 5 Overview of effects of umbilical cord blood-derived mesenchymal stem cells after spinal cord injury

| Source of MSC | Main pathological features improved/repared | Limitations/recommendations/conclusions | Ref. |
|---------------|--|--|--|
| Human | Stem cells migrated to injured areas, functional recovery | hUCB may be a viable source of stem cells for treatment of neurological disorders | Saporta <i>et al</i> ^[69] , 2003 |
| | Axonal regeneration, functional recovery | HUCBs and BDNF reduced the neurological function deficit to a moderate degree for SCI rats | Kuh <i>et al</i> ^[70] , 2005 |
| | Stem cells secrete neurotrophic hormones and remyelinating proteins, axonal remyelination | Studies on long-term survival of hUCBSC and remyelination are recommended. | Dasari <i>et al</i> ^[71] , 2007 |
| | Repair and maintenance of structural integrity of the injured spinal cord, downregulation of apoptosis, functional recovery | Role of hUCBSC in maintaining structural integrity and thereby promoting the long-term survival of neurons and oligodendrocytes in the injured spinal cord | Dasari <i>et al</i> ^[72] , 2008 |
| | Downregulation of neuronal apoptosis | Modulation of the micro environment of the injured spinal cord by application of hUCBSC might be a potential therapeutic modality | Dasari <i>et al</i> ^[73] , 2009 |
| | Downregulation of elevated tPA activity/ expression in SCI rats | tPA is involved in secondary pathogenesis following spinal cord injury | Veeravalli <i>et al</i> ^[76] 2009 |
| | Upregulation of MMP2, reduction of glial scar | hUCBSC treatment after SCI upregulates MMP-2 levels and reduces the formation of the glial scar | Veeravalli <i>et al</i> ^[77] , 2009 |
| | GDNF and VEGF were secreted in the injured spinal cord after transplantation of CD34 ⁺ cells | CD34 ⁺ cell therapy may be beneficial in reversing the SCI-induced spinal cord infarction and apoptosis and hindlimb dysfunction | Kao <i>et al</i> ^[78] , 2008 |
| | Serum IL-10 levels increased, TNF- α levels decreased, functional recovery | Recovery of SCI-induced hind limb dysfunction is by increasing serum levels of IL-10, VEGF and GDNF in SCI rats. | Chen <i>et al</i> ^[79] 2008 |
| | Infarct size and blood vessel density at the injured site were significantly different in the treated group, functional recovery | Transplantation of CD34(+) HUCBCs during acute phase could promote functional recovery better than during subacute phase after SCI by raising blood vessel density | Ning <i>et al</i> ^[80] , 2013 |

MSC: Mesenchymal stem cell; SCI: Spinal cord injury; IL: Interleukin; TNF- α : Tumor necrosis factor- α ; hUCB: Human umbilical cord blood; hUCBSC: Human umbilical cord blood-derived mesenchymal stem cell.

after the transplantation of hUCBSC, thereby promoting angiogenesis and neuronal regeneration. Recently, Ning *et al*^[80], showed that transplantation of CD34⁺ HUCBCs during the acute phase could promote functional recovery better than during the subacute phase after SCI by raising neovascular density. These multifaceted protective and restorative effects from hUCB grafts may be interdependent and act in concert to promote therapeutic recovery for SCI (Table 5). Nevertheless, clinical studies with hUCBSC are still limited due to concerns about safety, mode of delivery, and efficiency. Among these concerns, the major histocompatibility in allogeneic transplantation is an important issue that needs to be addressed in future clinical trials for treating SCI^[16].

HUMAN WHARTON'S JELLY/UMBILICAL CORD MATRIX CELLS

There are two main populations of cells with a mesenchymal character within the human umbilical cord: Wharton's jelly mesenchymal stem cells (WJ-MSCs) and human umbilical cord perivascular cells (HUCPVCs)^[81]. Wharton's jelly cells (WJ-MSCs) can proliferate more rapidly and extensively than adult BMSCs (for a detailed review refer to Vawda and Fehlings, 2013). Yang *et al*^[82], examined the effects of human umbilical mesenchymal stem cells (HUMSC) transplantation after complete spinal cord transection in rats. They observed that transplanted HUMSCs survived for 16 wk and produced large amounts of human neuro-

Table 6 Overview of effects of Wharton's jelly/umbilical cord matrix cells after spinal cord injury

| Source of MSC | Main pathological features improved/repaired | Limitations/recommendations/conclusions | Ref. |
|---------------|---|---|---|
| Human | Survival of transplanted HUMSCs 16 wk, secretion of human neutrophil-activating protein-2, neurotrophin-3, basic fibroblast growth factor, glucocorticoid induced tumor necrosis factor receptor, and vascular endothelial growth factor receptor 3 in the host spinal cord | Transplantation of HUMSCs is beneficial to wound healing after SCI in rats | Yang <i>et al</i> ^[82] , 2008 |
| | Axonal regeneration, neuroprotective action by grafted cells, functional recovery | Co-grafted HUMSCs and BDNF may be a potential therapy for SCI | Zhang <i>et al</i> ^[83] , 2009 |
| | hUCMSCs survive, migrate, and produce GDNF and neurotrophin-3, functional recovery | Studies on dose-dependent effects of hUCMSCs transplantation on SCI are required | Hu <i>et al</i> ^[84] , 2010 |
| | Increased intensity of 5-HT fibers, increased volume of spared myelination, decreased area of cystic cavity, functional recovery | NT-3 enhanced therapeutic effects of HUMSCs after clip injury of the spinal cord. | Shang <i>et al</i> ^[85] , 2011 |

MSC: Mesenchymal stem cell; SCI: Spinal cord injury; hUCMSC: Human umbilical cord blood-derived mesenchymal stem cell.

phil-activating protein-2, neurotrophin-3, basic fibroblast growth factor, glucocorticoid induced tumor necrosis factor receptor, and vascular endothelial growth factor receptor 3 in the host spinal cord. Zhang *et al*^[83], used an animal model of transected SCI to test the hypothesis that co-grafted human umbilical mesenchymal stem cells-derived neurospheres (HUMSC-NSs) and BDNF can promote morphologic and functional recoveries of the injured spinal cord. Recovery of hindlimb locomotor function in SCI rats was significantly enhanced in human umbilical cord mesenchymal stem cells-grafted animals at five weeks as compared to control sham-grafted animals^[84]. Using a rat model for clip SCI, Shang *et al*^[85], showed that Neurotrophin-3 (NT-3) genetically modified human umbilical mesenchymal stem cells (NT-3-HUMSCs) promoted the morphologic and functional recovery of injured spinal cords (Table 6). Although these studies involved thoracic SCI model, these positive findings will most likely apply to cervical SCI as well^[3].

CONCLUSION

Therapeutic application of MSCs represents a promising approach in the treatment of spinal cord injury. Nevertheless, cell-based therapy for SCI in its nascent stages is facing several challenges including translational clinical issues, regulatory and ethical concerns, as well as modalities of transplantation, timing, safety and efficacy of the transplanted cells. A better understanding is also needed of the mechanisms of action and the behavior of stem cells in the pathological environment after transplantation in order to determine the best time frame and the most efficient routes for cell delivery after the injury^[86]. Although several clinical trials utilize MSCs transplantation for the treatment of SCI, the ultimate value of a translational approach needs continued exploration of basic scientific knowledge of SCI and proven therapeutic efficacy *via* rigorous controlled, randomized, double blind, multi-center clinical trials.

ACKNOWLEDGMENTS

We thank Diana Meister for manuscript review. The

authors wish to thank the editors of the Journal of Neurotrauma, Neurobiology of Disease and Stem Cells and Development for permission to use the figures and Tables 3 and 4, which appear in this article.

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P- Reviewers: Ho I, Kan L, Miller RH **S- Editor:** Song XX

L- Editor: A **E- Editor:** Zhang DN



WJSC 6th Anniversary Special Issues (2): Mesenchymal stem cells

Brain mesenchymal stem cells: The other stem cells of the brain?

Florence Appaix, Marie-France Nissou, Boudewijn van der Sanden, Matthieu Dreyfus, François Berger, Jean-Paul Issartel, Didier Wion

Florence Appaix, Marie-France Nissou, Jean-Paul Issartel, Didier Wion, INSERM U836, Grenoble Institut des Neurosciences, Bâtiment Edmond J Safra, Université Joseph Fourier, CHU Michallon, 38042 Grenoble, France

Boudewijn van der Sanden, Matthieu Dreyfus, François Berger, INSERM U1167 CLINATEC, Centre de Recherche Edmond J Safra, MINATEC Campus CEA, 38054 Grenoble, France

Author contributions: All authors contributed to the writing, the illustrations and the revision of the manuscript.

Supported by INSERM and the Ligue contre le Cancer Isère-Rhône Alpes

Correspondence to: Didier Wion, PhD, INSERM U836, Grenoble Institut des Neurosciences, Bâtiment Edmond J Safra, Université Joseph Fourier, CHU Michallon, 38042 Grenoble, France. didier.wion@ujf-grenoble.fr

Telephone: +33-456-520645 Fax: +33-456-520639

Received: November 12, 2013 Revised: January 16, 2014

Accepted: February 20, 2014

Published online: April 26, 2014

Abstract

Multipotent mesenchymal stromal cells (MSC), have the potential to differentiate into cells of the mesenchymal lineage and have non-progenitor functions including immunomodulation. The demonstration that MSCs are perivascular cells found in almost all adult tissues raises fascinating perspectives on their role in tissue maintenance and repair. However, some controversies about the physiological role of the perivascular MSCs residing outside the bone marrow and on their therapeutic potential in regenerative medicine exist. In brain, perivascular MSCs like pericytes and adventitial cells, could constitute another stem cell population distinct to the neural stem cell pool. The demonstration of the neuronal potential of MSCs requires stringent criteria including morphological changes, the demonstration of neural biomarkers expression, electrophysiological recordings,

and the absence of cell fusion. The recent finding that brain cancer stem cells can transdifferentiate into pericytes is another facet of the plasticity of these cells. It suggests that the perversion of the stem cell potential of pericytes might play an even unsuspected role in cancer formation and tumor progression.

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Key words: Stem cell; Mesenchymal stem cell; Pericyte; Brain; Cell plasticity; Cancer stem cell; Glioma; Neurodegenerative disease

Core tip: Mesenchymal stem cells (MSCs), in addition to their potential to differentiate into cells of the mesenchymal lineage, have immunomodulatory properties and can transdifferentiate to generate neural cells at least *in vitro*. These stem cells are found in almost any adult tissue, including brain. The existence of similarities between MSC and pericytes points to brain pericytes as the other stem cell population of the adult brain in addition to neural stem cells. This raises fascinating perspectives on the potential of brain pericytes in nervous system maintenance and repair. The recent finding that brain cancer stem cells transdifferentiate into pericytes is another facet of the plasticity of these cells. It suggests that the perversion of the stem cell potential of pericyte might play an even unsuspected role in cancer formation and tumor progression.

Appaix F, Nissou MF, van der Sanden B, Dreyfus M, Berger F, Issartel JP, Wion D. Brain mesenchymal stem cells: The other stem cells of the brain? *World J Stem Cells* 2014; 6(2): 134-143 Available from: URL: <http://www.wjgnet.com/1948-0210/full/v6/i2/134.htm> DOI: <http://dx.doi.org/10.4252/wjsc.v6.i2.134>

INTRODUCTION

The history of multipotent mesenchymal stromal cells started when colony-forming unit fibroblastic cells (CFU-F) with osteogenic potential were obtained from bone marrow cultured cells^[1-3]. Accordingly, CFU-F cells were defined as self-renewing non-hematopoietic bone marrow stromal stem cells (BMSCs). They were isolated on the basis of their plastic adherence, and characterized both by their ability to form colony when plated at low-density and to differentiate into osteoblasts^[3]. Thereafter, BMSCs were shown to differentiate *in vitro* and *in vivo* into other cells of mesenchymal lineage including chondrocytes and adipocytes^[4]. Cells similar to BMSCs are also isolated from non-marrow fetal tissue such as placenta, cord blood, fetal liver and lung, as well as from adult tissues including muscle, adipose tissue, dental pulp, lung and brain^[5-8]. These fetal and adult stem cells have the same ability as BMSCs for self-renewal and for differentiation into osteoblasts, chondrocytes and adipocytes *in vitro*. They also exhibit, at least *in vitro*, transdifferentiation capacity (see below). These cells are referred as mesenchymal stem cells or as multipotent mesenchymal stromal cells (MSCs). However, the question remains if these ubiquitous cells behave *in vivo* as genuine stem cells or if their stem cell potential is a cell culture artifact^[9]. The existence of these MSCs in virtually all postnatal organs does not necessarily mean that these cells behave as stem cells during development. For example, their physiological function could be limited to postnatal regenerative processes. Hence, the concept of mesenchymal stem cell, initially well-defined and restricted to a multipotent progenitor for skeletal tissues and residing within the bone marrow has progressively evolved towards an all-encompassing concept including multipotent perivascular cells of almost any tissue^[9]. Importantly, there is not an exclusive and universal marker for immunophenotyping MSCs. Therefore, their immuno-characterization relies on a combination of both positive and negative markers. Positive markers can include CD11b, CD13, CD19A, CD73, CD105, CD146, CD271, nestin, nerve/glia antigen 2 (NG2), platelet-derived growth factor receptor β (PDGFR- β), while negative markers usually are endothelial, and hematopoietic stem cell proteins (Table 1)^[10-12]. An additional remarkable feature is that MSCs lack or have a low expression of MHC class II and of the costimulatory molecules CD40, CD80, CD86, CD134 and CD142^[13]. In relation to this, MSCs have strong anti-inflammatory and immunomodulating potentials^[14]. MSCs exert their inhibitory effects on T-cell proliferation by mechanisms involving both cell to cell contact between MSC and T lymphocytes, and secreted factors such as prostaglandin E2 (PGE2), inoleamine 2,3-dioxygenase and nitric oxide^[14]. As in many biological processes, this immunosuppressive effect is dose dependent and depends on the ratio between MSCs and T cells. Indeed low ratios of MSCs can even enhance T cell proliferation^[14]. In addition, MSCs prevent the differentiation of monocyte into dendritic cells, and modulate natural killer cell activ-

ity by the release of inhibitory factors such as PGE2 and transforming growth factor- β ^[14]. MSCs also have anti-inflammatory action by reducing the production of tumor necrosis factor (TNF)- α and interleukin (IL)-12 and by increasing the synthesis of IL-10 by macrophages^[14]. These anti-inflammatory and immuno-modulatory capacities of MSCs are already exploited *in vivo*. MSC-based treatment is beneficial in several models of graft-vs-host disease and in auto-immune diseases such as collagen-induced arthritis, experimental autoimmune encephalomyelitis, type 1 diabetes mellitus disease and inflammatory bowel disease models^[14-17]. Clinical trials are currently underway for these different pathologies^[15,18]. The ability of MSCs to home in damaged tissues, associated with their capacity to secrete bioactive molecules such as growth factors, and their immunosuppressive and anti-inflammatory properties, suggest that these cells protect tissues from damage and facilitate tissue repair independently of their capacity to generate differentiated cells^[18].

For all these reasons, MSCs became the focus of intense researches in tissue engineering and regenerative medicine. These cells could provide an answer both to the ethical concerns raised by the therapeutic use of human embryonic stem cells and to their scarce availability. Furthermore, as MSCs are easily isolated from adult tissues, they offer the advantage to allow autologous transplantation. Importantly, experimental studies performed with MSCs revealed an additional property: MSCs have a greater differentiation plasticity potential than previously envisioned. For example, they can transdifferentiate into urothelial, myocardial, and epithelial cells^[19-21]. Numerous studies also report the *in vitro* transdifferentiation of MSCs into neural and glial cells^[22-30]. At the moment, the potential of MSCs to regenerate human tissues *in vivo* is not clearly defined. Current research is ongoing to resolve this critical issue by improving MSC culture engineering and cell transplantation technology. A better characterization of the therapeutic potential of MSCs according to their tissue of origin is also a critical issue.

WHEN MSCs TRANSDIFFERENTIATE INTO NEURAL CELLS: FACTS AND ARTIFACTS

The observation that MSCs transdifferentiate into neurons was first obtained with bone MSCs, and then extended to MSCs isolated from different adult tissues including adipose tissue, bone marrow, and brain^[5,31-34]. Brain implanted marrow stromal cells also differentiate into glial cells^[25]. Importantly, grafting MSCs in several brain lesion models reduces neuronal deficits^[35-42]. However, current evidence suggests that in the experimental models used, the repair and functional improvements reported are primarily mediated by paracrine or cell-cell interactions rather than by the successful engraftment and the *in situ* transdifferentiation of implanted MSCs into neural cells^[43-47]. Regarding MSC transdifferentia-

Table 1 Major positive and negative markers used for identifying bone marrow mesenchymal stem cells and pericytes

| Markers | MSCs | Pericytes | EC | HSPCs | NSPCs |
|---------|-----------------|-----------|---------|---------|-----------|
| CD10 | + [12] | + [12] | | | |
| CD13 | + [12] | + [12] | | | |
| CD29 | + [12] | + [12] | | + [91] | + [92] |
| CD44 | + [12] | + [12] | | + [93] | + [92] |
| CD73 | + [12] | + [12] | + [94] | | |
| CD90 | + [12] | + [12] | | + [95] | + [92,96] |
| CD105 | + [12] | + [12] | + [97] | | |
| CD140B | + [12] | + [12] | | | |
| CD146 | + / low [12,90] | + [12] | + [98] | | |
| CD166 | + [12] | + [12] | | + [99] | |
| NG2 | + [12] | + [12] | - [11] | - [100] | |
| Nestin | + [101,102] | + [72] | | | + [103] |
| CD14 | - [12] | - [12] | | | |
| CD31 | - [12] | - [12] | + [104] | | |
| CD34 | - [12] | - [12] | + [105] | + [105] | |
| CD45 | - [12] | - [12] | | | |
| CD133 | - [12] | - [12] | - [106] | + [107] | + [108] |
| CD117 | - [12] | - [12] | | + [109] | + [110] |
| CD144 | - [12] | - [12] | + [111] | | |
| vWF | - [112] | - [113] | + [114] | | |

In the absence of any universal and specific marker to define mesenchymal stem cells, their immunophenotyping relies on the use of combinations of both positive and negative markers. Note that MSCs profile may vary depending on the cell culture conditions^[88], or with their *in situ* localization^[89]. Expression of the cell surface antigens CD73, CD90, CD105 and non-expression of CD14, CD34, CD45 are useful criteria to define bone MSCs and pericytes. MSCs: Mesenchymal stem cells; EC: Endothelial cells; HSPCs: Hematopoietic stem and progenitor cells; NSPCs: Neural stem and progenitor cells.

tion into neural cells, a notable controversy arose when it was reported that, (1) the rapid *in vitro* morphological differentiation of MSC into neuron-like cells following administration of DMSO or cAMP elevating agents such as forskolin or IBMX can be linked to actin depolymerization resulting in cytoplasm retraction and not through neurite extension^[48-50]; and (2) the transformation of MSCs into neurons *in vivo* can result from the fusion of MSCs with brain cells rather than to MSC transdifferentiation^[51]. Therefore, additional criteria are now applied when studying MSC transdifferentiation. For example, reporting neuronal differentiation of MSCs now requires observation of morphological changes, the demonstration of neural biomarkers expression, neurotransmitter responsiveness or electrophysiological recording, and absence of cell fusion^[28,33,49,52,53]. Note however, that all MSCs are not equal and that their differentiation potential can be related to their tissue of origin^[6]. This suggests that brain-derived MSCs could have a greater potential for neural differentiation than bone MSCs. Hence, the difficulty to obtain functional mature neurons by differentiating bone MSC can be explained both by their origin and by cell culture conditions which are far to provide the cues found in the brain microenvironment. Accordingly, recent experiments using brain derived MSCs instead of bone marrow MSCs, provide additional evidence on the potential of brain MSCs to transdifferentiate into neuro-

nal cells at the clonal level and on the basis of stringent criteria^[54]. A notable point is that these observations are made *in vitro*. Therefore, it remains to establish whether the transdifferentiation of MSCs is a cell culture artifact with potential applications in cell replacement therapies for implanting pre-differentiated neurons, or is it also a physiological process contributing to brain development or repair. Part of the answer might be given by determining where MSCs reside in the organism and which cell behaves as MSC *in vivo*. Recent findings show that MSCs are perivascular cells such as pericytes^[11,55,56].

MSCS ARE PERIVASCULAR CELLS

Pericytes are perivascular cells, or more strictly speaking peri-endothelial vascular mural cells (Figure 1). Pericytes form an incomplete layer on the abluminal surface of capillary endothelial cells. They wrap capillary endothelial cells and both cell types are surrounded by the basal lamina^[57] (Figure 2). For many years, pericytes have been viewed as supportive vasculature cells involved in the regulation of capillaries blood flow and contributing to the blood-brain barrier^[58]. Nowadays, known functions of pericytes also include a role in angiogenesis, in matrix proteins and bioactive molecules synthesis (vascular endothelial growth factor, placental growth factor, leukemia inhibitor factor, CXCL12, basic fibroblast growth factor, nerve growth factor, platelet-derived growth factor B...), in vessel stabilization and in the regulation of vascular tone^[59]. Importantly, these cells are now considered as a potential reservoir of stem or progenitor cells for adult tissue repair. Regarding this stem cell potential, it has been known as early as 1995 that pericytes can differentiate into an osteogenic phenotype^[60]. Ten years after, perivascular cells were also demonstrated to differentiate into adipocytes^[61]. The definitive proof that MSCs are perivascular cells such as pericytes was done in 2008 in two landmark studies showing that a subset of perivascular cells from adult tissues, identified on CD146, NG2 and PDGF-R β expression, exhibit in culture the same osteogenic, chondrogenic, adipogenic and myogenic potentials than MSCs^[11,55]. In addition, these perivascular cells express MSC markers including CD10, CD13, CD44, CD73, CD90 and CD105^[11,12]. A consequence of the demonstration of a perivascular origin for MSCs was a burst of interest in pericyte research with the number of annual entries in PubMed for the keyword “pericyte” increasing from 83 in 1993 to 445 in 2013. With hindsight, the finding that some MSCs are pericytes is not incongruous^[11,56]. Stem cells must reside in a specialized environment (the stem cell niche), and the presence of MSCs in almost all adult tissues suggests a ubiquitous distribution for MSC niches. This is consistent with the omnipresence of capillary blood vessel mural cells. In addition, this perivascular location allows the rapid recruitment of MSCs to the site of focal lesions where they could act as microenvironmental regulators for tissue regeneration^[62]. Since tissue regeneration requires functional blood ves-

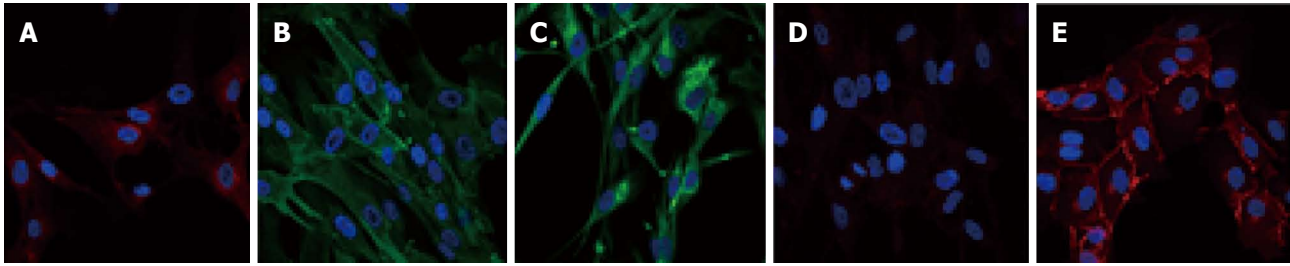


Figure 1 Pericyte immunophenotyping. Pericytes express antigens allowing their identification. However, there is currently no specific marker to identify them. Therefore, to distinguish pericytes from other cell types, both positive and negative markers are used. For example, pericytes are known to be positive for platelet-derived growth factor receptor β (PDGFR- β)/CD140b (A), Alanine aminopeptidase N/CD13 (B), and for the stem cell protein nestin (C). Pericytes are also negative for VE-Cad/CD144 (D) that is detected in human brain endothelial cells (E). Specific antigenic labeling is in green or red and nuclei are 4',6-diamidino-2-phenylindole stained (blue).

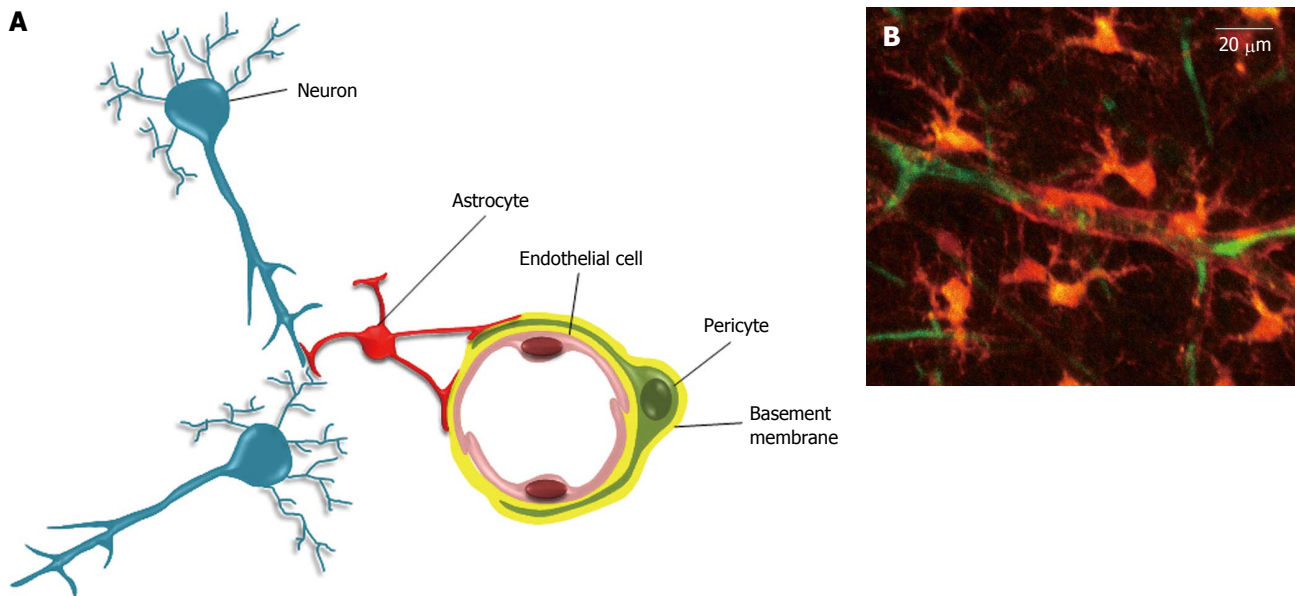


Figure 2 The neurovascular unit. A: The neurovascular unit. In the neurovascular unit, pericytes are located on the abluminal side of endothelial cells (EC). Both cells are ensheathed by the basement membrane (BM). The covering of EC by pericytes is incomplete, and interruptions in BM can allow direct contacts between pericyte and EC. These contacts occur through peg and socket structures, and adherent and gap junctions (not shown)^[59]. The abluminal side of the basement membrane is also contacted by astrocytes endfeet. In addition to these cells, the neurovascular unit also includes neurons, and microglial cells (not shown); B: Two-photon microscopy of a neurovascular unit. Following injection in the rat tail vein, the sulforhodamine-B dye crosses the blood brain barrier and stains astrocytes and pericytes in orange (reproduced from^[115]). The blood plasma is shown in green after *iv* injection of FITC-dextran (Mw 70 kDa). Neurons, endothelial and microglial cells are not shown here.

sels, associating MSCs with endothelial cells in a same “regenerative/healing unit” makes sense. Note that in addition to capillaries, MSCs are also detected in the adventitia of large vessels^[63-65].

CNS PERICYTE AND THE NEUROVASCULAR UNIT

With a human brain capillary network estimated to 400 miles length^[66], and a ratio of about one pericyte for three brain endothelial cells, the human brain pericyte population is far from negligible. Pericytes cover more than 30% of the cerebral capillary surface^[67]. These cells are well-known to be involved in the regulation of angiogenesis, vascular tone and blood brain barrier function. They constitute with endothelial cells, astrocytes and neurons a

critical brain structure named neurovascular unit (NVU). The NVU, in addition to selectively supplying nutrients and oxygen through the blood brain barrier structure, provides a permissive environment for neural stem cell homing and for their proliferation^[68-70]. Note that if most pericytes are of mesoderm origin, forebrain pericytes originate from the neural crest^[71]. The demonstration that MSCs originate at least in part from pericytes raises the question of the stem cell potential of brain pericytes. At a clonal level these cells have the potential to differentiate *in vitro* into adipocytes, chondroblasts and osteoblasts^[54]. Moreover these cells are also able to differentiate *in vitro* toward a neuronal phenotype depending on cell culture conditions^[33,54,72,73]. These observations revive the idea that CNS perivascular cells such as pericytes might contribute to brain repair either directly by generating new

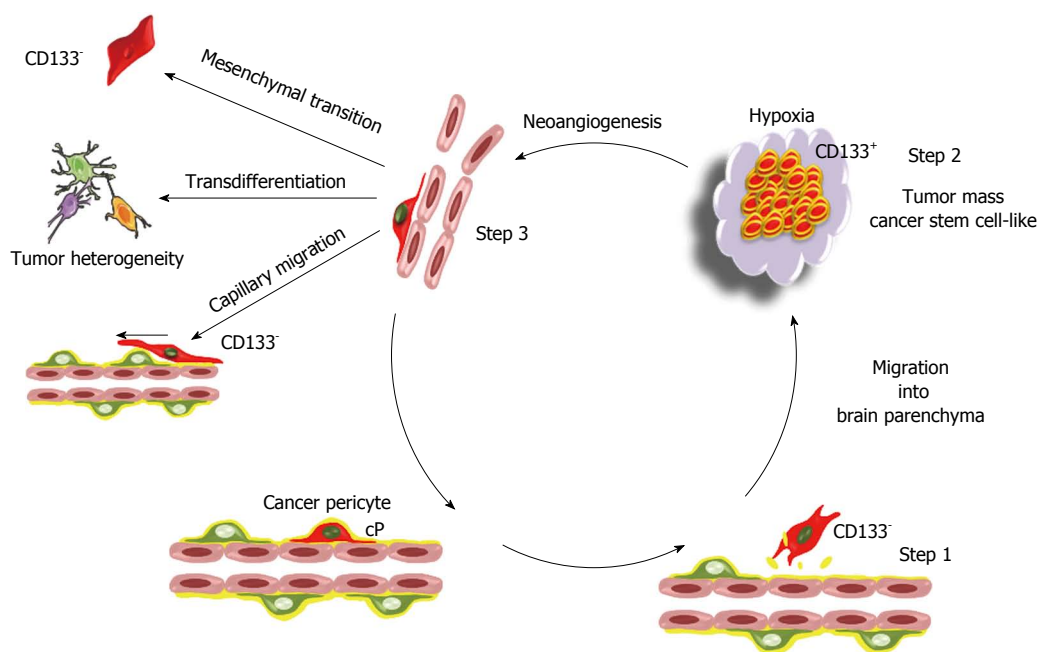


Figure 3 The cancer pericyte model: A perpetuum mobile. The proposed model of brain tumor is based on the mesenchymal stem cell potential of pericytes. In this model, the brain cancer initiating cell is a cancer pericyte (cP) harbouring oncogenic alterations and located on a brain capillary. After disruption of the basement membrane by proteases, it detaches from the vessel wall and migrates into brain parenchyma as normal pericytes do following injury^[76] (step 1). During the passage from a vascular to a neural environment the pericyte acquires a CD133⁺ neural stem cell-like phenotype, as observed *in vitro* for non-transformed pericytes^[72]. Such a transition towards a neural stem cell phenotype is already observed for non-transformed pericytes at least *in vitro*. This generates the CD133⁺ cancer stem cell pool (step 2). Amplification of the cancer stem cell pool generates hypoxia that triggers neoangiogenesis and the migration of endothelial cells towards the lesion as well as the migration of cancer stem cells towards endothelial capillaries^[83] (step 3). Cancer stem cells within this new vascular microenvironment reacquire a CD133⁺ pericyte-like phenotype. At this stage, they can either integrate into the tumor neovasculature and reinitiate a new cycle generating a perpetuum mobile, or migrate along capillaries and invade brain as previously described^[116,117]. Alternatively, due to the mesenchymal stem cell potential of pericytes, these pericyte-like cancer cells can acquire mesenchymal traits and progress towards a more aggressive mesenchymal phenotype. The transdifferentiation potential of pericyte-like cancer cells could in turn participate to the cellular heterogeneity found in glioblastoma multiforme. Since CD133 is not detected in pericytes, the existence of CD133⁺ pericyte-like cancer stem cells provides an issue to the controversy regarding the existence in glioma tumors of both CD133⁺ and CD133⁻ cancer stem cells^[118,119]. Note that this model is not exclusive. The transformation of a glial or neural stem cell might also generate cancer initiating cells.

neurons or indirectly *via* their immunomodulatory properties or the secretion of neurotrophins^[74]. Consistent with this idea is the observation that pericytes migrate away from the vascular wall and could generate neurons in response to injury^[75,76].

THE CANCER PERICYTE MODEL: A PERPETUUM MOBILE

The NVU also plays a critical role in brain cancer since a contingent of brain cancer stem cells is found near the capillaries^[77-79]. Importantly, glioblastoma stem cells are able to transdifferentiate into pericytes^[80]. According to the function of pericytes in vessel formation, these cancer pericytes contribute to the glioblastoma microvasculature^[80,81]. The recent finding that MSCs are pericytes, and that glioblastoma cells generate cancer pericytes, suggests that the stemness potential of pericytes could play a yet unsuspected role in cancer formation and progression. In the synthetic hypothetical model depicted in Figure 3, a transformed dormant pericyte harboring oncogenic mutations and lying in its vascular niche is activated and released from its vascular location as a consequence of the up-regulation of matrix proteases (Figure 3, step 1).

This activation can be triggered by inflammation or can occur following a local injury as observed *in vivo* with normal pericytes^[76,82]. In the proposed model, and in accordance with the similarities between pericytes and MSCs, this *cancer pericyte* behaves as a cancer mesenchymal stem cell. In accordance to the described potential of MSCs to generate neural stem cell-like cells^[30,72], cancer pericyte cells acquire a neural stem cell-like phenotype during their migration in brain parenchyma. This generates the cancer stem cell pool found in the tumor mass (Figure 3, step 2). Proliferation of these cancer stem cells generates hypoxia and triggers the angiogenic switch. Cancer stem cells are then recruited to develop vessels by endothelial cell-secreted cytokines such as CXCL12^[83-85] (Figure 3, step 3). In this novel vascular microenvironment made of chaotic vessels, cancer stem cells reacquire a pericyte-like phenotype as described^[80,81]. These pericyte-like cancer cells not only participate to tumor vascularization^[80,81], but also re-express their mesenchymal potential by undergoing a mesenchymal transition reminiscent to the epithelial mesenchymal transition. This generates the perpetuum mobile described in Figure 3. Indeed, MSCs have already been characterized as cancer initiating cells in gastric cancer^[86].

CONCLUSION

Since the first observation of pericyte cells by Rouget^[87], it has been a long road and winding road to get here. For many years, pericytes have been largely under-recognized and considered only as supportive cells of the vasculature. Their active role in angiogenesis and in cell-cell interactions with endothelial cells and astrocytes, as well as their *in vitro* stem cell functions, has only recently emerged. However, much remains to be done for a better understanding of the *in vivo* pericyte potential. For example, can pericytes/MSCs be considered as mobile “drugstores” migrating and delivering factors at the sites of injury^[88]? Is the pericyte/MSC transdifferentiation potential an *in vitro* artifact or is it physiologically relevant? Is it an ancient feature of more primitive organisms which has been lost during the course of evolution and which is now reactivated *in vitro*? Alternatively, could it be an emerging evolutionary trait already engaged *in vivo* in some regenerative processes? Is the neural transdifferentiation potential of brain pericyte/MSC only efficient for repairing micro-lesions, which could explain why our current experimental paradigms which generate large infarcts might not be adequate to detect this potential? Do brain pericytes/MSCs behave like “sleeping beauties” awaiting the right physiological or pharmaceutical inducers for expressing their transdifferentiating and regenerative potentials? Conversely is the perversion of this potential involved in some brain tumors? The answers to these questions promise to be fascinating.

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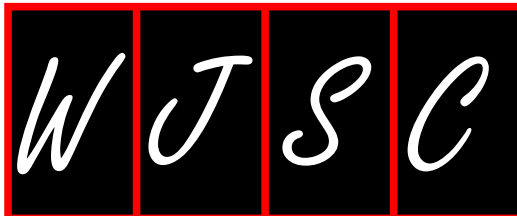
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P- Reviewers: Cardinale V, Gassler N **S- Editor:** Gou SX
L- Editor: A **E- Editor:** Zhang DN





WJSC 6th Anniversary Special Issues (2): Mesenchymal stem cells

Adipose mesenchymal stem cells in the field of bone tissue engineering

Cecilia Romagnoli, Maria Luisa Brandi

Cecilia Romagnoli, Maria Luisa Brandi, Metabolic Bone Diseases Branch, Department of Surgery and Translational Medicine, University of Florence, Florence 50139, Italy

Author contributions: Romagnoli C conceived and designed the paper, reviewed the literature and wrote the paper; Brandi ML revised and approved the final version of the paper.

Supported by The Regione Toscana-POR CRO FSE 2007-2013 and I.F.B. STRODER s.r.l.

Correspondence to: Maria Luisa Brandi, MD, PhD, Metabolic Bone Diseases Branch, Department of Surgery and Translational Medicine, University of Florence, Largo Palagi 1, Florence 50139, Italy. marialuisa.brandi@unifi.it

Telephone: +39-55-7946304 Fax: +39-55-7946303

Received: November 19, 2013 Revised: December 19, 2013

Accepted: March 3, 2014

Published online: April 26, 2014

Abstract

Bone tissue engineering represents one of the most challenging emergent fields for scientists and clinicians. Current failures of autografts and allografts in many pathological conditions have prompted researchers to find new biomaterials able to promote bone repair or regeneration with specific characteristics of biocompatibility, biodegradability and osteoinductivity. Recent advancements for tissue regeneration in bone defects have occurred by following the diamond concept and combining the use of growth factors and mesenchymal stem cells (MSCs). In particular, a more abundant and easily accessible source of MSCs was recently discovered in adipose tissue. These adipose stem cells (ASCs) can be obtained in large quantities with little donor site morbidity or patient discomfort, in contrast to the invasive and painful isolation of bone marrow MSCs. The osteogenic potential of ASCs on scaffolds has been examined in cell cultures and animal models, with only a few cases reporting the use of ASCs for successful reconstruction or accelerated healing of defects of the skull and jaw in patients. Although these reports

extend our limited knowledge concerning the use of ASCs for osseous tissue repair and regeneration, the lack of standardization in applied techniques makes the comparison between studies difficult. Additional clinical trials are needed to assess ASC therapy and address potential ethical and safety concerns, which must be resolved to permit application in regenerative medicine.

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Key words: Adipose-derived stem cells; Bone tissue engineering; Osteogenic differentiation; Scaffold; Regenerative medicine

Core tip: The complex and dynamic process of bone tissue engineering is a challenging field in regenerative medicine. Current research is focused on the optimization and facilitation of bone regeneration by combining growth factors and mesenchymal stem cells with the many types of materials that have been studied as scaffolds. This review presents an overview of ideal scaffold properties and discusses the application of adipose-derived stem cells in bone tissue engineering and translational medicine.

Romagnoli C, Brandi ML. Adipose mesenchymal stem cells in the field of bone tissue engineering. *World J Stem Cells* 2014; 6(2): 144-152 Available from: URL: <http://www.wjg-net.com/1948-0210/full/v6/i2/144.htm> DOI: <http://dx.doi.org/10.4252/wjsc.v6.i2.144>

INTRODUCTION

Recent progress in the field of bone tissue engineering has led to new and exciting research concerning regenerative medicine. This interdisciplinary field is focused

on the development of biological substitutes that restore, maintain or improve tissue function by applying the principles of engineering and the life sciences^[1]. The primary target of clinical therapeutic strategies is the regeneration of bone for skeletal reconstruction of large bone defects created by trauma, infection, tumor resection and skeletal abnormalities, or cases in which the regenerative process is compromised, including avascular necrosis, atrophic non-union and osteoporosis. Strategies that stimulate bone healing to reduce or treat complications are becoming more important, due to the increase in life expectancy and ageing of the world population.

Autologous grafts represent the “ideal graft bone substitutes” and are currently the gold standard therapeutic strategy as they combine all essential components to induce bone growth and regeneration, including osteogenic cells, osteoinductive growth factors and bone-supporting matrix. Autografts are non-immunogenic and histocompatible, as they are the patient’s own tissue. Although they reduce the likelihood of immunoreaction and transmission of infection^[2], autografts are limited and commonly result in donor site morbidity as a result of the additional surgical harvesting procedures, and are accompanied by the risk of infection, hematoma and chronic pain, which can all lead to implant failure^[3-7]. An alternative approach involves the use of allogenic bone grafts obtained from human cadavers or living donors, which bypasses the complications associated with harvesting and quantity of graft materials. However, allogenic grafts are limited by tissue matching, disease transmission, batch variability and an inability to survive and integrate following implantation^[8-10].

The limited success of auto- and allografts in some clinical situations has stimulated the investigation of a wide variety of biomaterials to be used as scaffolds, and the development of promising clinical therapies^[11]. Advantages to utilizing sophisticated bone scaffolds include the elimination of the risk for disease transmission, fewer surgical procedures, and reduced risk of infection or immunogenicity. Moreover, there is an abundant availability of synthetic or natural biomaterials that can be employed, including collagen, hydroxyapatite (HA), β -tricalcium phosphate (β -TCP), calcium phosphate cements and glass ceramics. The concept of bone substitution involves the replacement of bone structure to allow the migration, proliferation and differentiation of bone cells and to promote vascularisation, thus utilizing the body’s natural biological response to tissue damage in conjunction with engineering principles. Current models of *in vitro* bone formation are based on the idea that the same factors known to play a role during embryonic development can be used to induce cellular differentiation and function in the process of regeneration^[12]. In order to engineer an environment supporting bone formation, combinations of biochemical and biophysical signals need to be presented to the cells in a three-dimensional setting in a way that allows interactions between the surrounding cells and the extracellular matrix. The complexity of signal-

ing, with temporal and spatial gradients of molecular and physical factors affecting bone morphogenesis, presents significant challenges to engineering fully viable, functional bone. This “diamond concept” has allowed the scientific community to consider more complex interactions between scaffolds, cells and growth factors in order to induce tissue regeneration in bone defects^[13]. This article presents a concise review regarding the main properties of scaffolds, the most recent progress in bone tissue engineering using human adipose-derived stem cells and current models used for bone regeneration.

PROPERTIES OF ENGINEERED BONE SCAFFOLDS

An ideal scaffold must address multiple physical and biological requirements in order to optimize bone regeneration. One of the most important stages of bone tissue engineering is the design and processing of a porous, biodegradable three-dimensional (3D) structure. This scaffold provides a structural and logistical template for developing tissue, which can markedly affect cell behavior. The properties of scaffolds that are important for bone formation include the size, distribution and shape of the pores, the surface roughness, the presence of cell attachment sites and the biomechanics of both the material and the scaffold structures^[14-17]. The most suitable scaffolds for bone formation are those made of osteoconductive materials, such as bone proteins and HA, with mechanical properties similar to those of load-bearing native bone that stimulate osteogenesis and have large and interconnected pores to facilitate cell infiltration and matrix deposition, and rough inner surfaces to promote cell attachment. Additionally, scaffolds should be anisotropic structures that can be fashioned into anatomically correct shapes that also have the capacity for vascularization. Scaffolds should also incorporate and control the delivery of bioactive molecules, such as growth factors or drugs that regulate cellular function, accelerating healing and preventing pathology^[18,19]. Furthermore, as scaffolds will be replaced over time by new formed bone, they should be comprised of resorbable materials, or materials that degrade in an enzymatic or hydrolytic way, such as polymers, or can be dissolved by cells such as osteoclasts^[20,21].

The majority of studies are currently focused on the development of 3D structures that mimic the anatomical and biochemical organization of cells and native matrix in order to achieve suitable mechanical properties for bone tissue^[22]. Numerous materials have been shown to support *in vitro* bone formation by human cells, including bioceramics like HA, β -TCP, bio-glasses and biodegradable polymers^[23,24], and natural or synthetic collagen, fibrin, chitosan or polyesters^[25,26]. Scaffolds containing composites of these materials provide an optimized and convenient alternative as they combine the advantages of both bioactive ceramics and biodegradable polymers^[27-31].

OSTEOINDUCTIVE BIOMOLECULES

One of the most challenging tasks for the development of bone graft substitutes is to produce scaffolds with osteoinductive properties, which can involve the application of biologically active molecules. Growth factors that naturally occur within a healthy bone matrix or are expressed during fracture healing can be used to direct the development of structures, vascularization and differentiation of bone cells^[19]. Growth factors, such as cytokines, are endogenous proteins that act on a wide variety of cells and direct their actions by binding to and activating cell-surface receptors. As developmental bone formation is an orchestrated cellular process tightly controlled by actions of growth factors, their use in engineered scaffolds is an obvious strategy when the bone integrity is compromised and bone tissue needs to be repaired^[52,33]. This strategy aims to enhance the local presence of bone-depositing osteoblasts, either by attracting the cells to the repair site or by inducing the proliferation of local undifferentiated precursor cells, followed by the transformation of precursor cells into an osteoblastic phenotype^[34].

The introduction of specific biomolecules has been shown in animal models to enhance the union of non-union type (a fracture that does not heal by itself after several months) bone fractures^[32]. Many growth factors that have been used in bone repair with some degree of success include mitogens such as platelet-derived growth factors, metabolic regulators such as insulin-like growth factors, angiogenic proteins such as basic fibroblast growth factors, and morphogens such as bone morphogenetic proteins (BMPs)^[35-39]. BMPs, which are members of the transforming growth factor beta (TGF- β) superfamily, have been the most extensively studied, as they are potent osteoinductive factors that induce the mitogenesis and differentiation of mesenchymal stem cells and other osteoprogenitors^[35,11]. They are a very promising candidate for the treatment of bone diseases and defects, as a number of experimental and clinical trials demonstrate their safety and efficacy^[40-42]. However, the clinical application of BMPs is currently limited to the use of BMP-2 for open tibial fractures and spinal fusion, and BMP-7 (OP-1) for non-unions with limited indication for spinal fusion^[43,44], which were approved by the U.S. Food and Drug Administration in 2004. The clinical and scientific utility of bone tissue engineering largely depends on the ability to create scaffolds with specific characteristics that predictably direct cells to differentiate into the right phenotypes in a spatially and temporally defined pattern guided by molecular and physical factors.

HUMAN ADIPOSE-DERIVED MSCS

The combination of engineered scaffolds with recent developments in the emerging field of stem cell science may allow the use of stem cells to repair tissue damage and, eventually, to replace organs. MSCs are non-hematopoietic cells of mesodermal derivation that are present

in a number of postnatal organs and connective tissues. The stroma of bone marrow contains bone marrow mesenchymal stem cells (BMSCs) capable of differentiating into osteogenic, chondrogenic, adipogenic and endothelial lineages^[45-48], and thus is the most well studied source of MSCs for bone regeneration. Bone marrow transplantation is also being used clinically in combination with osteoconductive materials to augment bone healing^[9].

In the last few years, MSCs have been isolated from other tissue sources including trabecular bone^[49], synovium^[50], umbilical cord^[51], periodontal ligament^[52] and other dental tissues^[53], skeletal muscle, cord blood and skin^[54-56]. Although the stem cell populations derived from these sources are valuable, common problems include limited amounts of available tissues and low numbers of harvested cells, which necessitate at least some degree of *ex vivo* expansion or further manipulation before preclinical or clinical use. In contrast, a promising population of MSCs has been identified within adipose tissue, termed adipose-derived stem/stromal cells (ASCs) by the regenerative medicine community during the Second Annual International Fat Applied Technology Society Meeting in 2004. Human adipose tissue is ubiquitous and can easily be obtained in large quantities with little donor site morbidity or patient discomfort^[45], in contrast to the invasive and painful procedure for isolating BMSCs. Moreover, stem cell yields are greater from adipose tissue than from other stem cells reservoirs, a significant factor for use in regenerative medicine. As many 1×10^7 ASCs can routinely be isolated from 300 mL of lipoaspirate, with greater than 95% purity. ASCs comprise 2% of nucleated cells in processed lipoaspirate, with a yield of 5000 fibroblast colony-forming units (CFU-F) per gram of adipose tissue, compared with estimates of about 100-1000 CFU-F per milliliter of bone marrow^[57,58]. In general, cell isolation protocols include density gradient centrifugation of the collagenase-digested tissue (lipoaspirate or minced adipose tissue)^[57-61], followed by the seeding of the pelleted stromal vascular fraction (SVF) on monolayer culture plastics. The adherent cell population can then be expanded and used in a variety of assays.

Although the study of human ASCs (hASCs) is emerging, the standardization of isolation and culture procedures could improve quality control and facilitate comparisons between different studies. There are discrepancies in the results of studies from different laboratories due to differences in the methods and quality of hASC isolation, which can affect the composition of the initial cell culture, as well as in the procedures used to culture the cells. Cell culture basal medium, generally containing 10% fetal bovine serum, is often supplemented with epidermal growth factor, fibroblast growth factor-2 and/or TGF- β ^[58,62,63]. In addition, some protocols may recommend differing initial cell seeding densities, though evidence suggests that low seeding densities and subconfluent passaging are recommended^[64,65]. Other variables that may affect the composition of the initial isolated cell culture cannot be standardized, such as donor age, gen-

der, body mass index, ethnicity and medical history^[66]. It is therefore important to standardize hASC isolation and culturing methods to maximize the reliability and reproducibility of results from different laboratories.

COMPOSITION AND CHARACTERIZATION OF CULTURED hASCs

The SVF that is obtained from processed adipose tissue contains a highly heterogeneous cell population, including non-adherent cell populations. A complete characterization of SVF cell populations was done by Yoshimura *et al.*^[64] in which they identified endothelial cells, pericytes, blood-derived cells, fibroblasts, vascular smooth muscle cells and preadipocytes, in addition to the potential hASCs. Although the adherence of hASCs allows for their selection from the SVF during subsequent tissue culture passages, other cell types, such as fibroblasts, can also adhere to the culture plastic. Thus, other cell types, or subpopulations, may compromise the proliferation and/or differentiation potential of hASCs.

To reduce the heterogeneity of cultured ASCs, a washing procedure in the beginning of the cell culture can be used, as various cell types adhere to the plastic at different time points^[66]. Additionally, flow cytometric sorting or immunomagnetic separation with specific cell surface markers can be used to isolate and purify specific subpopulations of hASCs. However, there is considerable heterogeneity in commonly analyzed hASC surface markers, which can be modified by the culturing procedure. The cell phenotype can also be influenced by differences in the cell purification procedure and by the number of passages^[66-70]. Mitchell *et al.*^[59] identified hematopoietic lineage cells from the SVF using flow cytometry based on their expression of CD1, CD14, CD45 and other markers, which were lost with progressive passages. The loss of these markers indicates that they do not represent the adherent population. Moreover, SVF cells exhibit low levels of classic stromal cell markers (CD13, CD29, CD44, CD73, CD90, CD105, CD166) in the earliest stages of isolation, and assume a more homogeneous profile with consistently high levels of stromal markers after four to five passages, a temporal expression pattern that resembles what has been reported in human BMSCs^[54]. Work from Rada *et al.*^[71] demonstrated the complexity of hASC populations by showing that they are composed of several subpopulations that express different levels of hASC markers and exhibit distinctive differentiation potentials. In their study, hASC subpopulations were isolated using immunomagnetic beads specific for CD29, CD44, CD49, CD73, CD90, CD105, p75 and STRO-1, and cultured with specific chondrogenic or osteogenic media in order to evaluate their differentiation potential into these lineages. Among all the hASC subpopulations isolated, STRO-1-containing populations had the highest osteogenic potential, with the highest chondrogenic differ-

entiation potential in populations expressing CD29 and CD105. These data clearly demonstrate that SVF from adipose tissue is comprised of several stem cell subpopulations that exhibit *in vitro* chondrogenic and osteogenic differentiation profiles. Therefore, these subpopulations should be studied in order to select those most suitable for application in bone and cartilage regenerative medicine.

APPLICATION OF hASCs AND SCAFFOLDS FOR BONE TISSUE ENGINEERING

Since the discovery of hASC osteogenic differentiation, substantial progress has been made toward the use of these cells as an optimal source for bone regeneration. Although initial applications involved the direct administration of stem cells into the target fracture site, current paradigms using scaffolds loaded with stem cells are preferred as they provide support for cell colonization, migration, growth and differentiation^[72]. Combined with the support of a scaffold, the directed osteogenesis of hASCs confirms that adipose tissue is a promising autologous source of osteoblastic cells for bone regeneration. Utilization of hASCs in scaffolds for bone tissue engineering has been heralded as the alternative strategy of the 21st century to replace or restore the function of traumatized, damaged or lost bone.

In the last ten years, several cell characterization studies have extensively described the differentiation potential and function of hASCs *in vitro*^[58,62,67,69]. Many types of materials have been used to confirm these positive hASC characteristics, which have become available for scaffold-assisted bone regeneration in a variety of tissue engineering strategies. The importance of the scaffold in hASC osteogenesis has been demonstrated in a number of studies that recommend the use of different materials, including ceramics^[73], titan alloys^[74,75], natural and synthetic polymers^[76,77], and natural or semi-synthetic grafts^[78,79], with variable porosity, roughness, and methods of fabrication for future regenerative applications. A clear trend has emerged toward the use of composite scaffolds due to their superior properties and structures^[80-82] derived from the combination of two or more materials^[83-87].

The study of hASCs for bone regeneration has largely involved the insertion of biomaterials in rat and nude mouse models^[88-92]. Furthermore, a femoral defect in nude rats is available and calvarial defect models have been described for other species, to demonstrate the application and optimization of hASCs in regenerative medicine^[93-97]. However, relatively few reports are available concerning the utilization of hASCs for human bone tissue regeneration (Table 1). The first compelling evidence supporting the clinical application of an hASC scaffold to promote fracture healing was reported by Lendeckel *et al.*^[98] in 2004. In this work, a combination of autologous hASCs obtained from the gluteal region

Table 1 Summary of current representative bone tissue engineering models combined with human adipose-derived stem/stromal cells

| Scaffold origin | Type of scaffold | Active molecule | Study type | Differentiation pre-implant | Implant area | Species | Ref. |
|-----------------|------------------------------|------------------|-------------------------|-----------------------------|-------------------|---------|-------|
| Synthetic | BCP | - | <i>In vitro</i> | Yes | - | - | [73] |
| Synthetic | Ti6Al4V | - | <i>In vitro</i> | Yes | - | - | [74] |
| Synthetic | Ti6Al4V | - | <i>In vitro</i> | Yes | - | - | [75] |
| Semi-synthetic | CMCA | Sr ²⁺ | <i>In vitro</i> | Yes | - | - | [76] |
| Semi-synthetic | MPLA/CNC | - | <i>In vitro</i> | - | - | - | [77] |
| Semi-synthetic | Silk/fibroin | - | <i>In vitro</i> | Yes | - | - | [79] |
| Semi-synthetic | Apatite-coated CH/CS | rhBMP-2 | <i>In vitro</i> | Yes | - | - | [80] |
| Synthetic | Bioactive glass | - | <i>In vitro</i> | Yes | - | - | [81] |
| Synthetic | PCL | - | <i>In vitro</i> | Yes | - | - | [82] |
| Synthetic | PLA/ β -TCP | - | <i>In vitro</i> | Yes | - | - | [83] |
| Synthetic | PLA/ β -TCP | - | <i>In vitro</i> | Yes | - | - | [84] |
| Synthetic | BCP | - | <i>In vitro/In vivo</i> | Yes | Femur | Rat | [86] |
| Semi-synthetic | Collagen/PCL | - | <i>In vitro</i> | Yes | - | - | [87] |
| Synthetic | PEG/PCL | - | <i>In vitro/In vivo</i> | - | Subcutaneous | Rat | [88] |
| Synthetic | HA | - | <i>In vitro/In vivo</i> | - | Subcutaneous | Rat | [89] |
| Synthetic | HA/ β -TCP | - | <i>In vitro/In vivo</i> | - | Subcutaneous | Mouse | [90] |
| Synthetic | PCL/ β -TCP | - | <i>In vivo</i> | - | Subcutaneous | Rat | [91] |
| Synthetic | PLA | - | <i>In vivo</i> | Yes | Palate | Rat | [92] |
| Synthetic | HA/ β -TCP | - | <i>In vivo</i> | - | Femur | Rat | [93] |
| Synthetic | Apatite-coated PLGA | rhBMP-2 | <i>In vivo</i> | - | Calvaria | Mouse | [94] |
| Semi-synthetic | ABB/titanium | - | <i>In vivo</i> | - | Calvaria | Rabbit | [95] |
| Natural | Fibrin matrix | BMP-2 | <i>In vivo</i> | - | Femur | Rat | [96] |
| Synthetic | Carbon nanotube | rhBMP-2 | <i>In vitro/In vivo</i> | Yes | Subcutaneous | Mouse | [97] |
| Natural | Fibrin glue | - | <i>In vivo</i> | - | Calvaria | Human | [98] |
| Synthetic | β -TCP/titanium | rhBMP-2 | <i>In vivo</i> | - | Maxilla | Human | [99] |
| Synthetic | β -TCP | rhBMP-2 | <i>In vivo</i> | Yes | Mandibula | Human | [100] |
| Natural | ABB | PRP | <i>In vivo</i> | Yes | Maxilla/mandibula | Human | [101] |
| Synthetic | β -TCP/bioactive glass | rhBMP-2 | <i>In vivo</i> | Yes | Craniofacial | Human | [103] |

BCP: Biphasic calcium phosphate ceramics; Ti6Al4V: Titanium alloy; CMCA: Amidate carboxymethylcellulose; PLA: Poly(L-lactic acid); MPLA/CNC: Maleic anhydride grafted PLA/cellulose nanocrystals; CH/CS: Chitosan/chondroitin sulfate; PCL: Polycaprolactone; β -TCP: β -tricalcium phosphate; PEG: Polyethylene glycol; HA: Hydroxyapatite; PLGA: Poly(L-lactic acid-co-glycolic acid); ABB: Anorganic bovine bone; Sr²⁺: Strontium ion; rhBMP-2: Recombinant human bone morphogenetic protein; PRP: Platelet-rich plasma.

and bone grafts from the dorsal iliac crest was used for the treatment of a multi-fragment calvarial fracture in a 7-year-old girl. An autologous fibrin glue was applied using a spray adapter to keep the cells in place, and post-operative healing was uneventful after three months. In 2009, Mesimäki *et al.*^[99] described a novel method to reconstruct a major maxillary defect in an adult patient using autologous hASCs that were produced in clean room facilities free of animal-derived reagents, combined with recombinant human BMP-2 and β -TCP granules. The patient's healing was also clinically uneventful in this case, thus paving the way for extensive clinical trials using ASCs in custom-made implants for the reconstruction of bone defects. Moreover, the use of autologous cells, handled and prepared without animal-derived materials with good manufacturing practices in standard clean rooms, demonstrates that these cells can be considered safe for applications in tissue regeneration, according to the clinical cell therapy safety standards of the European Union.

Defects of the skull and jaws have been successfully reconstructed or their healing has been accelerated by the use of hASCs^[98-102], extending our limited knowledge regarding the potential use of hASCs for osseous tissue repair and regeneration. Work published in 2012 by Sándor demonstrates the synergistic effect of hASCs, resorbable

scaffolds (β -TCP and bioactive glass) and growth factors (BMP-2), in the treatment of 23 patients with craniofacial osseous defects^[103]. He has established the utility of hASCs in combination with biomaterials in 85% of the cases followed after bone reconstruction, though the long-term success of this procedure needs to be verified using a large sample.

CONCLUSION

The emerging application of hASCs on engineered scaffolds for bone tissue regeneration represents the most exciting challenge for the scientific community in future translational medicine. The ability to obtain a large quantity of MSCs from easily accessible adipose tissue, combined with the growing research on new biomaterials incorporating bioactive molecules such as drugs and growth factors, opens the way to new therapeutic applications. Although clinical trials have demonstrated the use of hASCs for the reconstruction of craniofacial defects in humans, there are many aspects that need to be examined and resolved. Further investigations are needed to standardize procedures for harvesting, isolating, cultivating and preparing hASCs for clinical applications. The differences in currently applied techniques make

comparisons across studies difficult. Moreover, the lack of guidelines for the proper utilization of different bone scaffold materials may provoke safety concerns, impeding clinical trials and the translation of scaffold technologies to the clinical environment. Prospective randomized clinical trials are needed to identify clear indications for and to demonstrate clinical outcomes of the hASC therapies. Ethical and safety concerns must be resolved to prevent human testing as the first stage in novel scaffold development.

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P- Reviewers: Jun Y, Liu L, Maraldi T **S- Editor:** Ma YJ

L- Editor: A **E- Editor:** Zhang DN



WJSC 6th Anniversary Special Issues (2): Mesenchymal stem cells

Purinergic receptors and nucleotide processing ectoenzymes: Their roles in regulating mesenchymal stem cell functions

Sonia Scarfi

Sonia Scarfi, Department of Earth, Environment and Life Sciences, University of Genova, 16132 Genova, Italy

Author contributions: Scarfi S solely contributed to this paper. Correspondence to: Sonia Scarfi, PhD, Assistant Professor of Molecular Biology, Department of Earth, Environment and Life Sciences, University of Genova, Via Pastore 3, 16132 Genova, Italy. soniascarfi@unige.it

Telephone: +39-010-35338227 Fax: +39-010-35338227

Received: October 28, 2013 Revised: February 26, 2014

Accepted: March 11, 2014

Published online: April 26, 2014

Abstract

Human mesenchymal stem cells (MSCs) are a rare population of non-hematopoietic stem cells with multi-lineage potential, originally identified in the bone marrow. Due to the lack of a single specific marker, MSCs can be recognized and isolated by a series of features such as plastic adherence, a panel of surface markers, the clonogenic and the differentiation abilities. The recognized role of MSCs in the regulation of hemopoiesis, in cell-degeneration protection and in the homeostasis of mesodermal tissues through their differentiation properties, justifies the current interest in identifying the biochemical signals produced by MSCs and their active crosstalk in tissue environments. Only recently have extracellular nucleotides (eNTPs) and their metabolites been included among the molecular signals produced by MSCs. These molecules are active on both ionotropic and metabotropic receptors present in most cell types. MSCs possess a significant display of these receptors and of nucleotide processing ectoenzymes on their plasma membrane. Thus, from their niche, MSCs give a significant contribution to the complex signaling network of eNTPs and its derivatives. Recent studies have demonstrated the multifaceted aspects of eNTP metabolism and their signal transduction in MSCs and

revealed important roles in specifying differentiation lineages and modulating MSC physiology and communication with other cells. This review discusses the roles of eNTPs, their receptors and ectoenzymes, and the relevance of the signaling network and MSC functions, and also focuses on the importance of this emerging area of interest for future MSC-based cell therapies.

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Key words: Mesenchymal stem cell; Purinergic receptors; Ectoenzymes; ATP; β -NAD; Adenosine; cADPR

Core tip: The multifaceted aspects of extracellular nucleotide metabolism (mainly ATP and β -NAD) on mesenchymal stem cell (MSC) surface has been addressed by basic researchers only recently, sometimes revealing unexpected pivotal roles for these molecules in specifying differentiation lineages and modulating MSC physiology and communication with other cells. This review discusses the roles of extracellular nucleotides, their receptors and ectoenzymes, and the relevance of their signaling network and MSC functions, and also focuses on the importance of this emerging area of interest for future MSC-based cell therapies.

Scarfi S. Purinergic receptors and nucleotide processing ectoenzymes: Their roles in regulating mesenchymal stem cell functions. *World J Stem Cells* 2014; 6(2): 153-162 Available from: URL: <http://www.wjgnet.com/1948-0210/full/v6/i2/153.htm> DOI: <http://dx.doi.org/10.4252/wjsc.v6.i2.153>

INTRODUCTION

Human mesenchymal stem cells (MSCs, also known

as marrow stromal cells) are a rare population of non-hematopoietic stem cells with multilineage potential originally identified in the bone marrow (BM)^[1,2]. BM-derived MSCs (BM-MSCs) are still considered the gold standard for MSC applications; nevertheless, the BM has several limitations as a source of MSCs, such as low frequency in this compartment, a painful isolation procedure and the loss of differentiation potential with donor's increasing age. Thus, there is growing interest in identifying alternative sources for MSCs. To this end, MSCs obtained from the adipose tissue^[3], dental pulp^[4], placenta and Wharton's jelly^[5] have gained much attention in recent times since they can be easily isolated from tissues without any ethical concerns and which would be otherwise discarded.

Due to the lack of a single specific marker, MSCs can be recognized and isolated by a series of features such as plastic adherence, a panel of surface markers, the clonogenic and differentiation abilities^[2,6,7]. They can be expanded *in vitro* for several passages without losing their lineage properties and are commonly considered the precursors of mesodermal cell types such as osteocytes, adipocytes and chondrocytes. Whether MSCs can differentiate to non-mesodermal cell types such as hepatocytes or neurons is still under debate^[8-10].

In the BM, MSCs play a key role in providing hemopoietic progenitors (HPs) with soluble factors essential to their proliferation and differentiation^[11]. Furthermore, MSCs possess immunoregulatory functions^[12]. Actually, a number of clinical trials are currently exploring the use of MSCs in cell-based therapies of various pathological conditions, such as graft *vs* host disease, renal, neurological and cardiovascular diseases^[13,14]. The clinical benefit of MSC-based cell therapy seems mostly related to MSC-derived soluble factors possessing immunomodulating, growth-supporting and/or antiapoptotic activities, as demonstrated on animal models^[12]. Furthermore, their differentiation and tissue regeneration potential have already been used in therapeutic clinical approaches involving tissue engineering and gene therapy^[15,16].

In vitro differentiation of MSCs requires the activation of specific transcription factors, regulatory genes and signal cascades^[17,18]. Adipogenesis induction gives rise to preadipocytes with cytoplasmic accumulation of lipid droplets and release of adipokines and extracellular matrix-associated proteins^[19]. On the other hand, osteogenesis-induced osteoblasts secrete mineralized extracellular matrix, with high levels of calcium phosphate forming hydroxyapatite crystals^[20]. Since both osteoblasts and adipocytes originate from a common MSC precursor, it seems obvious that osteoblast and adipocyte differentiation pathways are regulated jointly^[21].

Although a plethora of studies^[22-24] have shown that many substances, as well as mechanical agents, are causally related to these differentiation processes, the mechanisms involved are not yet completely defined. However, a large body of evidence supports the idea that there is an inverse relationship between the differentiation of MSCs to osteoblasts or to adipocytes, *i.e.*, conditions favoring

the differentiation towards one lineage impair the differentiation to the other lineage. This seems to occur during attainment of peak bone mass^[25,26] for instance, when adipogenesis in the BM is inhibited, favoring osteogenesis, or in aging population^[27], when the BM adipocytes are predominant in respect to other cells of mesodermal origin.

MSCs regulate their fate through the complex integration of autocrine and paracrine extracellular signals (*i.e.*, hormones, cytokines, nucleotides, xenobiotics) enabling the cells to sense the external milieu and to establish a fine communication with the surrounding cell population. Hence, they calibrate their response (differentiation, immunomodulation, proliferation, migration) on the basis of the necessities of the tissue in which they reside or on the organism's physiopathological conditions.

From an evolutionary point of view, nucleotides are considered among the most ancient molecules with biological activity and they are in fact used by living organisms for many different purposes: energy metabolism, storage of genetic information, signal transduction and extracellular communication. Nucleotides can be released or leaked into the extracellular milieu by virtually every cell in the body. Extracellular nucleotides (eNTPs) comprise both extracellular purines (ATP, ADP, β -NAD, ADPR and cADPR) and extracellular pyrimidines (UTP and UDP). Once outside the cell, they either serve as signaling molecules by binding specific P2 purinergic receptors (P2X or P2Y) or are converted into other active nucleotides^[28] and finally degraded to the related nucleosides. Nucleosides, mainly adenosine, can then bind different types of P1 purinergic receptors^[29]. Nucleotide extracellular metabolism is mediated by special proteins located on the outer surface of the plasma membrane that possess an enzymatic domain in the extracellular region, called ectoenzymes^[30]. Currently, there is an accumulating body of evidence indicating that the various ectoenzymes work in concert to dismantle eNTPs. Thus, in whatsoever milieu, the balance between nucleotides and nucleosides relies on the direct outflow of such molecules from transporters and channels in the plasma membrane^[31-33], as well as on the activity of the specific ectoenzymes present on the cell surface.

It is now well established that eNTPs mediate intercellular communication in virtually all tissues. They are one of the most important indicators of cell stress in the pericellular environment^[34] and the network of extracellular nucleotides/nucleosides serves multiple functions in a balanced and finely tuned fashion^[35-37].

MSCs possess a significant display of purinergic receptors and ectoenzymes on their plasma membrane^[38-40] and these cells have been reported to actively release nucleotides such as ATP and β -NAD upon certain stimuli^[39-42] (Figure 1). Thus, from their niche, these cell types give a significant contribution to the complex network of signaling involving eNTPs and its derivatives, and accumulating literature indicates that MSC functions are also autocrinally influenced by eNTPs affecting their differ-

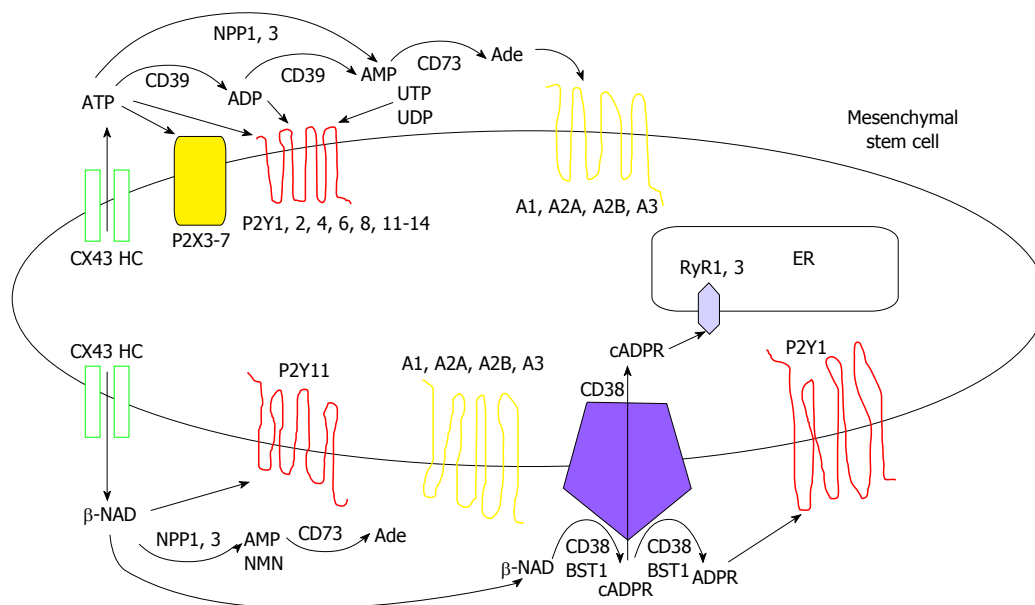


Figure 1 Surface network of purinergic receptors and nucleotide ectoenzymes on mesenchymal stem cells. On the basis of the recent findings, all the purinergic receptors and ectoenzymes whose presence has been ascertained on mesenchymal stem cells through qPCR analyses and/or demonstration of a clear physiological function (see text for references) are shown. Furthermore, both ATP and β -NAD stimulation mechanisms and metabolisms are summarized as an example of the finely tuned extracellular balance between nucleotides and nucleosides and their pleiotropic effects. CX43 HC: CX43 hemichannels; Ade: Adenosine; NMP: Nicotinamide monophosphate; ER: Endoplasmic reticulum; RyR1,3: Ryanodine receptors 1 and 3.

entiation properties as well as their immunomodulatory activity.

Here, the role of eNTPs, its receptors and converting ectoenzymes and the relevance of this signaling network in MSC functions are discussed, also focusing on the importance of this emerging area of interest for future MSC-based cell therapies.

P1 RECEPTORS IN MSC

Purinergic receptors (PRs) are plasma membrane receptors specific for adenosine, purine and pyrimidine nucleotides, which are expressed throughout the mammalian organism in all cell types. Upon their physiological agonist, Ps can be classified into P1 receptors, whose natural ligand is adenosine, and P2 receptors, whose recognized natural ligands are nucleotides (mainly ATP and UTP, see Figure 1)^[29]. The adenosine receptors are G protein-coupled seven-transmembrane proteins, further classified into the A₁R, A_{2A}R, A_{2B}R and A₃R subtypes^[29]. In particular, the P1 signaling pathway involves cyclic adenosine monophosphate (cAMP) synthesis upon A_{2A}R and A_{2B}R activation, or cAMP inhibition upon A₁R and A₃R activation^[29].

Adenosine can be directly released by cells^[31,32] or generated by the dephosphorylation of adenine nucleotides, which in many tissues are dephosphorylated to AMP by the ectonucleoside triphosphate phosphohydrolase (CD39). AMP is then further dephosphorylated to adenosine by ecto-5'-nucleotidase (CD73)^[30]. The resulting adenosine has an essential role in the attenuation of inflammation and in damaged tissue healing. Furthermore, it mediates diverse cardioprotective, neuroprotective, vasodilatory and angiogenic responses^[43-46], in many

cases counteracting the ATP inflammatory/stress signal triggered by P2 purinergic receptor activation.

Several studies in the last decade have established the presence of both P1 and P2 receptor family members on MSC surface (Figure 1), trying to elucidate their role in the homeostasis and differentiation properties of this cell type both *in vitro* and *in vivo*.

Adenosine receptor presence and function on MSC surface was first evidenced by Evans and coworkers^[47], demonstrating the formation of extracellular adenosine by an osteoprogenitor cell line and by MSCs for the first time. On that occasion, the presence of all four adenosine receptor subtypes, especially A_{2B}R, was ascertained, demonstrating a causal role of their activation in active secretion of the inflammatory cytokine IL-6 and of the osteoclastogenesis inhibitory factor osteoprotegerin. These data indicate that adenosine production, as well as its activity through adenosine receptors, could be a potential target for pharmacological interventions in the bone for many diseases, including osteoporosis^[48].

A further study^[49] demonstrated that adenosine signaling affects proliferation and development of BM-MSCs. Perhaps the most significant finding of this work is the demonstration that adenosine A_{2A}R deletion or blockade diminishes the number of colony-forming unit-fibroblasts (CFU-F) in cultured BM-MSCs. Thus, the authors speculated that adenosine, targeting the A_{2A}R, could increase the proliferation of MSCs, as also reported for other cell types^[50,51]. Alternatively, they suggest that since A_{2A}R stimulation has been shown to diminish apoptosis in other cell types^[52,53], an increased survival of MSCs could enhance CFU-F yield from freshly isolated adult stem cells. Interestingly, they confirmed that A_{2A}R and

CD73 are coordinately regulated in MSCs as in other cell types^[54], strengthening the idea of an active crosstalk in adenosine signaling between the adenosine receptor and the ectoenzymes able to generate the nucleoside in the pericellular space.

More recently, both *in vitro*^[55] and *in vivo*^[56,57] studies have evaluated the contribution of adenosine signaling in MSC differentiation. Gharibi *et al*^[55] in particular investigated the *in vitro* expression of adenosine receptor subtypes and the adenosine metabolism as they differentiated MSCs into osteoblasts or adipocytes. They found differential expression of the adenosine receptor subtypes during differentiation as well as in mature cells. Differential expression was related both to the progression of lineage specificity (A_{2B}R dominant in osteoblast differentiation; A₁R and A_{2A}R in adipogenic differentiation) and to the maintenance of specialized features in the two lineages (A_{2A}R essential to ALP expression in osteoblasts; A₁R involved in lipogenic activity in adipocytes).

These data suggest that useful strategies could include the targeting of the adenosine signaling pathway in cases of diseases associated with an imbalance in the differentiation and function of these two lineages. This research will be useful in preventing or treating conditions with insufficient bone or excessive adipocyte formation^[25-27].

Finally, an essential role of adenosine signaling through A_{2B}R in *in vivo* osteoblast differentiation and bone formation seems to be definitely confirmed in recent reports^[56,57]. Both studies suggest that the pharmacological stimulation of this signaling pathway may enhance bone density and bone fracture healing in variously compromised situations, such as non-healing fractures in osteoporosis^[56] and osteolytic bone lesions in multiple myeloma^[57]. In general, all the above-mentioned studies confirm an essential, functional role of extracellular adenosine and its signaling pathway in MSC physiology, homeostasis and intervention in bone and adipose tissue reconstitution, allowing the identification of new pharmacological targets.

P2 RECEPTORS IN MSC

Extracellular nucleotides have been definitely recognized as autocrine/paracrine signaling molecules^[58] released from cells in response to physiological and pathological stimulation, such as mechanical stress, hypoxia, inflammation and other agonists. The mechanisms of nucleotide release comprise exocytosis, ATP-binding cassette transporters, connexin hemichannels and voltage-dependent anion channels^[33]. Many signaling roles for nucleotides have been demonstrated in several tissues, including: neurotransmission^[33]; rhythm regulation in the myocardium^[59]; gastrointestinal and liver function^[60]; regulation of epithelial cell responses^[61]; blood flow distribution, oxygen delivery and endothelial barrier integrity^[62,63]; immune responses^[43,64]; and activation of platelets at sites of vascular injury^[65]. Besides acute signaling events, there is increasing evidence that purines and pyrimidines also

have potent long-term roles in cell proliferation and growth^[34], induction of apoptosis and anticancer activity^[43] and atherosclerotic plaque formation^[66]. These effects are mediated by extracellular stimulation of P2 purinergic receptors, of which two major subfamilies, P2X and P2Y, have been described. The ionotropic P2X receptors are ligand-gated channels that gate extracellular cations in response to ATP and comprise seven receptor subtypes (P2X₁-P2X₇)^[29]. Conversely, the metabotropic P2Y receptors are G-protein-coupled proteins that alternatively couple to G_q (P2Y₁₋₂, P2Y₄, P2Y₆ and P2Y₁₁) and therefore activate phospholipase C- β , or to G_i (P2Y₁₂₋₁₄), that inhibit adenylyl cyclase and regulate ion channels^[29]. Notably, P2Y₁₁ receptor is dually coupled to phospholipase C and adenylyl cyclase stimulation.

P2Y receptors can be divided into: (1) adenine nucleotide-preferring receptors, mainly responding to ATP and ADP (P2Y₁, P2Y₁₁₋₁₃); (2) uracil nucleotide-preferring receptors (P2Y₄ and P2Y₆) responding to both UTP and UDP; (3) receptors of mixed selectivity (P2Y₂); and (4) nucleotide sugar-preferring P2Y₁₄ receptor responding to UDP-glucose and UDP-galactose^[29]. Finally, the P2Y₁ and P2Y₁₁ receptors have also been described as β -NAD receptors with diverse functional activities^[64,67,68]. In particular, P2Y₁ is also a receptor for ADPR, a β -NAD metabolite generated by the cycling/hydrolyzing activity of CD38 and BST1/CD157 ectoenzymes^[36,68].

P2 receptors and the related activating nucleotides have been the object of investigation in relation to MSC functions (Figure 1) only recently. In earlier reports^[41,42], the spontaneous release of ATP from MSCs *via* gap junction hemichannels was assessed, on one occasion demonstrating a direct stimulation of P2Y₁ receptor triggering intracellular Ca²⁺ oscillations^[41], while showing the concurrent activation of P2X and P2Y receptors by ATP in another, resulting in a modulation of the proliferation rate at early passages of MSC cultivation^[42].

The presence of the G-protein coupled P2Y₂ receptor has also recently been demonstrated on rat MSCs, as well as its activation by the preferred agonist UTP inducing intracellular Ca²⁺ oscillations or elevating Ca²⁺ levels depending on cell density, and suggesting that these different Ca²⁺ responses in MSCs may be correlated with cell cycle progression^[69].

More recently, different investigations have been directed to the pleiotropic effects of P2 receptor activation by ATP, focusing on MSC functionality in the hematopoietic niche and on the differentiation properties of these cells^[70-73]. In a recent paper analyzing the effects of ATP on MSC functions, Ferrari and collaborators^[70] observed a downregulation of genes related to cell proliferation and anti-inflammatory cytokines and concurrently an upregulation of pro-inflammatory cytokines and cell migration related genes. These data confirm the *in vitro* inhibitory activity of ATP on MSC proliferation, as already observed in a previous work^[42], and demonstrate an *in vivo* potentiated homing capacity to the BM of ATP-pretreated MSCs that could be useful in supporting thera-

pies for BM engraftment.

The role of ATP during MSC differentiation has also been addressed in the last years^[38,71-73]. The related studies indicate that: (1) a variety of metabolically active P2X (P2X3-7) and P2Y (all subtypes) receptors are detectable in MSCs (Figure 1) and are up- or downregulated during adipogenic and osteogenic differentiation. In particular, P2Y₄ and P2Y₁₄ seem to be important for the onset of MSC commitment (regulated both in adipogenic and in osteogenic differentiation), P2Y₁ and P2Y₂ are downregulated in osteogenic differentiation, while P2Y₁₁ is significantly upregulated in adipogenic commitment^[38]; (2) significant ATP release by MSCs, especially observed during shockwave treatment, is able to promote osteogenic differentiation through P2X₇ receptor activation with a significant positive impact in bone healing^[71]; and (3) ATP treatment modulates the expression of several genes governing adipogenic and osteogenic differentiation of MSCs which can be tuned from one lineage to the other by specific culture conditions in the presence of this nucleotide^[72]. In addition, evidence from Ciciarello and coworkers^[72] seems to indicate that ATP is able to promote adipogenesis through its triphosphate form, while osteogenic differentiation seems to be induced by its nucleoside adenosine, as also proposed by others^[55-57], resulting from ATP degradation by the CD39/CD73 system or directly released by cells. Thus, based on these findings, it is proposed that adipogenic differentiation is mainly mediated by activation of P2Y₁ and P2Y₄ receptors, while stimulation of the adenosine receptor subtype A_{2B}R is involved in osteogenic differentiation. In another recent investigation, P2Y₁₃ receptor has been implicated in *in vivo* osteogenic differentiation through the study of impaired bone turnover in a P2Y₁₃-KO mouse model^[73]. In this study, P2Y₁₃ activation and consequent osteogenic induction, at the expenses of adipocyte differentiation, seems to be orchestrated by ADP stimulation and not ATP, thus complicating the picture of nucleotide involvement in the MSC differentiation process.

Together, all these data provide new insights into the molecular regulation of MSC differentiation and demonstrate the necessity to further deepen this topic of investigation in order to better understand the pleiotropic effects of ATP and its derivatives on MSC differentiating abilities and to finally merge current, sometimes contrasting, observations.

Besides ATP and its derivatives, the dinucleotide β -NAD has also been shown to activate P2 receptors (P2Y₁ and P2Y₁₁), its effects mainly investigated in cell types of the immune system and in neuromuscular transmission^[64,67,68]. Interestingly, it has been recently demonstrated that this nucleotide also has a significant impact on MSC functions^[39]. In particular, β -NAD can be released in the extracellular milieu upon stimuli able to open CX₄₃ hemichannels in MSCs (*i.e.*, low extracellular calcium, shear stress, inflammatory stimuli) and this release is functional to increase MSC proliferation, migration and production of immunomodulatory cyto-

kines without compromising the differentiation abilities of these cells. Such effects are observable in MSCs in the presence of β -NAD, both extracellularly added or autocrinally released, and are dependent on P2Y₁₁ activation (Figure 1). Thus, as for adenosine and its preferential receptors, β -NAD through its specific P2Y₁₁ target can also exert a beneficial role in modulating cell protective functions relevant to MSC-based cell therapies.

NUCLEOTIDE-DEGRADING

ECTOENZYMES IN MSC

Ectoenzymes are a family of cell surface molecules whose catalytic domain lies in the extracellular region. A subset of this family, the nucleotide-metabolizing ectoenzymes, are key components in the regulation of the extracellular balance between nucleotides and nucleosides, together with equilibrative transporters and channels enabling direct outflow of these molecules^[31-33].

Following the signal transduction, eNTPs need to be rapidly inactivated, mainly to adenosine which in turn has other pharmacological/counteracting properties. Nucleotide hydrolyzing enzymes include the nucleoside triphosphate diphosphohydrolase (NTPDase) family^[74], the nucleotide pyrophosphatase/phosphodiesterase (NPP) family^[75,76] and ecto-5'-nucleotidase^[77].

NTPDases are capable of hydrolyzing a broad range of nucleoside tri and diphosphates, but not monophosphates. Namely, half of the eight different NTPDase genes (NTPDase1, 2, 3 and 8) are expressed as cell surface-located enzymes. The prototypic member of the NTPDase family is the cell activation antigen CD39 (NTPDase1)^[78] whose expression has been demonstrated on a variety of cells, vascular endothelial and smooth muscle cells^[79], exocrine pancreas^[80], dendritic cells^[81], lymphocytes^[82] and recently MSCs^[40] (Figure 1). On the other hand, The NPP family consists of seven related ectoenzymes possessing surprisingly broad substrate specificity capable of hydrolyzing pyrophosphate and phosphodiester bonds generating, for instance, AMP from ATP, or AMP and NMN (nicotinamide monophosphate) from β -NAD^[83]. The first three members of this family, NPP1-3, hydrolyze various nucleotides and are therefore relevant in the purinergic signaling cascade^[75]. In particular, human NPP1 is highly expressed in bone and cartilage and less in other organs and tissues^[75]. In bone tissue, NPP1 acts as a P_{Pi}-generating ectoenzyme ensuring normal bone matrix mineralization and soft tissue calcification^[84]. The presence and enzymatic activity of NPP1 and NPP3 has been recently demonstrated in MSCs^[39] (Figure 1), attesting to the existence of an active and complex extracellular nucleotide metabolism in these cells once more.

Extracellular AMP, generated either from ATP or from β -NAD degradation, can be further metabolized by the ecto-5'-nucleotidase CD73 releasing adenosine^[77]. CD73 is expressed to a variable extent in different tissues, with abundant expression in the colon, kidney, brain,

liver, heart, lung and large vessel endothelium^[77,85,86]. Notably, CD73 is coexpressed with CD39 on the surface of CD4⁺ T_{reg} cells, being an important constituent of the suppressive machinery that converts ATP to the anti-inflammatory mediator adenosine with subsequent inhibition of T cell proliferation and cytokine secretion^[82]. Interestingly, this situation closely resembles that of MSCs whose immunomodulatory activity has also been recently related to the CD39/CD73 enzymatic axis actively producing extracellular adenosine, also with paracrine/immunosuppressive effects in these cells^[40] (Figure 1). These data may indicate a key role of adenosine in switching the stem cell properties of MSCs towards an immunomodulatory/pro-healing phenotype which in so many occasions has demonstrated its utility^[14], suggesting a possible pharmacological use of adenosine in potentiating these features in cell-based therapies.

Although CD73 is one major cell surface marker defining MSCs according to the International Society for Cellular Therapy (ISCT), it is surprising how little is known about the enzymatic function of CD73 in these cells^[87]. Notably, CD73 expression is regulated by Wnt- β -catenin signaling, one of the major pathways in stem cell and bone homeostasis^[88]. Recently, CD73 has been reported to be involved in osteogenic differentiation where loss of this ectoenzyme causes a lower bone mineral content in mouse trabecular bone with decreased osteocalcin serum levels and reduced expression of osteogenic mRNA markers^[89]. Little is known about the role of CD73 in chondrogenesis, except that CD73 is downregulated during differentiation^[90,91]. In a recent investigation, further insights into CD73 in relation to osteogenic/chondrogenic differentiation have been added to the literature^[92] using an *in vitro* model of MSCs differentiated after cyclic-compressive loading. In these conditions, Ode *et al.*^[92] observed increased chondrogenic differentiation accompanied by a decreased CD73 expression; in addition to that, they found that inhibition of CD73 enzymatic activity further increased chondrogenic matrix deposition. In contrast, in the same experimental setting but in conditions of osteogenic induction and in the presence of a CD73 inhibitor, MSCs showed a reduction of osteogenic marker expression and of mineral matrix deposition, suggesting that CD73 and its metabolite adenosine, as well as P1 receptors, belong to alternative differentiation pathways in MSCs whose expression enhance (osteogenic) or inhibit (chondrogenic) specific cell lineages. So far, and to our knowledge, no investigations have been undertaken to test the role of CD73 as an ectoenzyme during adipocyte differentiation in MSCs. Since it is known that this protein is expressed on mature adipocytes and that CD73-derived adenosine is functionally involved in body fat homeostasis, mainly inhibiting lipolysis^[93], it is highly probable that this topic will be eventually addressed in the near future, hopefully adding new bricks to the comprehension of adipose tissue formation mechanism and complex homeostasis.

Another well-known class of ectoenzymes are β -NAD-

consuming surface proteins, primarily represented by the CD38-BST1 system^[36]. The CD38 gene codes for a type II transmembrane protein distributed in a broad range of cell types^[36]. The other member of the family is BST1/CD157, which differs in structure and tissue distribution^[36]. The dual cycling/hydrolyzing metabolism of β -NAD by CD38 leads to the generation of potent intracellular Ca²⁺ mobilizing compounds, including cADPR (from cycling activity) and ADPR (from both cycling and hydrolyzing activities)^[94].

It has been recently demonstrated that MSCs show both a significant β -NAD release from CX43 hemichannels and an active extracellular metabolism of this dinucleotide due not only to NPP1/3 and CD73 degradation to adenosine, but also to CD38-BST1 secondary metabolite production^[39] (Figure 1). The release of β -NAD in the BM milieu from MSCs is essential not only for autocrine physiological and immunomodulatory functions^[39], but also for HP proliferation and stem cell niche maintenance^[95-97]. Thus, the bilateral nucleotide network generated upon β -NAD release from MSCs in the BM comprises the following enzymatic steps and functional effects: (1) β -NAD released in the BM milieu directly stimulates MSC and HP functions through the purinergic receptor P2Y₁₁^[39,98]; (2) extracellular β -NAD can be a substrate of various ectoenzymes present either on MSCs, possessing both NPP-CD73 and CD38-BST1 ectoenzymes, or on HP displaying the CD38 activity^[39,99-101]; and (3) these enzymatic activities are able to release secondary metabolites in the BM milieu, namely adenosine, ADPR and cADPR, which again can exert autocrine and paracrine regulatory effects on MSCs and HPs^[28,39,99-102]. Indeed, nanomolar/low micromolar concentrations of cADPR, such as those produced by variously stimulated CD38-BST1 positive BM cells^[99,100], significantly increase the *in vitro*^[99-102] and *in vivo*^[96,103] proliferation and engraftment of human HPs and MSCs, indicating a relevant role for this network of nucleotide-responding and nucleotide-metabolizing proteins in the BM.

CONCLUSION

The increasingly recognized role of MSCs in the homeostasis of mesodermal tissues through their proliferation/differentiation properties and in the regulation of hemopoiesis and cell-degeneration protection through the production of paracrine signals justifies the current interest in identifying the biochemical signals produced by MSCs and their active crosstalk in tissue environments. Only recently, such signals have been shown to also belong to the network of eNTPs and their metabolites produced by specialized ectoenzymes^[39,40,87,89-92,99] and active on both ionotropic^[41,71] and metabotropic receptors^[38,39,42,69,70,72,73] in MSCs (Figure 1). Researchers have just begun to uncover the multifaceted aspects of the eNTP network on MSCs, sometimes revealing unexpected pivotal roles for these molecules and their derivatives in specifying differentiation lineages and in modulating MSC physiology and

signaling towards other cells.

Thus, while extracellular β -NAD and cADPR signaling seem to be more related to MSC homeostasis/proliferation and to the maintenance of an optimal stem cell niche for the harmonious growth of HPs and MSCs in the BM^[39,95-97,99-103], ATP and adenosine demonstrate more pleiotropic roles affecting both the immunomodulatory properties of these cells and their lineage commitment. In particular, the nucleotide has been more frequently associated with inhibition of proliferation^[42,69], pro-inflammatory and cell migration properties^[70], as well as to an enhancement of both adipogenic and osteogenic differentiation^[38,71-73] in MSCs. Conversely, adenosine has been associated with an autocrine protective^[49] as well as a paracrine immunosuppressive^[40] activity counteracting ATP stimulation. Furthermore, in MSCs, adenosine seems to have a significant role in alternative lineage specification by concomitant promotion of bone formation^[55-57,72,90-92] and inhibition of cartilage production^[92]. In agreement with this, it has been suggested that the positive effect of ATP on osteocyte differentiation could be just a consequence of adenosine production on MSCs through surface activity of degrading ectoenzymes^[72].

The prosecution of these studies, on the basis of what has been discovered until now and is summarized in this review, seems to be essential for a thorough comprehension of MSC physiology and in the future will enable researchers to precisely define the involvement of these cells in tissue repair and to finally address the current clinical issues related to their use in cell-based therapies.

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P- Reviewers: Li GR, Pedata F, Yue JB **S- Editor:** Song XX

L- Editor: Roemmele A **E- Editor:** Zhang DN



WJSC 6th Anniversary Special Issues (2): Mesenchymal stem cells

Mesenchymal stem cells help pancreatic islet transplantation to control type 1 diabetes

Marina Figliuzzi, Barbara Bonandrini, Sara Silvani, Andrea Remuzzi

Marina Figliuzzi, Barbara Bonandrini, Sara Silvani, Andrea Remuzzi, Department of Biomedical Engineering, IRCCS-Istituto di Ricerche Farmacologiche Mario Negri, 24126 Bergamo, Italy

Andrea Remuzzi, Department of Industrial Engineering, University of Bergamo, 24044 Dalmine, Bergamo, Italy

Author contributions: All the authors collaborated in the manuscript draft and approved the final text.

Correspondence to: Dr. Marina Figliuzzi, Department of Biomedical Engineering, IRCCS-Istituto di Ricerche Farmacologiche Mario Negri, Via Stezzano, 87, 24126 Bergamo, Italy. marina.figliuzzi@marionegri.it

Telephone: +39-35-4213311 Fax: +39-35-319331

Received: October 29, 2013 Revised: December 20, 2013

Accepted: March 3, 2014

Published online: April 26, 2014

Key words: Mesenchymal stem cell; Islet transplantation; Type 1 diabetes; Vascularization; Immune modulation

Core tip: Type 1 diabetes is caused by a cell-mediated autoimmune destruction of pancreatic β cells. The transplantation of pancreatic islets provides a cure for this disorder. In this review, we first summarize the current knowledge on the pathogenesis of type 1 diabetes and on the therapeutic options for this disorder. Next we discuss the impact of mesenchymal stem cells on vascularization and immunomodulation of islet transplantation.

Figliuzzi M, Bonandrini B, Silvani S, Remuzzi A. Mesenchymal stem cells help pancreatic islet transplantation to control type 1 diabetes. *World J Stem Cells* 2014; 6(2): 163-172 Available from: URL: <http://www.wjgnet.com/1948-0210/full/v6/i2/163.htm> DOI: <http://dx.doi.org/10.4252/wjsc.v6.i2.163>

Abstract

Islet cell transplantation has therapeutic potential to treat type 1 diabetes, which is characterized by autoimmune destruction of insulin-producing pancreatic islet β cells. It represents a minimal invasive approach for β cell replacement, but long-term blood control is still largely unachievable. This phenomenon can be attributed to the lack of islet vasculature and hypoxic environment in the immediate post-transplantation period that contributes to the acute loss of islets by ischemia. Moreover, graft failures continue to occur because of immunological rejection, despite the use of potent immunosuppressive agents. Mesenchymal stem cells (MSCs) have the potential to enhance islet transplantation by suppressing inflammatory damage and immune mediated rejection. In this review we discuss the impact of MSCs on islet transplantation and focus on the potential role of MSCs in protecting islet grafts from early graft failure and from autoimmune attack.

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INTRODUCTION

Type 1 diabetes results from the autoimmune destruction of insulin-producing pancreatic islet β cells and is usually diagnosed in children and young adults. β cell replacement therapies using either pancreas or islet transplantation represent a therapeutic alternative to the administration of exogenous insulin.

Islet transplantation is advantageous compared with whole pancreas transplantation because it is relatively non-invasive. However, a decline in islet cell survival, after transplantation, remains a significant obstacle in successful islet transplantation. It has been suggested that the complete lack of islet vasculature and hypoxic environment in the immediate post-transplantation period contribute to the acute loss of islet by ischemia^[1]. Moreover, graft failure continues to occur because of

immunological rejection, despite the use of potent immunosuppressive agents.

Mesenchymal stem cells (MSCs) are non-hematopoietic multipotent stromal cells that can differentiate in a variety of tissues^[2]. The ability of MSCs to secrete trophic and angiogenic factors may help to prevent early islet damage and assist islet engraftment. MSCs may also attenuate autoimmunity through their immunomodulatory properties while secreting cytokines to control autoreactive T cells. All these properties could be used for *in vivo* co-transplantation to improve islet engraftment^[3]. Here we discuss the impact of MSCs on islet transplantation from both early graft failure and from autoimmune attack, to prevent immune rejection and promote long-term islet allograft survival.

PATHOGENESIS OF TYPE 1 DIABETES

Type 1 diabetes is a fast-growing global problem with enormous social, health, and economic consequences. This metabolic disorder is characterized by the irreversible destruction of insulin-secreting β cells. Death of the pancreatic β cells is associated with hyperglycaemia, which is the main determinant of long-term complications in diabetic patients. Exogenous insulin administration is required to control glucose levels in the blood. The pancreatic islets are the targets of an autoimmune assault, where islets are invaded by mononuclear cells that cause an inflammatory reaction called “insulinitis”, leading to loss of most of β cells. β cell death in the course of insulinitis is probably caused by direct contact with activated macrophages and T-cells, and/or exposure to soluble mediators secreted by these cells, as cytokines, nitric oxide (NO), and oxygen free radicals^[4].

Type 1 diabetes is a multifactorial disease where a genetic predisposition combines with environmental factors to induce the activation of the specific autoimmune destruction of β cells. Different known genetic risk factors can predict type 1 diabetes but autoantibodies are the most important preclinical markers. Autoantibodies include: islet cell autoantibodies, autoantibodies to insulin, autoantibodies to GAD (GAD 65), and autoantibodies to the tyrosine phosphatases IA-2 and IA-2 β . In 85%-90% of patients affected by juvenile diabetes, these autoantibodies are detectable^[5]. Several genetic loci have been associated with type 1 diabetes but the HLA (human leukocyte antigen) region, located on chromosome 6p, with its multiple genes is the strongest link to immune-mediated diabetes susceptibility. More than 200 identified genes are located in the HLA region, over half of which are predicted to be expressed^[6]. Non-genetic factors also contribute to the risk of type 1 diabetes. This is supported by the fact that the overall concordance rate for type 1 diabetes among monozygotic twins is only about 10%-40%^[7]. Environmental factors play a substantial role in the development of type 1 diabetes. They include specific infectious agents, dietary factors,

perinatal factors, socioeconomic factors, and psychosocial factors^[8].

THERAPEUTIC OPTIONS FOR TYPE 1 DIABETES

The treatment of type 1 diabetes mellitus includes different therapeutic approaches. The aim of clinical intervention is to arrest or prevent the β cell destruction due to autoimmunity, reverse this process and restore normal blood glucose level and immune homeostasis. Insulin therapy was the first therapy and represented the primary breakthrough treatment for type 1 diabetes, however, frequent hyper- and hypo-glycaemia episodes seriously affect the quality of life of these patients. Recent technological innovations such as insulin analogue formulation, devices for insulin delivery and glucose monitoring systems have allowed diabetic patients to improve their glycaemic control^[9]. Intensive insulin therapies *via* insulin pens, subcutaneous or intraperitoneal insulin infusions using pumps reduce the onset and progression of diabetic complications, risks of hypo- or hyper-glycaemia, and increase the patient's quality of life.

β cell replacement is the only way to restore euglycaemia and ameliorate the progression of diabetic complications. Pancreas or pancreatic islet transplantation represents therapeutic alternatives to the administration of exogenous insulin to treat patients with type 1 diabetes. At the current time pancreas transplantation can produce long-term exogenous insulin independence, however, it remains a major surgical undertaking, associated with sizeable early morbidity and mortality, and with mandatory life-long immunosuppression^[10]. Islet transplantation also offers glycaemic control and prevention of hyperglycaemia without the need for exogenous insulin administration^[11]. As islets make up only 1%-2% of the pancreas, islet transplantation provides a much smaller transplant mass than whole pancreas transplant and is therefore a much less invasive procedure, and presents a smaller load of immunogenic tissue.

New therapeutic strategies for type 1 diabetes focus on three important points: residual β cell prevention, β cell restoration and β cell immune protection^[12]. An achievable goal could be to develop a new cellular source for β cell. Different studies focus on immortalization and expansion of β cells from deceased donor pancreas^[13,14], reprogramming or transdifferentiation of other pancreatic cells to β cells^[15], differentiation from pancreatic progenitor cells in the adult pancreas^[16] and differentiation and maturation from embryonic stem cells and induced pluripotent stem cells^[17]. All these cellular sources appear promising in developing potential new candidates for beta-cell substitution and therapy for patients.

Immunoprotection strategies include immunomodulatory therapies and immunoisolation techniques. Immunotherapies aim to down-regulate the autoimmune

response to pancreatic self-antigens and arrest beta-cell destruction. Ideally, this type of technique would induce prolonged remission from type 1 diabetes and achieve a cure^[18]. As regards the separation of implanted cells from the host immune system, this has been recognized as an experimental strategy to prevent immunorecognition, rejection and avoid lifelong immune suppression. A bioartificial pancreas tries to create a barrier to immune cells while allowing sufficient oxygen and nutrients transfer. Immunoisolation strategies facilitate islet transplantation without the need of immunosuppression^[19].

Islet transplantation as a cure for type 1 diabetes

Transplantation of pancreatic islets is a less invasive procedure than pancreas transplantation, with a shorter hospital stay and lower morbidity. This therapeutic option is reserved for patients with severe glycaemic variability, progressive diabetic complications and life threatening hypoglycemia^[19]. Successful islet transplantation was established in the early 70s in diabetic rats^[20] and rhesus monkeys^[21]. Najaran *et al.*^[22] reported the first significant case of human islet transplantation in patients with chronic pancreatitis. These patients underwent total or near total pancreatectomy, followed by autologous islet transplantation which prevented the development of diabetes. Thereafter, allograft was attempted in selected patients with type 1 diabetes. Unfortunately, out of the 237 allografts transplanted from 1990 to 1999, only 16% have resulted in insulin-independence for more than 1 week, and only 11% for more than 1 year^[23]. Important progress was made thanks to improvements in techniques for isolating human islets^[24,25] and to the availability of new and more effective immunosuppressive agents.

A positive turn in islet transplantation occurred in 2000, when James Shapiro and his colleagues treated 7 diabetic patients with severe hypoglycemia with allogeneic islets and a novel immunosuppressive regimen, obtaining insulin-independence in all the transplanted patients at a median follow-up period of 11.9 mo^[11]. This success was due to several changes in the transplantation procedure, such as the large number of infused islets (from 2-4 donors for each recipient), an immunosuppressive regimen with inclusion of sirolimus and without glucocorticoids and the limited cold ischemia time of the recovered pancreases. A follow-up report monitored 65 transplant recipients for a period of 5 years. This study showed that 80% of the transplanted patients remained insulin-independent at 1 year, but only 10% retained an insulin-free status at 5 years. However partial graft function allowed improvement of glycaemic control with a decreased occurrence of hypoglycemic episodes. Recent results for islet transplantation demonstrate major improvement in outcomes. Analysis of transplantations performed by Collaborative Islet Transplant Registry (CITR) from 1999 to 2010 showed that the insulin independence rate at 3 years after transplantation increased from 27% in 1999-2003 to 44% in 2007-2010 and at 4

years approximately 90% of the grafts showed some degree of function^[26].

All these studies indicated that islet transplantation is a promising strategy for treatment of type 1 diabetes. However, there are several challenges limiting widespread application. The disadvantages of the current approach is the limited supply and suboptimal yields of procurement and isolation of islets, graft failure and relatively high requirements to achieve prolonged insulin independence and glucose stability^[27]. Poor vascularization and hypoxia of the transplanted islets^[28], destruction by autoimmunity and allorejection^[29] and exposure to the toxic effects of immunosuppressive agents^[30] are thought to contribute to early graft failure. Better protection of the transplanted islets and improved immunosuppression are current strategies under investigation that could substantially advance islet transplantation as an acceptable alternative treatment. Mesenchymal stromal cells have been proposed to be one possible means to enhance islet transplantation protocols^[31].

ROLE OF MSCS IN VASCULARIZATION AND IMMUNOMODULATION OF ISLET TRANSPLANTATION

MSCs are multipotent, self-renewing cells that reside mainly in the bone marrow, representing only 0.001%-0.01% of nucleated marrow cells. They can be also isolated from other tissues, including skeletal muscle^[32], adipose tissue^[33], amniotic fluid^[34] and umbilical cord blood^[35] and expanded for several passages without losing their self-renewing capacity^[36,37]. The International Society for Cellular Therapy has defined criteria to define the MSC population, including adherence to plastic in culture, expression of cell surface markers, such as CD105, CD73 and CD90, and lack of expression of CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA-DR surface molecules^[38]. MSCs have been well characterized for their ability to differentiate into several cell types of mesenchymal origin, such as osteoblasts, adipocytes and chondrocytes^[38], but it has been also demonstrated that they have the capacity to differentiate into cell types of endodermal and ectodermal lineages, including lung epithelial cells^[39], retinal pigment^[40], skin^[41], sebaceous duct cells^[42], renal tubular cells^[43], neural cells^[44], hepatocytes^[45] and insulin producing cells^[46]. However, an intense debate about the contribution of MSCs to form functional tissue through transdifferentiation processes is still open^[47]. Aside to their ability to differentiate into many types of cells, MSCs can also have a reparative effect through the migration to the site of injury^[48] and the release of paracrine factors that affect cell migration, proliferation and survival of the surrounding cells^[49]. In addition, MSCs have been shown to contribute to repair processes through the secretion of pro-angiogenic molecules, thus promoting the formation of new blood vessels *in vivo*^[50]. Moreover, MSCs have

emerged as a useful cell population for immunomodulation therapy thanks to their ability to secrete a large amount of bioactive molecules that affect immune and inflammatory responses^[51]. The combination of tissue regenerative potential and immunomodulatory or immunosuppressive activity has prompted therapeutic interest.

MSCs promote islet graft revascularization

Normally pancreatic islets have a rich vascular supply within the pancreas to support their metabolic activity and to facilitate rapid dispersal of secreted hormones. Large islets are supplied by 1-3 arterioles that penetrate the B cell-rich islet core and distribute into a dense network of sinusoidal capillaries connected to venules in the islet periphery^[52]. Islets receive considerably more blood flow than surrounding pancreatic exocrine tissue^[53] and islet capillaries are much more permeable than exocrine capillaries due to the presence of 10 times as many small pores within their endothelial cells^[54]. Relatively strong expression of VEGF by islets is probably responsible for the high degree of vascularization and fenestration. Depletion of VEGF in β cells in mice reduces vascular density and permeability to the level of exocrine tissue and partly impairs insulin secretion^[55]. The islet vasculature degenerates during the process of isolation and transplantation and the islets must initially rely on diffusion of oxygen and nutrients from the culture medium and from vessels in the transplant environment for their survival^[56,57]. This condition leads to prolonged hypoxia that, at the early post-transplant stages, is considered a major reason for early islet graft loss. The vessel density and oxygen tension in transplanted islets are less than half compared with islets in the native pancreas^[58]. Further compromising islet graft survival in this context is their vulnerability to oxidative stress, a consequence of relatively low expression of antioxidant enzymes^[59]. Thus, transgenic islet expression of antioxidant enzymes, such as glutathione peroxidase, could be a possible solution. However, a potential drawback of this approach is that glutathione peroxidase removes H₂O₂, an inducer of VEGF synthesis^[60], and thus may further impair islet graft revascularization. The net result of oxidative and other challenges is that more than 70% of islets transplanted intraportally fail to become stably engrafted^[61].

VEGF is a multi-functional angiogenic regulator that stimulates blood vessel formation, endothelial cell survival and epithelial cell proliferation^[62]. The receptors of VEGF are predominantly expressed on vascular endothelial cells^[62] and are also expressed in pancreatic islets^[63]. Several lines of evidence indicate that insufficient expression of VEGF limits the rate and extent of islet graft revascularization. Transplanted islets show a significant reduction of VEGF expression at day 3-4 after transplantation^[64] while an over expression of VEGF markedly improves the degree of revascularization and function of islet grafts. Mouse islets transfected with an adenovirus carrying the cDNA for the human VEGF₁₆₅ isoform were transplanted under the kidney capsule of diabetic nude mice. Vascular

endothelial growth factor (VEGF) expression resulted in an increase in both islet graft mass and revascularization and, unlike vector-transfected grafts, rapidly returned recipient to stable normoglycaemia^[65].

Several bone marrow subpopulations, such as endothelial progenitor cells and MSCs may be able to differentiate into one or more of the cellular compartments of the vascular bed^[66,67]. MSCs are known to secrete VEGF and other growth factors and to enhance proliferation of endothelial cells and smooth muscle cells^[68]. MSCs release a wide array of cytokines that support hematopoietic stem and progenitor cell development, as well as the secretion of other cytokines that are relevant to increasing blood flow to ischemic tissue^[69]. Moreover, MSCs secrete several important arteriogenic cytokines, including VEGF and monocyte chemoattractant protein-1 (MCP-1). In mice undergoing distal femoral artery ligation, a model of hind limb ischemia, local injection of MSCs increased adductor muscle levels of VEGF and fibroblast growth factor (FGF) proteins compared with controls, and co-localization of VEGF and transplanted MSCs within adductor tissue was demonstrated^[68].

Recently it has been reported that in animal models, MSCs are able to enhance survival and function of islet graft by increasing islet revascularization^[70]. Consistent with these studies, our group showed that cultured MSCs express high level of VEGF and that transplantation of those MSCs elicited a robust host angiogenic response leading to neovascularization of syngeneic islet grafts in diabetic rats. This effect may serve to increase local perfusion of the islets and ameliorate their metabolic activity^[71]. Similar results were obtained in a preclinical model by Berman *et al.*^[72] that demonstrated enhanced islet engraftment and function at 1 mo post-transplant in a cynomolgus monkey model of allogeneic islet-MSCs transplantation. The authors hypothesized that MSCs enhance islet engraftment by staying in proximity to the islets at the time of cotransplantation, providing revascularization and regenerative signals. MSCs provided an important approach for enhancement of islet engraftment, thereby decreasing the numbers of islets needed to achieve insulin independence^[72].

In summary, MSCs cotransplanted with islets in type 1 diabetic recipients can facilitate islet revascularization, engraftment and improved islet function: Consequently, the presence of MSCs permit to reduce the islet number required for reversal of diabetes. Therefore, cotransplantation of MSCs with islets could facilitate islet engraftment and improve islet graft function in clinical islet transplantation.

Immunomodulation of islet transplantation by MSCs

One of the most promising aspects of MSCs regards their dynamic role in modulating the immune system. MSCs are not only immunoprivileged cells, due to the low expression of class II Major Histocompatibility Complex (MHC-II) and co-stimulatory molecules in their cell surface, but they also interfere with different

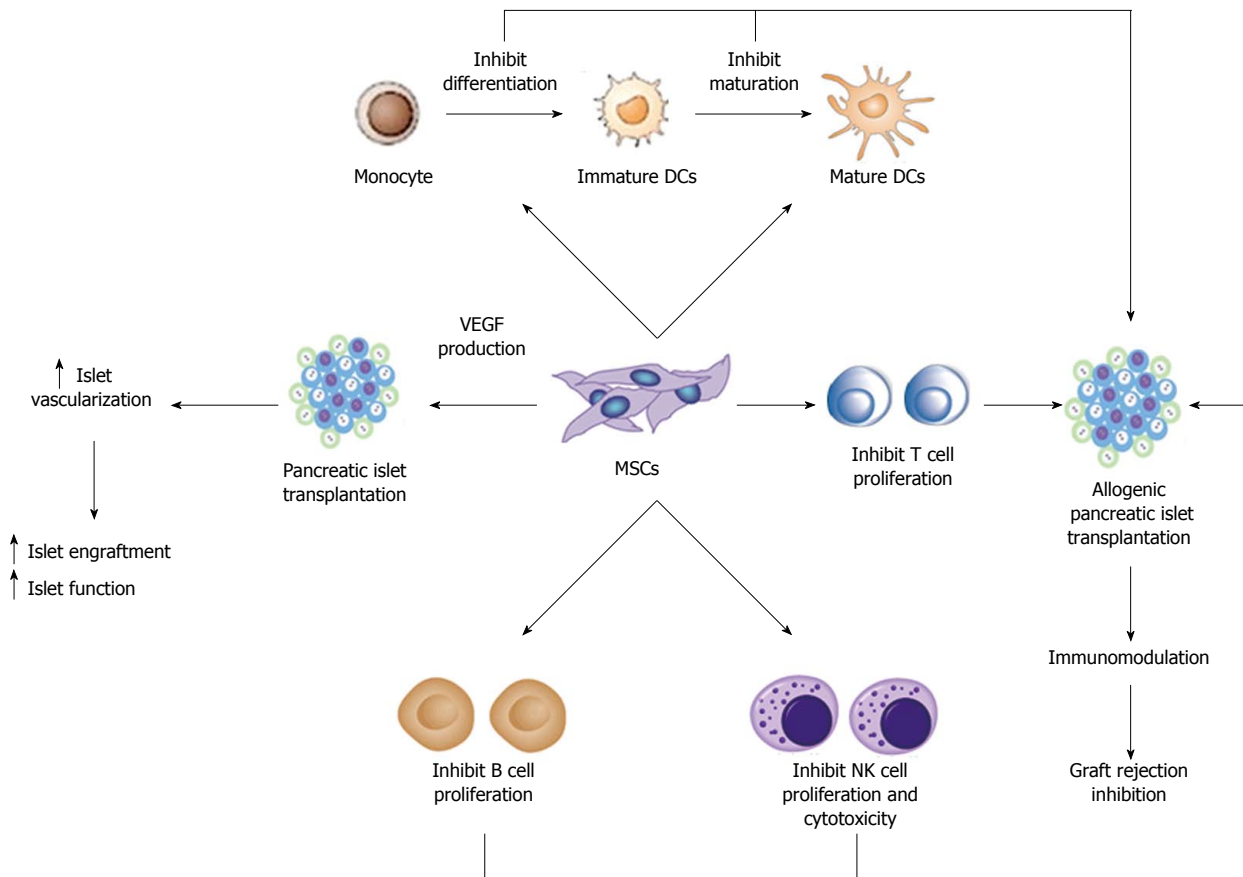


Figure 1 Schematic representation of the role of mesenchymal stem cells in islet transplantation. MSCs: Mesenchymal stem cells; VEGF: Vascular endothelial growth factor; DCs: dendritic cells.

pathways of the immune response by means of direct cell-to-cell interactions and soluble factor secretion. As schematically represented in Figure 1, it is well established that MSCs can exert immunosuppressive activity on T cells^[73] and interfere with dendritic cell (DC) maturation^[74]. Furthermore, MSCs may modulate natural killer (NK) cell cytotoxic activity, B cell proliferation and immunoglobulin production.

MSCs have been shown to suppress autoreactive T-cell responses in models of autoimmunity such as experimental autoimmune encephalomyelitis^[75], collagen-induced arthritis^[76] and autoimmune enteropathy^[77]. Type 1 diabetes is one of the most prevalent autoimmune diseases in childhood. The effector mechanisms of immune-mediated destruction of islet β cells are complex, but an essential early event is the activation of islet cell antigen reactive T cells. Recently, the therapeutic benefit of MSCs has been evidenced in the treatment of type 1 diabetes. Lee *et al.*^[78] used immunodeficient recipient mice chemically induced by streptozotocin to study the effect of human MSCs in the development of diabetes. Infusion of hMSCs reduced glycaemic levels and increased peripheral insulin levels. In the pancreas of these mice the islets appeared larger compared with islets from untreated diabetic mice^[78]. In experimental mouse models, intravenously infused MSCs are capable of migrating to pancreatic islets^[48]. However, the role of MSCs in β cell

replacement is controversial. Some evidence suggests the possibility that MSCs differentiate into islet β cells^[48]. In addition, similar results were reported by Ezquer *et al.*^[79] in a model of streptozotocin-induced diabetes. Reversion of hyperglycemia and glycosuria was observed after injection of MSCs, with increased morphologically normal β pancreatic islets. Other reports have contradicted these findings suggesting that MSCs could be feeder cells for islet differentiation, proliferation and vascularization, but do not differentiate into β cells^[80].

MSCs may also offer therapeutic opportunities in transplantation by directly targeting alloreactive T cells. MSCs are immunosuppressive *in vitro* and, in mixed-lymphocyte reactions, suppress T-cell proliferation^[73] through soluble factors, including 2,3-dioxygenase (IDO), prostaglandin-E2 (PGE2), nitric oxide, transforming growth factor β (TGF β) and hepatocyte growth factor (HGF)^[81,82]. Neutralizing antibodies against TGF β and HGF can restore the MSC-induced suppression of T cell proliferation^[73]. In a model of allogeneic pancreatic islet transplantation, the administration of MSCs resulted in the prolonged survival of islets and led to long-term stable normoglycemia^[83]. In this study MSCs were colocalized at the graft site where they locally produced immunosuppressive matrix metalloproteinase-2 and -9 that block the activation and expansion of alloreactive T cells^[83]. In a most recent paper, using

a rat model of streptozotocin induced diabetes, the authors found that MSCs significantly improved glycemic control and reduced graft infiltration by immune cells in either allogeneic or syngeneic pancreatic islet transplantation^[84]. They found that MSCs were effective when administrated either locally or systemically. The modulation of acute rejection that the authors observed after islet transplantation may indicate that soluble factors are released by MSCs to several organs after their systemic administration.

Additional studies revealed that MSCs might produce this anti-proliferative effect *via* induction of anergy in the T cell population^[85], T cell tolerance^[75], or by inducing proliferation of regulatory T cell populations^[86]. Berman *et al.*^[72] first reported increased numbers of Treg in a MSC allogeneic islet transplant preclinical model. MSCs treatment significantly enhanced islet engraftment and function at 1 mo post-transplant, as compared with animals that received islets without MSCs. Additional infusions of donor or third-party MSCs resulted in reversal of rejection episodes and prolongation of islet function. Stable islet allograft function was associated with increased numbers of regulatory T-cells in peripheral blood^[72].

The immune response is related not only to T cells, but to the interaction between DC cells and T cells^[87]. DCs are antigen-presenting cells (APCs) capable of stimulating both naïve and memory T cells. MSCs affect the differentiation, maturation and function of DCs at different levels^[74,88]. MSCs have also been shown to alter the cytokine secretion profile of DCs toward up-regulation of regulatory cytokines, such as IL-10, and down regulation of inflammatory cytokines such as IFN γ , IL-12 and TNF α , inducing a more anti-inflammatory or tolerant dendritic cell phenotype^[74,89]. Studies in animal models suggest that DC based immunotherapeutic strategies might also be utilized to facilitate islet transplant tolerance^[90,91]. Li *et al.*^[92] demonstrated that in mice with combined transplantation of pancreatic islets and MSCs, the expression of CD11c (DCs phenotype derived from monocytes) and CD83 (mature DCs phenotype) was down regulated markedly. This finding showed that MSCs inhibit the maturation of DCs and the stimulation of T cell was weakened, resulting in survival of transplanted pancreatic islets.

Autoimmunity also involves B cells by antibody production. The interaction between MSCs and B cells is not yet completely understood. However, co-culture experiments with these two cells using both mouse and human cells showed that MSCs inhibit B cell proliferation^[93]. They also observed that MSCs affect chemotactic properties of B cells while B-cell co-stimulatory molecule expression and cytokine production were unaffected by MSCs.

Finally, NK cells are key effector cells of innate immunity. MSCs alter the function of NK cells by suppressing their proliferation, and cytotoxicity. Spaggiari *et al.*^[88] demonstrated that cytokine induced proliferation of

freshly isolated NK cells was inhibited in the presence of MSCs.

Thanks to their interactions with many different types of immune cells, MSCs administrated in conjunction with islet cell transplantations could prevent immune rejection and promote long term islet allograft survival.

CONCLUSION

In summary, current data suggest that MSCs have the potential to aid in the treatment of type 1 diabetes and overcome some of the current limitations to islet transplantation. These cells may exert beneficial pro-angiogenic and immunomodulatory effects when co-transplanted with pancreatic islets. The pro-angiogenic effects result from the release of angiogenic factors from MSCs that have been shown to improve islet vascularization and graft function in islet transplantation. The immunomodulatory properties of MSCs may help in reducing inflammatory damage to the islets in the early peritransplant period. MSCs may also reduce autoimmunity through their capacity to inhibit T cell proliferation and suppress differentiation and maturation of dendritic cells.

These data encourage further preclinical co-transplantation of MSCs and pancreatic islets to improve the outcome of allogeneic islet transplantation in the treatment of type 1 diabetes. However, some key issues need to be addressed before MSC based therapies become a safe option for clinical studies. Most importantly, it is unclear if co-transplanted MSCs engraft and differentiate at the implantation site. Thus, the long-term stability of MSC activity and function after transplantation should be assessed *in vivo*. In addition, the selection of a suitable donor MSC source may differ if the treatment aims at modulating the autoimmune disease or enhancing pancreatic islet engraftment and vascularization. Therefore, whether autologous or allogeneic MSCs are suitable as a donor source should be selected according to the specific aim of the study.

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P- Reviewers: Chang FC, Sanlioglu AD, Sumi S
S- Editor: Song XX **L- Editor:** O'Neill M **E- Editor:** Zhang DN



WJSC 6th Anniversary Special Issues (2): Mesenchymal stem cells

Mesenchymal stem cells in treating autism: Novel insights

Dario Siniscalco, James Jeffrey Bradstreet, Nataliia Sych, Nicola Antonucci

Dario Siniscalco, Department of Experimental Medicine, Second University of Naples, 16-80138 Napoli, Italy

Dario Siniscalco, Centre for Autism, La Forza del Silenzio, 81036 Caserta, Italy

Dario Siniscalco, Cancellautismo, 50132 Florence, Italy

James Jeffrey Bradstreet, Brain Treatment Center, Buford, GA 30518, United States

Nataliia Sych, Clinical Department, Cell Therapy Center Em-Cell, 04073 Kiev, Ukraine

Nicola Antonucci, Biomedical Centre for Autism Research and Treatment, 70126 Bari, Italy

Author contributions: Siniscalco D designed the paper; Siniscalco D and Bradstreet JJ wrote the paper; Sych N contributed to the paragraph on MSCs; Antonucci N contributed to the paragraph on autism; Bradstreet JJ edited the English language.

Correspondence to: Dario Siniscalco, ChemD, PhD, Department of Experimental Medicine, Second University of Naples, Via S Maria di Costantinopoli, 16-80138 Napoli, Italy. dariosin@uab.edu

Telephone: +39-81-5665880 Fax: +39-81-5667503

Received: November 11, 2013 Revised: December 19, 2013

Accepted: March 17, 2014

Published online: April 26, 2014

Abstract

Autism spectrum disorders (ASDs) are complex neurodevelopmental disorders characterized by dysfunctions in social interactions, abnormal to absent verbal communication, restricted interests, and repetitive stereotypic verbal and non-verbal behaviors, influencing the ability to relate to and communicate. The core symptoms of ASDs concern the cognitive, emotional, and neurobehavioural domains. The prevalence of autism appears to be increasing at an alarming rate, yet there is a lack of effective and definitive pharmacological options. This has created an increased sense of urgency, and the need to identify novel therapies. Given the growing awareness of immune dysregulation in a significant portion of the autistic population, cell therapies have been proposed and applied to ASDs. In particular, mesenchymal stem cells (MSCs) possess the immunological properties which make them promis-

ing candidates in regenerative medicine. MSC therapy may be applicable to several diseases associated with inflammation and tissue damage, where subsequent regeneration and repair is necessary. MSCs could exert a positive effect in ASDs through the following mechanisms: stimulation of repair in the damaged tissue, *e.g.*, inflammatory bowel disease; synthesizing and releasing anti-inflammatory cytokines and survival-promoting growth factors; integrating into existing neural and synaptic network, and restoring plasticity. The paracrine mechanisms of MSCs show interesting potential in ASD treatment. Promising and impressive results have been reported from the few clinical studies published to date, although the exact mechanisms of action of MSCs in ASDs to restore functions are still largely unknown. The potential role of MSCs in mediating ASD recovery is discussed in light of the newest findings from recent clinical studies.

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Key words: Autism spectrum disorders; Autism treatment; Cell therapy; Mesenchymal stem cells

Core tip: Autism spectrum disorders are still untreatable pathologies. Mesenchymal stem cells possess the immunological properties which make them promising candidates as a novel therapeutic option.

Siniscalco D, Bradstreet JJ, Sych N, Antonucci N. Mesenchymal stem cells in treating autism: Novel insights. *World J Stem Cells* 2014; 6(2): 173-178 Available from: URL: <http://www.wjgnet.com/1948-0210/full/v6/i2/173.htm> DOI: <http://dx.doi.org/10.4252/wjsc.v6.i2.173>

AUTISM SPECTRUM DISORDERS

Autism spectrum disorders (ASDs) are complex neuro-

developmental disorders. Indeed, this term refers to a heterogeneous group of varied conditions characterized by dysfunctions in social interactions, skills, and communication, restricted interests, and repetitive stereotypic verbal and non-verbal behaviors, influencing the ability to relate to others. Cognitive, emotional and neurobehavioral abnormalities characterize the core symptoms^[1,2]. The prevalence of these disorders has dramatically increased in the last years, with present rates of 11.3 per 1000 (one in 88) children aged 8 years in the United States, according to Centers for Disease Control^[3]. ASDs are presumed to be a lifelong disability with multiple impacts on child and adult health. Indeed, adult autistic individuals show limited independence because of their learning disability. In adulthood, communication is still impaired, as reading and spelling abilities are poor. Stereotyped behaviors and restricted interests still persist. The children affected require special and intensive parental, school, and social support^[4]. ASD results in a substantial impact on a person's quality of life and that of their family^[5]. Given the total lifetime societal cost of caring for one individual with autism, estimated in \$3.2 million^[6], autism should be considered as an urgent public health priority^[2].

Together with the cognitive, emotional and neurobehavioral abnormalities, ASDs are disorders characterized by a broad range of biochemical, toxicological and immune involvement, including: oxidative stress, endoplasmic reticulum stress, decreased methylation capacity, limited production of glutathione, mitochondrial dysfunction, intestinal dysbiosis, increased toxic metal burden, and immune dysregulations including autoimmunity^[7].

Currently, only a handful of medications are licensed for treating a limited number of autism-related symptoms^[8]. Moreover, prescribed pharmaceuticals (*i.e.*, antipsychotics) fail to address the ASD core symptoms, have the potential of markedly adverse effects, and are at best palliative^[9-12]. The alternative treatments for ASDs are diverse and include: behavioral, nutritional, and biomedical approaches. Thus the need for a definitive and effective therapy is an unfulfilled priority for autism research.

MESENCHYMAL STEM CELLS

Presently, cell therapies and cell-based biopharmacies offer a valid intervention for several otherwise untreatable human diseases. Stem cells appear to represent the greatest potential for the future of molecular and regenerative medicine^[13,14]. Among the various stem cell subtypes, mesenchymal stem cells (MSCs) provide a useful tool for the treatment of several diseases associated with inflammation, tissue damage, and subsequent regeneration and repair^[15].

MSCs are multipotent stem cells that possess the capacity to differentiate *in vivo* or *in vitro*, under specific conditions, into cells of connective tissue lineages, including bone, fat, cartilage and muscle^[16]. They are distinct from the hematopoietic lineage, and were initially described by

Alexander Friedenstein in the 1960s after he extracted MSCs from bone marrow^[17]. It is common practice for clinical and research applications, to acquire MSCs from bone marrow aspirates of the superior iliac crest under local anesthesia. The cells are then isolated by their adherence to plastic and amplified through passage in culture, where they exhibit a great replicative capacity^[18].

In order to achieve a detailed classification of this type of stem cell, the International Society for Cellular Therapy has proposed the following minimal criteria to identify human MSCs: they must grow in standard, plastic-adherent culture conditions; must express the cyto-specific markers CD73, CD90 and CD105, without expression of CD45, CD34, CD14 or CD11b, CD79alpha or CD19 and HLA-DR surface molecules; and must be capable of *in vitro* differentiation into osteoblasts, adipocytes and chondroblasts^[19].

Interestingly, MSCs seem to be the most promising clinical candidate for immune-modulatory cell-based therapy^[20]. MSCs show immunomodulatory capacities, as they are able to induce tolerance in immunocompetent allotransplants or even xenotransplant recipients^[21]. Interacting with a wide range of immune cells, probably through a cell-to-cell contact mechanism^[22], MSCs are able to modulate T-cell phenotype and immune-suppress the local environment^[23].

Their unique properties of immunomodulation, multipotency, and rapid self-renewal proliferation rate, distinguish them as useful tools for application in immunomodulatory therapy and neurological disorders. In addition, other desirable characteristics of MSCs, *e.g.*, genetic stability, stable phenotype, and easy procedures for collection, storage and shipping from the laboratory to the bedside^[24], direct us to MSC-based therapies as a potent intervention.

In clinical settings, MSCs can be transplanted directly without genetic modification or pretreatments (*i.e.*, immunosuppressants). No host *vs* graft rejection has been observed^[25]. Importantly, there is an absence of uncontrollable growth or tumorigenesis with MSCs, in contrast to the potential problems intrinsic to embryonic stem cells^[26]. Crucially, MSCs create no moral objection or ethical-religious controversies, unlike embryonic or fetal stem cells^[27].

MESENCHYMAL STEM CELLS IN TREATING AUTISM: THE RATIONALE

The potential application of cell therapy, in particular MSCs, for ASDs has already been discussed by our group^[28,29]. After a brief description of MSC-mediated ameliorative effects in ASDs, we will review recent and ongoing clinical trials using MSC transplantation in ASD patients.

We hypothesize that MSCs exert a positive effect in ASDs through the following mechanisms: stimulation of the plastic response in the host damaged tissue (*e.g.*, inflammatory bowel disorders); synthesizing and releas-

ing anti-inflammatory cytokines and survival-promoting growth factors (paracrine and biopharmacy); integrating into existing neural and synaptic network (engrafting), and restoring plasticity^[28,29]. Following transplantation, MSCs target and migrate to the site of injury. In some cases these cells respond to the local environment with appropriate secretion of soluble factors to ameliorate inflammation and promote repair^[30]. This paracrine mechanism offers potential in ASD treatment.

ASDs are characterized by a coexistent, if not etiological, immune system dysregulation^[31]. Changes in innate and adaptive immune responses have been reported in ASD patients^[32]. Characteristically, ASD cases show alterations in both T cell- and B cell-mediated immunity, as well as an imbalance in CD3⁺, CD4⁺, and CD8⁺ T cells and natural killer (NK) cells^[33]. On these bases, the regulatory effects mediated by MSCs present an optimal way to restore immune balance, which cannot otherwise be obtained through pharmaceutical interventions. Through inhibition of the proliferation of CD8⁺/CD4⁺ T lymphocytes and NK cells, suppression of the immunoglobulin production by plasma cells, and inhibition of the maturation of dendritic cells (DCs), MSC transplantation appears ideally suited to provide a unique therapeutic application for ASDs^[34,35].

In addition, MSCs are able to inhibit T lymphocyte pro-inflammatory cytokine production^[36]. MSCs function as an implanted biopharmacy: after homing in to the targeted tissue site, they synthesize and release a broad range of bioactive molecules^[35,37], *i.e.*, anti-inflammatory cytokines, trophic and growth factors, interleukin (IL)-6, IL-7, IL-8, IL-11, IL-12, IL-14, IL-15, macrophage colony-stimulating factor, Flt-3 ligand, and stem-cell factor^[38], which in turn could be responsible for activating endogenous restorative mechanisms within injured tissues. This strong paracrine activity of MSCs seems to be the most plausible and reasonable mechanism for the functional benefit derived from MSC transplantation. Furthermore, transplanted MSCs can induce the host tissue to upregulate the production of anti-inflammatory molecules, such as IL-10, in this way restoring the pro-inflammatory processes noted in ASDs^[39,40].

MESENCHYMAL STEM CELLS IN TREATING AUTISM: CLINICAL EVIDENCE

Despite insufficient pre-clinical models of MSC therapy for ASDs^[41], several clinical studies on humans have been conducted. Recently, a non-randomized, open-label, controlled, single-center phase I / II clinical trial to examine the treatment safety and efficacy of transplantation of human cord blood mononuclear cell (CBMNCs) and/or human umbilical cord-derived mesenchymal stem cells (UCMSCs) in children with autism has been performed^[42]. Stem cell administration was carried out via intravenous and intrathecal infusions. Autistic children transplanted with cells were followed for 24 wk. According to the authors, the cell treatment was safe, well toler-

ated without immediate or long term side effects, and no allergic, immunological reactions or other serious adverse events were observed at the time of injection or during the whole follow-up period. The cell transplantation showed efficacy; improvements were observed in visual, emotional and intellectual responses, body use, adaption to change, fear or nervousness, non-verbal communication and activity level, as measured by Childhood Autism Rating Scale, as well as in lethargy/social withdrawal, stereotypic behavior, hyperactivity and inappropriate speech evaluated by the Aberrant Behavior Checklist^[42]. They noted that the group receiving CBMNCs and UCMNCs demonstrated a more robust therapeutic effect than the group receiving mono-cell line therapy, which may be attributed to the action of CBMNCs and UCMSCs in synergy. It has been proposed the synergistic mechanism is related to increased cell-mediated perfusion in brain areas and/or the regulation of immune dysfunction.

Intrathecally transplanted autologous bone marrow-derived mononuclear cells were efficacious in improving the quality of life in a 14-year-old boy with severe autism^[43]. A detailed cell-sorting analysis was not done, but the cell extract contained a percentage of MSCs. We know bone marrow is comprised of a heterogeneous population of stem cells, encompassing hematopoietic stem cells, MSCs, endothelial progenitor cells, and very small embryonic-like stem cells. The bone marrow cell transplantation was safe, the patient had no noted side-effects and showed some immediate improvements within a week (eye contact and attention, fine motor activities). Significant improvements were observed over a period of 6 mo to 1 year (social interaction and emotions, impulse control, reading skills, tracing, recognition of all shapes and following commands, and hyperactivity). Interestingly, comparisons of pre/post cell therapy brain positron emission tomography scans revealed a markedly increased uptake in bilateral temporal lobes and bilateral calcarine cortices with mild increased uptake in the left medial pre-frontal cortex^[43].

Transplanted stem cells therefore seemed to ameliorate neural hypoperfusion in the previous case report. Hypoperfusion may be a consequence of focal inflammation and would likely result in low-grade ischemic consequences: hypoxia, abnormal metabolites, neurotransmitters dysregulation, and potential neural tissue damage.

In the light of these encouraging, but limited observations, the authors launched an open-label proof-of-concept study using autologous bone marrow-derived mononuclear cell transplantation in 32 patients with autism^[44]. The average number of intrathecally injected cells was 8×10^7 cells. Cell treatment was determined to be safe and adverse events were transient (hyperactivity). They hypothesize that the intrathecal administration route is able to enhance homing of the transplanted cells into the central nervous system. Clinical improvements after cellular therapy were observed in social relationships and reciprocity, emotional responsiveness, communication and behavior. As a putative mechanism of action,

the authors further hypothesized that cellular transplantation was able to restore function to ASD patients by neuroprotection, neural circuit reconstruction, neural plasticity, neurogenesis, and immunomodulation.

The hypothesis that intrathecal administration increases the efficacy of stem cell therapies is not actually evaluated by these various studies. Clearly, it is a testable hypothesis and future studies should include arms with and without intrathecal administration to compare the therapeutic efficacy of the more invasive intrathecal implantations.

PROBLEMS

Despite these early clinical trials with MSCs, there are no apparent pre-clinical studies on the use of MSCs in ASD models^[41]. Thus, more research into the mechanisms of action post transplantation is required to adequately understand the route, dosing and safety. However, the parental perspective is unlikely to wait on more detailed scientific studies. Stem cells are readily available from many centers in numerous countries, with various cell types and methodologies being utilized. Families recognize the devastating nature of autism on their children and are already seeking stem cell therapies. Based on a simple scan of the internet sites advertising cell therapies, it appears hundreds of ASD children have already been treated.

Another complexity in the research arises from stem cell sourcing. Some protocols use allogeneic (derived from a different person or collection of donors), while others use autologous donor (self-derived) cell types^[15]. Some protocols for ASD also use expanded autologous MSCs (United States Patent Application: 20060182724). This adds another dimension to the discussion and a potential source of laboratory contamination. Expansion requires medium for growth from which the cells must then be isolated, and any medium washed sufficiently to prevent a reaction in the recipient. Typically bovine serum is used. This creates the further risk of prion infection of the medium. To avoid this, one group has proposed using pooled human serum^[45]. This xeno-free methodology has many desirable features, but retains the concerns about human pathogen transmission. The group, however, screened extensively for contamination and it appeared they were able to ascertain that the samples were free of any disease vectors. This process should be considered for any use of expanded MSCs for ASD therapies.

Another challenge in the standardization of dosing derives from the varying efficacy amongst allogeneic donors in terms of: vitality, potency, and expansion potential. Every donor is different and this could affect efficacy and also paracrine effects. Indeed, it seems that the secretion of bioactive molecules could differ by a factor of 10 between different donors of matched age and gender^[15]. Recently, in order to increase the adequate supply of stem cells from donor tissues, it has been demonstrated that a 3D co-culture system with murine-derived hematopoietic stem cells co-cultured with MSCs produces 3D-microag-

gregates of stem cells. These 3D-microaggregate systems support the expansion of approximately twice as many hematopoietic stem cell candidates as the 2D controls. In addition, the MSCs maintained in 3D aggregates are able to express significantly higher levels of hematopoietic niche factors compared with 2D cultures^[46].

Finally, there are complex hurdles to overcome from the legal and regulatory restrictions placed by governments seeking to control cell therapies^[27]. Several countries (*i.e.*, United States and EU area) have attempted to create uniformity within the regulations governing cell trials, while creating very stringent regulations on cell culture conditions, diseases to be treated, and patient safety. However, in some other countries (*e.g.*, Ukraine, China, Dominican Republic, Panama, and Mexico) the access to cell therapy is more readily available.

CONCLUSION

The rapidly increasing prevalence of ASDs worldwide is creating an urgent need for effective restorative therapies. The lack of safe and effective psychopharmaceuticals and other definitive medical therapies, together with the limited understanding of the pathophysiology, has created an urgency to identify novel and more effective therapies^[47]. MSCs appear to offer a greater potential in regenerative medicine for complex disorder like autism than existing pharmaceutical protocols. Promising and impressive early results have been achieved from a few clinical studies, although the exact restorative mechanisms of action of MSCs in ASDs are still largely unknown.

ACKNOWLEDGMENTS

The authors gratefully thank Mr. Enzo Abate, Ms. Giovanna Gallone, and the nonprofit organizations “La Forza del Silenzio” and “Cancellautismo,” Italy for their useful assistance.

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P- Reviewers: Shawcross SG, Tanabe S, Yao CL

S- Editor: Gou SX **L- Editor:** Cant MR **E- Editor:** Zhang DN



WJSC 6th Anniversary Special Issues (2): Mesenchymal stem cells

Neurotrauma and mesenchymal stem cells treatment: From experimental studies to clinical trials

Ana Maria Blanco Martinez, Camila de Oliveira Goulart, Bruna dos Santos Ramalho, Júlia Teixeira Oliveira, Fernanda Martins Almeida

Ana Maria Blanco Martinez, Camila de Oliveira Goulart, Bruna dos Santos Ramalho, Júlia Teixeira Oliveira, Fernanda Martins Almeida, Laboratory of Neurodegeneration and Repair, Institute of Biomedical Sciences, Health Science Center, 21941-902, Rio de Janeiro, Brazil

Ana Maria Blanco Martinez, Camila de Oliveira Goulart, Bruna dos Santos Ramalho, Fernanda Martins Almeida, Pathology Department, Faculty of Medicine, Federal University of Rio de Janeiro, 21941-902, Rio de Janeiro, Brazil

Fernanda Martins Almeida, Federal University of Rio de Janeiro, Campus Macaé, 27930-560, Rio de Janeiro, Brazil

Author contributions: Martinez AMB and Almeida FM conceived and designed the manuscript; all authors contributed equally to the acquisition and analysis of data and the manuscript writing; Martinez AMB and Almeida FM revised and approved the final version of the manuscript.

Correspondence to: Ana Maria Blanco Martinez, MD, PhD, Laboratory of Neurodegeneration and Repair, Institute of Biomedical Sciences, Health Science Center, bloco F - sala 12, 21941-902, Rio de Janeiro, Brazil. martinez@histo.ufrj.br
Telephone: +55-21-25626431 Fax: +55-21-25626431

Received: October 29, 2013 Revised: February 26, 2014

Accepted: March 11, 2014

Published online: April 26, 2014

Abstract

Mesenchymal stem cell (MSC) therapy has attracted the attention of scientists and clinicians around the world. Basic and pre-clinical experimental studies have highlighted the positive effects of MSC treatment after spinal cord and peripheral nerve injury. These effects are believed to be due to their ability to differentiate into other cell lineages, modulate inflammatory and immunomodulatory responses, reduce cell apoptosis, secrete several neurotrophic factors and respond to tissue injury, among others. There are many pre-clinical studies on MSC treatment for spinal cord injury (SCI) and peripheral nerve injuries. However, the same is not true for clinical trials, particularly those concerned

with nerve trauma, indicating the necessity of more well-constructed studies showing the benefits that cell therapy can provide for individuals suffering the consequences of nerve lesions. As for clinical trials for SCI treatment the results obtained so far are not as beneficial as those described in experimental studies. For these reasons basic and pre-clinical studies dealing with MSC therapy should emphasize the standardization of protocols that could be translated to the clinical set with consistent and positive outcomes. This review is based on pre-clinical studies and clinical trials available in the literature from 2010 until now. At the time of writing this article there were 43 and 36 pre-clinical and 19 and 1 clinical trials on injured spinal cord and peripheral nerves, respectively.

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Key words: Neurotrauma; Stem cell therapy; Mesenchymal stem cells; Pre-clinical studies; Clinical trials

Core tip: Basic and pre-clinical studies have highlighted the positive effects of mesenchymal stem cell (MSC) treatment after spinal cord injury (SCI) and nerve trauma. There are many pre-clinical studies on MSC treatment for SCI and nerve injuries. However, the same is not true for clinical trials, particularly those concerned with nerve trauma. As for clinical trials for SCI, the results obtained so far are not as beneficial as those described in experimental studies. For these reasons basic and pre-clinical studies dealing with MSC therapy should emphasize the standardization of protocols that could be translated to the clinical set with consistent and positive outcomes.

Martinez AMB, Goulart CO, Ramalho BS, Oliveira JT, Almeida FM. Neurotrauma and mesenchymal stem cells treatment: From experimental studies to clinical trials. *World J Stem*

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SPINAL CORD LESION: MECHANISMS OF DEGENERATION AND REGENERATION

Spinal cord injury (SCI) causes motor and sensory deficits that impair functional performance, and significantly impacts expectancy and quality of life of affected individuals. The estimated annual global incidence of SCI is 15-40 cases per million inhabitants^[1]. In addition to the sensory and functional deficits, spinal cord injury also causes great economic impact on the whole society and it is estimated that this impact is greater than 4 billion dollars per year^[2].

SCI results from primary and secondary injury mechanisms. Primary injury refers to the immediate physical injury to the spinal cord as a consequence of laceration, contusion, compression, and contraction of the neural tissue^[3]. Pathological changes resulting from primary injury mechanisms include severed axons, direct mechanical damage to cells, and ruptured blood vessels. Secondary injury is responsible for the expansion of the injury site which, in turn, limits the restorative process^[4,5]. Specific secondary sequel include alterations in local ionic concentrations, loss of regulation of local and systemic blood pressure, reduced spinal cord blood flow, breakdown of the blood-brain barrier, penetration of serum proteins into the spinal cord, inflammatory responses (alterations in chemokines and cytokines), apoptosis, excitotoxicity, calpain proteases activation, neurotransmitter accumulation, production of free radicals/lipid peroxidation, and imbalance of activated metalloproteinases. These changes lead to demyelination, ischemia, necrosis, and apoptosis of spinal cord parenchyma^[5]. These intrinsic responses to tissue injury contribute to an environment that is inhibitory to axonal regrowth^[6]. As a consequence of these negative influences when axons in the central nervous system (CNS) are damaged they mount a poor regenerative response.

An injury in the central nervous system generally leads to transection of some nerve fibers as well as damage to the surrounding tissues. The distal ends of the damaged axons form dystrophic growth cones that are exposed to a glial hostile microenvironment. During the initial phase of lesion, inhibitors associated with intact myelin oligodendrocyte and myelin debris, such as Nogo (no go), MAG (myelin associated glycoprotein) and OMgp (oligodendrocyte myelin glycoprotein) proteins can restrict axonal growth^[7]. In addition, the recruitment of inflammatory cells and astrocytes, in an attempt to restore the blood-brain barrier, leads to the formation of glial scar, which is usually accompanied by cavities filled with astrocytes secreted substances, such as chondroitin sulfate proteoglycans, which also acts as axon growth inhibitory molecules^[8,9]. Furthermore, there is also a lack of trophic

factors in the lesion milieu due to intrinsic changes in neurons such as atrophy and death after axonal injury. Together, all these inhibitory molecules form a glial microenvironment which is hostile to axonal repair^[2,4,10,11].

Although effective treatments for SCI remain limited, there have been many studies in recent years that have promised for the future from a clinical translational perspective. In general, basic science, preclinical, and clinical studies are aimed at overcoming the factors that are involved in impeding recovery from SCI. Current research is aimed at preventing secondary injury, promoting regeneration, and replacing destroyed spinal cord tissue. In particular, a variety of therapies have been addressed to alter neuro-inflammation^[12-14], reduce free radical damage^[15-17], reduce excitotoxic damage to neurons^[18,19], improve blood flow^[20,21], and counteract the effects of local ionic changes^[20,22-25]. Current experimental studies and the knowledge of clinical situations provide us with a better understanding of the complex interaction of the pathophysiological events after SCI. Future approaches should involve strategies aimed at blocking the multiple mechanisms of progressive pathogenesis in SCI and therefore promoting neuroregeneration.

Methylprednisolone (MP), a glucocorticoid, is the only current pharmacotherapy approved for SCI in the human clinic. Although therapy with methylprednisolone has been shown to be protective, its efficacy is limited and it only marginally improves outcomes^[14]. Recent advances in SCI research have led to a variety of novel experimental therapeutic strategies. The approach based on cell therapy using various lineages of stem cells has been considered as the most potential for the treatment of spinal cord injuries^[26]. Cell transplantation after spinal cord injury has several goals, among them, filling the cavity of the lesion to make a bridge that joins the edges of conserved areas, restore dead cells (neurons or myelinating cells) and make a favorable environment for axonal regeneration. Our laboratory employed *in vivo* experiments using predifferentiated embryonic stem cells^[27], human dental pulp stem cells^[28], and mesenchymal stem cell (MSC) (data not published) as a therapy for compressive spinal cord injury in mice, and our results show that these treatments lead to positive and similar functional and morphological responses. Among these lineages, mesenchymal stem cells have strengths such as easy extraction and cultivation, and do not involve ethical and moral issues, making them one of the favorite lineages for spinal cord injury treatment.

MSC THERAPY FOR SPINAL CORD LESION: FROM EXPERIMENTAL STUDIES TO CLINICAL TRIALS

MSC transplantation has been extensively investigated by several groups and these cells can be considered a feasible candidate for treatment of central nervous system diseases because they have characteristics that address the multifactorial events that occur after SCI. These cells have anti-inflammatory, immunomodulatory^[29] and neu-

roprotective^[30] effects. It has also been shown that MSC can secrete trophic factors thus exerting a paracrine effect that can stimulate axon regeneration contributing to functional recovery enhancement.

Concerning the paracrine effect, some groups have identified the ability of these cells in secreting pro-survival factor such as insulin-like growth factor (IGF) brain-derived neurotrophic factor (BDNF), vascular endothelial growth factor (VEGF), granulocyte-macrophage colony stimulating factor (GM-CSF), fibroblast growth factor-2 (FGF2) and transforming growth factor beta (TGF- β)^[31-33]. In addition, MSC can be combined with gene therapy, by introducing genes to generate molecules with great therapeutic potential in promoting neuron survival and regeneration^[34]. Table 1 is a summary of preclinical studies using MSC for spinal cord injury, from 2010 until now.

Sources of MSC

MSC reside in a range of adult tissues that are easily accessible such as bone marrow, adipose tissue, skin, and even peripheral blood^[34]. Most of the studies in SCI use MSC derived from bone marrow and adipose tissue, but it is also possible to get MSC from a perinatal source like umbilical cord blood, umbilical cord matrix^[74], amniotic fluid and placenta^[75-77]. MSC can be extracted from these tissues and plated to be used in autologous transplantation, minimizing the rejection risk.

Studies using MSC extracted from bone marrow in rodents have demonstrated a beneficial effect of cell transplantation after SCI. The beneficial effect of MSC is usually attributed to secretion of neurotrophic factors^[78,79] and anti-inflammatory cytokines^[71,80,81]. Studies performed with pigs^[82] and monkeys^[83] showed that MSC can promote axonal growth and sprouting, corroborating the previous results in rodents, thus supporting the clinical use of MSC.

MSC extracted from adipose tissue is considered an attractive source of cells due to easiness of isolation, obtention of a large amount of cells per donor, and also due to the fact that this tissue is usually discarded after liposuctions. In SCI models, treatment with these cells have resulted in cell survival, neuroprotection, attenuation of secondary damage, axonal regeneration, decrease of gliosis, angiogenesis and enhanced functional recovery^[61,84-90]. A comparative study using MSC extracted from both bone marrow and adipose tissue after SCI found that both sources of MSC expressed similar surface protein markers, but animals that received adipose tissue cells presented higher levels of tissue BDNF, increased angiogenesis, higher number of preserved axons and a decrease in the number of macrophages, suggesting that the use of MSC extracted from adipose tissue is a better candidate for SCI treatment^[41]. However, this is not a consensus and should be further investigated because in another comparative study published in 2012, the authors did not find any difference between animals that received MSC derived from bone marrow or adipose tissue, in

terms of axonal regeneration, neuroprotection and functional recovery after a compression lesion in dogs^[51].

Despite being less investigated in terms of SCI treatment, MSC extracted from perinatal tissues also present a therapeutic potential. Human umbilical cord blood cells (hUCBC) transplantation in rats submitted to an injury, resulted in differentiation of these cells into neural cells and downregulation of the fas/caspase-3 pathway in neurons and oligodendrocytes, and also increased levels of anti-apoptotic proteins^[91,92].

The umbilical cord matrix, also known as Wharton's jelly, possesses a stem cell population that present some advantages in comparison to other sources because they can proliferate more rapidly and extensively than adult MSC^[76,93] and also because they are easily obtained after normal and cesarean births, with low risk of viral contamination^[94,95]. Other advantage is the possibility of using them for allogenic transplantation because they act by suppressing immune response and are, therefore, considered non-immunogenic cells^[96]. Some studies using umbilical cord matrix-derived MSC indicated that these cells can survive in the injury site and promote repair and recovery after SCI. This improvement is attributed to immunomodulatory and trophic effects through secretion of glial-derived neurotrophic factor (GDNF), BDNF and nerve growth factor (NGF) which are known as supporters of cell survival and regeneration^[54,97].

The amniotic fluid cells constitute another source of MSC, which are obtained from discarded post-partum tissue, without any ethical objections about their use. They present similar proliferation and differentiation patterns in comparison to adult MSC^[98,99]. According to few studies, these cells are able to enhance cell survival and axon myelination and improve hind limb function, after transplantation in SCI models^[100]. Some studies have also demonstrated the immunomodulatory effect and trophic support provided by these cells after SCI^[101,102].

Issues regarding the quantity and best via of administration of MSC for SCI

Two important questions that should be addressed when we discuss MSC and its efficacy in treating central nervous system disorders are: the ideal quantity of cells and the best administration *via*. Concerning the cell quantity, the literature presents several studies using different amount of cells. In terms of cell administration, most transplantation is delivered directly into the injury site or adjacent to it, by injecting few microliters of cell suspension^[103]. Attempts have been made to inject cells intravenously or intraperitoneally in order to decrease tissue damage and, thus, avoiding subjecting the individual to another surgical intervention.

There are several studies that injected different quantity of cells with similar results. Apart from the difference on the quantity of cells, there are other points that make the comparison among these studies difficult, such as the diversity of lesion models, animal types and route of cell administration. For example, Cizkova and colleagues^[104]

Table 1 Summary of pre-clinical studies using mesenchymal stem cell for spinal cord injury

| Animal | Lesion type | Cells source | Route of administration | Effects on CNS regeneration | Ref. |
|--------|--|---|---|---|------------|
| Rat | Contusion | Human mesenchymal precursor cells | Lesion site | Improvement in functional recovery and tissue sparing and reduction of cyst volume | [35] |
| Rat | Contusion | Human bone marrow-MSC | Lesion site , intracisternal, intravenous | Improvement in functional recovery | [36] |
| Rat | Hemisection | Bone marrow-MSC induced into Schwann Cells | Lesion site | Improvement in locomotor and sensory scores, axonal regeneration and remyelination | [37] |
| Rat | Contusion | Bone marrow-MSC | Lesion site , intravenous | Improvement in locomotor scores and NGF expression | [38-40] |
| Rat | Transection to the dorsal columns and tracts | Bone marrow-MSC, adipose derived-MSC | Lesion site | Improvement in locomotor scores, increased angiogenesis, preserved axons, decreased numbers of ED1-positive macrophages and reduced lesion cavity formation | [41] |
| Rat | Hemisection | Human umbilical cord-derived MSC | Lesion site | Suppress mechanical allodynia, and this effect seems to be closely associated with the modulation of spinal cord microglia activity and NR1 phosphorylation | [42,43] |
| Rat | Hemisection | Human bone marrow-MSC | Lesion site | Improvement in locomotor scores, shorter latency of somatosensory-evoked potentials and differentiation into various cells types | [44] |
| Rat | Hemisection | Bone marrow-MSC | Lesion site | Improvement in locomotor scores and reduced lesion cavity formation | [45] |
| Mouse | Compression | Bone marrow-MSC | Lesion site | Improvement in locomotor and sensory scores and reduced lesion volume | [46] |
| Rat | Contusion | Human bone marrow-MSC | Lesion site | Improvement in functional recovery, tissue sparing and reduction in the volume of lesion cavity and in the white matter loss | [35,47-49] |
| Rat | Contusion | Human umbilical cord-MSC | Lesion site | Improvement in functional recovery, reduction of the extent of astrocytic activation and increased axonal preservation | [50] |
| Dog | Compression | Bone marrow, adipose, Wharton's jelly, umbilical cord derived-MSC | Lesion site | Improvement in functional recovery, increased numbers of surviving neurons, smaller lesion sizes and fewer microglia and reactive astrocytes in the epicenter of lesion | [51] |
| Rat | Compression | Bone marrow-MSC | Intravenous | Improvement in functional recovery, increase of NGF expression, higher tissue sparing and density of blood vessels | [52] |
| Rat | Contusion | Human umbilical cord-MSC | Lesion site | Improvement in functional recovery, endogenous cell proliferation and oligogenesis, and smaller cavity volume | [53,54] |
| Rat | Transection | Human-MSC | Lesion site | Improvement in functional recovery, increased amplitude of motor-evoked potentials, differentiation into neural cells | [55,56] |
| Rat | Contusion | Bone marrow-MSC | Lesion site | Improvement in functional recovery, preservation of axons, less scar tissue formation and increase in myelin sparing; higher levels of IL-4 and IL-3 and higher numbers of M2 macrophages, and reduction in TNF- α and IL-6 levels, and in numbers of M1 macrophages | [57-60] |
| Dog | Compression | Neural-induced adipose derived-MSC | Lesion site | Improvement in functional recovery and neuronal regeneration, and reduction of fibrosis | [61] |
| Mouse | Transection | Bone marrow-MSC | Lesion site | Improvement in functional recovery and neuronal survival, reduction of cavity volume and attenuation of inflammation, promotion of angiogenesis and reduction of cavity formation | [62-64] |
| Rat | Compression | Bone marrow-MSC | Lesion site | Improvement in functional recovery, up-regulation of VEGF mRNA expression, increase in angiogenesis and prevention of tissue atrophy | [65-67] |
| Rat | Compression | Human umbilical cord-MSC | Lesion site | Improvement in functional recovery, increase in the intensity of 5-HT fibers and in the volume of spared myelination; decrease in the area of the cystic cavity | [68] |
| Dog | Compression | Umbilical cord-MSC | Lesion site | Improvement in functional recovery, promotion of neuronal regeneration and reduction of fibrosis | [69] |
| Dog | Compression | Human umbilical cord-MSC | Lesion site | Improvement in functional recovery and remyelination | [70] |
| Rat | Contusion | Bone marrow-MSC | Intrathecal | Improvement in functional recovery | [71] |
| Rat | Contusion | Human bone marrow-MSC | Lesion site , lumbar puncture | Improvement in functional and sensory recovery | [72] |
| Rat | Contusion | Neural differentiated and undifferentiated MSC | Lesion site | Improvement in functional recovery and reduction of cavitation | [73] |

CNS: Central nervous system; MSC: Mesenchymal stem cell; TNF: Tumor necrosis factor; IL: Interleukin; NGF: Nerve growth factor; VEGF: Vascular endothelial growth factor.

demonstrated cell survival and enhancement in locomotor performance after MSC transplantation delivered by intravenous injection (one million cells in a volume of 0.5 mL of DMEM) in a model of balloon compressive injury in rats, while Sheth *et al.*^[105] performed cell transplantation (600000 cells in a volume of 6 μ L) directly into the injury site after contusive injury in rats, and also observed an enhancement in locomotor function and a decrease in the lesion volume, indicating a neuroprotective effect of these cells. Thus, it is still difficult to determine the ideal quantity of cells and the best *via* for stem cell transplantation after SCI. The questions that arise from these studies are: Is there a minimum number of transplanted cells that can be used and yet giving the best results in terms of functional recovery? Can we get similar results with cells injected systemically in comparison to local injection? Studies using the same type of lesion and different amount of cells and administration *via* should be further undertaken in order to better clarify this issue.

Time point for cell transplantation

Other crucial issue that should be further addressed here is the time point for cell transplantation after lesion. This is important because the environment created after SCI is hostile for regeneration and can negatively influence cell survival and differentiation. Thus, depending on the time that the treatment is performed the results can be completely different. Most studies have been performed in acute or sub-acute phases, which means immediately or 1-2 wk after injury, respectively^[35,103]. There are fewer studies in the SCI chronic phase, when cells are delivered in later stages, when the glial scar is already present^[38,41].

Clinical trials

The clinical trials conducted for SCI comprise three different phases with human participation in all phases. The phase 1 trial begins with the administration of the cell transplants to a human subject with the aim to investigate the presence of adverse or toxic effects and treatment safety. People who participate in these trials may experience some risks and have limited benefits. In phase 2, the objective is to determine the potential and variability of a therapy in comparison with a control group. The participants are usually recruited and randomly assigned to the groups (experimental or control) and both, participants and investigators, do not know to which study they have been assigned to. The phase 3 clinical trials are usually the definitive clinical trial. The aim is to confirm the preliminary results obtained at the phase 2, with a statistically significant clinical benefit of the therapeutic intervention. The number of subjects is also larger and multiple study centers are involved^[106,107]. The majority of the studies using MSC transplantation after spinal cord injury are in phase 1 or 2.

At the time of writing this article there were twenty clinical trials being either completed, ongoing or in the recruitment stage, using either adult or perinatal sources of mesenchymal stem cells in different phases of the dis-

ease, and most of them use autologous transplantation to minimize the risk of rejection. Table 2 list the clinical trials listed on the clinical trials.gov.

The number of clinical trials using MSCs for treatment of SCI is increasing, indicating that despite several questions that still need to be addressed at basic and pre-clinical levels, the MSC are considered potentially beneficial for translational studies.

According to PubMed database, in the last three years only three studies were published in “clinical trials” category, using MSC transplantation after SCI. One of them transplanted autologous bone marrow-derived MSC into the cerebrospinal fluid of patients with complete SCI. The authors described that 45% of the patients showed a recovery, but there was no difference between these patients and those from control groups; they emphasized that despite the fact that results were not positive, the transplantation was a feasible and safe technique, since patients did not present any adverse reaction^[108]. On the other hand, Park *et al.*^[109] using the same cell source, and repeated cells injections directly into the spinal cord, demonstrated that three of ten patients presented a motor improvement, and significant magnetic resonance changes and electrophysiological results. These results are similar to those obtained by Dai *et al.*^[110] who also demonstrated a clinical improvement in patients that received autologous MSC transplantation. The results of these studies are not conclusive, and, unfortunately, not as good as those obtained in pre-clinical experiments. In spite of that, all of them emphasize mesenchymal stem cell clinical potential.

WALLERIAN DEGENERATION AND NERVE REGENERATION IN THE PERIPHERAL NERVOUS SYSTEM

Traumatic injury to nerves in the peripheral nervous system (PNS) is a large-scale problem annually affecting more than one million people worldwide. These injuries often result in pain and disabilities, owing to reduction in motor function and sensory perception. Moreover, the trauma can cause emotional, social and work-related disorders, and the affected individuals undergo a reduction in their quality of life^[111,112].

While it is widely accepted that the PNS has an inherent potential for regeneration, functional recovery after a lengthy peripheral nerve injury (PNI) remains unsatisfactory^[113]. After an extensive traumatic nerve injury with a large gap between the proximal and distal nerve stumps, a long period of time is required for regenerating axons to cross that gap. During that time, the ability of axotomized neurons to regenerate declines and Schwann Cells (SC) can no longer support regenerating neurons and their axons. As a result, regenerating axons fail to reach their target organs and the injury cannot be successfully repaired. In order to accelerate the rate of axonal growth many therapeutic strategies are being developed and in-

Table 2 Summary of clinical trials studies using mesenchymal stem cell for spinal cord injury

| Title | Lesion type | Cells source | Phase of the study | Status | Effects on CNS regeneration |
|--|---|--|-------------------------|----------|--|
| Clinical study of treatment for acute SCI using cultured bone marrow stromal cells | Cervical SCI | Autologous Bone marrow-MSc | Terminated | 1/2 | Rapid and remarkable recovery of ASIA B and C patients, but gradual or limited in ASIA A patients. |
| Autologous mesenchymal stem cell in SCI patients | Complete cervical or thoracic SCI | Autologous bone marrow-MSc | Enrolling by invitation | 2 | Not informed |
| Different efficacy between rehabilitation therapy and umbilical cord derived MSCs transplantation in patients with chronic SCI in China | Traumatic SCI | Umbilical cord derived-MSc | Recruiting | 3 | Not informed |
| A phase III/IV clinical trial to evaluate the safety and efficacy of bone marrow-derived MSC transplantation in patients with chronic SCI | Cervical SCI | Autologous bone marrow-MSc | Recruiting | 1/2 | Not informed |
| Phase I / II trial of autologous bone marrow derived MSCs to patients with SCI | Traumatic thoracic or lumbar SCI | Autologous bone marrow-MSc | Recruiting Completed | 1/2 1 | Not informed |
| Safety of autologous adipose derived MSCs in patients with SCI | Clinical diagnosis of SCI (ASIA A to C) | Autologous Adipose derived-MSc | | | |
| The effect of intrathecal transplantation of autologous adipose tissue derived MSCs in the patients with SCI, phase I clinical study | Clinical diagnosis of SCI | Autologous Adipose derived-MSc | Recruiting | 1 | Not informed |
| Phase I, single center, trial to assess safety and tolerability of the intrathecal infusion of ex-vivo expanded bone-marrow derived MSCs for the treatment of SCI | Clinical diagnosis of SCI (ASIA A) | Autologous bone marrow-MSc | Active, not recruiting | 1 | Not informed |
| Study the safety and efficacy of bone marrow derived autologous cells for the treatment of SCI | Clinical diagnosis of SCI | Autologous bone marrow-MSc | Recruiting | 1/2 | Not informed |
| Surgical transplantation of autologous bone marrow stem cells with glial scar resection for patients of chronic SCI and intra-theal injection for acute and subacute injury-a preliminary study | Complete spinal cord trans-section | Autologous bone marrow-MSc | Completed | 1/2 | Not informed |
| To study the safety and efficacy of autologous bone marrow stem cells in patients with SCI | SCI below C5 (ASIA A to C) | Autologous bone marrow-MSc | Recruiting | 1/2 | Not informed |
| Safety of autologous stem cell treatment for SCI in children | Clinical diagnosis of SCI (ASIA A to D) | Bone marrow-MSc | Recruiting | 1 | Not informed |
| Autologous bone marrow derived cell transplant in SCI patients | Traumatic SCI | Autologous bone marrow-MSc | Completed | 1/2 | Not informed |
| Phase 1 study of autologous bone marrow stem cell transplantation in patients with SCI | Traumatic thoracic or lumbar SCI | Autologous bone marrow-MSc | Not informed | 1 | Not informed |
| Phase I pilot study to evaluate the security of local Administration of autologous stem cells obtained from the bone marrow stroma, in traumatic injuries of the spinal cord | Traumatic SCI between C3 and L1 | Autologous bone marrow-MSc | Recruiting | 1 | Not informed |
| Feasibility and safety of umbilical cord blood cell transplant into injured spinal cord: an open-labeled, dose-escalating clinical trial | Chronic SCI between C5 and T11 (ASIA A) | Umbilical cord blood mononuclear derived-MSc | Active, not recruiting | 1/2 | Not informed |
| Efficacy difference between rehabilitation therapy and umbilical Cord derived transplantation in patients with acute or chronic SCI in China | Clinical diagnosis of SCI | Umbilical cord derived-MSc | Not informed | 2 | Not informed |
| Safety and feasibility of umbilical cord blood cell Transplant Into Injured Spinal cord: an open-labeled, dose-escalating clinical trial | Chronic SCI between C5 and T11 (ASIA A) | Umbilical cord derived-MSc | Active, not recruiting | 1/2 | Not informed |
| Safety and effect of lithium, umbilical cord blood cells and the combination in the treatment of acute and sub-acute spinal cord injury : a randomized, double-blinded placebo-controlled clinical trial | Acute or Subacute traumatic SCI between C5 and T11 (ASIA A) | Umbilical cord derived-MSc | Active, not recruiting | 1/2 | Not informed |

MSC: Mesenchymal stem cell; CNS: Central nervous system; SCI: Spinal cord injury.

vestigated. The identification of crucial elements responsible for successful regeneration in injured peripheral nerves will be quintessential in improving regenerative outcomes after peripheral and central nerve injuries.

Nerve trauma elicits a cascade of molecular, cel-

lular, and ultrastructural responses which are necessary for degeneration and posterior regeneration, including: disruption of axonal conduction; increase in cell body metabolism and protein synthesis; degeneration of the distal stump of injured axons; dedifferentiation and pro-

liferation of SC; degradation of the myelin sheath; recruitment of macrophages to the site of injury^[114], as well as the release of cytokines, neurotrophins and growth factors^[115-117]. These events will allow rapid and efficient removal of the growth inhibitory cellular debris present in the injured peripheral nerve milieu, producing a favorable microenvironment for axonal growth^[118].

After an injury the axon is divided into two segments: a proximal stump that remains in contact with the cell body, and a distal stump which is separated from the rest of the neuron. The distal nerve stump undergoes a cascade of events called "Wallerian degeneration"^[119,120], which is initiated within 24 to 48 h by the entry of calcium in the axoplasm. Calcium influx activates proteases, such as calpains that promote cytoskeletal degradation and disintegration of axoplasm, myelin and axolemma^[121,122]. The rupture of the blood-nerve barrier allows the entry of macrophages into the site of injury and, together with SC, these cells initiate intense phagocytosis and removal of degenerating axon and myelin debris. The barrier permeability decreases two weeks after the injury and then, in the fourth week, increases again in order to regain homeostasis after Wallerian degeneration^[118].

Immediately after injury, the SC in the distal stump of the nerve begin the process of dedifferentiation. Even before axonal degeneration occurs, SC can modify its gene expression^[123] and 48 h after injury, they decrease myelin protein expression, acquire a non-myelinating phenotype and begin to express genes related to regeneration, like growth associated protein 43 (GAP-43), neurotrophic factors and their receptors, neuroregulins and their receptors, and assume an intense proliferative activity^[124,125]. About four days after injury SC reach their proliferation peak. These proliferative cells are confined within the tube formed by its own basal lamina and align forming the so called bands of Büngner. These bands columns will form a supportive substrate, providing clues that will guide axon growth toward the target organ, through the release of trophic factors. When SC contact the regenerating axons, the process of re-myelination is started^[126].

The injury also causes a rapid arrival of signals from the damaged axons to the neuronal body resulting in an extraordinary change from a transmitting to a growth promoting phenotype. Cell body suffers a process called chromatolysis, which is characterized by swelling of the neuronal body and by dispersion of Nissl corpuscles^[127,128]. These changes reflects variations in the metabolic activity of neurons which, as a result, fail to synthesize proteins required for neurotransmission, and start producing substances that are important for axonal sprouting and growth^[129]. The regeneration that follows occurs *via* different mechanisms: the elongation of the distal end of injured axons and the growth of collateral axons from nodes of Ranvier in the proximal stump. However, the success of regeneration and target organ reinnervation depends mostly on the enhancement of the number of regenerating axons, the velocity of axon

growth and on the ability of affected neurons to survive and acquire a regenerative phenotype.

In the clinical settings, reconstruction of transected peripheral nerve requires accurate microsurgical repair that connects the proximal and distal stumps of the nerve in a tension-free manner. In cases of injury with tissue loss, autologous peripheral nerve grafts, *i.e.*, autografts, is considered by neurosurgeons the gold standard technique, but unfortunately, even in these cases, the clinical results remain disappointing and, therefore, the search for better strategies is an urgent necessity. In cases of digital nerve lesions, biodegradable artificial nerve conduits are being used in the clinical settings, but their use is still limited to these thin nerves. An advantage of the use of these conduits is that they can be combined with other pro-regenerative strategies, such as the local injection of neurotrophic factors and cells.

New therapeutic approaches should have as a goal an increase of the intrinsic regenerative capacity of transected nerve fibers and a decrease of the extrinsic factors that limit regeneration of severed nerve fibers, thus creating an appropriate environment in which, axon elongation, remyelination and proper reinnervation of target organ may occur. A stem cell-based therapy represents an important new strategy to manage peripheral nerve injury. In the next part of this review we will discuss the potential use of mesenchymal stem cells, in promoting nerve regeneration.

MSC THERAPY IN PNS: FROM EXPERIMENTAL STUDIES TO CLINICAL TRIALS

A number of experimental studies have shown the potential of MSC to improve peripheral nerve regeneration following traumatic injuries^[130-135]. These cells may act on nerve regeneration mainly by paracrine, neuro/axonoprotective, or immunomodulatory effects; by transdifferentiation into SCs; by cell-to-cell contact; or even by a combination of the above mechanisms^[134]. However, most of the beneficial effects exerted by the MSC are strongly correlated with the production of neurotrophic substances, such as FGF, NGF, ciliary neurotrophic factor, BDNF, GDNF among others^[132,133,136,137].

Our group showed the presence of high levels of NGF-b in the in MSC *in vitro* suggesting that they are also able to express this potent neurotrophic factor *in vivo*; this result could represent one mean by which these cells acted on the enhancement of axon regeneration and remyelination, consequently contributing to the observed return of motor function^[133]. In agreement with these findings, bone marrow-MSC locally injected in the mouse ischiatic nerve resulted in improvement of regeneration of sensory and motor axons^[134]. Because these authors also observed that these cells were capable of increasing neurite outgrowth *in vitro* through NGF releasing, and that they presented low potential to differentiate into SC

in vivo, they suggested that the beneficial effects exerted by the implanted cells were mainly dependent on their trophic activity rather than their stemness potential^[134]. In another work, our group also observed the benefits of bone marrow-MSC locally injected in the mouse median nerve following transection and conduit repair. This cell system was capable of increasing the number of both myelinated and unmyelinated fibers, preventing the muscle atrophy and, most importantly, improving functional performance^[130].

It is also possible that MSC can act indirectly on nerve regeneration by modulating cellular behaviors such as inducing SC to survive, proliferate, produce neurotrophic factors and promote remyelination. A coculture system with rat bone marrow-MSC conditioned media and SC demonstrated cell-cell interactions despite no direct contact between the two population of cells. MSC not only favored survival and proliferation of SC but also induced them to express NGF, BDNF and NGF receptors^[138]. This is an important MSC feature as it might indicate that MSC can relay and magnify neurotrophic function from stem cells to glia cells, thus improving peripheral nerve regeneration.

Besides rodents, larger animal models have also been used to investigate the effects of MSC-based therapy on more challenging nerve gaps. Few authors have shown the successful bridging of a 30 mm-long ischiatic nerve defect by means of a biodegradable conduit in dogs^[139]. After six months of MSC implantation, they observed the reconstruction of ischiatic nerve trunk with restoration of nerve continuity, functional recovery for conducting electrical impulses and transporting materials, and muscle re-innervation, which lead to improvement of locomotion activities. Even more challenging, using a two-fold nerve gap in a similar experimental model but with addition of autologous MSC, the same group^[140] demonstrated that the cellular treatment improved nerve regeneration and functional recovery in a manner comparable to the autograft-treated animals, which is considered by neurosurgeons the current gold standard for peripheral nerve repair.

As aforementioned, the great majority of the experimental studies of mesenchymal stem cell-based therapy on the peripheral nerve regeneration use rodents (mainly mice and rats) as animal models^[130,133,134,138], perhaps because they are small size mammals and, consequently, easy to handle; also, they have been extensively used in the field of genetic engineering for a diversity of experimental trials of gain and loss of function as well as reporter assays. However, there are few studies using non-human primates such as cynomolgus and rhesus monkeys, which share high level of sequence homology with human genome, that have confirmed the feasibility of this cell system for improving nerve regeneration after severe nerve lesions. MSC transplantation into either allogeneic nerve grafts^[141] or artificial conduits^[142] for bridging severe upper extremity nerve defects in higher primates yielded structurally and functionally regenerated

nerves; these studies proved to be safe and effective, thus giving great insight into the use of MSC in human clinics.

MSC obtained from human subjects have also been used in pre-clinical studies for promoting nerve regeneration, yielding promising results^[143-145]. These studies are of great relevance because they address human MSC properties, clarifying their mechanisms of action, and also provide insight into their effects on peripheral nervous tissue recovery. Interestingly, the authors of these studies demonstrated that human MSC-based therapy improved peripheral nerve regeneration as well as functional recovery. However, McGrath *et al.*^[145] showed that MSC survived in the conduit and enhanced axonal regeneration only when transplantation was combined with the immunosuppressive treatment, cyclosporine A. As these results provide evidence of the nerve regeneration potential of human MSC, and taking into account that one of the great advantages of MSC is the possibility of auto transplantation without donor-site morbidity, they might encourage the use of this cell system for treating human peripheral nerve trauma.

Thus, the results of pre-clinical studies highlighting the improved outcomes yielded by using MSC with the aim to repair a large nerve gap may increase the feasibility of translation of MSC-based therapy to clinical trials for peripheral nerve applications.

Table 3 summarizes the studies using MSC for nerve injuries, either in pre-clinical or clinical trials, since 2010 until now. To date, only one clinical trial has used autologous bone marrow mononuclear cells within silicone tubes to repair human median or ulnar nerves^[146]. In this study scores for motor function, sensation and the effect of pain on function were better than those obtained from individuals that had the tubular nerve repair only; However, a possible limitation in this study is the fact that there was a difference between groups regarding the age of individuals and the length of follow-up after treatment, which could represent biases in this study. So, the interval between injury and treatment was always longer than 75 d, which could possibly limit the positive effects exerted by the cells on the nerve regeneration process. Another possible disadvantage of this work is that nerve conduits were made of silicone, a non-biodegradable material, thus requiring a second surgery to remove the conduit. In spite of these limitations cells-treated patients presented a better recovery compared to the untreated. The results of this study will, hopefully, encourage subsequent clinical studies to be conducted safely, with fewer biases, and with the association of the cellular treatment with suitable biodegradable conduits, thus preventing discomfort and complications generated from the use of silicone material.

Although important advances have been achieved in the use of stem cells for improving nerve regeneration, they are still limited to basic and pre-clinical trials. In addition, there are several variables among these studies, such as tissue source; methods of cell isolation, expansion and characterization; route of cell delivery; number

Table 3 Summary of pre-clinical and clinical studies using mesenchymal stem cell for peripheral nerve injury

| Animal | Nerve | Lesion type | Cells source | Route of administration | Ref. |
|--------|-----------------|-------------|---|--------------------------|-------------------|
| Mouse | Median | Transection | Bone marrow-MSC | Local | [147] |
| Mouse | Ischiatic | Transection | Bone marrow-MSC | Local | [148] |
| Mouse | Ischiatic | Transection | Embryonic stem cell derived-MSC | Local | [144] |
| Mouse | Ischiatic | Crush | Adipose derived-MSC | Intravenous | [149] |
| Mouse | Ischiatic | Transection | Bone marrow-MSC | Local | [131] |
| Rat | Ischiatic | Crush | Amniotic fluid derived-MSC | Local | [150] |
| Rat | Ischiatic | Crush | Amniotic fluid derived-MSC | Intravenous | [151-153] |
| Rat | Facial | Transection | Bone marrow-MSC | Local | [154,155] |
| Rat | Ischiatic | Crush | Bone marrow-MSC | Local | [156] |
| Rat | Ischiatic | Transection | Adipose derived-MSC | Local | [157-159] |
| Rat | Ischiatic | Transection | Umbilical cord derived-MSC | Local | [160,161] |
| Rat | Ischiatic | Transection | Bone marrow-MSC | Local | [145,158,162-169] |
| Rat | Cavernous | Traction | Bone marrow-MSC | Intracavernous injection | [170] |
| Rabbit | Facial | Transection | Bone marrow-MSC | Local | [171] |
| Rabbit | Ischiatic | Traction | Bone marrow-MSC | Local | [172,173] |
| Pig | Ischiatic | Transection | Bone marrow-MSC | Local | [174] |
| Dog | Ischiatic | Transection | Bone marrow-MSC | Local | [140,175] |
| Dog | Ischiatic | Transection | Adipose derived-MSC | Local | [176] |
| Monkey | Median | Transection | Bone marrow-MSC | Local | [142] |
| Human | Median or ulnar | Transection | Bone marrow mononuclear cell fraction-MSC | Local | [146] |

MSC: Mesenchymal stem cell.

of transplanted cells; therapeutic time window; animal and nerve models; type of injury; number of transplanted cells; and immunogenicity. These variables represent an important obstacle for comparing and contrasting study outcomes from different groups, thus hindering progress in the field.

In 2006, The International Society for Cellular Therapy proposed the development of a set of minimal criteria (adherence to plastic in standard culture conditions, expression of a number of markers and multipotent differentiation potential into osteoblasts, adipocytes and chondroblasts) for defining the MSC for research purposes^[177]. Although this action represented a great attempt to allow for comparison of scientific studies among different groups, the criteria for mesenchymal cells from different species should be further considered and well-defined, in particular the non-human and human primate MSC.

CONCLUSION

Pre-clinical studies have shown the beneficial effects of MSC therapy in the neurotrauma field. Unfortunately, these effects are not usually seen in the clinical trials, and the results are far from being as good as those described in experimental studies. Therefore, there is an urgent need to seek for standardization of protocols in terms of source of cells, culture conditions, time of treatment after injury, number and *via* of administration of cells, plasticity and capability of human MSC after extraction and expansion in culture, among other concerns. Basic and pre-clinical studies focusing on these important points will, hopefully, be of great help in terms of their successful implementation in clinical trials.

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P- Reviewers: Kita K, Zocchi E **S- Editor:** Song XX **L- Editor:** A
E- Editor: Zhang DN



WJSC 6th Anniversary Special Issues (2): Mesenchymal stem cells

Umbilical cord-derived mesenchymal stem cells: Their advantages and potential clinical utility

Tokiko Nagamura-Inoue, Haiping He

Tokiko Nagamura-Inoue, Haiping He, Department of Cell Processing and Transfusion, The Institute of Medical Science, The University of Tokyo, Tokyo 108-8639, Japan

Haiping He, Division of Molecular of Therapy, Center for Advanced Medical Research, The Institute of Medical Science, The University of Tokyo, Tokyo 108-8639, Japan

Author contributions: Both authors contributed to this work.

Correspondence to: Tokiko Nagamura-Inoue, MD, PhD, Department of Cell Processing and Transfusion, Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan. tokikoni@ims.u-tokyo.ac.jp

Telephone: +81-3-54495688 Fax: +81-3-5449 5438

Received: October 31, 2013 Revised: January 21, 2014

Accepted: February 20, 2014

Published online: April 26, 2014

Abstract

Human umbilical cord (UC) is a promising source of mesenchymal stem cells (MSCs). Apart from their prominent advantages, such as a painless collection procedure and faster self-renewal, UC-MSCs have shown the ability to differentiate into three germ layers, to accumulate in damaged tissue or inflamed regions, to promote tissue repair, and to modulate immune response. There are diverse protocols and culture methods for the isolation of MSCs from the various compartments of UC, such as Wharton's jelly, vein, arteries, UC lining and subamniotic and perivascular regions. In this review, we give a brief introduction to various compartments of UC as a source of MSCs and emphasize the potential clinical utility of UC-MSCs for regenerative medicine and immunotherapy.

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Key words: Umbilical cord; Mesenchymal stem cells; Wharton's Jelly; Multipotency; Immunotherapy

Core tip: Human umbilical cord (UC) is a promising source of mesenchymal stem cells (MSCs). UC-MSCs have shown the ability of faster self-renewal and to differentiate into three germ layers, to accumulate in damaged tissue or inflamed regions, to promote tissue repair, and to modulate immune response. There are diverse protocols and culture methods for the isolation of MSCs from the various compartments of UC, such as Wharton's jelly, vein, arteries, UC lining membrane and subamniotic and perivascular regions. In this review, we introduce various compartments of UC and discuss the potential clinical utility of UC-MSCs for regenerative medicine and immunotherapy.

Nagamura-Inoue T, He H. Umbilical cord-derived mesenchymal stem cells: Their advantages and potential clinical utility. *World J Stem Cells* 2014; 6(2): 195-202 Available from: URL: <http://www.wjgnet.com/1948-0210/full/v6/i2/195.htm> DOI: <http://dx.doi.org/10.4252/wjsc.v6.i2.195>

INTRODUCTION

Mesenchymal stem cells (MSCs) originate in the human embryo and are considered multipotent stem cells. MSCs are a heterogeneous subset of stromal stem cells, which can be isolated from the bone marrow^[1], mobilized peripheral blood^[2], cord blood^[3], umbilical cord (UC)^[4,5], placenta^[6], adipose tissue^[7], dental pulp^[8], and even the fetal liver^[9] and lungs^[10]. UC contains two umbilical arteries (UCAs) and one umbilical vein (UCV), both embedded within a specific mucous connective tissue, known as Wharton's jelly (WJ), which is covered by amniotic epithelium (Figure 1). UC is considered medical waste and the collection of UC-MSCs is noninvasive; furthermore, the access to UC-MSCs has not been encumbered with ethical problems. UC-MSCs, similarly to MSCs derived

from other sources, have distinct capacity for self-renewal while maintaining their multipotency, *i.e.*, the ability to differentiate into adipocytes, osteocytes, chondrocytes, neurons and hepatocytes, although some differentiation abilities are known to be partial^[11-13]. Moreover, UC-MSCs have also attracted great interest because of their immunomodulatory properties. Nowadays, UC-MSCs are proposed as a possible versatile tool for regenerative medicine and immunotherapy.

HISTORY OF UC-MSCs

During pregnancy, the fetus and mother are connected by UC. UC prevents umbilical vessels from compression, torsion and bending, while providing good blood circulation. McElreavey *et al.*^[4] for the first time reported isolation of fibroblast-like cells from WJ of human UC in 1991. The UC-derived cells have the similar surface phenotype, plastic adherence and multipotency as those of MSCs derived from other sources. It was 3 years earlier that the first cord blood (CB) transplantation was performed in France in 1988^[14]. After that, together with the development of CB banking, CB transplantation has become the alternative source of hematopoietic stem cells. Although CB-derived MSCs cannot be consistently isolated^[15-18], MSCs were considered to circulate in the blood of preterm fetuses and able to be successfully isolated and expanded^[3]. Where these cells home at the end of gestation is not clear^[13]. Thus, UC has inevitably become a focus of attention as a source of MSCs because it contains CB^[18]. One key study appeared concerning CB-derived MSCs appeared around 2003^[19]. Mitchell *et al.*^[20] successfully isolated matrix cells from UC WJ using explant culture and Romanov *et al.*^[19] isolated MSCs-like cells from the subendothelial layer of UCV.

ADVANTAGES OF UC-MSCs

Stem cell populations can be isolated from embryonic, fetal and adult tissues. Embryonic stem cells (ESCs) are a leading candidate for tissue engineering because of their high self-renewal capacity and pluripotency (ability to differentiate into all germ layers) *in vitro* and *in vivo*. Nonetheless, in addition to ethical restrictions, the clinical applications of ESCs are severely limited by technical difficulties with the depletion of immature cells that may result in the formation of a teratoma.

In contrast, adult stem cells, such as those in the skin, bone marrow (BM) and adipose tissue, may have wider clinical applications. BM-MSCs have been used for autologous and allogeneic purposes. Recently, successful clinical application of autologous BM-MSCs was reported for conditions such as cardiac infarction^[21], graft-versus-host disease (GVHD)^[22,23], Crohn's disease^[24] and bone tissue engineering^[25]. On the other hand, the autologous use was sometimes limited by cell numbers and age-related changes such as decreased growth and differentiation capacity^[26,27].

Compared with BM-MSCs and ES cells, UC-MSCs show a gene expression profile more similar to that of ESCs and faster self-renewal rather than BM-MSCs^[11,12].

It is easy to obtain a substantial number of UC-MSCs after several passages and extensive *ex vivo* expansion^[28]. The most appreciable advantage is that the collection procedure is noninvasive and ethically acceptable.

Similar to BM-MSCs, UC-MSCs can be considered for autologous and allogeneic use. Autologous UC-MSCs might be used as gene therapy for genetic diseases and as regenerative or anti-inflammatory therapy for neonatal injury, such as cerebral palsy or hypoxic brain damage. On the other hand, allogeneic UC-MSCs can be expanded and cryopreserved in a cell bank for patients in need. The only disadvantage is that physicians need to confirm the baby's health as a donor because it cannot be ascertained in advance whether the donor will grow normally without health problems; thus, genomic or chromosomal tests need to be performed. In contrast, in the case of a BM donor, the physician can directly see and examine the donor and then decide to collect BM. In the case of CB banking, many CB banks monitor the baby's health after birth. Thus, it is important to know the advantages and disadvantages of UC-MSCs for each clinical application.

DIFFERENT METHODS FOR ISOLATION OF MSCs FROM DIFFERENT COMPARTMENTS OF UC

There are two methods to obtain MSCs from various UC compartments or from the whole UC: the explant method and the enzymatic digestion method.

The explant method

UC, or its compartments, is manually minced into small fragments 1-2 mm³. These fragments are aligned and seeded regularly on the tissue culture-treated dishes. After the tissue fragments are attached to the bottom of the dish, the culture medium is poured slowly and gently, so as not to detach the fragments, and the culture is started^[29-31]. The culture medium is replaced every 3-7 d for 2-4 wk until fibroblast-like adherent cells reach 80%-90% confluence. The adherent cells and tissue fragments are rinsed once with PBS and detached using a trypsin solution, followed by washing with the medium. The cells and tissue fragments are filtered to remove the tissue fragments.

The disadvantage of this method is that the fragments often float in the medium, resulting in poor cell recovery. No MSCs can be obtained from the floating fragments. To collect a consistent number of MSCs each time, it is important to prevent the exfoliation of the tissue fragments from the bottom of plastic dishes.

The enzymatic digestion method

WJ is either directly exposed to enzymatic solutions to

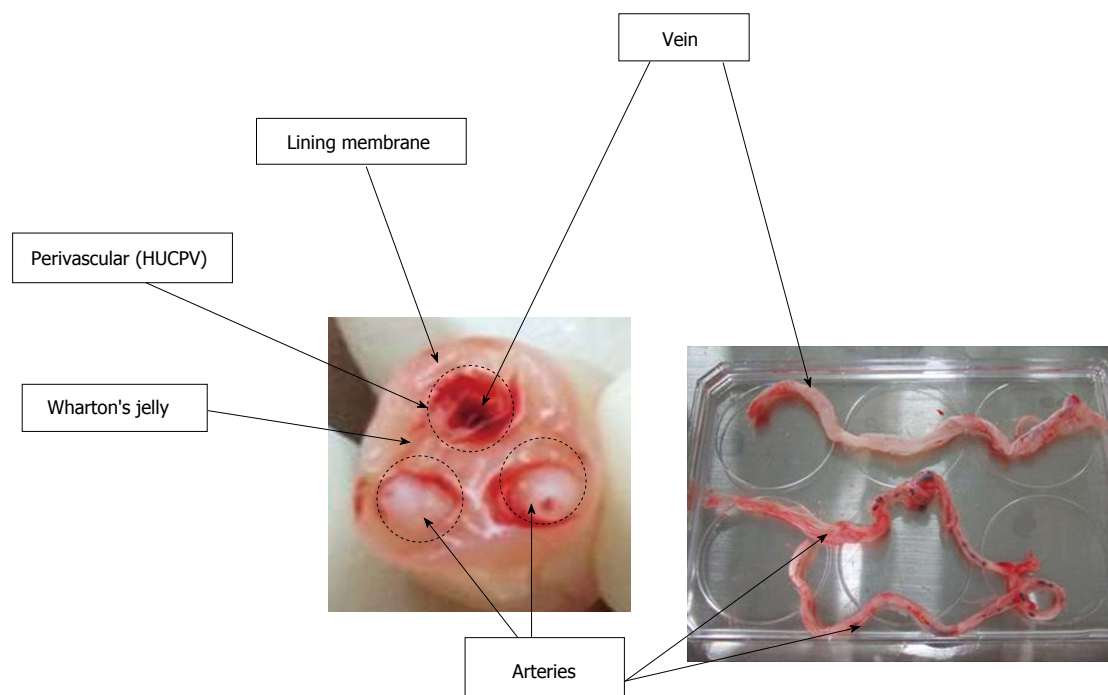


Figure 1 Various compartments of umbilical cord from which mesenchymal stem cells can be isolated. HUCPV: Human umbilical cord perivascular.

release the cells or it is cut into small pieces followed by enzymatic digestion. The enzymes used for digestion vary from simple collagenase^[31,32] to a combination of either collagenase and hyaluronidase with or without trypsin^[33,34] or collagenase, dispase II and hyaluronidase^[33]. The digestion time and concentrations varied by researchers.

There are four compartments of UC as a source of MSCs: (1) Whole UC-MSCs: the whole UC is cut into smaller pieces followed by either an explant procedure or enzyme digestion^[30,35,36]; (2) UCWJ-, UCA- and UCV-MSCs: UCWJ-MSCs are obtained after removing umbilical vessels. Umbilical vessels [two arteries (UCAs) and one vein (UCV)] can also be minced into 1-2 mm³ fragments. The fragments are aligned regularly on the plate and cultured until MSCs start growing; (3) UC lining and subamniotic-derived MSCs: the subamniotic region of UC lining membrane is removed with a razor blade and then cut into small pieces. These fragments are plated in plastic culture dishes until MSCs start growing (explant method). With this method, however, it might be difficult to remove the adjacent region underneath the amniotic epithelium completely^[37,38]; and (4) Human UC perivascular stem cells (HUCPVC): the vessels are extracted from UC and tied at both ends into loops. The loops are then placed into an enzymatic solution for a defined period of time to allow the cells to separate from the perivascular region. The detached cells are cultured and collected as HUCPVCs^[26,28,39].

It is still controversial whether the isolation of the cells from the whole or some compartment is superior to others with respect to their proliferation ability, differentiation ability and immunosuppressive capacity.

Proliferation assays

The frequency of colony-forming unit fibroblasts (CFU-F)

is significantly higher in whole UC-derived MSCs than in BM-MSCs with limiting dilution^[26,30,40]. The authors first compared UCWJ-MSCs, UCA-MSCs and UCV-MSCs. UCV-MSCs exhibited a significantly higher frequency of CFU-F than UCWJ-MSCs and UCA-MSCs, but the doubling time was not different among these cell types^[5]. The Mennan group also reported that there are no significant differences among the various compartments of UC, although the cells derived from any UC compartment proliferate significantly faster than BM-MSCs, with mean doubling times of 2-3 d at P0 through P3^[41]. Depending on the purpose, researchers need to select either a compartment or the whole UC.

Biomarkers of UC-MSCs

The immunoprofile of UC-MSCs is analyzed using flow cytometry, according to the standard definitions for MSCs described by the position paper of the International Society for Cellular Therapy^[42]. There are no single specific markers that can be used to identify multipotent MSCs. MSCs are positive for adhesion markers such as CD29 and CD44; mesenchymal markers such as CD90, CD73 and CD105; and human leukocyte differentiation antigen class I (HLA-ABC). MSCs are negative for endothelial cell marker CD31; hematopoietic cell markers such as CD34, CD45 and CD117; and human leukocyte differentiation antigen class II (HLA-DR)^[43]. Among the different UC compartments, UCWJ-, UCV- and UCA-derived MSCs show a similar fibroblast-like spindle shape and the MSCs from these three types of tissues demonstrate no significant differences in the immunoprofile. These cells are positive for CD13, CD29 (integrin β 1), CD73 (SH3), CD90 (Thy-1), CD105 (SH2; endogrin) and HLA-ABC at the cellular frequency greater than 90% and are negative

for CD34, CD45, CD133 and HLA-DR, with the cellular frequency less than 1%^[5]. Mennan *et al.*^[41] also confirmed that MSC immunophenotypes showed no significant differences among different sources: BM, umbilical cord arteries, vein, WJ and UC lining membrane. Even although the authors could not find any major differences in their immunophenotypes, the cell populations derived from the different compartments may consist of different proportions of multipotent stem cells. Karahuseyinoglu *et al.*^[44] demonstrated that CD73 is expressed throughout the vessels and endothelium and is absent in the perivascular region, but the strongest expression is observed in the epithelial and subepithelial regions of WJ. CD90 is positive in most compartments but negative in the endothelial lumen lining. A high expression of vimentin, CKs (1, 4, 5, 6, 8, 10, 13, 18 and 19), desmin and SMA has been detected in the subamniotic layer and the perivascular region. Schugar *et al.*^[45] reported that CD146 (endothelial progenitor marker) is expressed in the vessel walls (100%) and the perivascular region of UC (62%) but is no longer expressed in UCWJ-MSCs^[26,46]. These markers might aid in determining the multipotency of the isolated cell population. Phenotypic characterization of UC-MSCs might be influenced by the culture passage number, culture medium and culture method.

Furthermore, ESC markers such as Oct4, Nanog, Sox-2 and KLF4 are expressed only at low levels in UC-MSCs^[47]. This suggests that MSCs are primitive stem cells, somewhere between ESCs and mature adult stem cells. Nonetheless, a precise isolation of pluripotent MSCs using specific markers remains a challenge.

The role of SSEA3 and SSEA4 in MSCs remains controversial. Gang *et al.*^[48] reported that SSEA4⁺ cells proliferate predominantly when the culture is initiated from primary BM cells in the medium supplemented with special cocktails of cytokines. In contrast, the authors failed to reproduce the same phenomena in UCWJ-MSCs in the medium consisting of α -MEM and 10% FBS. Furthermore, SSEA4 expression in UCWJ-MSCs significantly correlates with the FBS concentration in the culture medium, whereas SSEA3 expression was inversely correlated. We concluded that SSEA4 in UCWJ-MSCs is not a marker of either proliferation capacity or multipotency^[31]. Schrobback *et al.*^[49] assessed SSEA4 expression in human articular chondrocytes, osteoblasts and BM-derived MSCs and characterized their differentiation potential. Their results showed that SSEA4 levels in these cells are not related to the capacity for chondrogenic and osteogenic differentiation and the proliferation potential *in vitro*^[49].

THE ABILITY OF UC-MSCs TO DIFFERENTIATE INTO ADIPOGENIC, CHONDROGENIC AND OSTEOGENIC LINEAGES

UC-MSCs originating from the extraembryonic meso-

derm and their capacity for differentiation into adipogenic, chondrogenic and osteogenic lineages have been extensively studied^[50]. Regarding the osteogenic differentiation ability, Hsieh *et al.*^[11] demonstrated that the gene profiles of UC-MSCs are close to ESCs; UC-MSCs show delayed and insufficient differentiation into osteocytes. On the other hand, BM-MSCs have already expressed an osteogenic gene profile and can easily differentiate into osteocytes. Among the three compartments, UCWJ, UCV and UCAs, UCWJ-MSCs demonstrate an obviously defective ability to differentiate into osteocytes, even although the expression of osteocyte-related genes is detected by reverse-transcriptase PCR, at levels similar to those in the other two tissues/compartments^[5]. Mennan *et al.*^[41] compared the osteogenic differentiation among cord regions in six samples and found that the best differentiation is seen with UCWJ-MSCs and whole UC-derived MSCs, rather than with UCA-, UCV- and UC lining MSCs.

As for adipocytic differentiation, Mennan *et al.*^[41] reported that UC-MSCs produce small lipid vacuoles, whereas BM-MSCs produce more mature adipocytes (unilocular lipid vacuoles). UC-MSCs might maintain their multipotency for longer periods than BM-MSCs can^[51], although there were no obvious differences among MSCs derived from UC compartments in our research^[5].

With respect to chondrogenic differentiation, UC-MSCs show no apparent differences among the different cord regions (sources)^[41]. Moreover, the comparison of the chondrogenic potential between BM-MSCs and UC-MSCs revealed that UC-MSCs produce thrice as much collagen as BM-MSCs; this finding indicates that the former may be a better option for fibrocartilage tissue engineering^[52].

In relation to other differentiation abilities, UCWJ-MSCs are the most studied cell type among various UC compartments and many papers have been published^[53,54]. In addition to differentiating into osteocytes, chondrocytes and adipocytes, UCWJ-MSCs can differentiate into cardiomyocytes (with the gene expression of N-cadherin and cardiac troponin I^[55]), neurons and glia^[20], oligodendrocytes^[56] and hepatocytes^[57]. Recently, clinical trials have been conducted using UC-MSCs for neurogenic disorders (spinocerebellar ataxia and multiple system atrophy of the cerebellar type)^[58] and liver disorders^[59,60].

IMMUNOSUPPRESSIVE PROPERTIES OF UC-MSCs

Immunosuppressive effects have now become the most popular property of MSCs for potential clinical use. First, MSCs themselves are weakly immunogenic owing to the lack of HLA-DR and low expression of MHC class I molecules. MSCs have been shown to have immunomodulatory properties *in vitro*^[61]. Furthermore, MSCs lack both CD80 and CD86 proteins^[36,62], which

are costimulatory molecules inducing T cell activation and survival. The lack of HLA-DR, CD80 and CD86 suggests that MSCs do not elicit acute rejection and are suitable for allogeneic cell-based therapy.

Second, UC-MSCs have immunosuppressive properties *in vitro* and *in vivo*. Many studies have been published about the immunosuppressive effect of UCWJ-MSCs^[63], UC lining-MSCs^[37,64], HUCPV^[65] and whole UC-derived MSCs^[66]. The immunosuppressive effect of UC-MSCs is mediated by soluble factors and cell-to-cell contacts. PGE2, galectin-1 and HLA-G5 are released from MSCs and serve as effective factors of immunosuppression^[67]. Among these factors, indoleamine 2,3-dioxygenase (IDO) is one of the most relevant because it is inducible by IFN- γ and catalyzes conversion from tryptophan to kynurenine^[62,68]. This depletion of tryptophan from the environment can suppress T cell proliferation. UCWJ-MSC-mediated immunosuppression may require preliminary activation by proinflammatory cytokines, such as IFN- γ , with or without TNF- α , IL-1 α or IL-1 β .

It was recently suggested that the inflammatory environment produced by the upregulation of cytokines such as IFN- γ and TNF- α might alter the biological activity of MSCs from immunosuppression to immunostimulation^[68]. In this case, UC-MSCs may not prevent GVHD *in vivo*. It is known that upon stimulation by activated immune cells or cytokines (priming), MSCs are primed and become functional immunosuppressors. The extent of immunosuppression is greater with UCWJ-MSCs than with BM-MSCs^[62]. Polchert *et al.*^[68] demonstrated that MSCs primed with IFN- γ are effective in a mouse GVHD model despite upregulated MHC class II molecules. In order to ensure the effective and safe therapeutic use of UC-MSCs, more *in vivo* experiments need to be conducted because of the many discrepancies with *in vitro* data.

CONCLUSIONS

Compared with the counterparts of other origins, UC-MSCs have attractive advantages as MSCs and as UC-derived cells: (1) a noninvasive collection procedure for autologous or allogeneic use; (2) a lower risk of infection; (3) a low risk of teratoma; (4) multipotency; and (5) low immunogenicity with a good immunosuppressive ability. It is still unclear which compartment in UC is the best for clinical use; nonetheless, the era of the clinical use of UC-MSCs is approaching quickly.

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P- Reviewers: Forte A, Kim SJ **S- Editor:** Wen LL
L- Editor: Roemmele A **E- Editor:** Zhang DN



WJSC 6th Anniversary Special Issues (2): Mesenchymal stem cells

Differentiation of mesenchymal stem cells into gonad and adrenal steroidogenic cells

Takashi Yazawa, Yoshitaka Imamichi, Kaoru Miyamoto, Akihiro Umezawa, Takanobu Taniguchi

Takashi Yazawa, Takanobu Taniguchi, Department of Biochemistry, Asahikawa Medical University, Hokkaido 078-8510, Japan

Takashi Yazawa, Yoshitaka Imamichi, Kaoru Miyamoto, Department of Biochemistry, Faculty of Medical Sciences, University of Fukui, Fukui 910-1193, Japan

Akihiro Umezawa, National Research Institute for Child Health and Development, Tokyo 157-8535, Japan

Author contributions: Yazawa T, Umezawa A and Miyamoto K designed the research; Yazawa T and Imamichi Y performed the research; Yazawa T and Taniguchi T wrote the paper.

Supported by Ministry of Education, Culture, Sports, Science and Technology of Japan, No. 23590329; the Terumo Life Science Foundation, and the Smoking Research Foundation

Correspondence to: Takashi Yazawa, Lecturer, Department of Biochemistry, Asahikawa Medical University, Midorigaoka Higashi 2-1-1-1, Asahikawa, Hokkaido 078-8510, Japan. yazawa@asahikawa-med.ac.jp

Telephone: +81-166-682342 Fax: +81-166-682349

Received: November 2, 2013 Revised: December 24, 2013

Accepted: January 17, 2014

Published online: April 26, 2014

Abstract

Hormone replacement therapy is necessary for patients with adrenal and gonadal failure. Steroid hormone treatment is also employed in aging people for sex hormone deficiency. These patients undergo such therapies, which have associated risks, for their entire life. Stem cells represent an innovative tool for tissue regeneration and the possibility of solving these problems. Among various stem cell types, mesenchymal stem cells have the potential to differentiate into steroidogenic cells both *in vivo* and *in vitro*. In particular, they can effectively be differentiated into steroidogenic cells by expressing nuclear receptor 5A subfamily proteins (steroidogenic factor-1 and liver receptor homolog-1) with the aid of cAMP. This approach will provide a source of cells for future regenerative medicine for the treatment of diseases caused by steroidogenesis

deficiencies. It can also represent a useful tool for studying the molecular mechanisms of steroidogenesis and its related diseases.

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Key words: Steroid hormone; Adrenal; Gonad; Steroidogenic factor-1; Liver receptor homolog-1; Mesenchymal stem cells; Differentiation

Core tip: Stem cells can be a potential source of cells for regenerative medicine for diseases caused by steroidogenesis deficiency. Among various stem cell types, mesenchymal stem cells have the potential to differentiate into steroidogenic cells both *in vivo* and *in vitro*. This system can also provide a powerful tool for studying the molecular mechanisms of steroidogenesis and its related diseases.

Yazawa T, Imamichi Y, Miyamoto K, Umezawa A, Taniguchi T. Differentiation of mesenchymal stem cells into gonad and adrenal steroidogenic cells. *World J Stem Cells* 2014; 6(2): 203-212 Available from: URL: <http://www.wjgnet.com/1948-0210/full/v6/i2/203.htm> DOI: <http://dx.doi.org/10.4252/wjsc.v6.i2.203>

INTRODUCTION

In mammals, steroid hormones are produced from cholesterol mainly in adrenal glands and gonads. Steroid hormones are essential for glucose metabolism, the stress response, fluid and electrolyte balance, sex differentiation and reproduction via binding to cognate receptors in target tissues. Therefore, a steroidogenesis abnormality can often be life threatening. Congenital adrenal hyperplasia (CAH) is one of the most common disorders caused by deficiency of any enzyme involved in steroidogenesis in adrenal glands^[1,2]. Impaired cortisol and aldosterone pro-

duction increases adrenocorticotrophic hormone (ACTH) secretion from the pituitary gland, leading to adrenal hyperplasia and accumulation of adrenal androgens. Female patients are prenatally virilized because of excess androgen and neonates of both genders may suffer from a life-threatening Addisonian crisis. Steroid hormone deficiency also occurs in aging people by hypogonadism. In males, testosterone concentrations decline with age, causing various clinical symptoms such as obesity and hypertension^[3-6]. Postmenopausal women often suffer from osteoporosis caused by estrogen deficiency^[7,8]. Hormone replacement therapy has been well established for the treatment of such patients, although they require hormone replacement for their entire lifetime. In addition, these patients suffer from various side effects (liver and kidney damage, immune system dysfunction) and risks associated with long-term replacement therapy (cancer). Therefore, another therapy is needed to resolve these problems. Stem cells represent an innovative tool for tissue regeneration and gene therapy, which could possibly solve these problems. In this review, we provide an overview of differentiation and regeneration of steroidogenic cells using mesenchymal stem cells (MSCs), preceded by a description of the development of steroidogenic organs. We also describe molecular events, such as coactivator function and epigenetic modifications, which occur during differentiation.

DEVELOPMENT OF STEROIDOGENIC ORGANS AND NUCLEAR RECEPTOR 5A SUBFAMILY

Steroidogenesis begins with conversion of cholesterol into pregnenolone in mitochondria by the P450 side chain cleavage enzyme (P450_{sc}/CYP11A1/Cyp11a1), a rate-limiting enzyme in the synthesis of all steroid hormones. Thereafter, various hormones are synthesized by tissue-specific P450 hydroxylases and hydroxysteroid dehydrogenases^[9,10]. Although adrenal glands and gonads produce various steroid hormones in adult life, they have a common developmental origin, a so-called adrenogonadal primordium (AGP) that mainly originates from the intermediate mesoderm and is localized on the coelomic epithelia of the developing urogenital ridge^[11-13]. As development proceeds, AGP separates into two distinct populations, adrenocortical and gonadal primordia, characterized by the existence of chromaffin cell precursors and primordial germ cells, respectively, which originate and migrate from other germ layers. During differentiation, adrenal glands and gonads synthesize tissue-specific steroid hormones by specific expression patterns of steroidogenic enzymes.

Steroidogenic factor-1 (SF-1, also known as Ad4BP) is one of the earliest markers of the appearance of AGP^[11,14]. Because SF-1 knockout mice fail to develop adrenal glands and gonads, SF-1 represents a master regulator of the development of these organs^[15-17]. SF-1/

Ad4BP is also important for steroidogenesis by regulating the transcription of steroidogenic genes. SF-1/Ad4BP was originally discovered by Keith Parker and Ken Morohashi as a transcription factor that binds to the Ad4 sequence in promoter regions of all cytochrome P450 steroid hydroxylase genes for transactivation^[18,19]. They concluded from the expression of SF-1 in steroidogenic cells and its regulation of all steroid hydroxylase genes that SF-1 is a determinant factor in cell-specific expression of steroidogenic enzymes. In addition to steroidogenic enzymes, diverse groups of SF-1 target genes, such as other steroidogenic genes, pituitary hormones and cognate receptors, and sex differentiation-related genes have been identified thus far^[17,20,21]. SF-1 belongs to the nuclear receptor (NR) superfamily. NRs are lipophilic ligand-dependent and independent transcription factors and essential for various physiological phenomena^[22,23]. A large number of family members have been identified from invertebrate to mammals. There are a total of 48 family members on the human genome. They share a common structural organization: zinc finger DNA-binding domain and a carboxyl-terminal ligand-binding domain. The NR superfamily can be broadly divided into four classes based on their characteristics (steroid hormone receptors, RXR heterodimers, dimeric orphan receptors and monomeric orphan receptors). SF-1 is categorized into monomeric orphan receptors, although Ingraham and colleague argued the possibility that phosphatidylinositols are ligands for SF-1^[24]. SF-1 is very similar to liver receptor homolog-1 (LRH-1). LRH-1 was originally identified in the liver^[25] and is known to function in metabolism, cholesterol and bile acid homeostasis by regulating the transcription of a number of genes^[26-29]. In addition to the liver, LRH-1 is highly expressed in tissues of endodermal origin. It is also expressed in gonads and involved in steroidogenesis; in particular, its ovarian expression levels are the most abundant among tissues^[30]. These factors constitute one of the NR subfamilies and are designated as NR5A proteins (Table 1, SF-1 is NR5A1 and LRH-1 is NR5A2). SF-1 and LRH-1 have various common characteristics, such as binding sequences, target genes and cofactors^[24,31-38].

Consistent with its role in steroidogenesis, SF-1 expression is detected in adults in three layers of the adrenal cortex (zona reticularis, zona fasciculata and zona glomerulosa), testicular Leydig and Sertoli cells, ovarian theca, granulosa cells and, to a lesser extent, in the corpus lutea^[39,40]. In the corpus lutea, LRH-1 rather than SF-1 is highly expressed and is important for progesterone production^[36,41,42]. LRH-1 is also expressed in testicular Leydig cells^[12,43,44].

SF-1 knockout mice die shortly after birth because of adrenal insufficiency and exhibit male-to-female sex reversal in external genitalia^[15]. These phenotypes are caused by the complete loss of adrenal glands and gonads. Although the initial stages of adrenal and gonadal development occur in the absence of SF-1, they regress and disappear during the following developmental stage.

Table 1 Summary of the characteristics of steroidogenic factor-1 and liver receptor homolog-1

| Nuclear receptor | Expressing tissues | Function | Phenotypes of knockout mice |
|--------------------------|--|---|---|
| SF-1/ Ad4BP/ NR5A1 | Testis, ovary, adrenal, | Steroidogenesis Sex differentiation Energy homeostasis | Adrenal and gonadal agenesis Sex reversal in external genitalia Impaired expression of pituitary gonadotropins Abnormality of ventromedial hypothalamic nucleus |
| LRH-1/ NR5A2 | Ovary, testis, liver, pancreas, intestine, early embryo | Steroidogenesis Ovulation Bile acid synthesis Glucose metabolism | Embryonic lethal around E6.5-7.5 d |

Because gonads disappear prior to male sexual differentiation, the internal and external urogenital tracts of SF-1 knockout mice are of the female type, irrespective of genetic sex. Heterozygous SF-1 knockout mice show decreased adrenal volume associated with impaired corticosterone production in response to stress^[45-47], whereas transgenic overexpression of SF-1/Ad4BP increases adrenal size and ectopic adrenal tissue in the thorax^[48,49]. Total SF-1 disruption in mice demonstrated that SF-1 is crucial for the determination of steroidogenic cell fate *in vivo*. It has also been shown in Leydig cell and granulosa cell-specific knockout (LCKO and GCKO, respectively) models that SF-1 plays important roles in steroidogenesis following the development of steroidogenic organs. In LCKO mice, testicular steroidogenic acute regulatory protein (StAR) and Cyp11a1 expression is impaired, indicating a defect in androgen production^[50]. Consistent with this hypothesis, the testes fail to descend (an androgen-dependent developmental process) and are hypoplastic. In GCKO mice, the ovaries are hypoplastic, adults are sterile and ovaries show reduced numbers of oocytes and lack corpora lutea^[51]. Gonadotropin-induced steroid hormone production are also markedly reduced in this model.

LRH-1 knockout mouse embryos die around E6.5-7.5 d^[52,53]. Moreover, heterozygous and GCKO models revealed the importance of LRH-1 in steroidogenesis^[41,54,55]. In heterozygous Lrh-1-deficient male mice, testicular testosterone production is decreased along with the expression of steroidogenic enzymes and the development of sexual characteristics^[54]. In addition, GCKO mice are infertile because of anovulation with impaired progesterone production^[41]. It has also been demonstrated that LRH-1 has a broader role beyond steroidogenesis in these cells as they fail to luteinize.

Although SF-1 and LRH-1-deficient models revealed a common function in gonadal steroidogenesis, both factors cannot compensate for the deficiency of the other factor, even in cells expressing both factors. These facts indicate that even although SF-1 and LRH-1 control transcription by binding to the same response sequences,

each has selective actions on the pattern of gene expression in the development of steroidogenic cells and steroidogenesis.

DIFFERENTIATION OF MSCS INTO STEROIDOGENIC CELLS

In an early study, forced expression of SF-1 has been shown to direct differentiation of murine embryonic stem cells (ESCs) toward the steroidogenic lineage and then Cyp11a1 mRNA was expressed after the addition of cAMP and retinoic acid^[56]. However, the steroidogenic capacity of these cells is very limited and they do not undergo *de novo* synthesis because progesterone is the only steroid hormone produced in the presence of the exogenous substrate, 20 α -hydroxycholesterol. In addition, major differences between these differentiated cells and natural steroidogenic cells have been shown in cholesterol delivery and the steroidogenic pathway, including deficiencies of StAR (cholesterol delivery protein from the outer to inner mitochondrial membrane in steroidogenic cells) and steroidogenic enzymes, except for Cyp11a1 and Hsd3b1^[56-58]. It is also very difficult to isolate clones expressing SF-1 from ESCs and induced pluripotent stem cells^[37,57,59] because SF-1 (and LRH-1) overexpression is cytotoxic to these cells. These studies clearly indicate that SF-1 initiates the fate-determination program of the steroidogenic lineage in stem cells, although it is not completed in pluripotent stem cells.

Based on these results, we focused on MSCs^[57], multipotent adult stem cells that have been shown to differentiate into mesodermal lineages, such as adipocytes, chondrocytes, osteoblasts and hematopoietic-supporting stroma, both *in vivo* and *ex vivo*^[60-63]. Furthermore, MSCs are able to generate cells of all three germ layers, at least *in vitro*. Although MSCs were originally discovered in bone marrow (BM-MSCs)^[60,64-66], they have also been isolated from various origins, such as fat, placenta, umbilical cord blood and other tissues^[62,63,67-69]. In addition to their multipotency, MSCs have attracted considerable interest for use in cell and gene therapies because they can be obtained from adult tissues and suppress immune responses^[70,71]. Indeed, their therapeutic applicability has been assessed in some cases and particularly in bone tissue engineering^[72,73].

Induction of MSC differentiation into steroidogenic cells *in vivo* and *in vitro*

To investigate the potential of MSCs to differentiate into steroidogenic cells, BM-MSCs from GFP-transgenic rats were transplanted into prepubertal testes (Figure 1A)^[57]. In testes, there are two different steroidogenic populations, fetal and adult Leydig cells^[74-76]. Even although the cells in these two populations share a common characteristic of producing androgen, they are different in their origin, ultrastructure, lifespan, steroidogenic pathway and its regulation. Fetal Leydig cells have multiple origins and

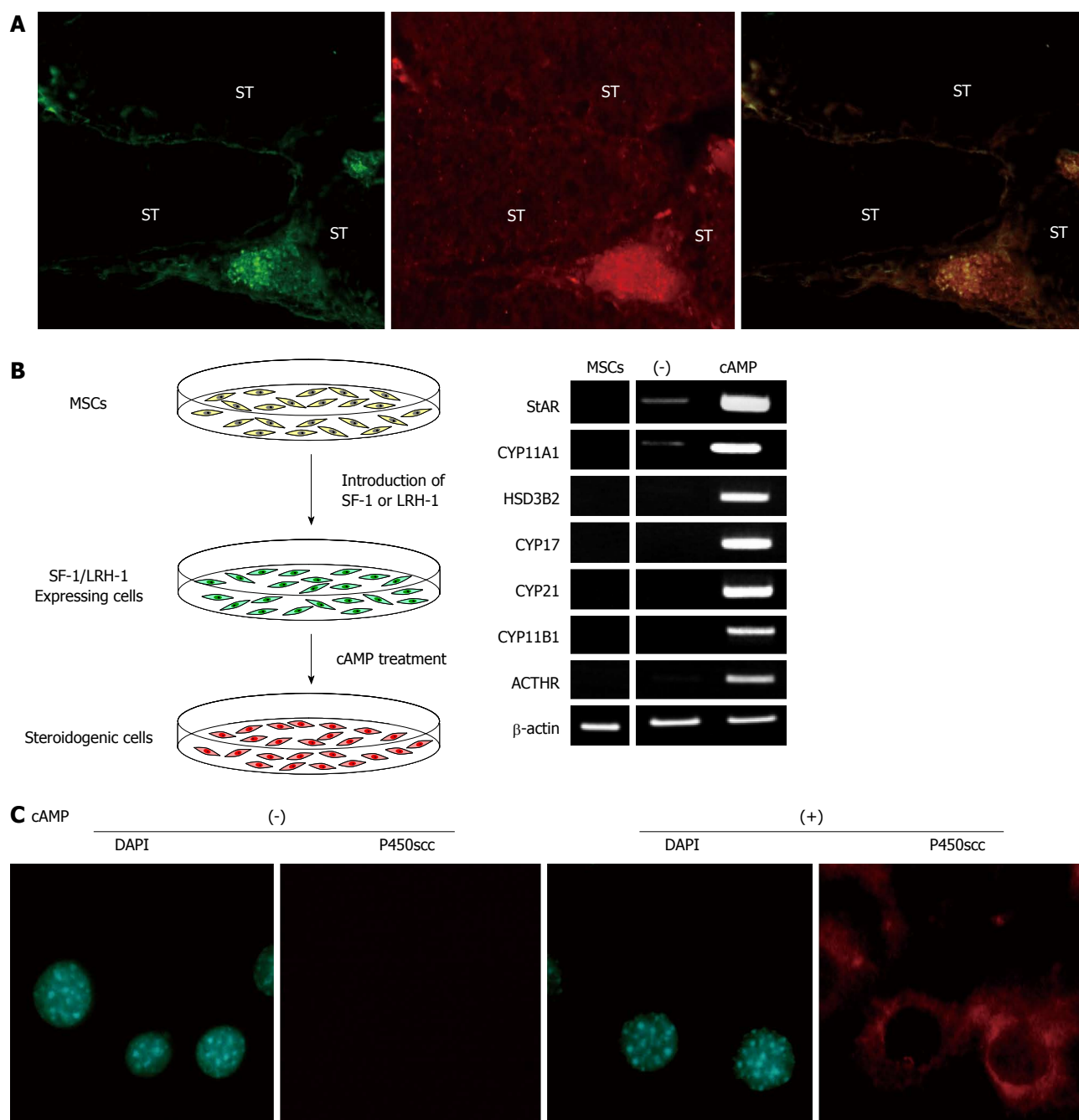


Figure 1 Differentiation of mesenchymal stem cells into steroidogenic cells. A: Transplantation of GFP-positive MSCs into prepubertal testis. Double staining of frozen sections from the testis 5 wk after MSC transplantation with anti-GFP and anti-P450scc antibodies; B: Protocol for generating steroidogenic cells from MSCs, and gene expression pattern of steroidogenic cells derived from hBM-MSCs; C: Fluorescence images of DAPI staining and P450scc immunostaining of SF-1 introduced BM-MSCs cultured with or without cAMP. ST: Seminiferous tubule. MSC: Mesenchymal stem cell.

appear in the interstitial space to induce sex differentiation just after the formation of the testis cord. Adult Leydig cells, which originate from mesenchymal precursor cells present in the testicular interstitium, appear to induce puberty. During the postnatal period, fetal Leydig cells are replaced by adult Leydig cells in prepubertal testis. Therefore, it should be possible to use transplanted BM-MSCs in such conditions *in vivo*. Indeed, after 3 wk, transplanted GFP-positive cells were located in the interstitium and expressed various steroidogenic enzymes for androgen production (P450scc/Cyp11a1, 3 β -HSD I and

Cyp17). These results indicate that MSCs have the capacity to differentiate into steroidogenic Leydig cells *in vivo*.

Although these data suggest that the injected stem cells differentiated into Leydig cells, the apparent stem cell plasticity may also be explained by possible cell-nuclear fusion between donor and recipient cells. However, purified murine BM-MSC lines spontaneously differentiate into steroidogenic cells *in vitro*^[57]. A human *CYP11A1* promoter-driven GFP reporter, which consisted of a 2.3-kb fragment that drives reporter gene expression selectively in adrenal and gonadal steroidogenic cells^[77],

Table 2 Properties of steroidogenic cells derived from mesenchymal stem cells induced by steroidogenic factor-1/liver receptor homolog-1 and cAMP

| Cells | Origin | SF-1/LRH-1 | Produced | Properties of differentiated cells |
|--------------------|-------------------------------|-----------------------|------------------------|------------------------------------|
| KUM9 | Mouse Bone marrow | Plasmid | Testosterone | Testicular leydig cells |
| hMSC- TERT-E6/7 | Human Bone marrow | Plasmid Retrovirus | Cortisol Cortisol | Adrenal fasciculata cells |
| UE7T-13 | Human Bone marrow | Retrovirus | Testosterone, cortisol | Fetal adrenal-like cells |
| UE6E7T-12 | | Retrovirus | Testosterone, cortisol | |
| UE6E7T-11 | | Retrovirus | Testosterone, cortisol | |
| UCB408E6E7T-33 | Human Umbilical cord blood | Retrovirus | Progesterone cells | Ovarian granulosa-luteal cells |

has been transfected into BM-MSCs to detect cell populations committed to the steroidogenic lineage. In some transfected cell lines, GFP fluorescence was detected in very small populations that were also positive for Cyp11a1. Further analysis showed that these cells expressed several Leydig cell markers, including 3β -HSD type I and VI and luteinizing hormone (LH) receptor. These observations further support the *in vivo* findings that MSCs have the capacity to differentiate into steroidogenic cells, even under the isolated condition. Therefore, part of population of MSCs can spontaneously differentiate into steroidogenic cells *in vitro*. Interestingly, SF-1 expression was also detected in the GFP-positive cells.

Differentiation of MSCs into steroidogenic cells induced by SF-1 and LRH-1

The above mentioned results strongly suggest that SF-1 can effectively direct the differentiation of MSCs into the steroidogenic lineage. Indeed, MSCs completely differentiate into steroidogenic cells and show their phenotype after stable expression of SF-1 (using plasmids or retroviruses) and cAMP treatment (Figure 1B)^[36,37,44,57,78,79]. SF-1 by itself induces morphological changes in BM-MSCs, such as the accumulation of numerous lipid droplets, although these cells hardly express steroidogenic enzyme genes or produce steroid hormones at detectable levels. However, SF-1 expressing cells strongly become positive for CYP11A1/Cyp11a1 after cAMP treatment (Figure 1C). These cells express many other steroidogenesis-related genes (*SR-BI*, *SLAR*, *3β -HSD* and other P450 steroid hydroxylases) and autonomously produce steroid hormones, including androgen, estrogen, progesterone, glucocorticoid and aldosterone. Notably, this approach differentiates human BM-MSCs into high cortisol-producing cells in response to ACTH, which are very similar to fasciculata cells in the adrenal cortex (Figure 1B). Adenovirus-mediated transient expression of SF-1 also differentiates BM-MSCs into steroidogenic cells with the capacity of *de novo* synthesis of various steroid hormones^[80-84]. After transplantation into animal models, these MSC-derived steroidogenic cells can improve symptoms of steroid hormone deficiencies caused by adrenalectomy. However, as mentioned above, these methods are not applicable to ESCs, embryonal carcinoma cells and terminally differentiated cells, such as fibroblasts and adi-

pocytes^[37,57,81]. These results indicate that MSCs are suitable stem cells for differentiation of steroidogenic cells. This hypothesis is supported by the fact that after pre-differentiation into MSCs, ESCs can also be subsequently differentiated into steroidogenic cells using SF-1^[37].

As in the case of SF-1, introduction of LRH-1 (using retroviruses) into BM-MSCs with the aid of cAMP induced the expression of steroidogenic enzymes and differentiation into steroid hormone-producing cells^[44]. Expression of SF-1 was never induced in LRH-1-transduced cells and vice versa. Therefore, LRH-1 could act as another master regulator for determining the MSC fate to the steroidogenic lineage. This phenomenon is likely to represent a situation of active progesterone production in human corpus luteum; LRH-1 is highly expressed, whereas SF-1 is expressed at very low levels^[36,42].

MOLECULAR MECHANISMS OF DIFFERENTIATION

Steroidogenic cells derived from various MSCs and their properties

In addition to BM-MSCs, various MSC types have been differentiated into steroidogenic cells by the above mentioned methods. However, their steroidogenic properties markedly vary and depend on the derivation tissues and species (Table 2)^[36,42,57,83,84]. For example, hBM-MSCs differentiated into cortisol-producing adrenocortical-like cells and umbilical cord blood (UCB)-derived MSCs differentiated into granulosa luteal-like cells, which produced high levels of progesterone^[36,57]. Gondo *et al.*^[83] also reported that steroidogenic profiles of adipose tissue-derived MSCs were markedly different from those of BM-MSCs prepared from the same mouse. However, the cell differentiation fate was consistent in each MSC. These findings suggest that the steroidogenic properties of the differentiated cells depend on the characteristics of the originating MSCs.

To determine the difference between BM-MSCs and UBC-MSCs, the fluctuations in gene expression were investigated by a DNA microarray^[36,85]. Among the identified genes, peroxisome proliferator-activated receptor γ coactivator-1 α (PGC-1 α) was expressed only in UBC-MSCs at relatively high levels. Consistent with these re-

sults, the expression of PGC-1 α was observed in ovarian granulosa cells. Overexpression of PGC-1 α in granulosa cells induced the genes essential for progesterone synthesis, whereas knockdown of PGC-1 α in granulosa cells attenuated the expression of these genes. These results demonstrate that PGC-1 α represents one of the important factors for progesterone production in luteinized granulosa cells.

Epigenetic regulation during differentiation

Differentiation of stem cells into specialized cells can be viewed as a process in which epigenetic changes result in alterations in genes expressed by the cell as it becomes more specialized^[86,87]. Thus, stem cell differentiation is a process that involves a series of epigenetic changes in the genome: histone and DNA modifications cause chromatin structural changes and affect the profiles of gene expression. In fact, such epigenetic modifications contribute to the induction of steroidogenesis-related genes when MSCs differentiate into steroidogenic cells^[44,88-90].

The histone code hypothesis predicts that post-translational modifications of histone tails, alone or in combination, function to direct specific and distinct DNA-templated programs^[91]. Histone acetylation is a positive marker of transcription, while histone methylation correlates with transcriptional activation (H3K4, H3K36) and repression (H3K9, H3K27) that are dependent on their amino acid residues^[92]. In hMSCs-derived steroidogenic cells, H3K27 acetylation and H3K4 dimethylation (active enhancer markers) increased in the regulatory regions of some steroidogenesis-related genes (glutathione S-transferase A and ferredoxin reductase) after the introduction of SF-1^[89,90]. Conversely, histone eviction, which has been reported in actively transcribed genes^[93], took place on the promoter and the enhancer regions of the *SLAR* gene^[88]. Because these modifications occurred around the SF-1 binding sites, recruitment of SF-1 to the regulatory regions is likely to induce recruitment of various transcriptional regulators and histone modifiers, which in turn alter chromatin structure and lead to the expression of steroidogenesis-related genes.

In addition to histone modifications, DNA methylation at cytosine residues of the dinucleotide sequence CpG, which induces gene silencing, is essential for differentiation and development^[94,95]. In MSC-derived steroidogenic cells, the DNA methylation status changes in the promoter regions of some steroidogenic genes during differentiation^[44]. In undifferentiated hBM-MSCs, the *CYP11A1* promoter region is hypomethylated, whereas the *CYP17A1* promoter region is highly methylated. In SF-1/LRH-1-introduced MSCs during cAMP treatment, this condition was almost completely unchanged in the *CYP11A1* promoter region, whereas the *CYP17A1* promoter region was progressively demethylated. These methylation patterns of the *CYP11A1* and *CYP17A1* promoters closely paralleled the induction patterns of both genes by cAMP. There is a time lag associated with the induction of steroidogenic enzymes by

cAMP treatment in SF-1/LRH-1-introduced MSCs^[44,57]. The order of induction of the enzymes is similar to the sequential order of the steroid hormone synthesis pathway; upstream enzymes (CYP11A1 and 3 β -HSD) were rapidly induced at earlier time points (6-12 h), whereas downstream enzymes (CYP17A1 and CYP11B1) were induced at later time points (24-48 h). Because this time lag disappeared by treatment with a demethylating agent, the status of DNA methylation in the promoter regions could be important for regulating the expression of steroidogenic enzymes in MSCs.

CONCLUSION

It is clear that SF-1 represents a master regulator, not only for the development of steroidogenic organs, but also for steroidogenesis following organogenesis. LRH-1 is also important for steroidogenesis in gonads. In addition, SF-1 and LRH-1 direct differentiation of non-steroidogenic stem cells into steroidogenic cells. Among the various stem cell types, MSCs are suitable stem cells for the differentiation of steroidogenic cells. After pre-differentiation into MSCs, pluripotent stem cells can also be subsequently differentiated into steroidogenic cells using SF-1. These cells may provide a source for regenerative and gene therapies, although various problems should be resolved in future studies. It is essential to delineate the conditions that allow the directed differentiation into specific steroidogenic lineages with the characteristics of testicular Leydig cells, ovarian granulosa and theca cells, as well as various types of adrenocortical cells (reticularis, fasciculata and glomerulosa). In addition, it is necessary to establish methods for inducing SF-1 and LRH-1 expression in stem cells without gene transfer. Further studies are required for the realization of regeneration of steroidogenic tissues.

MSC-derived steroidogenic cells also provide opportunities for investigating various phenomena involved in differentiation of steroidogenic cells and steroidogenesis. In addition to the molecular mechanisms of differentiation described herein, the conservation and evolution of the androgen metabolic pathway (11-ketotestosterone production) between teleost fish and mammals has been revealed^[78,96]. Genome-wide analyses of differentiated cells identified novel target genes regulated by SF-1 and LRH-1^[89,90,97,98]. In addition, they contributed to the elucidation of one of the causes of steroidogenesis disorders^[99-101]. Thus, progression of these studies is also important for the understanding of steroidogenesis and its related disorders.

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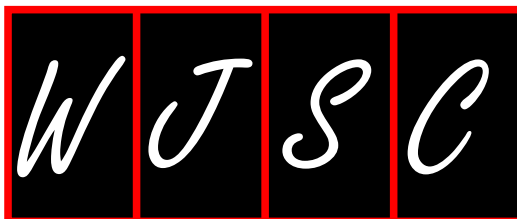
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P- Reviewers: Holan V, Lalli E, Pixley JS **S- Editor:** Qi Y
L- Editor: Roemmele A **E- Editor:** Zhang DN





WJSC 6th Anniversary Special Issues (2): Mesenchymal stem cells

Intestinal stem cells and celiac disease

Anna Chiara Piscaglia

Anna Chiara Piscaglia, Endoscopy and Gastroenterology Unit, State Hospital-Republic of San Marino, 47893 Borgo Maggiore, Repubblica di San Marino

Author contributions: Piscaglia AC designed and wrote this manuscript.

Correspondence to: Anna Chiara Piscaglia, MD, PhD, Endoscopy and Gastroenterology Unit, State Hospital-Republic of San Marino, Via Scialoja 20, 47893 Borgo Maggiore, Repubblica di San Marino. annachiarapiscaglia@hotmail.com
Telephone: +39-347-1015909

Received: October 30, 2013 Revised: March 1, 2014

Accepted: March 11, 2014

Published online: April 26, 2014

Abstract

Stem cells (SCs) are the key to tissue genesis and regeneration. Given their central role in homeostasis, dysfunctions of the SC compartment play a pivotal role in the development of cancers, degenerative disorders, chronic inflammatory pathologies and organ failure. The gastrointestinal tract is constantly exposed to harsh mechanical and chemical conditions and most of the epithelial cells are replaced every 3 to 5 d. According to the so-called Unitarian hypothesis, this renewal is driven by a common intestinal stem cell (ISC) residing within the crypt base at the origin of the crypt-to-villus hierarchical migratory pattern. Celiac disease (CD) can be defined as a chronic immune-mediated disease that is triggered and maintained by dietary proteins (gluten) in genetically predisposed individuals. Many advances have been achieved over the last years in understanding of the pathogenic interactions among genetic, immunological and environmental factors in CD, with a particular emphasis on intestinal barrier and gut microbiota. Conversely, little is known about ISC modulation and deregulation in active celiac disease and upon a gluten-free diet. Nonetheless, bone marrow-derived SC transplantation has become an option for celiac patients with complicated or refractory disease. This manuscript summarizes the "state of the art" regarding CD and ISCs, their niche and potential role in the de-

velopment and treatment of the disease.

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Key words: Intestinal stem cells; CD133; Lgr5; Celiac disease; Paneth cells; Gut microbiota; Gut barrier

Core tip: The intestinal epithelium has a high turnover rate since most of the epithelial cells are replaced every 3 to 5 d. This renewal is driven by intestinal stem cells residing within the crypt base at the origin of the crypt-to-villus hierarchical migratory pattern. Many aspects of the pathogenesis of celiac disease have been elucidated over the last years regarding the interactions among genetic and immunological factors, intestinal barrier and gut microbiota. Conversely, little is known about intestinal stem cell modulation and deregulation in celiac disease. The current knowledge regarding celiac disease and intestinal stem cells, and the potential role of stem cells in the development and treatment of the disease are summarized.

Piscaglia AC. Intestinal stem cells and celiac disease. *World J Stem Cells* 2014; 6(2): 213-229 Available from: URL: <http://www.wjgnet.com/1948-0210/full/v6/i2/213.htm> DOI: <http://dx.doi.org/10.4252/wjsc.v6.i2.213>

"Enthusiasm is that temper of the mind in which the imagination has got the better of the judgment" - William Warburton.

STEM CELLS AND THEIR POTENTIAL

Stemness can be defined as the capability of extensive self-maintenance and differentiation^[1,2]. Stem cells (SCs) are undifferentiated cells able to give rise to diverse mature progenies and to self-renew through the alternation of symmetrical and asymmetrical divisions. SCs play a

central role in tissue genesis, regeneration and homeostasis by providing differentiated cells that can increase tissue mass during pre- and post-natal growth and replace cell loss due to senescence or damage^[3,6].

SCs possess a hierarchy of potentialities: from the totipotency of the zygote and its immediate progeny, to the pluripotency of embryonic stem cells (ESCs), up to the multi/unipotency of adult SCs (ASCs)^[7].

ESCs are pluripotent cells derived from the inner cell mass of the blastocyst that can generate any differentiated phenotype of the three primary germ layers (endoderm, mesoderm and ectoderm), as well as germ cells. ESCs might constitute an easily available source to obtain a large number of transplantable cells for regenerative treatments. Nevertheless, ethical concerns and the possibility of immune rejection and teratoma/teratocarcinoma formation are major obstacles to the feasibility and safety of ESC clinical applications^[8].

Pluripotent stem-like cells could also derive from non-pluripotent cells-typically an adult somatic cell-by inducing a “forced” expression of specific genes. These induced pluripotent stem-like cells (iPS cells) are similar to ESCs in many aspects, such as the expression of certain SC genes, potency and differentiability, formation of embryoid bodies, teratomas and viable chimeras, even if the full extent of their relationship to natural pluripotent SCs is not fully elucidated; as a consequence, they cannot be currently considered a reliable and feasible source of SCs^[9,10].

Another population of SCs with high differentiation potential is represented by cells established from placental/cord tissues, which do not tend to form teratomas/teratocarcinomas and have a higher proliferation and differentiation potential than ASCs. In particular, the plasticity and accessibility of umbilical cord blood SCs (CBSCs) have given the rationale for the creation of CBSC unit banks where these cells can be collected and stored for future use^[7].

The least differentiation potential is possessed by ASCs, which persist indefinitely in the tissue of origin, allowing for local tissue regeneration and renewal^[11]. Despite the paradigm of unidirectional cell determination, recent studies have shown that ASCs are endowed with an unexpected plasticity as circulating adult progenitor cells can differentiate into mature cells of other tissue types^[5]. A particularly high degree of plasticity is shown by hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs).

HSCs are responsible for the renewal of blood cells^[12]. Commonly used markers for HSCs identification and isolation include two membrane phosphoglycoproteins: CD34 and AC133 (CD133, or “prominin1” in rodents)^[13]. It is generally accepted that the most primitive and long-term human HSCs are characterized by the expression of CD133, Thy1 (CD90) and VEGFR2 and by a variable expression of CD34 and CD38^[14,15]. Bone marrow (BM) resident HSCs can be mobilized into the peripheral blood under specific stimuli such as tissue in-

jury or administration of mobilizing agents^[1]. *In vitro* culture and *in vivo* transplantation assays have demonstrated that HSCs are able to give rise to a wide array of phenotypes, including blood, cartilage, fat, tendon, lung, liver, muscle, brain, heart and kidney cells^[1]. Moreover, it has been demonstrated that the number of circulating HSCs expressing early markers for muscle, nerve and hepatic differentiation increases following treatment with mobilizing agents. This phenomenon has led to speculation about the existence of BM-derived circulating pluripotent SCs which could migrate from the peripheral blood into every tissue and contribute to normal turnover and repair following injury^[16].

MSCs, also called “stromal stem cells”, “stromal precursors”, “mesenchymal progenitors” and “colony-forming unit-fibroblast cells”, are highly proliferating, adherent cells which reside in a perivascular niche within the BM and also in the wall of blood vessels within most organs^[17]. MSCs can differentiate into a variety of mesodermal cell lineages, including osteoblasts, chondroblasts, adipocytes, myocytes and cardiomyocytes, as well as non-mesodermal cells, such as hepatocytes and neurons^[18]. In addition to BM, MSCs have been isolated from various adult tissues, including muscle, adipose tissue, connective tissue, trabecular bone, synovial fluid and from perinatal tissues (umbilical cord, amniotic fluid and placenta). The presence of MSCs in peripheral blood is still being debated as some authors identified a circulating fibroblast-like population, whereas others failed^[19].

SCs colocalize with supporting cells in a physiologically limited and specialized microenvironment or niche that varies in nature and location depending upon the tissue type^[20]. The reciprocal interactions between SCs and their microenvironment, through cell-cell and cell-matrix connections as well as the secretion of soluble factors, influence SC behavior, regulating the balance between quiescence and dividing state under specific pathological or physiological conditions^[5]. Understanding the molecular signals which regulate SC behavior is critical for their therapeutic applications. In fact, the exogenous stimulation with specific growth factors or cytokines may be used to activate SCs *in vivo* and *in vitro*.

DEVELOPMENT AND TURNOVER OF THE INTESTINAL EPITHELIUM

The gastrointestinal tract surface derives from the endoderm. The embryonic stratified endodermal epithelium is subsequently converted into a monolayer overlying nascent villi while dividing cells segregate to the intervillous region. Intestinal crypts develop during the early postnatal period, becoming the niche for gastrointestinal SCs^[21]. Once completely structured, the epithelium along the gut is characterized by a heterogeneous cell population, in terms of morpho-functional properties and proliferation kinetics, reflecting the various functions of the different gastrointestinal components^[7]. The adult mammalian gut can be broadly segregated into two functionally distinct

parts: the small intestine and the colon, which present with marked architectural differences, reflecting their different functions. In particular, in the small intestine, the crypts of Lieberkuhn are associated with the intestinal villi that maximize surface area, endowing the small intestine with an excellent capacity to absorb dietary nutrients from the lumen. In contrast, the absence of villi within the colonic epithelium translates to a flatter morphology, highlighting its predominant role in stool compaction^[22].

As a consequence of its role in digestion, nutrient absorption and waste excretion, the gastrointestinal tract is constantly exposed to harsh mechanical and chemical conditions. Therefore, the intestinal tract has evolved mechanisms to cope with these assaults *via* a highly regulated process of self-renewal^[23]. Mucosal proliferation plays a fundamental role in the maintenance of the gut integrity. Most of the epithelial cells are replaced every 3 to 5 d which is a high proliferation rate, second only to the hematopoietic system^[7]. According to the so-called “Unitarian hypothesis”, first proposed by Cheng and Leblond in 1974^[24], this epithelial renewal is driven by a common intestinal stem cell (ISC) residing within the crypt base at the origin of the well established crypt-to-villus hierarchical migratory pattern^[25,26]. From their niche, ISCs give rise to transit-amplifying (TA) cells that migrate upwards and progressively lose their proliferative capability and mature to become fully-differentiated villous epithelial cells (absorptive enterocytes or secretory cells which include goblet cells, enteroendocrine cells, Paneth cells and Tuft cells). Each adult crypt harbors approximately 5 to 15 ISCs that are responsible for the daily production of about 300 cells; up to 10 crypts are necessary to replenish the epithelium of a single villus^[23]. Crypt-derived epithelial cells generally reach the villus tip after 3-5 d when they die and are exfoliated into the lumen^[27], except for Paneth cells (PCs) that evade this upward migration program, instead forcing their way to the base of the crypt^[28]. PCs are confined to the small intestine where they can live for up to 8 wk^[29]. PCs are also unique in that they appear after birth during crypt emergence^[30]. PCs secrete defensins, lysozyme and phospholipase A2 and play a central role in host defense against enteric pathogens; moreover, the antimicrobial peptides secreted by PCs shape the composition of gut microbiota and protect from bacterial translocation^[29]. In addition, crypts supply less common cell types such as the M cells and cup cells, although their lineages are poorly understood^[23].

ISCS AND THEIR NICHE

ISC hierarchy

Since the 1970s, several studies have supported the concept of ISCs. The ability of SCs to regenerate gut epithelium has been investigated in various animal models of intestinal injury. Such studies have led to the hypothesis of an ISC hierarchy organized in three main compartments and progressively recruited at various degrees of

damage in order to ensure an effective crypt regeneration^[7].

The initial location for the ISCs was deemed to be the fourth cell position from the bottom of the crypt (+4) where slowly cycling cells that show label-retention of BrdU (the so-called “+4 label retaining cells”, LRCs) were described by Potten *et al*^[31] in 1974.

A second theory regarding the location of the ISCs was formulated in the same year by Cheng and Leblond^[24]. In a series of electron microscopy studies on the small intestinal crypts, these authors described slender, immature, cycling cells wedged between PCs at the positions 1-4 of the crypt base. Upon ³H-thymidine treatment, these “crypt base columnar” (CBC) cells were able to phagocytose close damaged cells; subsequently, phagosome-labeled cells were found in all intestinal epithelial lineages, suggesting the role of CBCs as ISCs.

In 2007, a Wnt-target gene encoding a leucine-rich orphan G protein-coupled receptor named *Lgr5* was identified to specifically label CBCs in the mouse small intestine^[27]. Through a lineage tracing approach, Sato *et al*^[32] demonstrated that CBCs are able to give rise to all intestinal epithelial lineages and are a self-renewing population of multipotent SCs. Further proof that *Lgr5*+ cells are ISCs derived from *ex vivo* culture assays, where single *Lgr5*+ cells were able to form self-renewing epithelial organoids highly reminiscent of crypt/villus epithelial units *in vivo*, while cells that expressed low or no *Lgr5* were unable to form such structures. Unlike LRCs, *Lgr5*+ CBCs are resistant to radiation and are rapidly proliferating, thus challenging the previously held belief that all ASCs are quiescent or slowly cycling. In 2009, lineage tracing studies also showed that some *Lgr5*+ cells co-express prominin-1 (or CD133) and these CD133+ cells can generate the entire intestinal epithelium^[33,34].

In addition to *Lgr5* and CD133, other potential ISC markers have been identified in the last years, including *musashi1* (*MSI1*), expressed by both LRCs and CBCs; *olfactomedin 4* (*OLFM4*), expressed by *Lgr5*+ cells; *PTEN*, *AKT1*, *mTERT* and *BMI1*, predominantly expressed in LRCs (for extensive revision on this topic, see^[23,35]) (Figure 1).

In 2008, Scoville *et al*^[36] proposed the coexistence of two types of ISCs: the LRCs at the +4 location that are a “reserve pool” in a prolonged quiescent state and the actively cycling CBCs able to respond to stimulating signals from their microenvironment and to provide progenitor cells on an everyday basis. To support this hypothesis, Sangiorgi *et al*^[37] found that *Bmi1*+ cells corresponding to +4 LRCs can self-renew, proliferate, expand and like CBCs give rise to all the differentiated lineages of the small intestine epithelium. The authors concluded that +4 LRCs and CBCs are ISCs in different niches, able to migrate from one to the other^[37]. Recently, two independent groups showed a dynamic interplay between both cell populations: Tian *et al*^[38] demonstrated that *Lgr5*+ cells are dispensable for gut homeostasis and that *BMI1*+ cells are able to replenish the *Lgr5*+ cell compartment after its experimental ablation; Takeda *et al*^[39] suggested

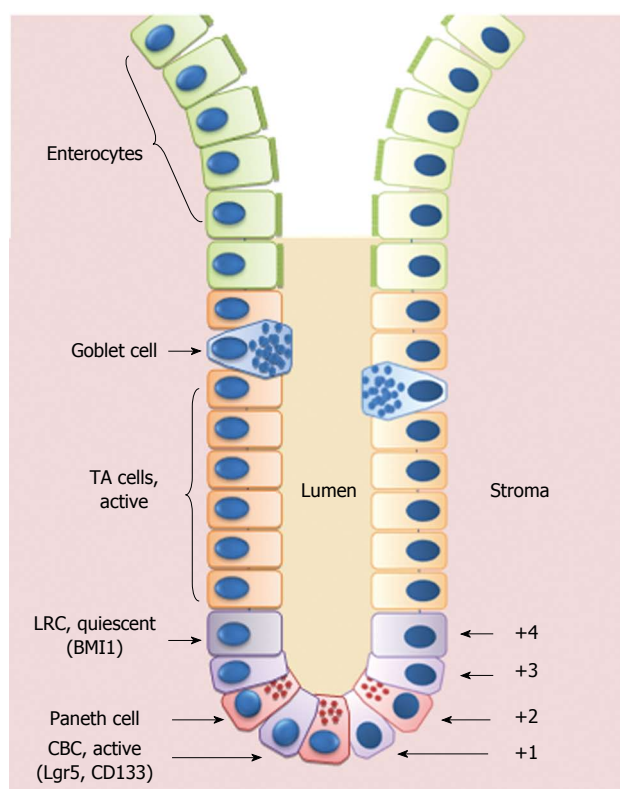


Figure 1 Schematic representation of the crypt/villus axis. Putative intestinal stem cells (ISCs) reside either at the crypt base, between Paneth cells, as Crypt Base Columnar Cells (CBCs), or in position +4 from the bottom of the crypt, as Label Retaining Cells (LRCs). ISCs give rise to Transit Amplifying (TA) cells that are able to migrate upwards and progressively mature losing their proliferative capability to become fully-differentiated villous epithelial cells.

a more complicated bidirectional relationship between *Lgr5*⁺ cells and +4 LRCs, the latter being able to either originate from or give rise to *Lgr5*⁺ cells. Whether the +4 LRCs and the CBCs truly are two distinct ISC populations and whether this is an intrinsic quality or the result of the different location within the ISC niche is still a matter of debate^[40].

A third potential source of ISCs is represented by circulating multipotent SCs of BM origin that can colonize the intestinal epithelium and contribute to its turnover and regeneration^[41-43]. BM stem cells may participate in gut repair by giving rise to ISCs through direct differentiation and also by providing supporting elements within the ISC niche, as demonstrated in different experimental models^[44-47]. However, the reduced levels of engraftment and the low rate of differentiation into intestinal cells reported in most of these studies discouraged the practical application of these cells in a clinical setting. Recently, efforts have been made to develop strategies to enhance the levels of engraftment. Zhang *et al.*^[48] demonstrated that transplantation with BM SCs genetically modified to express CXCR-4 resulted in levels of engraftment able to ameliorate radiation enteritis. Colletti *et al.*^[49] identified a marker (EphB2) for isolating and culturing an expandable subpopulation of human BM-derived SCs with enhanced intestinal homing and contribution to ISC region.

ISC niche

ISCs reside in a physiologically limited and specialized niche that dictates the mechanisms of tissue turnover and regeneration through cell-cell interactions and molecular signals^[5,50].

Traditionally, the underlying stromal cells (pericryptal myofibroblasts, enteric neurons, endothelial cells and intraepithelial lymphocytes) have been considered to constitute the niche for ISCs. Recently, it has been suggested that PCs are an essential component of the *Lgr5*⁺ ISC niche^[51]. Much evidence sustains this hypothesis. *In vivo*, the absence of PCs compromises the recovery ability, resulting in complete loss of the intestinal epithelial integrity^[52]. *In vitro*, the presence of PCs significantly increases the generation of epithelial organoids by *Lgr5*⁺ cells^[51]. PCs produce many growth factors involved in ISC maintenance and activation, including epidermal growth factor, Wnt3 and transforming growth factor- α ^[23]. The intimate relationship between PCs and ISCs seems to be involved in the response to nutritional status of the organism. Indeed, PCs can act as a “sensor” for nutritional status and enhance ISC function in response to caloric restriction^[53]. Finally, PCs seem essential to regulate ISC self-renewal by neutral competition between symmetrically dividing ISCs and a limited PC-defined niche within the crypt base^[54]. Thus, PCs serve as multifunctional guardians of ISCs by secreting bactericidal products and by providing essential niche signals. As a consequence, despite the fact that SC niches are typically portrayed as pre-existing sites to which SCs migrate^[55], ISCs are unique since they also receive niche support from their own specialized progeny of PCs.

The main molecular pathways involved in ISC regulation are Wnt, Notch, Hedgehog, Bmp and PTEN-PI3K-Akt.

Wnt signaling: Wnt signaling is based on the autocrine and paracrine interaction of secreted cysteine-rich Wnt-glycoproteins with a transmembrane Frizzled receptor (Fz). Binding of Wnt to its receptor activates the canonical pathway with stabilization and nuclear translocation of beta-catenin or the non-canonical pathway that encompasses the planar cell polarity and the Wnt/ Ca^{2+} pathway. The canonical pathway is the best characterized and most relevant in SC signaling: the binding of secreted Wnt-proteins to Fz induces nuclear translocation of beta-catenin that triggers Wnt-target gene transcription. Many studies have shown the importance of this pathway in the proliferation and differentiation of the gastrointestinal epithelium (revised in^[40,56]). Wnt signaling has different effects in different cell types, also depending on its localization along the crypt/villus axis.

Direct evidence of Wnt-activity in ISCs is their unique expression of *Lgr5*, a Wnt-target gene^[27]. Other Wnt-target genes associated with proliferation of TA-cells include c-myc and cyclin D1^[57,58]. R-spondins, glycoproteins likely secreted by enteroendocrine cells, amplify Wnt signaling, induce a proliferative response in human

intestinal epithelium, and are responsible for the expansion of organoid cultures^[59-61].

Wnt signaling is necessary for ISC proliferation and maintenance of the ISC phenotype. The previously reported PC-dependence of single Lgr5+ cells in plating efficiency can be overcome by the addition of Wnt-3 in culture^[51]. Conversely, a decreased Wnt signaling results in the loss of the proliferative compartment. Over-expression of Kruppel-like factor 4 (Klf4, a negative regulator of Wnt signals) induces cell cycle arrest, while its deletion leads to increased proliferation^[40,62,63].

Wnt signaling plays a pivotal role in cell differentiation: an overactive Wnt signaling impedes ISC differentiation and induces mislocalization of PCs, impaired goblet cell and enterocyte maturation; on the other hand, an underactive Wnt signaling induces depletion of progenitor cells, leading to the absence of properly differentiated cells^[40,62]. The development of PCs is also directly dependent on Wnt signaling^[64].

Wnt signaling is indispensable for intestinal morphogenesis and normal cell migration. Indeed, beta-catenin ensures the correct positioning of epithelial cells along the crypt/villus axis by regulating the expression of members of the Ephrin and Ephrin receptor (Eph) families^[65]. Ephrins and Eph, both membrane-bound proteins, are differentially expressed in intestinal mucosa, with Eph localized in the intestinal crypt region, while Ephrin proteins colonize the villi^[66,67]. A direct influence of EphB-signaling on ISC proliferation has been shown^[68] and it has been demonstrated that EphB3 is essential for PC downward migration^[69]. In addition to their role in promoting cell proliferation of the intestinal epithelium, tissue repair, acceleration of wound closure and maintenance of homeostasis of the intestinal barrier in adults, Ephrin/Eph signaling has been recognized to function as tumor suppressors by controlling cell migration and inhibiting tumoral invasive growth^[70-72].

Given its pivotal role within the ISC niche, it is not surprising that alterations in Wnt signaling play a pivotal role in the development of non-neoplastic gastrointestinal disorders, such as chronic inflammatory bowel disease and intestinal cancers (as reviewed elsewhere^[40,73]).

Notch signaling: Notch signaling is known to control cell fate decisions in the development of many tissues. The ligands Delta or Jagged bind the Notch receptor, thereby inducing its proteolytic cleavage; NCID, a cleavage fragment of Notch, translocates to the nucleus where it acts as a transcription factor, thus inducing the activation of molecular pathways involved in the control of proliferation and differentiation^[74]. Manipulations of the Notch signaling in experimental models revealed its role in intestinal epithelial differentiation. Hes1, the major Notch-target gene, colocalizes with Msi1 in both the CBCs and the +4 LRCs^[75,76].

Notch signaling plays a central role in preserving self-renewal in the intestinal progenitor cells by suppressing Atoh1^[40]. Notch signaling seems to trigger proliferation

of crypt progenitor cells in TA-cells and a regulated reduction of notch signaling in cooperation with activation of specific transcription factors (such as Atoh1 and neuroD) induces specific differentiation into the intestinal epithelial lineages^[40,56].

Hedgehog and BMP pathways: The morphogens Sonic Hedgehog (Shh) and Indian Hedgehog (Ihh) are secreted by epithelial cells, while their receptor, Patched (PTCH), is expressed by subepithelial myofibroblasts. In the intestinal epithelium, Ihh is mainly expressed at the base of the villi^[77]. Given the importance of the stromal-epithelial interactions in the regulation of the epithelial cell fate, hh signaling is indirectly involved in the ISC fate through its modulation of the maturation and localization of the underlying stromal cells that in turn generate signal molecules responsible for the maintenance of the ISC niche^[78]. Ihh down-regulates expression of TCF4 and beta-catenin, restricting Wnt signaling to the crypt base^[79]. Ihh also promotes maturation of the tolerogenic immune cells in the small intestine and is critical to the ability of the gut to respond to pro-inflammatory stimuli: disruption in the hh pathway may contribute to the pathogenesis of autoimmune diseases^[40,80].

Disturbed hh signaling results in severe developmental defects, enhancement of Wnt signaling, increased proliferation and structural abnormalities of crypts and villi. Such effects are mainly due to the reduced expression of bone morphogenetic proteins (BMPs) by stromal cells, which is normally triggered by hh^[81].

BMPs regulate differentiation, apoptosis and cell growth depending upon the specific cellular context. BMPs bind to BMP receptors, leading to phosphorylation of SMADs, which upon heterodimerization translocate to the nucleus and act as transcriptional factors^[82]. BMP pathway participates in the control of ISC numbers and self-renewal: active BMP signaling is found predominantly in differentiated intestinal epithelial cells, while its inhibition seems to confer intestinal stemness properties^[81]. Physiological inhibitors of BMP signaling, Noggin and Gremlin, induce Wnt signaling activation and are produced by myofibroblasts at the crypt base, ensuring a “BMP-free” ISC niche^[83]. Mesenchymal cells are the main target of BMP signaling which in turn down-regulates epithelial proliferation^[40]. Of note, the BMP pathway has a direct role in the differentiation of the intestinal epithelium toward secretory lineages (especially enteroendocrine cells), while it does not affect the absorptive phenotype^[84].

Mutations involving BMP signaling are associated with juvenile polyposis^[81]. BMPs stabilize PTEN, thereby leading to reduced Akt activity and subsequent reduction of nuclear beta-catenin accumulation^[56].

PTEN-PI3K-Akt pathway: PI3k activation leads to phosphorylation and subsequent activation of the kinase Akt, which induces cell survival, growth and proliferation programs. PTEN is a negative regulator of this pathway,

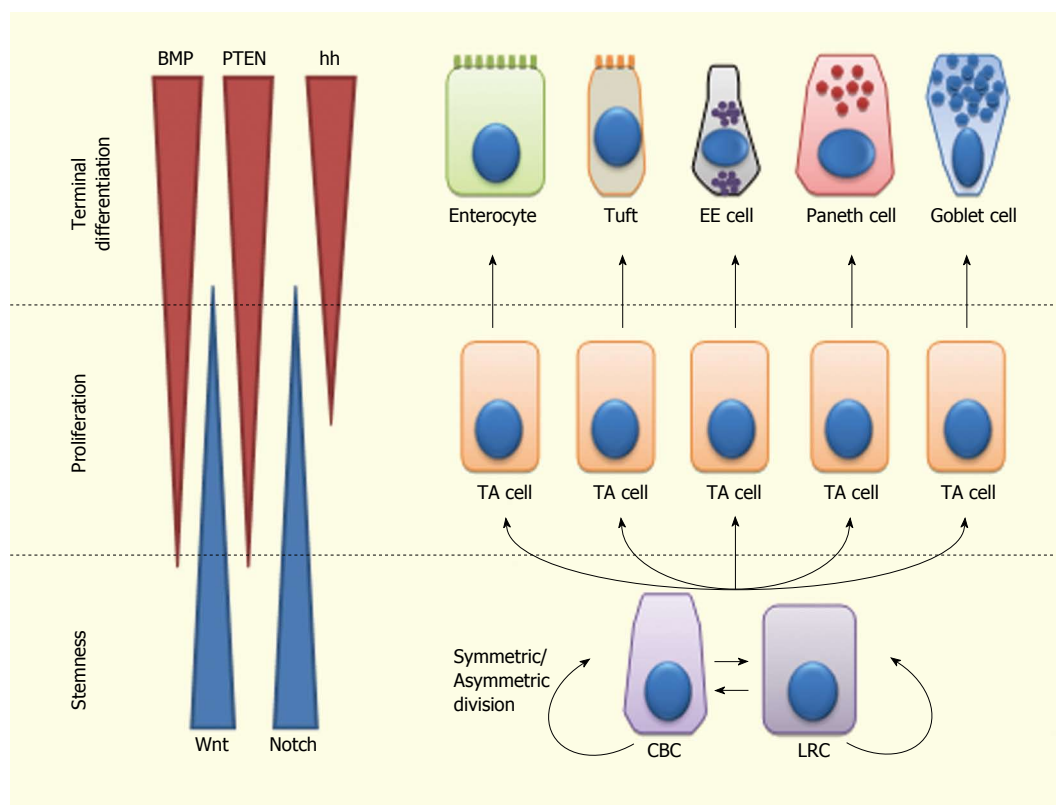


Figure 2 Lineage specification of intestinal stem cells. Intestinal stem cells (ISCs)-Crypt Columnar Cells (CBCs) and Label Retaining Cells (LRCs)-can divide asymmetrically or symmetrically to maintain the stem cell compartment. ISCs give rise to Transit Amplifying (TA) cells which actively proliferate and can further differentiate into enterocytes, tuft cells, enteroendocrine (EE) cells or goblet cells. Wnt signaling maintains the stem-like phenotype of ISCs, while Notch signaling maintains the proliferation of progenitor cells. In the upper crypt region, hedgehog (hh) triggers BMP expression in stromal cells which activates PTEN expression; all these factors inhibit Wnt signaling in the ISC niche.

thereby inhibiting Akt function^[85].

This pathway is activated in many human tumors, mainly as a consequence of PTEN inactivation^[86]. PTEN inherited mutations are responsible for hamartomatous polyps (Cowden syndrome)^[81].

As for the role of the PI3K pathway in ISC regulation, it has been demonstrated that it enhances ISC self-renewal, probably because p-Akt can increase the transcriptional activity of beta-catenin, the main effector of the canonical Wnt pathway^[87]. Moreover, PTEN might be involved in the restriction of the strong Wnt signaling to the crypt base^[88].

Overall, ISC fate is regulated by a complex balance among signals controlling stem cell maintenance, proliferation and differentiation^[40,56,89]. Wnt and Notch are mainly involved in ISC self-renewal and expansion. Moreover, Notch is involved in ISC differentiation, independently from Wnt. Notch inhibition leads to differentiation to a secretory phenotype, while Notch activation leads either to self-renewal within the ISC compartment or to differentiation towards an absorptive phenotype. The Wnt pathway is also implicated in PC differentiation and regulates cell migration along the crypt-villus axis, *via* Eph/Ephrin signaling. Hh effects on the ISCs are mainly indirect and occur through regulation of the BMP pathway. BMP signaling inhibits proliferation of ISCs, an-

tagonizing the Wnt pathway; this suggests a homeostatic function of BMP in keeping self-renewal within the ISC niche. Most likely, this interaction is mediated by PTEN inhibition of Akt, which in turn inhibits Wnt signaling. BMPs also support the differentiation of secretory cell lineages (especially of enteroendocrine cells) (Figure 2).

ISCs in GI diseases

Observations that mutations in the pathways involved in ISC maintenance occur in most colon cancers have led the majority of the research on ISC biology in humans. Alterations of ISC pathways have also been reported in inflammatory bowel diseases. In particular, decreased expression of TCF4 (Wnt target gene, correlated with defensin production) has been described in ileal Crohn's disease^[90]; increased activation of Notch and PC dysfunction have been reported in both ulcerative colitis and Crohn's disease^[29,40].

A better knowledge of ISC function and dysregulation in gastrointestinal diseases will help to understand the pathophysiology of such disorders and might also offer new insight into the development of SC-based therapies.

Theoretically, ISCs would be the best source for intestinal regeneration. Although ISCs can be expanded for multiple passages in the form of organoids, most of the

culture conditions provide little control over their self-renewal and differentiation. As a consequence, the inability to efficiently expand Lgr5+ SCs has so far considerably limited the translation to therapies as well as the study of intestinal epithelial biology. However, very recently Yin and co-workers identified small molecules (CHIR99021 and valproic acid) that target Wnt, Notch and BMP pathways to maintain the self-renewal of Lgr5+ ISC, resulting in nearly homogeneous cultures with high colony-forming efficiency and preservation of the multilineage differentiation ability^[91]. This might be a promising SC source for regenerative medicine, tissue engineering and drug screening.

So far, the only SCs that have “left the bench and reached the bedside” in gastroenterology are BM-derived SCs. BM SC transplantation has become an option for the treatment of selected cases of inflammatory bowel disorders (IBD). Experimental and clinical studies have suggested that both allogeneic and autologous BM SC transplants may be effective in inducing IBD remission^[92-94]. The mechanisms underlying this beneficial effect are still under investigation; they might include local immune-modulation and a direct contribution to tissue repair^[95,96].

BM SCs might also be used to cure other gastrointestinal pathologies, such as gastric ulcers or motility disorders, like gastroparesis, achalasia and chronic constipation^[97,98].

Finally, a promising application for SC-based therapy is celiac disease (CD). The following chapters will attempt to summarize the body of knowledge regarding CD physiopathology and clinical manifestations, as well as the status of the ISC compartment during the course of the disease and the possible SC-based treatments.

CELIAC DISEASE: FROM PATHOGENESIS TO CURRENT TREATMENT

CD likely first developed after the last ice age in the fertile crescent of the Middle East with cultivation of grains^[99]. The major breakthrough for the modern understanding of CD was the observation that bread shortages during World War II resulted in a dramatic decrease in death rate from celiac disease^[100]. Also known as “nontropical sprue”, “celiac sprue” and “gluten-sensitive enteropathy”, CD can be defined as a chronic immune-mediated disease that is triggered and maintained by dietary proteins (gluten) in genetically predisposed individuals. Patients affected by the disease display a specific autoantibody response, various degrees of intestinal inflammation and a broad range of clinical symptoms^[101,102].

Once considered a rare small bowel disease of childhood, CD is now recognized as a relatively common, systemic disease that may manifest at any age. CD affects 0.6%-1% of the population worldwide. The prevalence is up to 3-fold higher in women than in men; moreover, first-degree relatives of CD patients (10%-15%), individuals affected by autoimmune diseases, particularly type

1 diabetes (3%-16%) and Hashimoto's thyroiditis (5%), IgA deficiency (9%), Down's syndrome (5%) and Turner's syndrome (3%) are at increased risk of developing the disease. The disease is less common in Hispanic Americans and it is thought to be rare in central Africa and east Asia; the frequency of CD is increasing in many developing countries because of many factors, such as increased awareness of the disease, changes in wheat production and preparation and westernization of the diet. Interestingly, serological screening studies have shown that only a small proportion of cases of CD (up to 20%) are clinically recognized^[103].

Genetic background plays a pivotal role in the predisposition to CD: results from genetic linkage studies showed that CD is strongly associated with HLA-DQ genes (COELIAC1 locus, on chromosome 6p21). In particular, up to 90% of CD patients carry a variant of DQ2 (haplotype DQA1*0501/DQB1*0201), while about 5% of CD patients carry a variant of DQ8 (haplotype DQA1*0301/DQB1*0302); almost all of the remaining 5% of celiac patients have at least one of the two genes encoding DQ2^[101]. DQ2 and DQ8 haplotypes are necessary for the development of CD: DQ2 and DQ8, expressed on the surface of antigen-presenting cells, can bind activated (deaminated) gluten peptides, triggering an abnormal immune response. However, DQ2 is carried by approximately a third of the general population, thus suggesting that HLA is only partly the cause of the condition.

So far, more than 30 genes, mostly involved in inflammatory and immune response, have been linked to a CD predisposition^[104]. Non-HLA genes associated with CD include COELIAC2 (5q31-33) that contains cytokine gene clusters, COELIAC3 (2q33), encoding for the negative costimulatory molecule CTLA4, and COELIAC4 (19p13.1) that harbors an unconventional myosin able to alter cytoskeleton remodeling^[105,106].

Almost all patients with CD develop immunoglobulin IgA autoantibodies to the enzyme tissue transglutaminase 2 (TG), which is expressed by many cell types and is associated with the extracellular matrix (endomysium or reticulin fibers). TG targets certain glutamine residues in some extracellular and intracellular proteins, usually tethering them to a lysine residue of a second protein that results in cross-linking of both proteins. Alternatively, TG merely deaminates glutamines to negatively charged glutamine acid residues. Gluten proteins are preferred substrates for TG and once deaminated, they bind more strongly to HLA-DQ2 or DQ8 on the surface of antigen presenting cells^[105].

Serological tests are fundamental for CD screening. In patients with positive serology, a biopsy of the small intestine showing typical CD characteristics (increased number of intra-epithelial lymphocytes (IELs), elongation of the crypts and villous atrophy) is required to confirm the diagnosis. However, according to the most recent European guidelines, the confirmation biopsy is no longer required in children with predisposing HLA-genotypes, typical symptoms and a higher titer of anti-

TG (>10 times the upper limit of normal range)^[107].

CD is a “unique” model of autoimmune disease in that the key genetic components (HLA DQ2 and/or DQ8) are present in almost all patients, the autoantigen (TG) has been identified and the environmental trigger (gluten) is known. The central role of gluten in this cascade of events explains how the cornerstone of therapy for CD is a “gluten-free” diet (GFD).

Gluten is a protein complex composed of gliadins and glutenins that is responsible for the baking properties of wheat. Analysis of gliadin has identified more than one hundred components that can be grouped into four main types (omega5-, omega1, 2-, alpha/beta- and gamma-gliadins). The immunogenicity and toxicity of several gliadin epitopes has been established; although several gluten epitopes are immunostimulatory, an immunodominant peptide of 33 amino acids identified from the alpha-gliadin fraction has functional properties attributable to many proline and glutamine residues. Proline gives increased resistance to gastrointestinal proteolysis and causes a left-handed helical conformation which strengthens binding with DQ2 and DQ8 molecules on antigen-presenting cells. Additionally, glutamine residues are a preferred substrate for tissue transglutaminase-mediated deamination, which confers an enhanced immunogenicity. Storage proteins (prolamines), with similar amino acid composition to the gliadin fraction of wheat, have been identified in barley (hordeins) and rye (secalins) and show a close correlation to the taxonomy and toxic properties of wheat cereal^[102].

Gluten peptides can be transported across the intestinal epithelium either paracellularly, especially in presence of an impaired gut barrier, or *via* transcytosis or retrotranscytosis of secretory IgA through the transferrin receptor. Gluten can elicit an innate immune response in professional antigen-presenting cells (monocytes, macrophages and dendritic cells) that activates both IELs and intestinal epithelial cells. This immediate reaction might favor the development of adaptive immunity to gluten in HLA-DQ2 or DQ8 carriers^[108]. Innate immune activation of IELs by gluten induces expression of the non-classic class I molecule (MICA) on intestinal epithelium, which can in turn activate natural killer-like IELs, gamma-delta T cells and a subset of CD4+ and CD8+ T cells^[109]. Epithelial MICA and production of IL-15 by epithelial cells, macrophages and dendritic cells lead to enhanced proliferation of IELs and cytokine secretion in CD patients; moreover, IL-21, produced by CD4+ Th1 cells, acts in concert with IL-15 as an additional driving force of innate immunity in CD pathogenesis^[110].

Deamination or cross-linking of gluten by TG enhances the binding to HLA-DQ2 or DQ8 expressed by antigen presenting cells, leading to a more rigorous gluten-specific CD4+ Th1 T-cell activation^[105]. Activated gluten-reactive CD4+ T cells produce high levels of pro-inflammatory cytokines, thus inducing a Th1-pattern dominated by interferon (IFN)- γ . Th-1 cytokines promote extracellular matrix degradation and increase cyto-

toxicity of IELs and NK cells. Additionally, IFN-alpha released by dendritic cells perpetuates the inflammatory reaction by inducing CD4+ T cells to produce IFN- γ . Finally, the production of Th2 cytokines by activated CD4 T cells drives the clonal expansion of B cells and subsequent production of antigliadin and anti-TG antibodies that can form deposits in the basement membrane region of the mucosal layer, leading to cytoskeleton remodeling and subsequent epithelial damage^[102].

Clinical presentations of CD are extremely variable, reflecting the systemic nature of the disease. CD can be divided into 5 clinical subcategories: major (or classic), minor (or atypical), asymptomatic (or silent), latent and potential^[102,111].

Major CD has three distinctive features: malabsorption (diarrhea, weight loss, vitamin and nutrient deficiencies), positive serology and pathological findings of villous atrophy. A rare life-threatening manifestation of CD is the so-called “celiac crisis”, mostly observed in children that manifests with profuse diarrhea, hypoproteinemia, metabolic and electrolyte imbalances.

Minor CD may present with only trivial, transient and apparently unrelated symptoms (fatigue, anemia, abdominal discomfort, dyspepsia, altered bowel habits, cryptic hypertransaminasemia, osteoporosis, infertility, peripheral and central neurological disorders, short stature, dental enamel defects, dermatitis herpetiformis) or isolated symptoms of associated autoimmune diseases. Most of these patients are biopsied after a positive search for anti-TG and/or anti-endomysial antibodies.

Asymptomatic CD is recognized on biopsy specimens of patients with positive serology but without symptoms of disease.

Potential CD includes subjects with positive serology but normal small bowel mucosa on a gluten-containing diet in whom CD may develop later in life. Finally, the term “latent” has been attributed to a “preclinical state” of CD, usually recognized retrospectively, or to patients with an earlier presentation of CD who recover on a GFD and later remain silent when gluten is reintroduced into the diet. Potency and latency might be transient and these patients should be followed clinically since some degree of villous atrophy with variable symptoms may develop in the future in about 80% of cases^[102,111].

The only current treatment for CD involves a strict and life-long adherence to a GFD. With maintenance of a GFD, symptoms and serum celiac antibodies gradually disappear and healing of the intestinal damage typically occurs within 6 to 24 mo after initiation of the diet.

Refractory CD is diagnosed when there are persistent or recurrent malabsorptive symptoms and signs with villous atrophy detected on biopsy despite the maintenance of a strict GFD for more than 12 mo. Complications associated with untreated and/or refractory CD include ulcerative jejunoileitis, splenic hypofunction, enteropathy-associated T cell lymphoma and adenocarcinoma of the jejunum^[101].

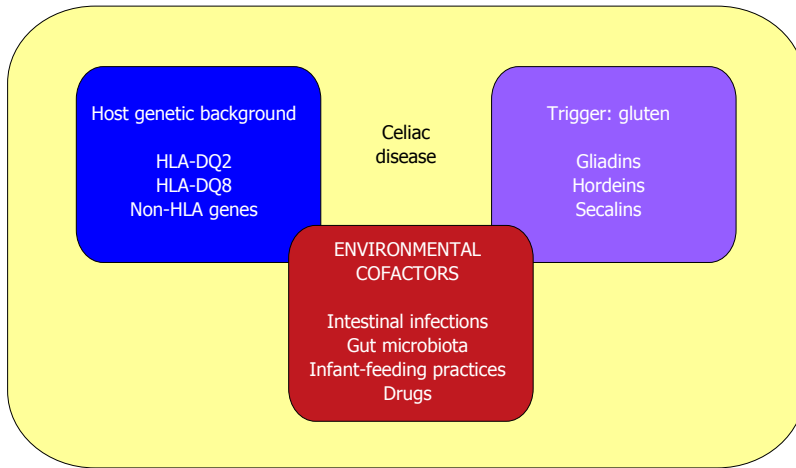


Figure 3 Causative factors in celiac disease. The pathogenesis of celiac disease involves: (1) host genetic background (HLA-DQ2 or DQ8 and other non-HLA genes); (2) an external trigger (gluten); and (3) environmental cofactors (such as intestinal pathogens, altered gut microbiota composition, infant-feeding practices and some immune-modulatory drugs).

GLUTEN EFFECTS ON EPITHELIAL BARRIER AND INTESTINAL HOMEOSTASIS

The presence of gluten in the mucosa is a prerequisite for the activation of gluten-reactive T-cells and the ensuing inflammation. However, gluten also affects the intestinal mucosa by non-immune mediated mechanisms.

It has been demonstrated that gliadin-derived cytotoxic peptides can induce oxidative stress, rearrangement of actin cytoskeleton, impairment of epithelial tight junction assembly and deregulation of the epithelial homeostasis in experiments on cultured epithelial cells and celiac mucosa^[112,113].

The oxidative stress induced by gliadin in epithelial cells might be responsible for the increased nuclear factor (NF)- κ B activity and subsequent interleukin (IL)-15 transcription that is present in the small intestinal mucosa of celiac patients^[114]. Epithelial NF- κ B activation in healthy hosts is normally suppressed by anti-inflammatory cytokines produced by underlying T lymphocytes, such as transforming growth factor (TGF)- β and IL-10. In active CD, the status of chronic inflammation and the direct toxic effects of gluten worsen the epithelial layer damage, thus causing activation of NF- κ B which leads to a vicious cycle of aberrant immune response, mucosal inflammation, increased mucosal permeability and impairment of the regenerative potential of the intestinal epithelium.

As for the alterations of the epithelial barrier, it is speculated that dysregulation of zonulin in many diseases may involve loss of cell junction integrity^[115]. The endogenous zonulin, which is functionally and immunologically related to zonula occludens toxin from *Vibrio cholera*, has been found to disassemble intercellular tight junctions *via* interaction with cell membrane receptors. Serum zonulin is up-regulated in active CD and decreases following GFD, suggesting a role for a “leaky gut” in the development of autoimmunity^[116]. Some gliadin peptides have

been shown to bind to the chemokine receptor CXCR3 on the surface of epithelial cells and induce tight junction permeability and zonulin release^[117].

Gliadin peptides can interfere with endocytic vesicle maturation and promote cell proliferation by prolonging epidermal growth factor receptor (EGFR) activation, which may correlate with the histological finding of crypt hyperplasia in CD^[118]. Interestingly, p31-43 gluten peptide stimulation on proliferation of epithelial cells *in vitro* is dependent on IL-15 activity^[108,118].

Furthermore, some toxic gliadin peptides have been reported to induce enterocyte apoptosis *via* the Fas-Fas ligand (FasL) pathway^[119]. IL-15 has also been shown to induce enterocyte MICA expression in CD patients and to trigger the anti-apoptotic pathway in human IELs, which can kill intestinal epithelial cells^[120].

ENVIRONMENTAL COFACTORS IN CELIAC DISEASE: GUT MICROBIOTA

Environmental cofactors that participate in the development and maintenance of CD include: intestinal pathogens that could enhance gluten immunogenicity and toxicity (*i.e.*, rotavirus infections^[121]); altered gut microbiota composition^[122,123]; infant-feeding practices (with a reported 50% lower risk among infants who are still being breast-fed at the time of gluten introduction^[124]); and some immune-modulatory drugs (*i.e.*, IFN- α ^[125]) (Figure 3).

In CD, the homeostatic mechanisms that allow coexistence of the host organism and the commensal microbiota are disrupted. Several studies have shown that celiac patients are characterized by a different composition of the gut microbiota when compared to healthy individuals. Rod shaped bacteria adhering to the small intestinal mucosa were frequently seen in patients with CD during the “Swedish CD epidemic”^[126]. Nadal *et al*^[127] demonstrated a higher proportion of total and gram-negative

bacteria, also including potentially pro-inflammatory bacteria (*Bacteroides-Prevotella* and *E. coli*), in active CD children *vs* symptom-free patients and controls. Schippa *et al*^[128] found a distinctive “microbial signature” in celiac patients, irrespective of the disease status. The duodenal mucosa of CD patients showed a higher diversity of associated bacteria population; *Bacteroides vulgatus* and *E. coli* were detected more often in celiacs than in controls^[128].

The changes detected in gut microbiota of CD patients could be either a consequence or a cause of the disease. In the first scenario, the damaged mucosa covered by immature enterocytes would facilitate gram-negative bacterial colonization to the detriment of gram-positive bacteria. In the second case, the predominant colonization of gram-negative bacteria in genetically predisposed individuals would contribute to the loss of tolerance to gluten. Indeed, changes in resident microbiota composition seem to precede the onset of the disease and as such, they might be a risk factor for the development of celiac disease in susceptible individuals. Of note, interplay has been observed between HLA genes and milk feeding practice for microbial colonization that could influence the manifestation of the disease. The PROFICEL study demonstrated that infants at high genetic risk have higher numbers of *B. fragilis* and *Staphylococcus spp.* and reduced numbers of *Bifidobacterium spp.*; breast-feeding promoted colonization of *Bifidobacteria*, while formula-feeding promoted that of *Bacteroides fragilis* and *E. coli*, among others. In breast-fed infants, the increased genetic risk was associated with increased *C. leptum* group numbers, while in formula-fed infants it was associated with increased *Staphylococcus* and *B. fragilis* group numbers. Finally, breast-feeding reduced the genotype-related differences in microbiota composition, which could partly explain the protective role attributed to breast milk in this disorder^[129].

Through a process of “cross-talk” with the mucosal immune system, gut microbiota negotiates mutual growth, survival and inflammatory control of the intestinal ecosystem. The intestinal mucosa is equipped with transmembrane and intracytoplasmic receptors referred to as pattern/pathogen recognition receptors (PRRs) that are defined by their ability to specifically recognize and bind distinctive microbial macromolecular ligands (microbial-associated or pathogen-associated molecular patterns, MAMPs or PAMPs), such as LPS, flagellin, peptidoglycans and formylated peptides. Subsequent signaling consists of an intricate and inter-relational pathway which determines the signaling output based on the initial perception of the triggering organism. Output can be a protective response to commensal microbiota, an inflammatory response to pathogenic organisms, or a trigger for apoptosis. Intestinal epithelial cells express high levels of the Toll-like receptor (TLR) inhibitor and Toll-interacting protein (TOLLIP). Expression of TOLLIP has been shown to correlate with the *in vivo* luminal bacterial load and is highest in healthy colonic mucosa; this inhibitory molecule is important in maintaining microbial homeo-

stasis.

Many studies have demonstrated that the expression of TLRs is deregulated in active CD, suggesting that microbiota-associated factors may be important in the development of the disease. Higher densities of TLR4+ cells were found in active CD patients *vs* controls^[130]. Recently, Kalliomäki *et al*^[131] demonstrated that expression of IL-8 mRNA (marker of intestinal inflammation) and TLR-2 mRNA significantly increased in duodenal biopsies of active celiacs compared with treated celiacs and controls, while expression of TOLLIP mRNA was down-regulated.

The CD-associated bacteria and the dysbiosis they might cause in the resident microbiota through TLR/PAMP interactions might contribute to the Th1 pro-inflammatory milieu characteristic of CD. Medina *et al*^[132] showed that gut microbiota from both active and treated CD patients increased TNF- α and IFN- γ production and decreased IL-10 production and CD4 expression in peripheral blood mononuclear cells compared with control samples. Interestingly, probiotics (*Bifidobacterium* strains) suppressed this pro-inflammatory cytokine pattern and increased IL-10 production. Similar beneficial effects of *B. longum* were found in an animal model of gliadin-induced enteropathy^[133].

ISC MODULATION IN CD

Despite the many achievements in understanding of the pathogenic interactions among genetic, immunological and environmental factors in CD, little is known about ISC modulation and deregulation during the course of the disease.

In the last years it has been observed that ISC differentiation towards PCs and goblet cells may be disturbed in active CD. This may result in a defective antimicrobial and mucus barrier which enables the intestinal bacteria to invade the mucosa and trigger the inflammation. Indeed, the expression of natural antibiotics such as defensins is limited in CD. In particular, it has been demonstrated that some beta-defensins are underrepresented among celiac patients and that their expression correlated negatively with the degree of villous atrophy and rose on GFD; this suggests that increased copy numbers could protect from CD, possibly by impeding bacterial infiltration more efficiently and preserving gut epithelial integrity^[134-136].

As for PC deregulation in CD, a number of studies have reported conflicting results. The earliest reports described the disappearance of PCs in patients with refractory CD and a significant decrease in patients with untreated and treated CD. However, later studies did not confirm a numeric reduction of celiac PCs and some authors even hypothesized that PCs would be increased in active CD given the high level of α -defensins found in untreated celiac mucosa^[137]. Di Sabatino *et al*^[137] adopted a multiple histochemical approach and showed no change of PC numbers in uncomplicated treated or untreated CD *vs* normal controls, while they observed a signifi-

cant decrease of PCs in patients with complicated CD; of note, this decrease did not correlate with the degree of mucosal damage or with the duration of GFD. The proliferative pattern of PCs was not statistically different among the various groups, while crypt enterocyte proliferation was significantly higher in uncomplicated, untreated CD in comparison with treated CD and control cases^[137]. More recently, Rubio found that in active CD patients, the normal production of PCs in the crypts is replaced by lysozyme-producing mucus cells. The author speculated that in CD, ISCs are re-programmed as an antimicrobial adaptation to signals generated by pathogenic duodenal bacteria^[138]. The molecular mechanisms behind the abrogation of PCs in duodenal crypts and their substitution with lysozyme-producing mucus cells in CD remain to be elucidated. Further studies are needed to clarify the exact entity of PC deregulation in CD, the underlying molecular pathways and its implications in terms of ISC fate.

The intriguing hypothesis that PC secretion might be involved in the control neoplasia, thus accounting for the low incidence of neoplasms in the small bowel, encourages further investigation of the relationship between PC deficiency and premalignant and malignant complications of CD, as well as other inflammatory bowel disorders.

Regarding the deregulation of goblet cells in CD, Cinova *et al*^[139] observed that gliadin fragments and/or IFN- γ were able to reduce the number of PAS-positive goblet cells and increase mucin secretion in rat intestinal loops; interestingly, these changes were more pronounced in the presence of potentially pathogenic enterobacteria, while the decrease in PAS-positive goblet cells by gliadin was reversed by probiotics (*B. bifidum* LATA-ES2).

The molecular mechanisms underlying the deregulation of ISC differentiation in CD are still being elucidated. Capuano *et al*^[140] assessed the miRNA-based modulation of gene expression in the celiac small intestine for genes involved in intestinal differentiation and proliferation. They found a downregulation of the Notch pathway and KLF4 signals in celiac patients, whereas more nuclear beta-catenin staining (a sign of Wnt signaling activation) and more Ki67 staining (a sign of cell proliferation) were present in crypts from celiacs than in controls. Moreover, they documented a reduction of the number of goblet cells in the small intestine of children with active CD and in those on a GFD compared to controls. The authors postulated that the Notch pathway could be constitutively altered in CD and that it could drive the increased proliferation and the decreased differentiation of ISCs towards the secretory goblet cell lineage^[140].

Another reported ISC niche alteration in CD regards the mucosal vasculature in the small intestine of active celiac patients that differs considerably from normal. Indeed, in celiac mucosa the capillary tufts are totally missing and the entire vasculature is disorganized. Myrsky *et al*^[141] reported that IgA and anti-TG from CD patients disturb several steps of angiogenesis (sprouting and migration of endothelial and vascular mesenchymal cells)

and also induce disorganization of the actin cytoskeleton *in vitro*. This disturbance of the angiogenic process could lead *in vivo* to the disruption of the mucosal vasculature seen in active CD^[141].

Finally, little is known about the contribution of BM-derived SCs in CD. Mastrandrea *et al*^[142] showed an increased traffic of circulating CD34+ HSCs in active CD patients *vs* healthy controls, but no correlation was found with anti-TG levels or histological severity. The authors postulated that this increased traffic of HSCs was more related to a defect shared by chronic inflammatory diseases than to a gliadin-specific Th1 local reaction. They hypothesized that the prevalence of apoptotic *vs* survival programs leading to excessive cell death in active CD might induce the mobilization of BM multipotent SCs as a supplementary source of ISCs for intestinal repair^[142].

The potential contribution of extra-intestinal SCs in gut regeneration offers new insights into the development of SC-based treatments against CD.

NOVEL THERAPIES FOR CD: A ROLE FOR STEM CELLS?

Adherence to a strict GFD might restrict social activities and limit nutritional variety. Additionally, it is costly and difficult to maintain in many countries. In the last years, alternative therapeutic strategies have been tested. These include intraluminal therapies (genetic modification of wheat and/or pretreatment of flours to reduce immunotoxicity, oral enzyme therapy, intraluminal binding of gluten peptides, neutralizing gluten antibodies), transepithelial treatments (inhibition of intestinal permeability through zonulin receptor antagonists) and subepithelial actions (TG inhibitors, gluten peptides that downregulate innate responses, HLA-DQ2 inhibitors, CCR9 and integrin antagonists, IL-15 antagonists, anti-IFN- γ antibody, anti-CD3, anti-CD4 and anti-CD25 antibodies). Such approaches have been tested in experimental models and in small clinical trials with inconclusive results overall in terms of efficacy; moreover, some of these treatments have a poor safety profile and their hypothetical use should be reserved for complicated forms of CD^[102,105].

Novel treatments for CD might derive from SCs. Indeed, the advancements in SC biology have led to the concept of regenerative medicine which is based on SC potential for therapies aimed at facilitating the repair of injured tissues^[143]. Such therapies require a deep knowledge of the dynamics underlying SC compartment regulation, both in physiological and pathological conditions.

A potential therapeutic avenue for CD is the discovery of epithelial mitogens that stimulates mucosa growth. Recently, R-spondin-1 has been shown to stimulate crypt cell growth, accelerate mucosal regeneration and restore intestinal architecture in experimental colitis in mice^[144]. In CD, the infusion of such mitogens might help to accelerate intestinal healing.

Another potential SC-based therapy for CD is transplantation of multipotent extraintestinal SCs of BM

origin that can contribute to intestinal repair. In the last two decades, BM-derived SC transplantation has become an option for patients with severe autoimmune diseases refractory to conventional treatments. Such a therapy has recently found an application in gastroenterology for the treatment of selected cases of complicated CD. Bishton *et al*^[145] reported the efficacy of autologous HSC transplantation preceded by conditioning in patients with enteropathy-associated T cell lymphoma: 4 out of 6 patients remained in a sustained complete remission for up to 4 years. Kline *et al*^[146] treated one celiac patient affected by acute myelogenous leukemia with allogeneic HSC transplant preceded by conditioning and achieved correction of CD despite the reintroduction of a gluten-containing diet. Similarly, Hoekstra *et al*^[147] reported that in one patient with severe aplastic anaemia and CD, allotransplant of SCs resulted in the cure of CD even after the return to a free diet. Recently, Ciccocioppo *et al*^[148] showed that in 2 patients affected by CD and β -thalassemia major who underwent successful myeloablative allogeneic HSC transplantation for the latter condition, the introduction of a gluten-containing diet did not cause the reappearance of clinical, serological and histological markers of CD in up to 5 years of follow-up. Al-Toma *et al*^[149] subjected patients with refractory CD type II to autologous peripheral blood SC transplant after conditioning: 6 out of 7 patients obtained a significant reduction in aberrant T cells in duodenal biopsies, associated with clinical improvement. Similar results were obtained by Tack *et al*^[150] in refractory type-II CD patients who showed an impressive clinical improvement upon auto-transplant of HSCs.

Despite these encouraging results, further studies and longer follow-up periods are required to confirm the efficacy of HSC infusion in refractory and complicated CD.

The molecular mechanisms underlying the beneficial effects of HSC transplantation in CD are still largely unknown. It has been postulated that the immune system ablation followed by HSC transplantation provides a reset of the host immune system imbalance; this effect is likely to be more pronounced and prolonged in cases of allogeneic transplantation when HSCs that do not carry any CD-predisposing polymorphism are infused upon conditioning. Moreover, BM cells might contribute to tissue repair by differentiating into epithelial cells and myofibroblasts and by facilitating the neoangiogenesis^[92,95].

CONCLUSION

SCs are the key to tissue genesis and regeneration. Given their central role in homeostasis, SC dysfunctions are involved in the pathogenesis of virtually all diseases, from cancers to degenerative disorders to chronic inflammatory pathologies. Ten years ago, SC research was compared to a “Pandora’s vase”, the opening of which could make it possible to clarify the nature and the pathophysiology of all human disease. If SCs are the source of all pathology, they can also be the ultimate cure; this is the precondition of regenerative medicine which is based on SC

potential for tissue renewal and regeneration^[2,7].

Despite the efforts made during the last 30 years, the body of knowledge of the physiology of ISCs and their involvement in bowel disorders appears fragmentary, incomplete and sometimes contradictory. As for CD, the role of ISCs and their niche in the development and maintenance of the disease is far from being elucidated and the clinical applications of SC-based treatments for CD are limited to a few case reports and uncontrolled trials, with small numbers of subjects affected by complicated disease.

Nonetheless, the expectations of the general population for SC-based therapies against CD are very high. Among the families that collect and bank cord blood for private storage, potential treatment of CD is the second most common motivation (19.7%)^[151]. This imposes a careful consideration: further studies are needed to clarify the complex interplay among gluten, gut microbiota, gut barrier, immune system and ISC modulation and deregulation in CD. Such knowledge should be the basis for any potential clinical application of SCs against CD in order to avoid an “excess of enthusiasm” that might get “the better of judgment”.

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P- Reviewers: Song LT, Sun J **S- Editor:** Song XX
L- Editor: Roemmele A **E- Editor:** Zhang DN



Glioblastoma stem cells: Molecular characteristics and therapeutic implications

Nermin Sumru Bayin, Aram Sandaldjian Modrek, Dimitris George Placantonakis

Nermin Sumru Bayin, Aram Sandaldjian Modrek, Dimitris George Placantonakis, Department of Neurosurgery, New York University School of Medicine, New York, NY 10016, United States

Nermin Sumru Bayin, Stem Cell Biology Training Program, New York University School of Medicine, New York, NY 10016, United States

Aram Sandaldjian Modrek, Medical Scientist Training Program, New York University School of Medicine, New York, NY 10016, United States

Dimitris George Placantonakis, Kimmel Center for Stem Cell Biology, New York University School of Medicine, New York, NY 10016, United States

Dimitris George Placantonakis, Brain Tumor Center, New York University School of Medicine, New York, NY 10016, United States

Author contributions: Bayin NS, Modrek AS and Placantonakis DG solely contributed to this paper.

Supported by Bayin NS received support from NYSTEM Institutional training grant, No. CO26880; Modrek AS received support from the Medical Scientist Training Program at NYU School of Medicine; Placantonakis DG received support from NIH/NINDS 1R21NS087241-01, NIH/NCI 2P30CA016087-33, NIH/NCATS UL1 TR000038; NYU Cancer Institute and NYU Clinical and Translational Science Institute

Correspondence to: Dimitris George Placantonakis, MD, PhD, Department of Neurosurgery, New York University School of Medicine, 530 First Avenue, Skirball 8R, New York, NY 10016, United States. dimitris.placantonakis@nyumc.org
Telephone: +1-212-2632441 Fax: +1-212-2638042

Received: January 1, 2014 Revised: January 25, 2014

Accepted: April 11, 2014

Published online: April 26, 2014

environment-mediated mechanisms to overcome current therapeutic approaches. They are, therefore, very important therapeutic targets. Although the functional criteria defining GSCs are well defined, their molecular characteristics, the mechanisms whereby they establish the cellular hierarchy within tumors, and their contribution to tumor heterogeneity are not well understood. This review is aimed at summarizing current findings about GSCs and their therapeutic importance from a molecular and cellular point of view. A better characterization of GSCs is crucial for designing effective GSC-targeted therapies.

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Key words: Glioblastoma; Glioblastoma stem cells; Self-renewal; Differentiation; Molecular markers; Therapy resistance

Core tip: Stem-like cells in glioblastoma, a malignant brain tumor, have increased tumorigenic capacity, generate tumor lineages and exhibit marked resistance to current therapies. A better understanding of these stem-like cells is necessary for designing new effective treatments. This review discusses the molecular characteristics of these cells and their therapeutic importance.

Bayin NS, Modrek AS, Placantonakis DG. Glioblastoma stem cells: Molecular characteristics and therapeutic implications. *World J Stem Cells* 2014; 6(2): 230-238 Available from: URL: <http://www.wjgnet.com/1948-0210/full/v6/i2/230.htm> DOI: <http://dx.doi.org/10.4252/wjsc.v6.i2.230>

GLIOBLASTOMA MULTIFORME

Glioblastoma Multiforme (GBM), classified by World Health Organization (WHO) as grade IV astrocytoma, is

Abstract

Glioblastoma Multiforme (GBM) is a grade IV astrocytoma, with a median survival of 14.6 mo. Within GBM, stem-like cells, namely glioblastoma stem cells (GSCs), have the ability to self-renew, differentiate into distinct lineages within the tumor and initiate tumor xenografts in immunocompromised animal models. More importantly, GSCs utilize cell-autonomous and tumor micro-

a deadly primary brain malignancy with more than 10000 new cases in the United States annually (<http://www.cb-trus.org>). Despite the aggressive treatment options involving surgery and concomitant chemoradiotherapy, median survival is 14.6 mo^[1]. The fact that survival has improved by only a few months over the past 50 years highlights the need for a better understanding of the disease and the design of informed therapies^[2].

GBM is a highly heterogeneous tumor with distinctive histologic hallmarks including high cell density, intratumoral necrosis, vascular hyperplasia and invasion through brain parenchyma^[3]. This heterogeneity is also displayed at the microscopic level, where a cellular hierarchy is dominated by the presence of stem-like cells, namely glioblastoma stem cells or GSCs^[4]. In this review we will discuss the molecular and phenotypic characteristics of GSCs and their therapeutic implications.

CANCER STEM CELL HYPOTHESIS AND GLIOBLASTOMA STEM CELLS

Within multi-cellular systems, cells specialize to undertake different responsibilities, in order to maintain homeostasis. As a consequence of this specialization, every cell is not equal in its self-renewal and differentiation ability. Some cells are more stem-like, meaning that they can self-renew and give rise to different progeny through more restricted intermediate progenitors (Figure 1A)^[5]. The extent of self-renewal is dictated by the developmental stage that cells are in and varies from tissue to tissue. For example, in tissues such as the gastrointestinal tract or hematopoietic system, where cellular turnover is high, adult stem cells self-renew more often, compared to more quiescent tissues such as the brain^[6,7]. On the other hand, as cells differentiate, their self-renewal ability decreases and they adopt properties related to their tissue (Figure 1A)^[8]. The differences in differentiation potential define a cellular hierarchy within these systems, where stem cells represent the top of this hierarchy. Lineage restriction and differentiation during physiological processes are mostly believed to be irreversible. However, pathologic conditions or experimental manipulations can cause de-differentiation^[4,9]. Therefore, it is important to understand how cellular hierarchy is established and maintained in tumors in order to understand tumor biology.

Guided from research in liquid tumors, the idea of cancer cells with stem-like properties has revolutionized the field of cancer biology^[10,11]. Although initially thought to be controversial, cancer stem cells (CSCs) are a proven concept for many liquid and solid tumors, including GBM.

In liquid tumors, cellular hierarchy is very well defined by the expression of surface markers. These hierarchically distinct populations were easily isolated by Fluorescence-Assisted Cell Sorting (FACS) *via* the expression of surface markers and their tumor formation ability was assessed *in vivo*^[10]. These surface markers were then investigated in many solid tumors and some of them are still among the

best-studied CSC markers.

Glioblastoma cells need to fulfill specific criteria to be classified as GSCs. In particular, they should be able to: (1) *self-renew* (Figure 1A); (2) differentiate into distinct lineages, a property termed *multipotency* (Figure 1A); and (3) *initiate tumors* in animal models, which recapitulate the original disease phenotype and heterogeneity (Figure 1A and B)^[12,13]. Self-renewal is assessed with *in vitro* tumor-sphere formation assay, a system borrowed from neural stem cell culture. In this assay, single cells are plated in suspension and their sphere formation ability is evaluated over serial passaging, which is an indicator of long-term self-renewal^[14]. *In vivo* self-renewal is assayed by serial xenograft tumor formation experiments^[11-13] (Figure 1B). The differentiation potential of GSCs is assessed *via* analysis of tumor-derived lineages *in vitro* and *in vivo*^[15-17].

Evidence for GSCs first came from Dirks and colleagues, who isolated cells from human GBM samples based on expression of the cell surface glycoprotein CD133 (Prominin1/PROM1)^[12,13]. They showed that these cells initiated orthotopic tumor xenografts in immunodeficient mice more efficiently than cells that did not express CD133.

Although the functional criteria defining GSCs are completely defined, the molecular characteristics of these cells are not understood. As expected by the heterogeneous histology of GBM, there is extensive cellular heterogeneity within GBM cells, and GSCs as well. The complex interplay of signaling pathways and lack of universal molecular markers identifying GSCs further complicate the study of these cells. More importantly, GSCs are resistant to chemoradiotherapeutic approaches and are, therefore, believed to cause tumor recurrence^[18-20]. Thus, it is of major importance to understand the biology of these cells and their contribution to tumorigenesis, in order to overcome the problems current therapeutic approaches encounter. This review will focus on GSC markers, their molecular signatures and the signaling pathways important for their biology. Finally, we will discuss the therapeutic importance of these cells.

MOLECULAR MARKERS

CD133, a pentaspan transmembrane protein of unknown function, is one of the best-studied GSC markers to date. CD133 expression has been observed during embryonic development, as well as in adult neural stem cells and ependymal cells. However, CD133 knockout mice only have a mild retinal phenotype^[21-23]. When isolated and injected into immunodeficient animals, CD133+ GBM cells are more tumorigenic than CD133- cells and produce xenograft tumors that phenocopy the original patient tumor^[13]. Furthermore, knockdown of CD133 with shRNA impairs GSC self-renewal^[24]. However, the facts that CD133- cells can also generate tumors and that some tumors do not have a CD133+ population suggest that CD133 is not a universal GSC marker^[25-31].

GSCs were also expected to share common markers with neural stem cells, their normal counterparts, based

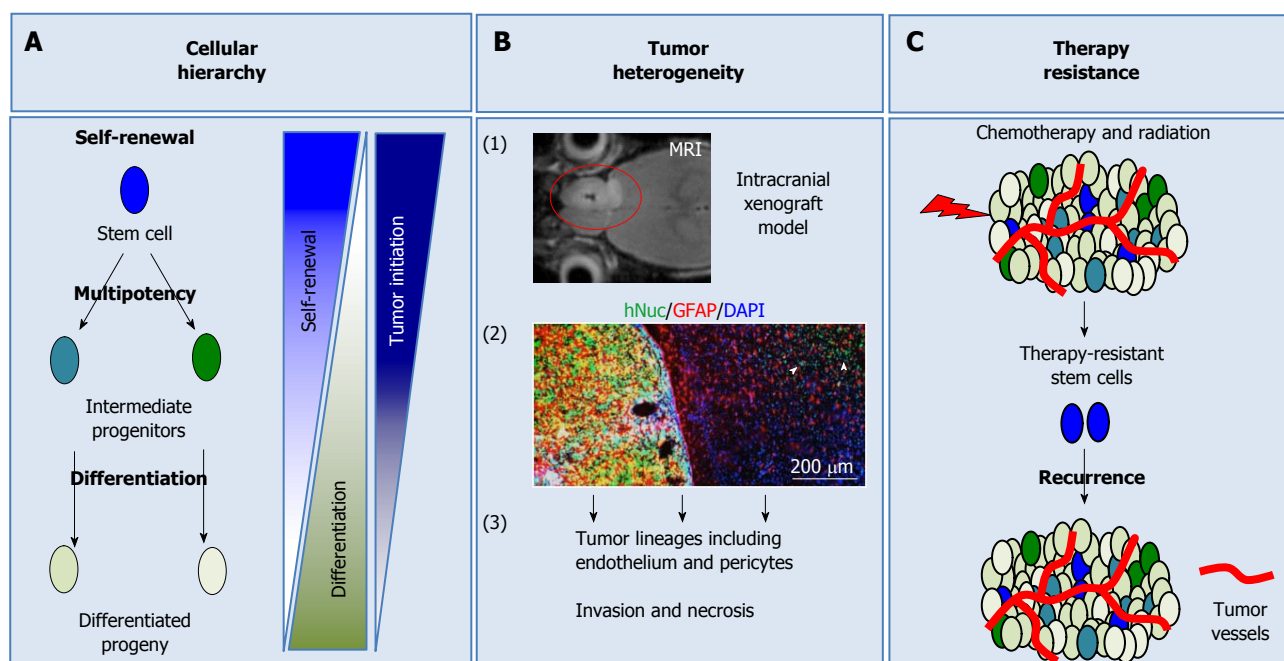


Figure 1 Biological significance of glioblastoma stem cells. A: Glioblastoma stem cells (GSCs) have the ability to self-renew and differentiate into distinct lineages through different intermediate progenitors, a property termed multipotency. Co-existence of cells with different differentiation capacities defines the cellular hierarchy within the tumor; B: GSCs have the ability to initiate tumors more efficiently than differentiated cells. Tumor initiation ability can be tested *via* intracranial xenograft models in immunodeficient animals. (1) These tumors can be imaged with Magnetic Resonance Imaging (MRI); (2) Microscopic analysis shows that xenografts maintain the histologic heterogeneity of the patient tumor, including the invasion of normal surrounding brain (arrowheads) (hNuc: human nuclear antigen marking human tumor cells in mouse brain, GFAP: Glial Fibrillary Acidic Protein, DAPI: nuclear counterstain); and (3) GSCs promote tumor heterogeneity by giving rise to distinct tumor lineages including tumor endothelium and pericytes, and maintain the phenotype of the parent tumor; C: GSCs are resistant to current therapeutic approaches causing relapse of the tumor.

on the concept of stem cells sharing common signaling pathways. With this rationale, expression of neural stem cell markers was analyzed in GBM tumors. GSCs were shown to have increased expression of Nestin, an intermediate filament expressed in neural stem cells in neurogenic niches^[18,32,33]. Besides Nestin, GSCs are enriched for Sox2, a transcription factor associated with multipotency and pluripotency^[34,35].

Comparative gene expression analysis led to identification of more GSC markers, including Oct4, SSEA-1/CD15, Bmi-1, Musashi-1, Nanog, integrin- α 6, L1CAM, A2B5 and ABC-type transporters, whose expression defines the side population (SP) on flow cytometric analysis, through the ability to extrude Hoechst dye^[25,35-40]. Interestingly, some of these markers are expressed in embryonic stem cells, suggesting GSC overlap not only with NSCs but also with less differentiated stem cells as well. However, none of these markers are universal. Furthermore, the intracellular localization of some of these markers makes them less desirable candidates for selective therapeutic targeting.

SIGNALING PATHWAYS REGULATING GSC BIOLOGY

In addition to oncogenic pathways globally important to tumor biology, signaling pathways that are important for maintenance of self-renewal and regulation of differentiation receive attention in cancer stem cell biology (Table

1). In the context of GSCs, pathways known to regulate neural development are of major interest. Various signaling pathways influence GSC biology by either maintaining self-renewal or regulating differentiation. However, certain pathways can regulate either self-renewal or differentiation in the appropriate context (Table 1).

Self-renewal

Studies of pathways involved in GSC self-renewal gained momentum when Fine and colleagues started culturing tumor cells in serum-free conditions^[41]. By using the mitogens epidermal growth factor (EGF) and fibroblast growth factor (FGF), they limited differentiation and promoted GSC self-renewal. These mitogens act through their receptor tyrosine kinases (RTKs) and induce activation of downstream pathways such as the Phosphoinositide 3-kinase/Akt (PI3K/Akt) and Mitogen-Activated Protein Kinase (MAPK), to induce proliferation, survival and tumorigenicity^[41,42]. Furthermore, blocking the PI3K/Akt pathway has been shown to impair GSC self-renewal and tumorigenicity. Finally, knockdown of CD133 in GSCs causes downregulation of Akt phosphorylation, further highlighting the role of the PI3K/Akt pathway in GSC biology^[43,44].

Originally identified in genetic screens in *Drosophila* as a master regulator of neurogenesis, Notch signaling plays diverse roles in nervous system development, including maintenance of self-renewal and regulation of fate decisions in neural and glial lineages^[45-47]. Upon bind-

Table 1 Major signaling pathways and their roles in glioblastoma stem cell biology

| Signaling pathway | Function | Ref. |
|---------------------------------|--|------------|
| Self-renewal | | |
| Notch Signaling | Maintenance of GSCs Tumorsphere formation Tumorigenesis Asymmetric division | [50-57] |
| TGF- β Signaling | Regulation of self-renewal Maintenance of perivascular GSCs | [34,58] |
| Sonic Hedgehog Signaling | Promotion of self-renewal and migration Upregulation of stem cell associated genes Tumorigenesis | [56,61-66] |
| Wnt/ β -catenin Signaling | Self-renewal and maintenance of GSCs Tumorigenesis Associated with bad prognosis | [15,66-71] |
| PI3K/Akt Signaling | Promotion of GSC self-renewal in vitro Proliferation and survival of GSCs Tumorigenesis | [41-44] |
| MAPK Signaling | Proliferation and survival of GSCs | [41] |
| Differentiation | | |
| BMP Signaling | Inhibition of asymmetric division Differentiation and proliferation block | [72-74] |
| Notch Signaling | Trans-differentiation to tumor-derived endothelium | [16] |
| TGF- β Signaling | Trans-differentiation to vascular pericytes | [17] |

GSC: Glioblastoma stem cell; TGF: Transforming growth factor.

ing to its ligands (Delta-like and Jagged), heterodimeric Notch receptors (Notch1-4) get cleaved by γ -secretase in the cytoplasm, releasing the Notch intracellular domain (NICD). NICD translocates into the nucleus where it acts as co-activator for transcription of the *Hes* and *Hey* families of genes^[48]. These genes are transcriptional repressors of neurogenic genes, thereby causing maintenance of stemness in activated cells^[49]. In GBM, Notch signaling is involved in several distinct processes in tumorigenesis, by regulating both self-renewal and differentiation of GSCs^[16,50,53]. Blockage of Notch signaling with γ -secretase inhibitors inhibits self-renewal, as assayed by tumorsphere forming ability, and causes depletion of the CD133+ GSC population^[54-56]. Furthermore, Numb, which prevents NICD from travelling to the nucleus and thus inhibits downstream signaling upon Notch activation, was shown to be asymmetrically distributed within GSCs and to promote asymmetric division. Asymmetric division of GSCs gives rise to two distinct daughter cells: a stem cell (GSC); and a more restricted and differentiated cell^[57]. These findings support a role for Notch signaling in the maintenance of GBM's stem cell compartment.

Inhibitors of Notch pathway components represent promising therapeutic candidates in GBM. However, the overlapping roles with normal neural and other adult stem cell maintenance raises the question of toxicity. Of note, there are ongoing phase II trials with Notch inhibitors in GBM patients (www.clinicaltrials.gov).

Transforming growth factor- β (TGF- β) signaling promotes GSC self-renewal through regulation of distinct mechanisms. First, it was shown to act through SRY-Related HMG-Box transcription factors Sox2 and Sox4, factors important for GSC biology, to induce self-renewal^[34].

Second, blockage of TGF- β signaling decreases perivascular CD44^{high}/Id1^{high} GSCs, *via* repression of inhibitors of DNA-binding proteins Id1 and Id3^[58].

Sonic Hedgehog (Shh-Gli) signaling, which is highly important for brain and spinal cord patterning during embryonic development, also plays crucial functions in GSC maintenance^[59,60]. It has been shown to promote GSC self-renewal and expression of stem cell genes, whereas its blockage leads to apoptosis, delay in tumorigenesis and inhibition of GSC self-renewal and migration^[56,61-66].

The Wnt/ β -catenin pathway induces proliferation of progenitor cells within gliomas^[15,67]. Some reports suggest that Wnt signaling is important for GSC self-renewal. Overexpression of Wnt ligands, Wnt3a and Wnt1, is observed in GSCs^[67]. Other Wnt pathway components were shown to promote GSC self-renewal and tumorigenicity. Some of pathway's downstream effectors such as β -catenin, Lgr5, Dishevelled 2 and Frizzled 4 are associated with negative prognosis^[66,68-70]. FoxM1, which promotes nuclear localization of β -catenin, was also shown to be critical for GSC maintenance and tumorigenesis^[71].

Differentiation

Bone morphogenic protein (BMP), a member of TGF- β superfamily, functions as a differentiation signal within GBM, as opposed to the previously discussed roles of other members of the TGF- β family in maintenance of self-renewal^[34,72]. The difference between BMP and TGF- β 's effects on GSC biology can be ascribed to distinct signaling cascades, even though they belong to the same superfamily of ligands. Also important for astrocytic differentiation in development, BMP4 treatment inhibits asymmetric division of GSCs, thereby blocking their self-renewal and depleting the stem cell compartment of the tumor^[73,74]. Treatment with BMP4 leads to differentiation and proliferation block. However, a subset of GSCs manages to escape this differentiation cue *via* epigenetic silencing of BMP receptor 1B (BMPR1B)^[74].

Although highly important for self-renewal, reports also suggest that Notch signaling is important for trans-differentiation of GSCs into tumor-derived endothelium^[16]. Similarly, TGF- β was shown to induce GSC differentiation into vascular pericytes, supporting vessel formation and leading to further tumor growth^[17].

MicroRNAs

An additional level of complexity in GSC biology is exhibited by regulatory non-coding RNAs, which are fine tuners of gene expression. Among them, microRNAs (miRNAs) have the ability to modify gene expression levels by specifically binding mostly to the 3'-UTRs of genes and causing their degradation through the RNAi machinery^[75]. Besides being highly important for regulation of pluripotency and reprogramming, miRNAs play important roles in GBM tumorigenesis and GSC biology. Similar to other molecular markers enriched in GSCs, miRNAs regulating neural stem cell biology are also of main interest in GSC biology. miRNAs upregulated

in GBM and particularly in GSCs have anti-apoptotic, anti-differentiation, pro-proliferative and pro-invasion properties^[40,76,77]. On the other hand, miRNAs promoting differentiation were shown to be downregulated in GBM, including miR-124, which is important for neural differentiation^[78-81].

STEM CELL NICHE AND TUMOR MICROENVIRONMENT

To better understand the interplay of different signaling pathways mentioned above and how they regulate GSC biology, we need to study the niches in which GSCs reside. Besides providing crucial signals for GSC maintenance, stem cell niches and the tumor microenvironment are critical factors in the response to therapy.

Vascular niche

Endothelial cells provide signals required for self-renewal of neural stem cells and many other adult stem cell populations^[82]. Similar to their normal counterparts, GSCs reside in a perivascular niche, where they maintain close contact with CD34+ endothelial cells^[83-85]. This close contact facilitates presentation of Notch ligands on the surface of endothelial cells. These ligands activate Notch signaling in GSCs, thereby promoting self-renewal^[85].

The perivascular niche is also subject to bidirectional cues coming from GSCs. CD133+ GSCs express higher levels of vascular endothelial growth factor (VEGF), leading to angiogenesis and increased vascularity of the tumor, when compared to their CD133- counterparts^[86].

New evidence for trans-differentiation of GSCs into endothelial cells and pericytes further suggests that GSCs play a central role in maintaining the tumor microenvironment and their own niches, when presented with appropriate signaling cues^[16,17].

Necrotic niche

As mentioned earlier, GBM is characterized not only by extensive vascular hyperplasia but also pronounced intratumoral necrosis. One of the main histologic hallmarks of GBM is a phenomenon called pseudopalisading necrosis (PPN), where densely packed tumor cells surround a necrotic area^[87]. Although the etiology and biological significance of these areas are not well understood, they are believed to be regions of active tumor growth and neo-vascularization. Considering the importance of hypoxia in promoting self-renewal in embryonic stem cells and NSCs, pseudopalisades represent plausible niches for GSCs^[88,89]. This hypothesis is further supported by studies showing immunoreactivity for CD133 in pseudopalisades^[90]. Furthermore, hypoxia leads to activation of angiogenesis and neo-vascularization through the upregulation of VEGF in GSCs^[91,92]. Some evidence also suggests that hypoxia reprograms CD133- GSCs to become CD133+ and induces Notch signaling, whose importance for GSC biology was mentioned above^[88,89].

Keeping these findings in mind, the possibility of a necrotic niche for GSCs is biologically intriguing and represents a therapeutic challenge for systemic drug delivery methods, since these areas are devoid of blood vessels.

Invasion

The most malignant feature of GBM is its invasion of brain parenchyma. GBM cells infiltrate normal brain tissue and can be found centimeters away from the tumor core^[93]. The vast majority of recurrence after surgery and chemoradiotherapy occurs within 2 cm of the resection cavity suggesting that these invading cells also have tumorigenic capacity^[94-96].

Expression of C-X-C chemokine receptor type 4 (CXCR4) and its ligand, stromal derived factor 1 α (SDF-1 α), which are important regulators of invasion of GBM cells, is enriched in GSCs^[91]. This signaling pathway also mediates recruitment of GSCs towards endothelium, causing further invasion, differentiation and endothelial cell proliferation *via* VEGF expression^[92].

GSCs AS THERAPEUTIC TARGETS

Standard care for GBM is surgical resection, followed by concomitant temozolomide, an alkylating agent, and radiotherapy. GSCs represent important therapeutic targets because they have intrinsic machinery that overcomes current chemoradiotherapeutic approaches (Figure 1C). Some of the molecular mechanisms underlying GSC resistance to chemoradiotherapy are discussed below.

Chemotherapy resistance

GSCs are believed to resist chemotherapy *via* several distinct mechanisms. One such mechanism involves the active transport of chemotherapeutic agents to the extracellular space *via* ABC-type transporters on the cell surface. This mechanism also defines the side population (SP) of GBM cells on flow cytometry, through the exclusion of Hoechst dye^[97]. Enrichment of stem cell markers such as CD133, CD117, CD90, CD71 and CD45 is observed in cells resistant to lethal doses of chemotherapeutic drugs^[98]. Furthermore, CD133 expression is increased in recurrent tumors. Transcriptional analysis of CD133+ GSCs showed that these cells have increased expression of anti-apoptotic genes, suggesting that GSCs have intrinsic mechanisms of chemoresistance^[36].

In line with these observations, more compelling evidence came from Parada and colleagues, who showed that a restricted Nestin+ GSC population was able to regenerate tumors after temozolomide treatment. Selective ablation of this population led to tumor growth arrest, consistent with the notion that GSCs resist conventional chemotherapy and cause relapse^[18].

Another mechanism for chemoresistance lies in the cell cycle profiles of GSCs. Most chemotherapeutic agents target actively cycling cells. However, GSCs are mostly dormant or slow-cycling cells, thereby resisting such therapies^[99].

Radioreistance

In addition to their chemoresistance, GSCs evade radiation, with radiation-resistant clones showing increased expression of GSC markers. More importantly, the Notch and TGF- β signaling pathways, which were mentioned earlier as critical for GSC self-renewal, promote radioreistance as well^[51,100]. GSCs have increased DNA repair capacity. CD133+ GSCs selectively activate Chk1 and Chk2 kinases upon radiation, making them less susceptible to radiation-induced apoptosis^[19].

CONCLUSION

In this review, we have summarized recent advances in understanding the biology of GSCs. We have focused on molecular markers commonly used to identify GSCs and signaling pathways that regulate important GSC characteristics, such as self-renewal, differentiation and therapy resistance. Due to their high tumorigenic potential and resistance to current therapies, GSCs represent critical drug targets. However, the lack of universal markers identifying GSCs, the complexity of signaling cascades regulating GSC biology and the large overlap between tumorigenic pathways active in both GSCs and normal stem cells complicate the development of GSC-targeted therapeutics. A better understanding of GSC biology and their contribution to cellular hierarchy and tumor heterogeneity is crucial for designing effective new therapies against gliomas and other brain malignancies.

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P- Reviewers: de la Serna IL, Kan L S- Editor: Qi Y
L- Editor: A E- Editor: Zhang DN



Familial Alzheimer's disease modelling using induced pluripotent stem cell technology

Lisa Mohamet, Natalie J Miazga, Christopher M Ward

Lisa Mohamet, Natalie J Miazga, Christopher M Ward, Stem Cell Biology Group, Core Technology Facility, Faculty of Human and Medical Sciences, The University of Manchester, Manchester M13 9NT, United Kingdom

Author contributions: Mohamet L, Miazga NJ and Ward CM contributed to research, writing and editing of paper.

Supported by United Kingdom Biotechnology and Biosciences Research Council, Engineering and Physical Sciences Research Council and the Technology Strategy Board

Correspondence to: Dr. Christopher M Ward, Stem Cell Biology Group, Core Technology Facility, Faculty of Human and Medical Sciences, The University of Manchester, Manchester, 46 Grafton Street, Manchester M13 9NT, United Kingdom. christopher.ward@manchester.ac.uk

Telephone: +44-161-2755182 Fax: +44-161-2755182

Received: December 4, 2013 Revised: February 7, 2014

Accepted: February 18, 2014

Published online: April 26, 2014

Abstract

Alzheimer's disease (AD) is a progressive neurodegenerative disease in which patients exhibit gradual loss of memory that impairs their ability to learn or carry out daily tasks. Diagnosis of AD is difficult, particularly in early stages of the disease, and largely consists of cognitive assessments, with only one in four patients being correctly diagnosed. Development of novel therapeutics for the treatment of AD has proved to be a lengthy, costly and relatively unproductive process with attrition rates of > 90%. As a result, there are no cures for AD and few treatment options available for patients. Therefore, there is a pressing need for drug discovery platforms that can accurately and reproducibly mimic the AD phenotype and be amenable to high content screening applications. Here, we discuss the use of induced pluripotent stem cells (iPSCs), which can be derived from adult cells, as a method of recapitulation of AD phenotype *in vitro*. We assess their potential use in high content screening assays and the barriers that exist to realising their full potential in predictive efficacy,

toxicology and disease modelling. At present, a number of limitations need to be addressed before the use of iPSC technology can be fully realised in AD therapeutic applications. However, whilst the use of AD-derived iPSCs in drug discovery remains a fledgling field, it is one with immense potential that is likely to reach fruition within the next few years.

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Key words: Human induced pluripotent stem cells; Alzheimer's disease; Neurodegenerative diseases; High-throughput screening assays; Cholinergic neurons; Drug discovery; Stratified medicine

Core tip: Alzheimer's disease (AD) affects 36 million people worldwide and is set to double by 2030. Progress in understanding AD has been hindered by a lack of suitable *in vitro* and *in vivo* models reflected in > 90% drug attrition rates. Induced pluripotent stem cells are an alternative source of neural cells that can be derived from patients' somatic cells and exhibit AD pathophysiological phenotypes. These cells are amenable to HTS formats required for drug discovery applications. Harnessing this combined potential would provide an unprecedented opportunity to significantly reduce timeframes and costs associated with developing novel therapeutics, ultimately improving patient outcomes.

Mohamet L, Miazga NJ, Ward CM. Familial Alzheimer's disease modelling using induced pluripotent stem cell technology. *World J Stem Cells* 2014; 6(2): 239-247 Available from: URL: <http://www.wjgnet.com/1948-0210/full/v6/i2/239.htm> DOI: <http://dx.doi.org/10.4252/wjsc.v6.i2.239>

INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegen-

erative disease in which patients exhibit gradual loss of memory that impairs their ability to learn or carry out daily tasks. The classic, post-mortem neuropathology exhibited in AD largely consists of amyloid plaques and neurofibrillary tangles^[1], however, there is significant controversy within the field as to the causative mechanism(s). Worldwide nearly 36 million people have AD or related dementia, with a reported 7.7 million new dementia sufferers worldwide per year. The global cost of neurodegenerative diseases was over United States \$600 billion in 2010 and affects people in all countries, with 58% living in low- and middle-income countries^[2]. In the United Kingdom alone, specific neurodegenerative diseases (including AD and Parkinson's disease), have a combined patient population in excess of 800000 and the cost for provision of care was an estimated £23bn in 2012^[3].

Diagnosis of AD is difficult, particularly in early stages of the disease, and largely consists of cognitive assessments, with only one in four patients being correctly diagnosed^[2]. Lack of knowledge of disease pathology is a major disadvantage in diagnosis and prescribing treatments since drug regimens are not the same for all dementias or patients. Moreover, development of a successful drug for the treatment of AD has, as yet, eluded pharmaceutical companies as current medicines treat only symptoms and not the cause(s) of AD. For example, in just over a decade there have been over 100 failed medicines for treatment of AD, including recent late stage failures of solanezumab and bapineuzumab with just five approved medications available to treat the symptoms of various stages of AD (three in United Kingdom). Therefore, a failure in pre-clinical to clinical development exists and can be attributed to several key factors; existing animal models or cellular models are inadequate, insufficient knowledge of drug action on human physiology and a lack of pharmacologically relevant biomarkers. Consequently, there is a pressing need for technologies that can provide definitive assays that can confirm disease pathology as well as predict novel or optimal drug regimens.

Since the creation of induced pluripotent stem cells (iPSCs) from human adult somatic cells in 2007^[4], the potential applications of stem cells in regenerative medicine are considerable. Human pluripotent stem cells (that include iPSCs and embryonic stem cells) are self-renewing, which permits them to be grown indefinitely, and retain the potential to give rise to all cell types of the body. iPSCs are an ideal alternative cell source as they can be derived (reprogrammed) from somatic cells from any individual and are genetically identical to the donor, making them invaluable for use in cell-based models for human disease (Figure 1). Reprogramming of somatic cells is a highly inefficient and lengthy methodology and, as such, certain parameters should be considered when making disease specific iPSCs. These include; source of somatic cells (*e.g.*, dermal fibroblast, blood cells), method

of cellular reprogramming (*e.g.*, retroviral, episomal) and the robustness of differentiation protocols for mature cell types. Here, we focus on AD-specific iPSCs and their derivatives to illustrate how they might be used in various applications in regenerative medicine. For a detailed overview of reprogramming, we refer the reader to another review^[5].

Crucially, previous research demonstrates that iPSC-derived neural cells harvested from individuals suffering from a range of neurodegenerative disorders exhibit similar abnormal disease characteristics *in vitro*^[6-9]. This observation presents an invaluable opportunity for the use of diseased cell lines in *in vitro* studies to further our understanding of disease modelling, early toxicity screening and in the development of novel therapeutics. Performance of a literature search using the NCBI database, PubMed, under specific search terms [disease modeling AND ips cells NOT "review" (Publication Type)] in original research publications reveals that the field of disease modelling using iPSCs has increased at a substantial rate since the creation of iPSCs in 2007 (Figure 2). A year-on-year increase in the number of publications from 2009 ($n = 20$) to 2011 ($n = 114$) is observed, however, in 2012 this trend appeared to slow. In 2013, a reduction in papers is recorded ($n = 52$) which could indicate that the field is maturing, whereby the initial raft of papers reflected high impact method-based publications (*i.e.*, the production of diseased iPSCs), whereas current work is focussed on disease modelling and drug discovery, which are lengthy studies. The number of original research articles containing iPSCs for disease modelling of AD patients was very small and there are only 8 research papers that have utilised AD-derived iPSCs between 2011-2013. This demonstrates that the use of iPSCs to model AD is still in its infancy and may reflect the difficulty of isolation of these cells and identification of appropriate donor patients. This review will discuss the pathology and cellular targets of AD, how we can utilise iPSCs as a model to investigate AD, applications and limitations of these cells in high throughput analyses and future opportunities in personalised medicine.

DISEASE PATHOLOGY

AD can be divided into familial or sporadic genetic events with early- or late-onset. Whilst the majority of AD cases manifest as late-onset sporadic form, familial cases present a unique opportunity to investigate the inheritance of genes contributing a higher risk of AD. The familial form of AD is associated with mutations in amyloid precursor protein (APP), presenilin-1 and presenilin-2. Risk of AD is also observed to be increased where mutations in apolipoprotein E4 (APOE4) or triggering receptor expressed on myeloid cells 2 (TREM2) are present. Genes associated with the pathology of AD include APP, which results in β -amyloid plaques (A β), and microtubule associated protein Tau (MAPT),

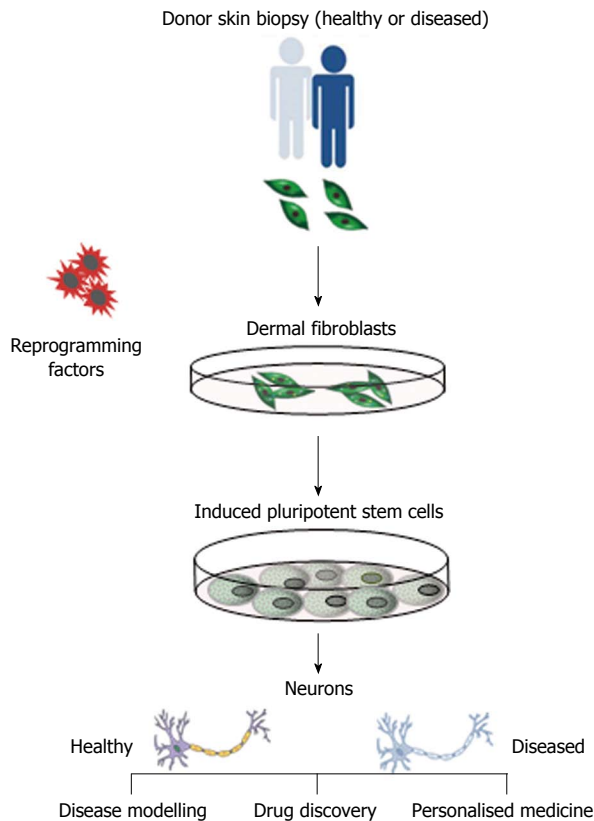


Figure 1 Isolation of disease specific induced pluripotent stem cells. Reprogramming of dermal fibroblasts from patients with Alzheimer's disease into induced pluripotent stem cells provides an infinite source of cells to apply directed differentiation protocols to generate disease-specific neurons that exhibit phenotypic disease traits. This presents a unique opportunity to utilise these cells in the exploitation of drug discovery, disease modelling and personalised medicine.

which results in hyperphosphorylated tau aggregates (tau tangles) within neurons of AD patients^[10]. Despite tau tangles being identified as a pathological feature of AD, mutations in this gene are unusual in such patients. AD is characterized by extracellular amyloid deposition, intracellular neurofibrillary tangle formation, and neuronal loss. Below, we discuss the contribution of these genes to the pathology of AD. Other confounding factors in AD include oxidative stress, mitochondrial function, inflammation and microglia function.

Amyloid precursor protein

A significant pathological feature of AD is the presence of extracellular plaques in the brain comprised of β -amyloid ($A\beta$) peptides derived from the amyloid precursor protein^[11,12]. APP is located on chromosome 21 in humans and is associated with dementia in Down syndrome patients, who exhibit a triplication of this chromosome (trisomy 21). Whilst APP in AD has been studied in significant detail, the events leading to $A\beta$ deposition are less well defined and likely to involve stimulation of APP expression *via* the neuroinflammation-promoting cytokines IL-1 and S100B^[12]. Drugs developed to target $A\beta$ deposits for the treatment of AD

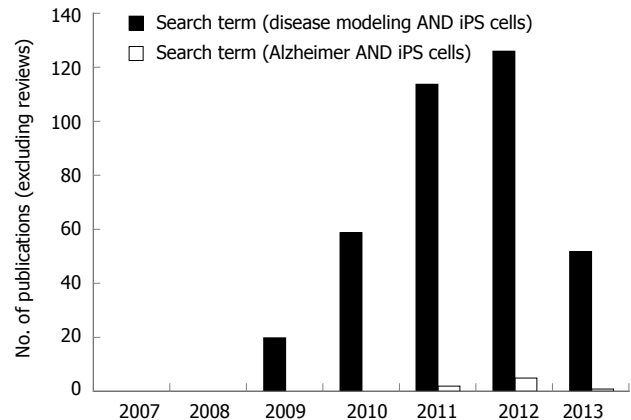


Figure 2 Publication statistics on original research papers using disease specific induced pluripotent stem cells between 2007 and 2013. Analysis of the search terms [disease modeling AND ips cells NOT "review" (Publication Type)] (blue bars) and (Alzheimer AND ips cells) (red bars) for research papers published on NCBI database (PubMed) between 2007 and 2013.

have proved relatively unsuccessful. This may be due to the fact that overexpression of APP is associated with other events, such as glial activation, suggesting that the deposition of $A\beta$ is associated with, rather than being a causal factor of, AD. As such, APP is now generally disparaged as a drug target for AD treatment with hyperphosphorylated tau aggregates now being a major focus.

Microtubule Associated Protein Tau

The Microtubule Associated Protein Tau (MAPT; Tau) functions to assemble and stabilize microtubules within neurons, playing an important part in regulation of neuronal polarity, axonal transport and neurite outgrowth^[10]. Phosphorylation of Tau allows regulation of binding and stability within neurons and aberrant phosphorylation or dephosphorylation in specific residues of the Tau protein lead to pathology, collectively known as tauopathies. The main component of the protein aggregates found in tauopathies is hyperphosphorylated tau protein within neurons. Although the exact mechanisms are unclear, the neurofibrillary tangles (NFT) associated with tauopathies may also involve conformational changes in Tau protein. Whilst tau in NFT forms the basis for pathology of tauopathies it has been suggested that tau oligomers act as a toxic species by providing a template for the misfolding of native tau and spreading from cell to cell leading to propagation of the disease^[13]. Research is now focused on the targeting of Tau oligomers for drug therapies for the treatment of AD.

Apolipoprotein E4

Apolipoprotein E consists of 3 isoforms of which apoE4 is a genetic risk factor for late-onset familial and sporadic forms of AD and is also associated with dementia in DS, Huntingdon's disease, vascular dementia and cerebrovascular disease^[14]. APOE4 exhibits multifunctionality in lipid and lipoprotein transport systems, mainly in the metabolism of dietary lipids^[15]. Carriers

of polymorphic variants of APOE4 are between 4- and 10-times more likely to exhibit late onset AD. In the CNS, APOE4 is produced by glial cells and interacts with receptors of the low-density lipoprotein family. APOE4 binds to A β peptide and onset of AD is likely to reflect the inability of APOE4 to aggregate and clear A β in the brain, although other factors such as the effect of APOE4 on synaptic plasticity, lipid transport, neuroinflammation may also account for this^[16]. Since the APOE4 isoform can be assessed prior to onset of neurodegeneration it is considered a promising target for drug therapy^[17].

Presenilin-1 and -2

Presenilin-1 (PSEN1) and PSEN2 are major components of the atypical aspartyl protease complex that is required for γ -secretase complex activity and cleavage of APP. Mutations in PSEN1 are the major cause of early onset AD and also account for the most severe forms of the disease^[18]. Early onset AD in PSEN1 mutation carriers can occur as early as 30 years of age, although the mean age of onset is over 58 years. More than 180 mutations have been described in PSEN1, of which the majority are missense mutations^[18]. PSEN2 mutations are less common and 14 specific mutations have been associated with AD^[19]. Mutations within the PSEN proteins affect APP synthesis and proteolysis leading to an increase in the ratio of A β 42 peptide compared to A β 40, the former a more toxic form of A β peptide that is more prone to oligomerisation and fibril formation^[19,20]. Drug treatments have focussed on γ -secretase modulators capable of decreasing the ratio of A β 42 to A β 40 peptides^[21].

Triggering Receptor Expressed on Myeloid Cells 2

Variants in Triggering Receptor Expressed on Myeloid Cells 2 (TREM2) have been identified that triple the risk of developing late onset AD^[22]. TREM2 is a cell surface receptor, which triggers activation of the immune response in association with DAP12^[23]. In the CNS, TREM2 is expressed by microglial cells and functions to activate phagocytosis in these cells and to suppress neuroinflammation and cytokine production^[22]. Several functions of TREM2 include aiding clearance of A β and synapse remodelling. Whilst the exact mechanism of TREM2 in late onset AD is unclear it is likely that mutations in this gene contribute to disease pathogenesis *via* insufficient clearance of A β and increased localised inflammation.

AD MODELLING USING HUMAN IPSCS

The single most important factor in the utility of iPSCs in AD modelling, is that mature cell type(s) affected by the disease, *e.g.*, neurons, exhibit phenotypic characteristics of the disease. Numerous studies have demonstrated that iPSCs can be used to model genetic diseases by showing that cells affected by the disease recapitulate

these traits *in vitro*. iPSC AD modeling is still in its infancy and only a few studies have demonstrated successful generation and characterization of AD patient-derived neurons (Figure 2). Five out of eight publications reported isolation of iPSC-derived neurons from patients with familial AD, however a key development in the field showed that reprogramming could similarly be used to recapitulate patient specific phenotypes *in vitro* of sporadic forms of the disease^[6,24,25]. iPSC-derived neurons generated from familial AD patients with mutation of the APP gene and sporadic AD showed, relative to non-demented controls, elevated levels of A β , phosphorylated tau and glycogen synthase kinase 3B^[6].

A known pathology of AD progression is significant neurodegeneration in the cortical regions, with all regions of the brain registering degenerative changes as the disease progresses. Initial reports using iPSC-derived neurons from patients with familial AD utilised heterogeneous neuron populations^[6,8]. Although results demonstrated an increase in A β 42 secretion from mutant PSEN1, PSEN2 and APP iPSC-derived neurons compared to control cells both studies observed inconsistencies in Tau expression. For example, no Tau expression or tangles were observed in the Yagi *et al*^[8] study, whereas increased levels of phosphorylated Tau were observed in both familial AD-derived neurons and one of the two sporadic AD-derived neurons compared to non-demented control neurons in the Israel *et al*^[6] study. In addition, a recent paper reported increased levels of intracellular neuron specific amyloid aggregates in cells derived from familial (APP-E693 Δ) and one of two sporadic AD derived neurons^[24]. These disparities may reflect the disparate differentiation periods used in the studies and differences in the proportion of cholinergic neurons within the populations. However, it is also possible that these differences reflect inherent variability of iPSCs, which is discussed further below.

In a seminal study, iPSCs derived from patients with Down's Syndrome (a model for early onset AD) were used to generate, highly enriched populations of cholinergic neurons in significant numbers. Following differentiation times of 28-100 d following neural induction of iPSCs, analysis of these cells showed production of neuron specific A β secretion, amyloid aggregate formation and altered Tau protein localisation and phosphorylation^[26,27]. Another key finding from this report (and others) demonstrates that early AD pathologies, such as the formation of A β 42 aggregates, occur in relatively short culture periods *in vitro* opposed to years *in vivo*. Furthermore, iPSC-derived neurons are able to respond functionally to various modulators highlighting their potential use in validation and identification in drug discovery^[8,25].

LIMITATIONS OF IPSCS AS MODELS OF DISEASE

At present, a number of limitations need to be ad-

dressed before the full potential of iPSC technology in predictive efficacy, toxicology and disease modelling can be realised. Human iPSCs are effectively man-made cells that are similar to embryonic stem cells, which themselves only exist *in vivo* for a matter of days. These nuances may be reflected in the challenges faced in the differentiation of pluripotent stem cells into mature cell derivatives, despite a good understanding of the molecular mechanisms that occur during development. In order to fully exploit opportunities in disease modelling, but in particular in HTS formats, robust, efficient and cost-effective methods are fundamental. Differentiation protocols that require cocktails of growth factors are costly and are susceptible to significant batch-batch variation, however, alternative methods to acquire differentiated phenotypes are being explored, such as the use of more cost effective small molecules^[28].

A significant research focus in the pluripotent stem cell field has been the development of robust differentiation protocols to enrich for specific mature cell types and populations. However, homogenous cell populations are difficult to obtain in practice and are unlikely to reflect the true pathophysiology of the disease. In addition, modelling complex, idiopathic diseases such as AD, likely requires exposing the cells to biological, chemical or environmental factors to reveal pathophysiological phenotypes. For example, Israel *et al.*^[6] demonstrated a favorably enriched neuron population (90%), however since neurons and synapses are largely dependent upon endocytic activity they found it necessary to co-culture with astrocytes.

In addition, it has been shown by hierarchical cluster analysis that AD-derived neurons are akin to fetal neurons and, therefore, not fully mature^[6]. Although, this is considered one of the major hurdles to overcome in modelling degenerative diseases, the recapitulation of a fetal phenotype presents an opportunity to isolate specific progenitors, which can be used to study developmental aberrations in congenital/developmental disorders. Conversely, for the study of late-stage onset diseases, such as sporadic AD, adult disease phenotypes might not be exhibited under standard differentiation conditions. As such, further work is necessary to identify appropriate differentiation methods for the derivation of adult neurons *in vitro*.

An advantage with the use of patient specific iPSCs means that each iPSC-derived cell reflects this genetic variation. Despite this being a clear advantage in the toxicological evaluation of patient populations to novel therapeutics, conclusions from studies using iPSCs from donors with different genetic backgrounds may be problematic. For example, are any phenotypic differences observed due to the mutation of interest or the genetic background of the patients? At present, parameters such as gender-, age- and ethnicity-matching are used in the selection of control donors, however, genome-wide studies show that each person has single nucleotide polymorphisms that may have disease relevance.

Therefore, a fundamental feature in the use of iPSCs in regenerative applications is careful consideration of appropriate control patients. A further aspect to consider is the reprogramming event required to derive iPSCs from donors. It is well known that epigenetic variations can, and often do, occur during the reprogramming stage of iPSC derivation. Therefore, iPSC clones must be fully characterised prior to use in therapeutic analysis.

HIGH THROUGHPUT SCREENING OF NOVEL THERAPEUTICS FOR AD: *IN VITRO* CLINICAL TRIALS

Development of novel therapeutics for treatment of disease is a lengthy and costly process with extremely high attrition rates of > 90%, in particular, CNS therapeutics exhibit one of the lowest success rates^[29]. Current practices involve evaluation of the safety and efficacy of new drugs in animal and *in vitro* models of relevant tissues and biological processes. Existing *in vitro* cell models attempt to recapitulate core pathologies or targets of AD. For example, Georgievska *et al.*^[30] recently described inhibition of Tau phosphorylation in response to AZD1080, an inhibitor of Glycogen synthase kinase-3 β , using a mouse 3T3 fibroblast cell line transfected with human Tau. Stable over expression of Tau has also been achieved in the human SH-SY5Y neuroblastoma cell line^[31], similarly, over expression of APP695wt in the SH-SY5Y cell line was used to determine A β 40 secretion in response to AZD3839 in pre-clinical studies^[32]. The use of animal cells, however, lacks human context and the cancer-derived SH-SY5Y cell line may not accurately reflect the cellular processes associated with AD. A recent paper highlighted the importance of the endoplasmic reticulum (ER) in protein catalysis and correlated the presence of amyloid- β plaques with age-related diminished ER function. The author went on to call for better drug discovery cell models which enable enhancement of ER function to be detected through embedding fluorescent reporter proteins within an exon of a target gene^[16]. In short, these methods of target validation focus on the recapitulation of only one key feature of AD in an often-irrelevant cell line, failing to account for other components of the signalling pathway. Primary neurons offer more relevant pre-clinical cell models and are capable of synapse formation, but are costly, difficult to transfect and are typically animal derived^[33]. Transgenic animal models and cell lines have undoubtedly aided our knowledge of AD mechanisms and predictive pharmacology, however, these are hindered by inter-species differences and lack of clinical relevance and genetic heterogeneity, which has resulted in poor clinical translation.

The derivation of iPSCs from patients with AD would, however, enable the applicable recapitulation of AD phenotype in a dish, since iPSCs retain the patient's genotype. Circumventing cross species differences and

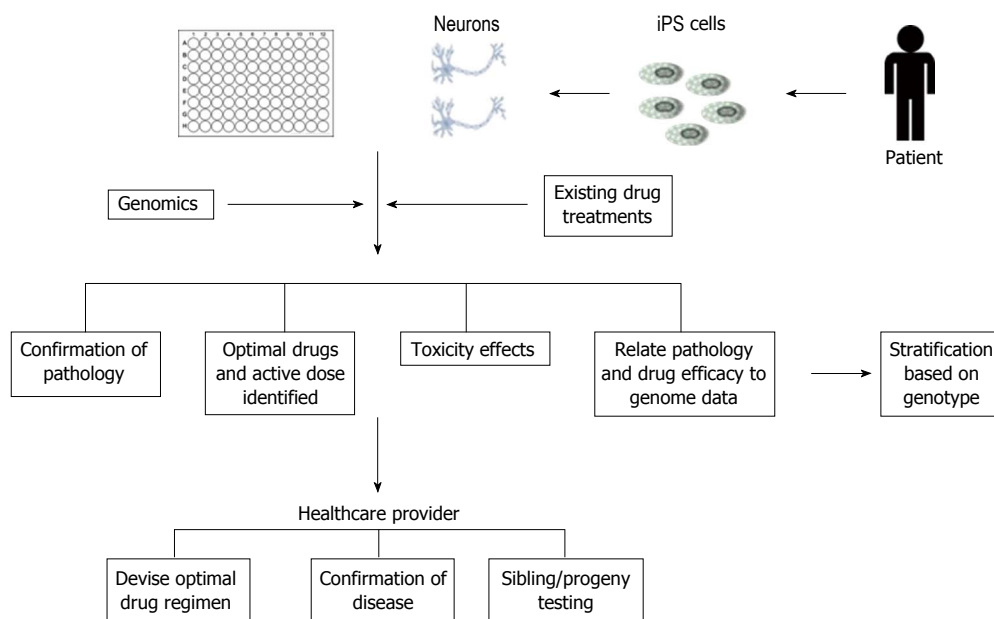


Figure 3 Example of some of the inputs and outputs in Alzheimer's disease high content screening applications. iPS: Induced pluripotent stem.

negating any ethical constraints associated with the use of human embryonic stem cells would create increased translational value. Indeed, neurons derived from disease specific iPSCs have been used to validate the potency of candidate drugs in the treatment of neurological pathologies^[34]. Of further importance, studies have shown treatment of AD iPSC-derived neurons with β -secretase inhibitors, but not γ -secretase inhibitors, causes significant reductions in phosphorylated Tau expression and GSK-3 β levels^[6,8,25]. The accessibility of iPSCs allows many compounds to be tested simultaneously, reflecting a real-life scenario of patients taking a variety of prescription and non-prescription drugs.

Harnessing this potential could provide an unprecedented opportunity to improve preclinical predictions by allowing therapeutics to be tested in multiple cell lines derived from a cohort of patients^[35]. This may also allow the repositioning, reprofiling or repurposing of old, failed and existing drugs. The use of patient-derived iPSCs could be highly amenable to high throughput screening (HTS) practices using multi-well formats to enable rapid analysis of thousands of compounds. Early identification of toxic or inefficacious compounds would, thus, prevent expensive animal studies and subsequent clinical failures. Traditional HTS techniques have focussed on biochemical assays measuring enzyme activity and protein interactions using absorbance, luminescence or fluorescence based readings. For example, Haugabook *et al*^[36] describe the use of a sandwich ELISA (in 96-well formats) to detect aggregation of amyloid plaques, a key contributor to the formation of senile plaques in AD. HTS assays have also been developed to enable detection of A β 42 aggregation using a GFP fusion construct expressed in *E. Coli*, in which compound inhibition of A β 42 aggregation resulted in the emission

of a fluorescent signal^[37,38]. As a result of these methods often lacking cellular context, high content screening (HCS) in whole cells has been recognised as a powerful tool for drug discovery and has been adopted largely by the pharmaceutical industry due to the large volume of multiparametric data that can be obtained^[39]. HCS encompasses the automated acquisition of fluorescent images and image analysis using mathematical algorithms to extract and quantify phenotypic information, including signal shape, intensity and cellular localisation, which can be statistically analysed^[40]. To increase throughput and reduce human error, additional processes such as compound storage, dosing and immunofluorescent staining can also be automated. The principle of HCS in neuronal cultures has already been demonstrated^[41-43]. Neurite loss is one of the core pathologies of AD and application of HCS to quantify neuronal outgrowth has already been achieved and proven to be faster than traditional manual tracing methods^[41,43]. Assessment of chemical toxicity has also been demonstrated by HCS in three neuronal cell lines, whereby proliferation was detected by BrdU incorporation (an indicator of actively proliferating cells) and cell counts were obtained with Hoechst 33342 nuclear dye in a 96-well plate format^[44]. HCS has applications in additional areas of neuroscience including neurogenesis, cell signalling and inclusion formation as reviewed by Dragunow^[45]. An example of HCS applications in AD therapeutics is shown in Figure 3.

Overall, powerful high-throughput and -content screening assays are in place that can be applied to multiple areas of drug discovery, but clinical success is hindered by a lack of relevant cell models in the pre-clinical stages. High throughput toxicity screening using human iPSC-derived cardiomyocytes has been reported

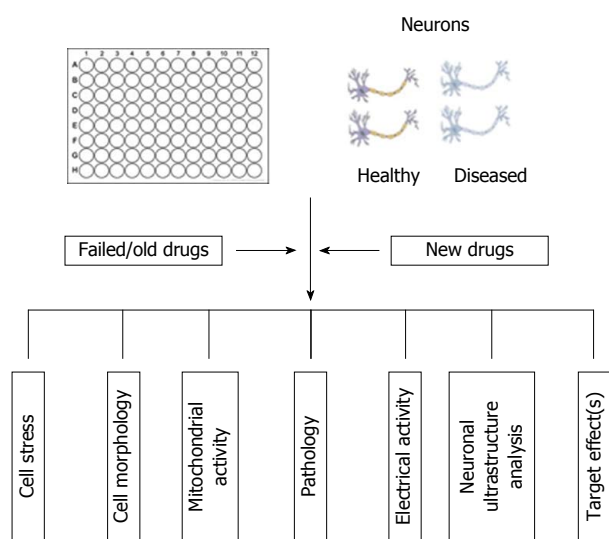


Figure 4 Example of how high content screening of patient-derived induced pluripotent stem cells could aid stratification of existing drug treatments and help identify genetic profiles associated with specific disease phenotypes.

using electrode sensors to acquire oscillating impedance measurements to detect the contraction and relaxation or beating of iPSC-derived cardiomyocytes in a 96-well plate format^[46]. Arrhythmia data obtained from iPSC-derived cardiomyocytes treated with cardiac modulators was qualitatively comparable to results obtained from more traditional, low throughput microelectrode arrays in parallel experiments. Therefore, the potential use of iPSC technology in high throughput drug discovery has been demonstrated but to date has not been described in the literature for iPSC-derived neurons. The UK Government and pharmaceutical industry have recognised the potential for iPSC AD models in HCS and by late 2013 several calls for funding such technology have been announced. As a result, we expect to see significant activity in this field and the development of HCS platforms for AD.

FUTURE PERSPECTIVES: PERSONALISED MEDICINE

The potential to use patient-specific cells to generate pluripotent cells, which can be maintained indefinitely and subsequently differentiated into desired cell types, presents a real opportunity for stratified (personalised) medicine applications (Figure 4). For example, this will allow scientists and clinicians to model, *in vitro*, the progression of AD (or other degenerative diseases) for each individual patient, perform “customised” pharmacologic screening to determine the optimal therapeutic regimes and implement genomic testing of large cohorts of patients, representing different ethnic/genetic backgrounds in order to inform pharma of susceptible populations. There is a clear unmet drug need for the treatment of AD and the utility of iPSC technology will provide a

more efficacious model to reassess (or rescue) former drug candidates that either have been withdrawn from use or aborted at a late stage of development for safety reasons. In short, the use of disease specific iPSC derived neural cells, in conjunction with high throughput/content screening methods, offer improved clinically relevant cell models that will significantly reduce time-frames and costs associated with the development of novel therapeutics, ultimately improving the number of new medicines to the market to treat patients with neurodegenerative diseases.

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P- Reviewers: Freter R, Perron M **S- Editor:** Ma YJ **L- Editor:** A
E- Editor: Zhang DN



Embryonic stem cell-derived neural progenitors as non-tumorigenic source for dopaminergic neurons

Mei-Chih Liao, Mihaela Diaconu, Sebastian Monecke, Patrick Collombat, Charles Timaeus, Tanja Kuhlmann, Walter Paulus, Claudia Trenkwaller, Ralf Dressel, Ahmed Mansouri

Mei-Chih Liao, Mihaela Diaconu, Ahmed Mansouri, Department of Molecular Cell Biology, Max-Planck-Institute for Biophysical Chemistry, 37077 Göttingen, Germany
Sebastian Monecke, Ralf Dressel, Department of Cellular and Molecular Immunology, University Medical Center Göttingen, 37073 Göttingen, Germany

Patrick Collombat, Université Nice Sophia Antipolis, UMR INSERM 1091/CNRS 7277/UNS, Diabetes Genetics Team, Institut de Biologie Valrose, 06100 Nice, France

Charles Timaeus, Walter Paulus, Ahmed Mansouri, Department of Clinical Neurophysiology, University Medical Center Göttingen, 37075 Göttingen, Germany

Tanja Kuhlmann, Institute of Neuropathology, University of Münster, 48149 Münster, Germany

Claudia Trenkwaller, Paracelsus-Elena-Klinik, 34128 Kassel, Germany

Patrick Collombat, Ahmed Mansouri, Genome and Stem Cell Center, GENKOK, Erciyes University, 38039 Kayseri, Turkey

Author contributions: Liao MC, Diaconu M, Monecke S, Collombat P, Timaeus C, Kuhlmann T, Dressel R and Mansouri A performed the experiments; Paulus W and Trenkwaller C contributed to the conception of the study; Dressel R and Mansouri A designed the study, wrote the manuscript and share senior authorship; all the authors approved the final version of the manuscript. Supported by The German Federal Ministry for Education and Research (BMBF), No. 01GN0818 and No. 01GN0819; the Max-Planck Society, and initially by the Dr. Helmut Storz Stiftung
Correspondence to: Ahmed Mansouri, PhD, Professor, Department of Molecular Cell Biology, Max-Planck-Institute for Biophysical Chemistry, Am Fassberg 11, 37077 Göttingen, Germany. amansou@gwdg.de

Telephone: +49-551-2011490 Fax: +49-551-2011504

Received: September 17, 2013 Revised: December 14, 2013

Accepted: January 13, 2014

Published online: April 26, 2014

METHODS: The human embryonic stem (hES) cell line H9 was used to generate human neural progenitor (HNP) cell lines. The resulting HNP cell lines were differentiated into dopaminergic neurons and analyzed by quantitative real-time polymerase chain reaction and immunofluorescence for the expression of neuronal differentiation markers, including beta-III tubulin (TUJ1) and tyrosine hydroxylase (TH). To assess the risk of teratoma or other tumor formation, HNP cell lines and mouse neuronal progenitor (MNP) cell lines were injected subcutaneously into immunodeficient SCID/beige mice.

RESULTS: We developed a fairly simple and fast protocol to obtain HNP cell lines from hES cells. These cell lines, which can be stored in liquid nitrogen for several years, have the potential to differentiate *in vitro* into dopaminergic neurons. Following day 30 of differentiation culture, the majority of the cells analyzed expressed the neuronal marker TUJ1 and a high proportion of these cells were positive for TH, indicating differentiation into dopaminergic neurons. In contrast to H9 ES cells, the HNP cell lines did not form tumors in immunodeficient SCID/beige mice within 6 mo after subcutaneous injection. Similarly, no tumors developed after injection of MNP cells. Notably, mouse ES cells or neuronal cells directly differentiated from mouse ES cells formed teratomas in more than 90% of the recipients.

CONCLUSION: Our findings indicate that neural progenitor cell lines can differentiate into dopaminergic neurons and bear no risk of generating teratomas or other tumors in immunodeficient mice.

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Key words: Human embryonic stem cells; Neural progenitor cells; Teratoma; Pluripotency; Dopaminergic

Abstract

AIM: To find a safe source for dopaminergic neurons, we generated neural progenitor cell lines from human embryonic stem cells.

neurons

Core tip: The use of pluripotent cells as a source for the generation of neuronal tissue for transplantation suffers from the risk of teratoma formation. To circumvent this problem, we have developed a simple and fast protocol to obtain human neural progenitor (HNP) cell lines from embryonic stem cells. These HNP cell lines have the potential to differentiate *in vitro* into dopaminergic neurons. After injection into immunodeficient SCID/beige mice, they did not form tumors even after 6 mo. These findings indicate that HNP cell lines can differentiate into dopaminergic neurons and bear no risk of generating teratomas in immunodeficient mice.

Liao MC, Diaconu M, Monecke S, Collombat P, Timaeus C, Kuhlmann T, Paulus W, Trenkwalder C, Dressel R, Mansouri A. Embryonic stem cell-derived neural progenitors as non-tumorigenic source for dopaminergic neurons. *World J Stem Cells* 2014; 6(2): 248-255 Available from: URL: <http://www.wjgnet.com/1948-0210/full/v6/i2/248.htm> DOI: <http://dx.doi.org/10.4252/wjsc.v6.i2.248>

INTRODUCTION

The derivation of human embryonic stem (hES) cells from human embryos^[1] has opened new perspectives for stem cell-based therapies of neurodegenerative disorders, such as Parkinson's disease, and for the development of new drug screening platforms. These scenarios have been stimulated by the recently established procedures to generate induced pluripotent stem (iPS) cells from human fibroblasts or other tissues^[2,3]. In fact, iPS cells may help to circumvent major ethical problems related to human embryonic stem cells. Similar to hES cells, iPS cells are pluripotent and therefore capable of differentiation into tissues of all three germinal layers *in vitro*^[2] and also *in vivo* as they can give rise to teratomas when injected into immunodeficient mice^[2].

In order to assess the potential of hES cells as a source for the derivation of tissues for cell replacement, several protocols have been established to generate various cell types from human embryonic stem cells, including subtypes of neuronal cells. However, it remains a matter of concern whether transplantation of hES cell-derived progenitors or even more differentiated cell types may lead to the formation of teratomas, a characteristic feature of pluripotent cells. It is assumed that most of these tumors observed following experimental transplantation of such *in vitro* differentiated cells are caused by a minor population or even single still pluripotent cells contaminating the grafts^[4,5]. Therefore we established a simple and fast protocol to derive human neural progenitors (HNP) from hES cells. These neural progenitors can be maintained in culture for several weeks and can be stored for at least five years in liquid nitrogen without losing their capacity to differentiate into midbrain dopaminergic neurons.

minergic neurons.

To examine whether hES cell-derived neural progenitor cells still have the risk to form teratomas, cells were injected subcutaneously into immunodeficient mice. Remarkably, no tumors were detected even six months after injection of up to 2×10^6 HNP cells.

MATERIALS AND METHODS

Cell culture

The Robert-Koch Institute in Berlin has approved working with hES cell lines H1 and H9 imported from WiCell (Madison, Wisconsin, United States) in compliance with German law (AZ. 1710-79-1-4-5). Human ES cells H9 were cultured as described previously^[1]. Briefly, cells were plated on mitomycin C-inactivated mouse fibroblasts (1.9×10^4 cells/cm²) in KnockOut medium (Life Technologies, Darmstadt, Germany) containing 20% KnockOut serum replacement (KSR) (Life Technologies), 2 mmol/L glutamine, 1 mmol/L non-essential amino acids (NEAA) (Life Technologies), 0.1 mmol/L beta-mercaptoethanol, 5 ng/mL basic fibroblast growth factor (bFGF) (Pepro Tech, Hamburg, Germany) and penicillin/streptomycin (P/S) (Life Technologies). Cells grown to 70% confluence were dissociated using accutase (PAA Laboratories, Cölbe, Germany) in the presence of Rock Inhibitor Y27632 (Sigma-Aldrich, Taufkirchen, Germany), and split 1 to 3 or 1 to 5. The neural induction medium consisted of KnockOut medium containing 15% KSR (Gibco, Life Technologies), 2 mmol/L glutamine, 200 ng/mL noggin (R and D Systems, Wiesbaden, Germany) or 2 μ mol/L dorsomorphin (Sigma-Aldrich), 1 mmol/L NEAA, 0.1 mmol/L beta-mercaptoethanol, and P/S. The HNP medium consisted of Neurobasal medium (Life Technologies) containing N2 and B27 supplements (Life Technologies), 20 ng/mL bFGF, 20 ng/mL epidermal growth factor (EGF) (Pepro Tech GmbH), 0.2 mmol/L ascorbic acid, and 2000 U/mL human leukemia inhibitory factor (LIF) (Merck Millipore, Darmstadt, Germany).

Dopaminergic neuron differentiation

HNP cells [$(5-7.5) \times 10^5$] were seeded on matrigel coated 3.5 cm culture dishes. The next day the cells were fed with neural differentiation medium (Neurobasal medium, 1 mmol/L NEAA, 1 \times P/S, 2 mmol/L glutamine, N2 and B27 supplements minus Vitamin A, 0.2 mmol/L ascorbic acid, 100 ng/mL fibroblast growth factor 8 (FGF8) (R and D Systems), 100 ng/mL Sonic hedgehog (SHH) (R and D Systems) or 1-2 μ mol/L purmorphamine (Cayman Chemical, Biomol, Hamburg, Germany). Medium was changed every other day. After two weeks, the cells were fed with neural differentiation medium containing 20 ng/mL glial cell-derived neurotrophic factor (GDNF) (Pepro Tech), 20 ng/mL brain-derived neurotrophic factor (BDNF) (Pepro Tech), 1 ng/mL transforming growth factor (TGF)- β 3 (R and D Systems), and 0.5 mmol/L dibutyryl-cAMP (dbcAMP) (Sigma Aldrich) without FGF8, SHH or purmorphamine.

to induce neuron maturation. Cells were analyzed after day 30 of the differentiation procedure. The HNP freezing medium consisted of the HNP medium with 10% dimethyl sulfoxide (DMSO). Medium for culture of PA6 cells was Dulbecco's Modified Eagle's Medium (DMEM) (Life Technologies) containing 10% fetal calf serum (FCS) (Life Technologies).

Immunofluorescence

For immunofluorescence staining, cells grown on glass coverslips (BD Bioscience, Heidelberg, Germany) were washed with phosphate-buffered saline (PBS), fixed for 10 min in 4% paraformaldehyde (PFA). Primary antibodies were used to detect nestin (MAB1259, 1:750; R and D Systems), musashi (ab21628, 1:200; Abcam, Cambridge, UK), CD133 (ab 19898, 1:200; Abcam), beta-III tubulin (TUJ1) (MMS-435P, 1:1000; Covance, Princeton, NJ, United States), tyrosine hydroxylase (TH) (AB152, 1:300; Millipore), and paired box protein 6 (PAX6) (PRB278P, 1:300; Covance). As secondary antibodies, we used Alexa 488-labeled donkey anti-rabbit IgG (A-21206, 1:750; Life Technologies), Alexa 488-labeled goat anti-rabbit IgG (A-11008, 1:750; Life Technologies), Alexa 488-labeled goat anti-mouse IgG (A-11001, 1:750; Life Technologies), Alexa 594-labeled donkey anti-rabbit IgG (A-21207, 1:750; Life Technologies), and Alexa 594-labeled goat anti-rabbit IgG (A-11012, 1:750; Life Technologies).

Quantitative real-time polymerase chain reaction

Neural progenitors and differentiated neurons were collected and total RNA extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA (1 µg) was used for cDNA synthesis with the QuantiTect Rev Transcription Kit (Qiagen) in 20 µL reaction volume. 20 µL cDNA were diluted in 60 µL RNase-free water and subsequently 2 µL were used for quantitative real-time polymerase chain reaction (q-RT-PCR) amplification. Each q-RT-PCR reaction was run in a 10 µL reaction volume containing 1 µL of the QuantiTect Primers (Qiagen) and 5 µL 2 × qPCR Master Mix (Kapa Biosystem, Woburn, MA, United States). The following QuantiTect Primers were used: glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) (QT01192646), LIM homeobox transcription factor 1 (*LMX1A*) (QT00048055), pituitary homeobox 3 (*PITX3*) (QT01006047), nuclear receptor related 1 protein (*NURR1*) (QT00037716), neurogenin-2 (*NGN2*) (QT00020447), paired box protein 6 (*PAX6*) (QT00071169), glial fibrillary acidic protein (*GFAP*) (QT00081151), tyrosine hydroxylase (*TH*) (QT00067221), and dopamine transporter (*DAT*) (QT00000231). All q-RT-PCRs were performed with a Mastercycler ep realplex (Eppendorf, Hamburg, Germany).

Animal experiments

Animal experiments were approved by the local government. Rats (LOU/c) were conventionally housed in the central animal facility of the University Medical Center Göttingen. Severe combined immunodeficient SCID/

beige (C.B-17/1crHsd-scld-bg) mice were kept under pathogen-free conditions as they lack T and B-lymphocytes and have no functional natural killer (NK) cells. A subgroup of rats received daily intraperitoneal injections of cyclosporine A (CsA) (10 mg/kg, Sandimmune, Novartis Pharma, Nürnberg, Germany) commencing two days before grafting. For the analysis of subcutaneous tumor growth, the cells were injected in 100 µL PBS into the flank of the animals. Tumor growth was monitored regularly by palpation. Animals were sacrificed 3 or 6 months after injection and autopsies were performed. Tumor tissue or subcutaneous tissue at the site of injection was immediately frozen in liquid nitrogen, later placed in phosphate-buffered 4% formalin for 16 h and then embedded in paraffin. Tissue sections (2.5 µm) were stained with hematoxylin and eosin (HE) for histological examination.

Statistical analysis

Teratoma frequencies were analyzed with contingency tables using WinSTAT software (R. Fitch Software, Bad Krozingen, Germany).

RESULTS

Derivation of neural progenitor cells from human ES cells

Using a monolayer of the stromal cell line PA6, mouse and human ES cells can be differentiated into neuronal cells with a high proportion of neurons displaying mesencephalic dopaminergic fate^[6]. We have established a procedure to culture mouse ES cells on PA6 cells to generate mouse neuronal progenitors (MNP) that can be frozen or maintained in culture for several passages^[7,8]. Human ES cells were first cultured for two passages in hES medium^[1] before subjection to neural differentiation. Cells were passaged using accutase in the presence of the Rock Inhibitor Y27632^[9]. Then, 9×10^4 hES cells were plated on a feeder layer of mitomycin C-inactivated PA6 cells on a 3.5 cm dish and cultured for 36 h in hES medium^[1]. Afterwards, the medium was replaced by neural induction medium containing noggin (200 ng/L) or dorsomorphin (2 µmol/L)^[10]. Half of the medium was replaced every other day. The onset of neuronal differentiation was monitored by the appearance of neural rosettes, the first of which was usually recognized at day 11 after plating hES cells on PA6 stromal cells. Neural rosettes were individually picked under the stereomicroscope. Cell aggregates were transferred to gelatinized 24-well-plates and cultured in HNP medium consisting of Neurobasal medium containing N2 and B27 supplements, ascorbic acid, NEAA, 10 ng/mL bFGF, 10 ng/mL EGF, and 2000 U/mL LIF. After 4–5 d, cells from each well were treated with accutase and passaged on two gelatinized wells of a 24-well-plate. When cells reached 60%–70% confluence, they were passaged to one gelatinized 3.5 cm plate. Only those cells were further processed that continuously formed neural rosettes. When confluent (60%–70%), cells

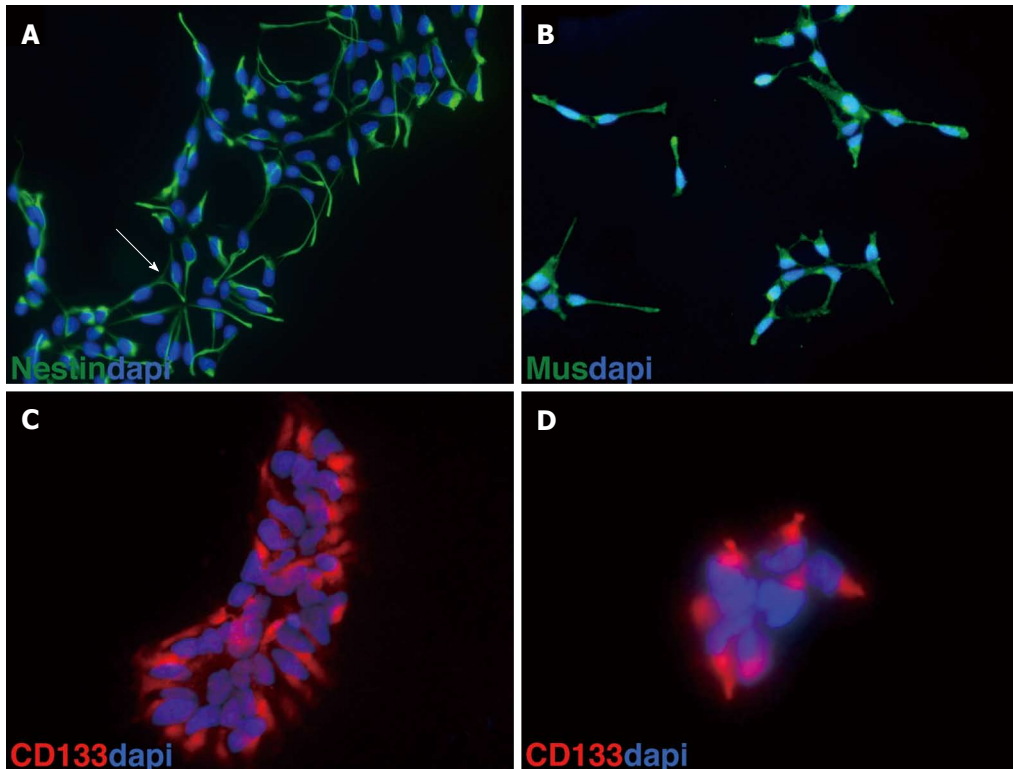


Figure 1 Immunofluorescence staining of human neural progenitor cells for stem cell markers. A: Human neural progenitor cells express the stem cell marker nestin (green, dapi: blue); B: Musachi (green), dapi (blue); C, D: CD133 (red), dapi (blue).

were further treated with accutase and stored in 1 mL of HNP freezing medium containing 10% DMSO in liquid nitrogen. Cells can be stored for years in liquid nitrogen. So far our cells have been in storage for 5 years. When thawed, frozen cells were brought to 37 °C in a water bath, transferred into 9 mL of HNP medium, and centrifuged for 5 min at 200 *g*. The cell pellet was resuspended in 2 mL of HNP medium and plated on a gelatin-coated 3.5 cm dish. Cells were usually split 1 to 3 every 4–5 d. So far, HNP cells were kept in culture for about two months (16 passages) without losing their capacity to differentiate into dopaminergic neurons. However, the percentage of dopaminergic neurons varied between different HNP clones. Each HNP clone originated from one neural rosette. HNP cells usually consist of a homogenous population expressing the stem cell markers nestin, musachi and CD133 (prominin 1) (Figure 1).

Generation of dopaminergic neurons from HNP cells

In order to generate dopaminergic neurons, HNP cells were dissociated into single cells and plated at a density of $(5-7.5) \times 10^5$ cells onto matrigel-coated 3.5 cm culture dishes or glass coverslips in HNS medium. At the second day after plating, the HNP medium was supplemented by FGF8 and SHH (or purmorphamine). After 2 wk, neuron maturation was induced by replacing FGF8 and SHH by GDNF, BDNF, TGF- β 3 and dbcAMP. Cells were analyzed after day 30 of the differentiation procedure.

The majority of the differentiated cells obtained from HNP4 cells at passage 16 expressed the neuronal marker

TUJ1 and a high proportion of these were positive for TH, indicating the development of dopaminergic neurons as shown in Figure 2. In addition, PAX6, a marker of midbrain tegmentum, was detected. Moreover, we performed q-RT-PCR to analyze the expression of several neuronal markers that were previously described to label midbrain dopaminergic neurons, such as *NGN2*, *PITX3*, *TH* and *DAT*^[11–13] (Figure 3A).

It has been reported that the number of dopaminergic neurons may decrease when progenitor cells are maintained for longer periods in culture. We therefore monitored midbrain dopaminergic markers in cultures obtained at different passages of HNP4 cells by q-RT-PCR. Neuronal marker genes, such as *NGN2*, *PITX3*, *PAX5*, *TUJ1* and *DAT*, were expressed at a similar level in cultures obtained from HNP4 cells at passages 6, 9 and 16 (Figure 3B). The expression of TUJ1 and TH proteins in neuronal cells differentiated from HNP4 cells at passage 16 were detected by confocal microscopy (Figure 4). Thus, HNP cells retain their capacity to differentiate into dopaminergic neurons even after a higher number of passages.

MNP and HNP cells do not induce teratomas in immunodeficient mice

To determine the risk of tumor growth after transplantation of mouse neural progenitor cells, we injected 1×10^6 MNP cells subcutaneously into B, T and NK cell deficient SCID/beige mice ($n = 9$). No tumors developed after injection of MNP cells (at passage 20) derived from

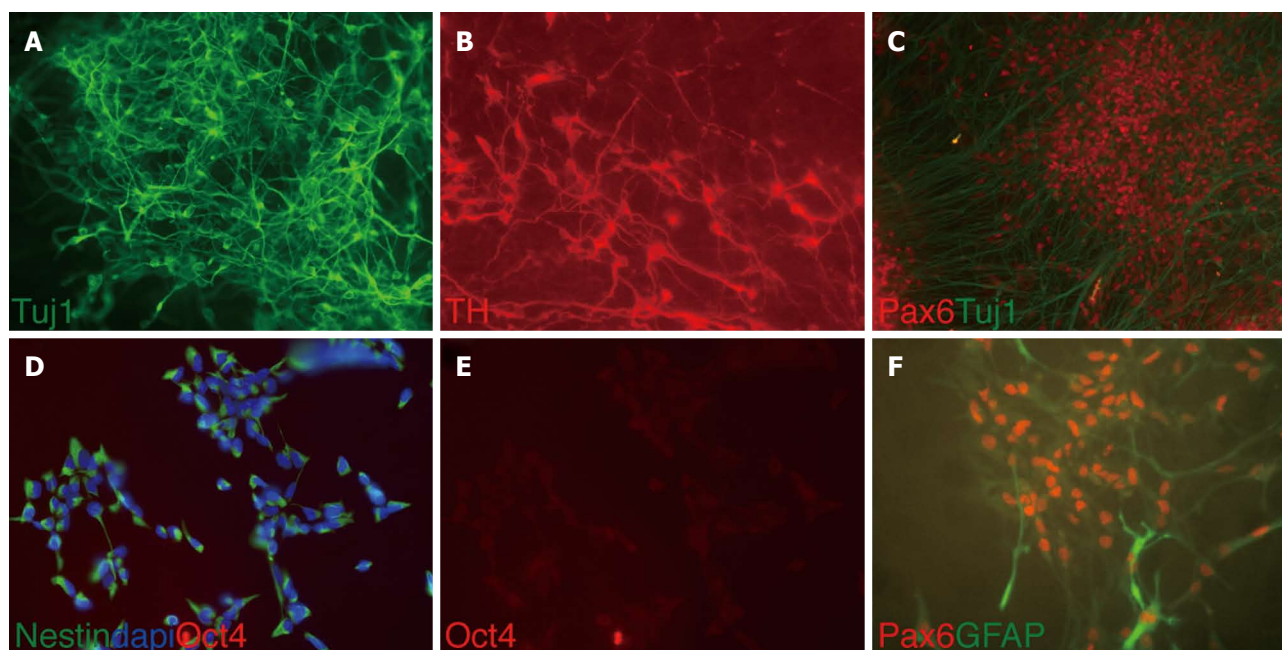


Figure 2 Immunofluorescence staining of neuronal cells differentiated from human neural progenitor cells. A: Neurons differentiated *in vitro* from embryonic stem cell-derived human neural progenitor (HNP4) cells express beta-III tubulin (TUJ1); B: Tyrosine hydroxylase (TH); C: PAX6; D: Neural progenitor marker Nestin; E: HNP4 cells do not express the pluripotency marker octamer binding transcription factor 4 (OCT4); F: Glial fibrillary acidic protein (GFAP).

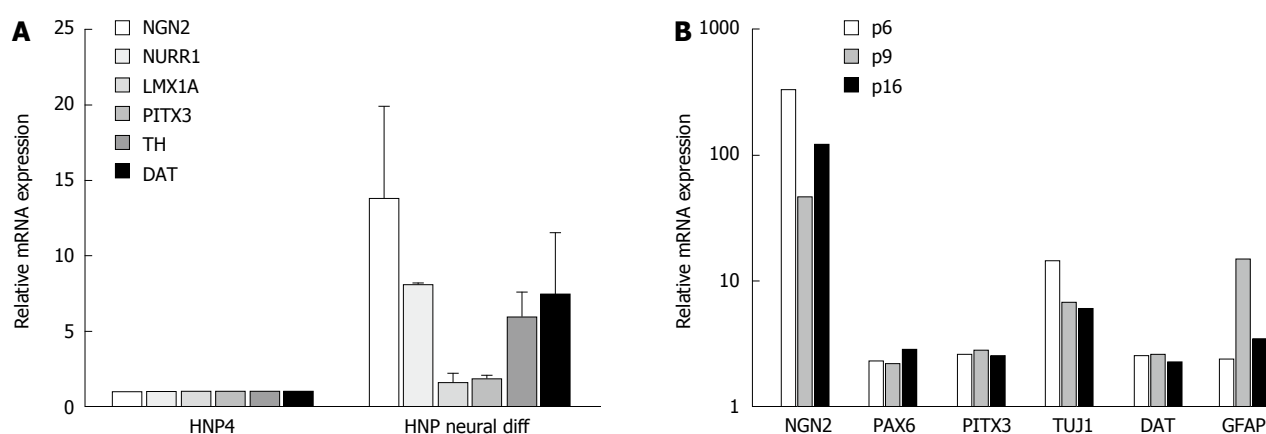


Figure 3 Characterization of dopaminergic neurogenesis in human embryonic stem (H9) derived neural progenitor cells (HNP4) by expression analysis of marker genes. A: The expression of several specific marker genes (*NGN2*, *NURR1*, *LMX1A*, *PITX3*, *TH* and *DAT*) is displayed as determined by q-RTPCR analysis. The relative gene expression of differentiated neuronal cells (HNP neural diff) at passage 16 was normalized to the house-keeping gene *GAPDH* and the neural progenitors (HNP4). Three independent experiments were performed and the means + SE are indicated; B: The expression of several specific marker genes (*NGN2*, *PAX6*, *PITX3*, *TUJ1*, *DAT* and *GFAP*) was analyzed 30 d after starting neuronal differentiation of HNP4 cells at passages (p) 6, 9 and 16 by q-RTPCR analysis. The relative gene expression of differentiated neuronal cells was normalized to the house-keeping gene *GAPDH* and the neural progenitors (HNP4).

mES cells (MPI-II) in the following 3 mo (Table 1). Importantly, injection of 1×10^6 mES cells or neuronal cells differentiated from mES cells resulted in teratoma growth in more than 90% of the recipients^[4]. Thus, the teratoma frequencies were significantly different after injection of these cell types ($P = 1.47 \times 10^{-7}$). Our previous studies revealed that the risk of teratoma growth could be higher after injection of differentiated cells as compared to undifferentiated mES cells when CsA-treated rats were used as recipients^[4]. Therefore, we also injected the MNP cells into rats receiving CsA (10 mg/kg per day) for immunosuppression. Again, no tumors were observed after three

months (Table 1), indicating a significantly reduced risk for tumor formation after injection of MNP cells compared to mES cells and neuronal cells differentiated from mES cells ($P = 1.9 \times 10^{-6}$). Similarly, two human HNP cell lines (HNP1 and HNP4) at passages between 10 and 21 did not form tumors in immunodeficient SCID/beige mice even within 6 mo after subcutaneous injection in contrast to hES cells H9 (Table 2). The teratoma frequencies were significantly different comparing mice injected with HNP1 or HNP3 and hES H9 cells ($P = 0.00013$). We did not find leftovers of the injected HNP cells, such as neural rosettes, in the subcutaneous tissue at the site

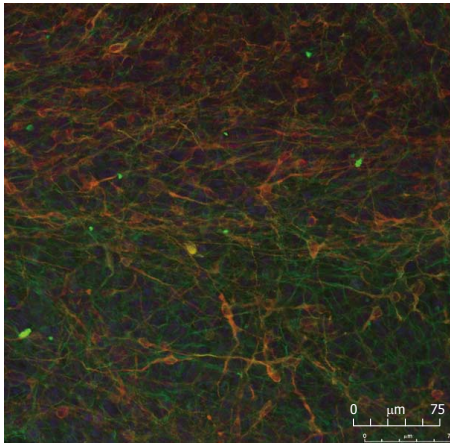


Figure 4 Generation of dopaminergic neurons from human neural progenitor 4 cells. Neurons differentiated *in vitro* from human neural progenitor 4 (HNP4) cells at passage 16 express beta-III tubulin (TUJ1) (green) and tyrosine hydroxylase (TH) (red) as shown by immunofluorescence staining.

of injection (data not shown), suggesting that the HNP cells did not survive.

DISCUSSION

The formation of tumors, either teratomas and teratocarcinomas or tumors with a more restricted tissue composition, after transplantation of cells or tissues derived from pluripotent stem cells remains a major problem for the therapeutic application of these cells in regenerative medicine^[14]. The assessment of the tumorigenicity of stem cell-derived grafts is complicated by the fact that it depends heavily on the immune response of the recipient^[15,15]. Grafts that do not form tumors in xenogeneic or allogeneic hosts due to immune rejection might form tumors in syngeneic recipients^[4]. The finding that cellular grafts derived from ES cells do not form tumors in xenogeneic recipients^[16] must not indicate that the graft is safe in an allogeneic or even syngeneic recipient^[4]. Moreover, in a previous study, we found that grafts obtained after neuronal differentiation of mES cells (MPI-II) for 14 d formed teratomas in CsA-treated rats (Table 1). Surprisingly, undifferentiated mES cells did not form tumors in these hosts^[4]. Mouse ES cells turned out to be highly susceptible to NK cells and were rejected by NK cells^[4,17]. Thus, differentiation cultures of mES cells apparently can give rise to cells that have, in certain hosts, an even stronger tumorigenic capacity than undifferentiated pluripotent stem cells.

It is a major challenge to produce cellular grafts directly from pluripotent stem cells and to avoid a contamination with tumorigenic cells. Strategies to remove tumorigenic cells from grafts include prolonged differentiation^[18], cell sorting or selection^[19-21], introduction of suicide genes^[22,23], and killing of remaining undifferentiated cells before transplantation^[24-26]. However, all grafts that are derived from pluripotent stem cells are in principle at risk of containing tumor-forming cells^[14]. As few as 2 mouse ES cells^[27] or 245 human ES cells^[28] were

Table 1 Tumor formation after subcutaneous injection of mouse neuronal progenitor cells in comparison to mouse embryonic stem cells and neuronal cells differentiated *in vitro* from mES cells

| | SCID/beige | LOU/c + CsA |
|--|-------------|-------------|
| MNP | 0% (0/9) | 0% (0/9) |
| mES cells | 93% (13/14) | 0% (0/25) |
| Neuronal cells differentiated from mES cells | 94% (17/18) | 61% (11/18) |

1×10^6 mouse neuronal progenitor (MNP), mouse embryonic stem (mES) cells (MPI-II)^[4] or neuronal cells differentiated *in vitro* for 14 d from the mES cells (> 95% beta-III tubulin -positive)^[4] were injected subcutaneously into the flank of SCID/beige mice or LOU/c rats treated with CsA (10 mg/kg per day). The percentage and number of animals is indicated in which tumors were found during autopsy at the site of injection 3 mo following injection.

Table 2 Tumor formation after subcutaneous injection of human neural progenitor cells in comparison to human embryonic stem cells

| | SCID/beige |
|---------------------------|------------|
| HNP1 p9, 1×10^6 | 0% (0/6) |
| HNP1 p19, 2×10^6 | 0% (0/3) |
| HNP4 p10, 1×10^6 | 0% (0/3) |
| HNP4 p21, 2×10^6 | 0% (0/9) |
| hES cells (H9) | 75% (3/4) |

1 or 2×10^6 human neural progenitor cells (HNP1 or HNP4) at passages 9 or 10 and 19 or 21, respectively, and 1×10^6 human embryonic stem (hES) cells (H9) were injected subcutaneously into the flank of SCID/beige mice. The percentage and number of animals is indicated in which tumors were found during autopsy at the site of injection 6 mo (HNP1 and HNP4) or 3 mo (hES cells H9) following injection.

reported to form teratomas in immunodeficient mice.

Therefore, it might be a more promising alternative to differentiate therapeutic grafts from pre-differentiated progenitor cell lines, which are not able to form tumors even in immunodeficient hosts. We have shown that mouse and human neural progenitor cells do fulfill this prerequisite. Both did not form tumors after injection in SCID/beige mice, which are deficient for T and B cells and that do not have functional NK cells. The mice were observed for 3 mo after injection of MNP cells and even 6 mo after injection of HNP cells before autopsy. MNP cells^[8] were compared with mES cells and neuronal cells directly differentiated from these mES cells (Table 1). Only MNP cells were safe and failed to form teratomas. Moreover, MNP cells also did not form tumors in CsA-treated rats, in which neuronal cells directly differentiated from mES cells formed teratomas in 61% of the animals^[4]. In this study, subcutaneous injections were performed to assess the tumor risk after injection of neuronal progenitor cell lines. The subcutaneous tissue usually does not promote the survival of neuronal cells over several months and we indeed did not detect leftovers of the HNP cells at the site of injection. Importantly, the results indicate that MNP and HNP cell lines do not have the capacity to form tumors. Thus, these cells are

apparently a much safer cell type for differentiation of neuronal grafts than ES cells, even without any selection strategy to remove the progenitor cells from differentiation cultures. The HNP cell lines were tested at passages between 9 and 21 for their capability to differentiate into dopaminergic neurons *in vitro* and to form tumors *in vivo*. The HNP cell lines differentiated with similar efficacy and did not form tumors at earlier and later passages. These data now encourage the testing of cell survival and therapeutic efficacy of dopaminergic neurons differentiated from these neuronal progenitor cell lines after intrastriatal transplantation in models of Parkinson's disease. The potential therapeutic efficacy of dopaminergic neurons derived from human ES cells has been recently demonstrated in xenotransplantation models using rats and rhesus macaques^[29]. However, this experimental setting cannot exclude a tumor risk after an allogeneic or even autologous transplantation of stem cell-derived human grafts^[5].

In conclusion, our findings clearly indicate that neural progenitor cells derived from mouse and human embryonic stem cells do not have the potential to generate teratomas or other tumors even up to six months following injection into immunodeficient animals. We think that this may also apply to iPS cell-derived neural progenitors. Our ongoing experiments using iPS cells derived from patients with Parkinson's disease support this notion. Thus, such hES cell-derived neural progenitors represent a strategy to circumvent safety concerns when used for potential future stem cell-based therapies. Moreover, HNP cells from iPS cells of patients with Parkinson's or other neurological diseases may be used for assessing alterations in neural differentiation properties or other defects that cannot be analyzed in patients.

ACKNOWLEDGMENTS

We would like to thank Thomas Schulz, Angelika Mönich and Leslie Elsner for excellent technical assistance.

COMMENTS

Background

The establishment of human embryonic stem cells has opened new perspectives for the development of cell-based therapies for treatment, e.g., of neurodegenerative disorders such as Parkinson's disease. The use of pluripotent cells as a source for the generation of tissue for transplantation suffers from the risk of teratoma formation, an inherent feature of pluripotent cells.

Research frontiers

Numerous strategies were suggested in the literature to deplete tumor-forming cells before grafting, including prolonged differentiation cultures. However, the authors of this study have shown previously that the risk of teratoma formation can even increase during differentiation culture due to an alteration of the immunological properties of the cells. To circumvent these problems, the authors of this study have developed mouse and human embryonic stem cell-derived neural progenitor cell lines.

Innovations and breakthroughs

The authors describe a new protocol to obtain human neural progenitor cell lines from embryonic stem cells which is fast and simple. These cell lines, which can be stored for several years, are shown to differentiate *in vitro* into dopaminergic neurons. Notably, human as well as mouse neuronal progenitor cell lines

did not form any tumors in immunodeficient mice.

Applications

Neural progenitor cell lines might be useful to differentiate dopaminergic neurons *in vitro* for transplantation in patients suffering from Parkinson's disease. The neural progenitor cell lines appear to be a safer alternative for the generation of grafts compared to embryonic stem cells since they did not form tumors in immunodeficient mice.

Terminology

Neural progenitor cell lines are cell lines derived from embryonic stem cells which can differentiate into neuronal cells, including dopaminergic neurons. Dopaminergic neurons are the neurons in the substantia nigra of the brain that are lost in Parkinson's disease. Teratomas are tumors containing derivatives of all three germinal layers which can occur after transplantation of pluripotent stem cells.

Peer review

In this manuscript, the authors generated both human and mouse neural precursor cell lines from embryonic stem cells. This study is very interesting and the writing style in this study was easy to follow.

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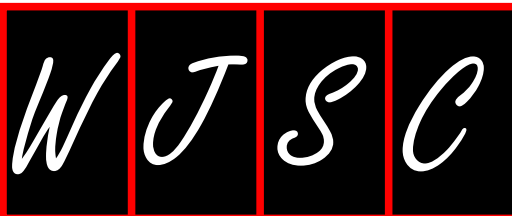
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P- Reviewers: Hwang DY, Petyim S **S- Editor:** Zhai HH

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WJSC covers topics concerning all aspects of stem cells: embryonic, neural, hematopoietic, mesenchymal, tissue-specific, and cancer stem cells; the stem cell niche, stem cell genomics and proteomics, and stem cell techniques and their application in clinical trials.

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World Journal of Stem Cells

ISSN

ISSN 1948-0210 (online)

Launch date

December 31, 2009

Frequency

Quarterly

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In press

- 3 Tian D, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

- 4 Diabetes Prevention Program Research Group. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

- 5 Vallancien G, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

- 6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

Volume with supplement

- 7 Geraud G, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

- 8 Banit DM, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

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- 9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

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- 10 Sherlock S, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

- 11 Lam SK. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)

- 12 Breedlove GK, Schorheide AM. Adolescent pregnancy. 2nd ed. Wiczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

- 13 Harnden P, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

- 14 Christensen S, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

- 15 Morse SS. Factors in the emergence of infectious diseases. *Emerg Infect Dis* serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: <http://www.cdc.gov/ncidod/eid/index.htm>

Patent (list all authors)

- 16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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