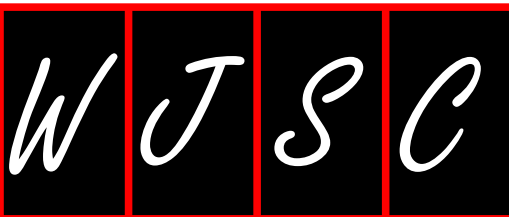


World Journal of *Stem Cells*

World J Stem Cells 2014 September 26; 6(4): 371-510





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AIM AND SCOPE

World Journal of Stem Cells (*World J Stem Cells*, *WJSC*, online ISSN 1948-0210, DOI: 10.4252), is a peer-reviewed open access academic journal that aims to guide clinical practice and improve diagnostic and therapeutic skills of clinicians.

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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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
Bimonthly

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PUBLISHER
Baishideng Publishing Group Inc
8226 Regency Drive,
Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
Help Desk: <http://www.wjgnet.com/esps/helpdesk.aspx>
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PUBLICATION DATE
September 26, 2014

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Haploidentical vs cord blood transplantation for adults with acute myelogenous leukemia

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Received: April 16, 2014 Revised: June 12, 2014

Accepted: July 17, 2014

Published online: September 26, 2014

Abstract

Hematopoietic cell transplantation is established as a curative treatment for patients with acute myelogenous leukemia. Haploidentical family donor and umbilical cord blood (UCB) are alternative sources of stem cells for patients lacking a matched sibling or unrelated donor. The early challenges of transplant complications related to poor engraftment and graft-vs-host disease have been overcome with new strategies such as using 2 units and increased cell dose in UCB and T-cell depletion and post transplantation cyclophosphamide in haploidentical transplantation. The outcomes of alternative transplantation for acute leukemia were compared to other traditional graft sources. For patients lacking a matched sibling or unrelated donor, either strategy is a suitable option. The choice should rely mostly on the urgency of the transplantation and the available cell dose as well as the expertise available at the transplant center. This manuscript reviews the options of alternative donor transplantation and highlights recent advances in each of these promising transplantation options.

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Key words: Umbilical cord blood transplantation; Hap-

loidentical transplantation; Leukemia

Core tip: Allogeneic hematopoietic cell transplantation is a curative treatment for patients with acute leukemia. Many patients lack a suitable matched donor and require another stem cell source. The choice between cord blood and mismatched relative is challenging as there is no direct comparison between the two transplantation modalities. This manuscript highlights the studies and current innovative approaches with either modality with an emphasis on the recent studies aiming at decreasing complications, enhancing engraftment and speeding immune recovery.

Solh M. Haploidentical vs cord blood transplantation for adults with acute myelogenous leukemia. *World J Stem Cells* 2014; 6(4): 371-379 Available from: URL: <http://www.wjgnet.com/1948-0210/full/v6/i4/371.htm> DOI: <http://dx.doi.org/10.4252/wjsc.v6.i4.371>

INTRODUCTION

Allogeneic hematopoietic cell transplantation (HCT) is a potential curative treatment for patient with leukemia. The preferable donor is a fully matched sibling; however, two thirds of patients needing transplant lack this donor option^[1]. In the absence of sibling donors, most centers choose a matched unrelated volunteer donor as the next option. Report from the National Marrow Donor Program's registry indicates an 8/8 HLA-matched adult unrelated donor is available for 51% of Whites, 30% of Hispanics, 20% of Asians and 17% of African-Americans^[2]. Hence, a 30% of all patients requiring HCT lack a suitable matched donor. The high relapse risk of many leukemia patients lacking a matched donor has led to the use of alternative sources of stem cells such as unrelated donor umbilical cord blood (UCB) and haploidentical

family donors.

First attempts with alternative HCT carried a high risk of mortality, engraftment complications and graft *vs* host disease^[3]. Progress in recent years has significantly improved the outcomes post alternative donor HCT. The improved outcomes are mostly credited to better donor selection, vigorous T-cell depletion in haploidentical transplantation, use of post infusion cyclophosphamide in haploidentical setting and use of 2 units in adult UCB transplantation (DUCBT) and introduction of more suitable conditioning regimens.

This article will review the recent advances in alternative donor HCT for acute leukemia in adults, describe the outcomes of HCT using these alternative donor sources and discuss ongoing studies in alternative HCT.

UMBILICAL CORD BLOOD TRANSPLANTATION

UCB offers several benefits over unrelated adult donors^[1,4]. UCB is safe for the donor as it is collected from the placenta during delivery. UCB units are readily available with less risk of transmission of infections, in particular cytomegalovirus (CMV), since most units are CMV negative. It permits a higher HLA disparity between donor and recipient when compared to MUD or related donors^[5,6]. Finally, UCB HCT may carry less risk of chronic GVHD compared to other cell sources^[7-10]. The main limitations associated with UCB are related to the small number of progenitor cells in each unit and the lack of access to donor lymphocytes for donor lymphocyte infusion (DLI), if needed. UCB constitutes a significant proportion of unrelated donor transplantations in children (40%) compared to only 10% in adults.

Laughlin *et al*^[3] reported on 68 patients [15 with acute lymphoblastic leukemia (ALL), 19 acute myeloid leukemia (AML) and 17 chronic leukemia] who received myeloablative UCB transplantation^[3]. Engraftment was better for patients with a nucleated cell dose $\geq 2.4 \times 10^7/\text{kg}$. Median time to engraftment was 27 d. Five patients experienced primary graft failure. CD34+ cell dose ($\geq 1.2 \times 10^5/\text{kg}$) was associated with a higher event free survival (EFS). EFS was not influenced by HLA matching (3-6/6) or patient age. This study established the safety of UCB transplantation in adults despite limited cell content and a more HLA mismatch than what has been reported in pediatrics^[11]. The COBLT study prospectively evaluated the outcomes of UCB transplantation^[12]. This study evaluated 34 adult subjects [AML = 19, ALL = 9, CML = 3, myelodysplastic syndrome (MDS) = 1]. Patients had a myeloablative conditioning (MA) with total body irradiation (TBI) plus cyclophosphamide and busulfan or melphalan with 4-6/6 HLA matched UCB units. The required cell dose was $> 1 \times 10^7$ nucleated cells/kg. Overall, 34 % had primary graft failure and 6 mo survival was only 30%. The reasons for higher mortality and complications with initial studies of UCB were due to patient selection and long duration from diagnosis to transplan-

tation. However, these reports established the importance of cell dose for successful UCB HCT and set the background for future studies of strategies to limit complications (*e.g.*, double umbilical cord blood transplantation, *ex vivo* expansion).

Double umbilical cord blood transplantation

The use of two UCB units was started at the University of Minnesota to overcome the cell dose limitation of single UCB units^[3,13,14]. DUCBT has yielded better engraftment, lower mortality and improved disease free survival comparable to other hematopoietic cell sources^[15].

A recent report from Minnesota group assessed 536 patients who received HCT with HLA MRD ($n = 204$), HLA allele matched or 1 antigen mismatched unrelated donor (MUD = 152, MMUD = 52) or HCT using 4-6/6 HLA matched two UCB units ($n = 128$) after myeloablative conditioning^[15]. Disease free survival (DFS) was similar for the different graft sources (UCB 51%, MUD 48%, MRD 33%, and MMUD 38%). UCB recipients had a lower relapse risk but a higher TRM. Another study from Minnesota suggested that using double UCB units carries a lower risk of relapse and a higher risk of acute GVHD when compared to single unit UCB transplantation^[16,17].

BMT-CTN 0501 is a myeloablative study that randomizes 1 *vs* 2 UCB grafts for children with leukemia. BMT-CTN 0604 study addressed RIC regimen in the DUCBT setting. Longer follow up from both studies will help improve our understanding of the use of DUCBT.

UCB outcomes

UCB has been compared to other donor sources in the myeloablative setting (Table 1). Transplant outcomes post UCB used to be inferior but recent series show similar outcomes for UCB when compared to other graft sources. The differences in outcomes between prior and current studies is related to many reason, the most important being an increase in the minimum acceptable cell dose in the cord unit to proceed with transplantation^[18].

Laughlin *et al*^[5] compared outcomes of 450 patients receiving 5-6/6 HLA matched unrelated donor transplants to 150 patients receiving 4-6/6 UCB transplants through the CIBMTR registry. The median time to neutrophil engraftment was delayed with UCB (27 d) compared to 18 d among 6/6 and 20 d among 5/6 HLA-matched unrelated bone marrow. Acute GVHD and relapse rates were similar between UCB and 6/6 MUD. UCB had higher TRM and poorer LFS. MUD had a better overall survival at 3 years (33% *vs* 23%) compared to UCB HCT. When UCB was compared to 5/6 MMUD, UCB was shown to have a lower risk of acute GVHD, but a similar risk of TRM, relapse, and LFS. Rocha *et al*^[6] on the other hand; reported that UCB had a lower risk of GVHD and similar rates of relapse, TRM and LFS. Both authors suggested UCB as a reasonable stem cell source in the absence of 6/6 MUD.

Single unit UCB transplantation outcomes were compared to MUD peripheral blood stem cells (PBSC) and

Table 1 Hematopoietic cell transplantation after myeloablative conditioning in adult patients comparing umbilical cord blood and other donor sources

Year	Graft type	Number of patients	Median age	ANC > 500/ μ L (median, d)	aGVHD II-IV(%)	Extensive cGVHD (%)	100 d TRM (%)	Relapse rate (%)	Survival (%)
2004 ^[5]	UCB	150	16-60	27	41	51	63	17 (3 yr)	26 (3 yr)
	MUD BM	367	16-60	20	48	35	46	23	35
	MMUD BM	83	16-60	18	51	40	65	14	20
2004 ^[6]	UCB	98	25	26	26	30	44	23 (2 yr)	36 (2 yr)
	MUD BM	584	32	19	39	46	38	13	42
2007 ^[19]	UCB	100	38	22	60	23	8	17 (3 yr)	NA
	MRD (BM and PB)	71	40	17	55	30	4	26	
2008 ^[10]	UCB	148	29	NA	NA	NA	41	26 (2 yr)	35 (2 yr)
	MUD PB	518	35	NA	NA	NA	27	30	45
	MMUD PB	210	NA	NA	NA	NA	42	24	36
	MUD BM	243	29	NA	NA	NA	26	28	48
	MMUD BM	111	NA	NA	NA	NA	37	26	38
2009 ^[62]	UCB AML	173	38	NA	32	8	32 (2 yr)	31 (2 yr)	43 (2 yr)
	MUD BM	311	38	NA	35	20	22	24	60
	UCB ALL	114	34	NA	28	10	24	31	49
	MUD BM	222	32	NA	42	17	25	24	57
2010 ^[15]	MRD	204	40	NA	65	47	24 (5 yr)	43 (5 yr)	NA
	MUD	152	31	NA	80	43	14	37	NA
	MMUD	52	31	NA	85	48	27	35	NA
	DUCB	128	25	NA	60	28	34	15	NA

ANC: Absolute neutrophil count; aGVHD: Acute graft versus host disease; cGVHD: Chronic graft versus host disease; TRM: Treatment related mortality; UCB: Umbilical cord blood; MUD: Matched unrelated donor; BM: Bone marrow; MMUD: Mismatched unrelated donor; NA: Not available; MRD: Matched related donor; PB: Peripheral blood stem cells; AML: Acute myeloid leukemia; ALL: Acute lymphoblastic leukemia; DUCB: Double umbilical cord blood.

bone marrow in a multiregistry study^[10]. Graft sources included 4-6/6 HLA matched single unit UCB ($n = 165$), 8/8 HLA matched PBSC ($n = 632$), 8/8 HLA matched bone marrow ($n = 332$), 7/8 HLA matched PBSC ($n = 256$) and 7/8 HLA matched bone marrow ($n = 140$). Endpoints included hematopoietic recovery, TRM, LFS and GVHD. Both acute Grade II-IV and chronic GVHD were lower in UCB than in PBSC MUD, while only chronic was lower in UCB than in 8/8 matched bone marrow patients. TRM was higher after UCB than after 8/8 allele matched PBSC (HR 1.62, $P = 0.003$) or bone marrow transplantation (HR = 1.69, $P = 0.003$). Overall, LFS was comparable between UCB and 7-8/8 allele matched unrelated donor.

A recent report from Minnesota and Fred Hutchinson group in Seattle showed that myeloablative DUCBT has comparable leukemia free survival as matched and 1 antigen mismatched unrelated donor.

UCB has also been compared to related donor transplantation. Takahashi *et al*^[19] reported on 171 adults who received single unit UCB ($n = 100$), 5-6/6 HLA matched related donor bone marrow transplant ($n = 55$) or 5-6/6 HLA matched related donor PBSC HCT ($n = 16$). UCB recipients had a delayed hematologic recovery and a lower incidence of grade III-IV acute and extensive chronic GVHD. Both UCB and related donor transplantation had similar relapse, TRM and DFS.

In summary, there is enough evidence to suggest UCB as an acceptable source of stem cells for patients requiring myeloablative HCT but lack a suitable matched donor.

UCB Transplantation after reduced-intensity conditioning

Older patients with AML requiring allogeneic HCT are at increased risk of complications with myeloablative conditioning. Studies with RIC UCB had variable TRM and this variability could be related to different study populations^[20-22]. Overall, most studies have reported OS and DFS that is similar to HCT using other stem cell sources.

A Minnesota study evaluated older patients after UCB transplantation and compared their outcomes to matched related donors, MUD and Mismatched URD. The TRM was higher (35% *vs* 27%), LFS was lower (28% *vs* 35%) and overall survival was lower (30% *vs* 43%) among UCB recipients when compared to MUD transplantation^[23]. This study and other reports establish the efficacy of UCB after RIC for patients who are not eligible for myeloablative conditioning.

Other factors in selecting cord blood units

The selection of cord blood units has been traditionally based on low resolution typing of HLA-A, B and high resolution at DRB1 and on the total nucleated cell dose. Recent studies have evaluated the importance of high resolution HLA typing, HLA-C match and KIR ligand status. Eapen *et al*^[24] found that patients who had units matched at HLA-A, B, DRB1 and HLA-C had better 3 year TRM (9%) and 3 year OS (57%) than patients who were matched at HLA-A, B, DRB1 but with mismatch at HLA-C (TRM 26%; OS 51%) and better outcomes than those with a mismatch on HLA-C with additional mismatch at HLA-A, B, DRB1 (TRM 31%, OS 37%)^[24].

Allele level typing was recently analyzed through a combined CIBMTR and Eurocord registry databases. The investigators showed that the frequency of neutrophil recovery was lower for recipients of mismatches at 3 or more alleles. Nonrelapse mortality was higher with units mismatched at 1 to 5 alleles compared with matched units. Overall mortality was not different except for those that received units mismatched at 5 alleles^[25]. The author concluded that cord blood transplantation with ≥ 3 allele level mismatches should be avoided.

When a fetus is exposed to non-inherited maternal antigen (NIMA) in utero, fetal T regulator cells are induced to that haplotype. It was hypothesized that recipients who are matched to donor NIMA may have lower mortality post transplantation. 5 year TRM was lower and OS was better among NIMA matched UCBT compared to NIMA mismatched UCBT (TRM 18% *vs* 32%, $P = 0.05$; OS 55% *vs* 38%, $P = 0.04$)^[26]. It was suggested that NIMA matching can be considered in a patient with multiple UCB units harboring adequate cell dose.

The role of Donor killer cell immunoglobulin-like receptor (KIR) ligand incompatibility has shown variable conclusions. A study from Eurocord showed that patients receiving UCB units mismatched at KIR-ligand had lower relapse and better leukemia-free survival^[27]. The results were significant for patients with AML, where recipients of KIR-ligand mismatched in the GVH vector had a better LFS (73% *vs* 38%, $P = 0.004$) and incidence of relapse (5% *vs* 36%, $P = 0.005$). This finding was not reproduced in a recent analysis by the Japan society for HCT^[28] or by an earlier study from Minneapolis in the myeloablative setting^[29]. In the same analysis, Minnesota group found that KIR ligand mismatch is associated with increased grade III-IV acute GVHD and increased risk of death in the reduced intensity setting.

Recent advances in UCB transplantation

Recent work in UCBT is aimed at achieving faster neutrophil engraftment and minimizing early TRM. Direct injection of stem cells into the marrow cavity was hypothesized to reduce systemic “wasting” of such cells. In one unit UCBT, intra-bone marrow injection was associated with lower risk of acute graft *vs* host disease with a sustained engraftment^[30]. These results were not reproducible in the DUCBT setting where one of the two units was injected directly into the bone marrow^[31].

New methods to enhance engraftment focus on *ex vivo* expansion and co-infusion of purified committed hematopoietic progenitors. One trial evaluated the effects of co-infusion of highly purified “of the shelf” CD34+ progenitors from healthy volunteers. The aim of this strategy was to assess if the additional CD34+ cells will help enhance neutrophil recovery without leading to long term engraftment. *Ex vivo* expansion is also receiving more support. One expansion method include cocultures of UCB derived CD34⁺CD38⁻ precursors with immobilized Notch I ligand^[32]. A study by de Lima *et al*^[33] reported on 31 patients who received *ex vivo* expanded

UCB with cocultures from mesenchymal stem cells. Time to engraftment was significantly improved at 15 d compared to 24 d for patients with unmanipulated cord infusion^[34]. The role of *ex-vivo* expansion in UCB transplantation is still an ongoing process.

Engraftment can also be improved by increasing stem cell homing. One such method include the use of complement fragment 3a and diprotein A^[34,35] that increase homing through stromal cell-derived factor 1 (SDF1). A recent study through the University of Minnesota established safety of infusing C3a primed units but failed to show effect on engraftment^[36].

HAPLOIDENTICAL FAMILY DONOR TRANSPLANTATION

Haploidentical transplantation has gained significant interest in the last few years with the introduction of new GVHD strategies such as T cell depletion with high CD34+ doses to overcome risk of graft failure^[37,38], and high dose cyclophosphamide post transplantation. Haploidentical donors are usually defined as having ≥ 2 HLA antigen mismatches at HLA-A, -B and -DRB1 loci. Some studies of haploidentical transplantation included family donors with one HLA antigen mismatch^[39]. There are several platforms for performing haploidentical transplantation including *ex vivo* T cell depletion prior to infusion, post infusion depletion with drugs such as cyclophosphamide and unmanipulated infusion with vigorous GVHD prophylaxis. With the choice of multiple available donors, selection can be based on factors such as sex, age, cytomegalovirus status (CMV) and killer immunoglobulin receptor (KIR) incompatibility. One advantage over UCB, is the availability of haploidentical donors for more cells if needed.

OUTCOMES OF HAPLOIDENTICAL TRANSPLANTATION IN ACUTE LEUKEMIA

Ex vivo T-cell depleted haploidentical transplantation

The Perugia group evaluated 104 adult leukemia patients who were conditioned with TBI, fludarabine, thiopeta and antithymocyte globulin (ATG)^[37]. Grafts were T-cell depleted using CD34+ immunoselection and no post-transplantation GVHD prophylaxis was used. Ninety-one percent of the patients engrafted, and for the seven patients who failed to engraft, engraftment was successful after a second transplant in six cases. Acute GVHD developed in 8% of patients (2% grade III-IV) and five patients developed chronic GVHD. 16/67 AML patients and 10/37 ALL patients relapsed. The event free survival for patients who were transplanted in complete remission was 48% for AML and 46% for ALL. Table 2 Summarizes studies that compared haploidentical transplantation to other donor sources.

Table 2 Haploidentical hematopoietic cell transplantation compared to transplantation from other graft sources

Year	Number of patients	Neutrophil engraftment (median d)	aGVHD II-IV (%)	cGVHD (%)	100 d NRM (%)	Relapse (2 yr)	Survival (%)
2002 ^[63]	MUD BM 81	16	42	57	23	25	58 (2 yr OS)
	MMUD BM 58	15	33	51	45	26	34
	Haplo 48	14	46	50	42	42	21
2005 ^[53]	Haplo-ALL 74	NA	8	NA	49	38	13 (2 yr LFS)
	UCB-ALL 91		26		41	23	36
	Haplo-AML 151		12		58	18	24
	UCB-AML 91		26		24	24	30
2009 ^[48]	Haplo 56	54/56 (13)	27	23	13	22	68 (2 yr LFS)
	MRD 51	48/51 (12)	14	31	8	17	76

aGVHD: Acute graft versus host disease; cGVHD: Chronic graft versus host disease; NRM: Non-relapse mortality; AML: Acute myelogenous leukemia; ALL: Acute lymphoblastic leukemia; NA: Not available; HCT: Hematopoietic cell transplant; MUD: Matched unrelated donor; BM: Bone marrow; MMUD: Mismatched unrelated donor; Haplo: Haploidentical family donor; OS: Overall survival; LFS: Leukemia free survival; UCB: Umbilical cord blood; MRD: Matched related donor.

Another T-cell depleted study evaluated 173 AML patients and 93 ALL patients who received a haploidentical transplantation^[40]. Patients received high dose of CD34⁺ cell with a median of 10×10^6 CD34⁺ cells/kg and 11.6×10^6 CD34⁺ cells/kg in AML and ALL patients, respectively. All patients received myeloablative conditioning containing TBI (74% AML and 92% ALL patients received TBI). Transplant related mortality was 66% for AML and 44% for ALL patients. Relapse incidence was 32% in AML and 49% in ALL patients. Among these patients with advanced disease, LFS was only 1% and 7% for AML and ALL, respectively. However, among patients transplanted in complete remission, the outcomes were more encouraging. Ninety-one percent of recipients engrafted with median time to engraftment of 12 d. The incidence of Grade II-IV GVHD was 5% and 18% among AML and ALL patients, respectively. In the AML group, recipients with a parent or sibling donor had lower TRM than other relatives (35% *vs* 65%, $P = 0.03$). The most common cause of TRM was infections, particularly viral infections such as adenovirus and CMV. Among these patients transplanted in remission, leukemia free survival at 2 years was 29% in AML and 23 % in ALL recipients. This multicenter study showed that infusion of high doses of immunoselected CD34⁺ cells without post-transplant immunosuppression can yield rapid and sustained engraftment and a low risk of GVHD.

A more selective T cell depletion can be performed by the Clini-MACS system. This system removes the α/β T cells and B cells, and keeps γ/λ T cells, natural killer and other cells. Locatelli *et al*^[41] reported on this method at the annual European BMT meeting where patients received myeloablative conditioning regimen of TBI, thiotepa, fludarabine and ATG followed by infusion of TCR α/β /CD19 T cell depleted grafts. This approach yielded sustained engraftment, faster immune reconstitution and low incidence of GVHD.

T-cell replete haploidentical transplantation

Di Bartolomeo *et al*^[42] studied the outcome of unmanipulated, G-CSF primed bone marrow haploidentical HCT for patients with high risk hematologic malignancies^[42].

The most common conditioning regimen used was thiotepa, busulfan and fludarabine in the myeloablative setting with GVHD prophylaxis comprised of 5 drugs: antithymocyte globulin, cyclosporine, methotrexate, mycophenolate mofetil and basiliximab. The 100 d incidence of grade III-IV acute GVHD was 5%, 1 year cumulative incidence of TRM was 36% and 3 year OS was 54% for standard risk patients^[42]. This study showed the feasibility of haploidentical transplantation without ex vivo T cell depletion by using a vigorous pre- and posttransplantation pharmacologic GVHD prophylaxis.

A group from china published results of unmanipulated G-CSF primed marrow haploidentical HCT followed by intensive immunosuppression. The incidence of grade III-IV acute GVHD was 13.4% and the 3 year LFS was 70.7% and 55.9% in standard and high risk AML^[43]. Another group from china published on the use of mismatched peripheral stem cells without conditioning regimen but post chemotherapy with cytarabine and mitoxantrone and showed an improvement of complete remission rate (80% *vs* 42.8%; $P = 0.06$) when compared to chemotherapy alone^[44].

Cyclophosphamide post haploidentical transplantation

A new Platform for RIC haploidentical transplantation was pioneered by John Hopkins university using high-dose post transplantation cyclophosphamide. Cyclophosphamide induced immune tolerance was first studied by Berenbaum *et al*^[45] who showed that mice treated with cyclophosphamide had a prolonged survival of mismatched skin graft if given up to the fourth day post grafting. The ability of post-transplant cyclophosphamide to prolong engraftment post a major histocompatibility mismatched skin graft, several immunologists became interested in developing durable chimerism before solid organ transplantation using post-transplant cyclophosphamide^[46]. These earlier studies established the fact that post-transplant cyclophosphamide kills T cells that undergo antigen driven proliferation and hence facilitates decrease risk of GVHD post transplantation.

Earlier phase II clinical studies with high dose cyclophosphamide were published in 2008 where cyclophos-

phamide 100 mg/kg given was administered over days +3 and +4 post RIC haploidentical marrow transplantation. The conditioning regimen included fludarabine, cyclophosphamide and TBI. Tacrolimus and mycophenolate were used for GVHD prophylaxis. Neutrophil engraftment was achieved at day 15 with very acceptable acute GVHD rates (grade II-IV GVHD was 35%). Relapse rate was 40%-50% at 1 year with DFS of 34%^[47]. Overall and EFS at two years were 36% and 26% respectively. A multicenter trial sponsored through the CIBMTR (CTN0603) using haploidentical BMT for high risk hematologic malignancies was run in parallel with another phase II trial (CTN 0604) using DUCBT. The probability of 1 year overall and PFS were 54% and 46% after DUCBT and 62% and 48% after haploidentical transplantation^[48].

Post-transplant cyclophosphamide was also applied in the myeloablative setting with peripheral blood cell source in the haploidentical setting. A study by the group in Philadelphia used a high dose TBI based conditioning with cytoxan 120 mg/kg given on days -3 and -2 followed by CD34 selected peripheral blood stem cells^[49]. The cumulative incidence of NRM was 22%, grade III-IV acute GVHD 7% and the 3 year survival was 27% for patients with active disease at the time of transplant. Other studies with myeloablative haploidentical transplantation using peripheral blood stem cell and post-transplant cyclophosphamide showed similar results of low incidence of acute GVHD and a 1 year of EFS in the range of 50%-60%^[50,51].

The use of peripheral blood as a source of stem cells in the nonablative haploidentical setting with post-transplant cyclophosphamide will allow wider applicability of this approach^[52].

Haploidentical transplantation vs UCB transplantation

The outcomes of 407 adult leukemia patients (AML = 242; ALL = 165) after UCB or haploidentical HCT were compared by the eurocord group^[53]. Compared to haploidentical HCT, recipients of UCB HCT had delayed neutrophil recovery, higher incidence of acute GVHD and similar incidence of relapse, LFS and TRM. A similar analysis among children with ALL showed that UCB HCT had higher rate of graft failure (23% *vs* 11%, $P = 0.07$). Both UCB and haploidentical HCT had similar TRM and DFS but more relapses were seen in the haploidentical group ($RR = 1.7$, $P = 0.01$)^[54]. These studies show that either UCB or haploidentical HCT is an acceptable option for both adult and children with leukemia in the absence of a fully matched sibling or unrelated donor.

A multicenter trial by the Clinical Trials network (BMT-CTN) is comparing the two stem sources in the reduced intensity setting for patients with acute leukemia. This study will hopefully help find some answers on the selective role of each of these procedures among leukemia patients.

Future strategies in haploidentical HCT

T-cell depletion has become the cornerstone of haplo-

identical transplantation. This usually leads to profound immunodeficiency lasting for 4-6 mo. Adoptive transfer of memory T lymphocytes helps protect against infections in the first months after transplantation. Infusion of virus-specific cell lines (CMV, Epstein-Barr virus, adenovirus and aspergillus) had inconsistent results in preventing and treating infections^[55,56]. Other strategies to hasten the post transplantation immune reconstitution without triggering GVHD have included infusion of donor T cells after engineering with a suicide gene^[57], photodynamic purging^[58], and the use of anti-CD25 monoclonal antibody to remove alloreactive cells^[59]. The Perugia group studied the infusion of haploidentical donor derived regulatory T cells followed by CD34 cells and donor mature T cells in the setting of T cell depleted haploidentical HSCT^[60]. With this approach, Perugia group was able to achieve a very low incidence of acute GVHD and a faster immune reconstitution.

More single centers are showing that usage of peripheral stem cell in the haploidentical RIC setting yields equivalent results to bone marrow infusion.

CONCLUSION

Patients with high risk acute leukemia requiring allogeneic HCT and lacking a fully matched related or unrelated donor have alternative options of stem cell sources. Either haploidentical or UCB is an acceptable option in this situation. The choice of best alternative donor is center dependent and several algorithms have been published to address donor selection^[40,61]. As studies continue to improve on engraftment rates in UCB, GVHD and relapse rates in haploidentical HCT, the order of donor choices will likely change with time.

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P- Reviewer: Porrata LF, Shimoni A, Tonks A, Yao CL

S- Editor: Ji FF **L- Editor:** A **E- Editor:** Lu YJ



Advances in haplo-identical stem cell transplantation in adults with high-risk hematological malignancies

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Received: November 28, 2013 Revised: July 4, 2014

Accepted: July 15, 2014

Published online: September 26, 2014

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Key words: Peripheral blood progenitors; Stem cell transplantation; Graft-versus-host disease; Haplo-identical donor; Hematological malignancies

Core tip: Timely donor availability remains a challenge for patients in need of an urgent stem cell transplant. The ability to obtain half matched stem cells from any family member represents a significant breakthrough in the field. This review summarizes some of the current strategies used to substantially improve the outcomes of patients undergoing haplo-identical stem cell transplantation.

Ricci MJ, Medin JA, Foley RS. Advances in haplo-identical stem cell transplantation in adults with high-risk hematological malignancies. *World J Stem Cells* 2014; 6(4): 380-390 Available from: URL: <http://www.wjgnet.com/1948-0210/full/v6/i4/380.htm> DOI: <http://dx.doi.org/10.4252/wjsc.v6.i4.380>

Abstract

Allogeneic bone marrow transplant is a life-saving procedure for adults and children that have high-risk or relapsed hematological malignancies. Incremental advances in the procedure, as well as expanded sources of donor hematopoietic cell grafts have significantly improved overall rates of success. Yet, the outcomes for patients for whom suitable donors cannot be found remain a significant limitation. These patients may benefit from a hematopoietic cell transplant wherein a relative donor is fully haplotype mismatched. Previously this procedure was limited by graft rejection, lethal graft-versus-host disease, and increased treatment-related toxicity. Recent approaches in haplo-identical transplantation have demonstrated significantly improved outcomes. Based on years of incremental pre-clinical research into this unique form of bone marrow transplant, a range of approaches have now been studied in patients in relatively large phase II trials that will be summarized in this review.

INTRODUCTION

Allogeneic bone marrow transplantation (BMT) offers a chance to cure patients that present with high-risk hematological malignancies. These include adults and children with acute myeloid leukemia (AML) in first or latter remission and acute lymphoblastic leukemia (ALL) employing BMT as post remission therapy in first or greater complete remission. Allogeneic BMT can also be considered in eligible patients with chronic lymphocytic leukemia, chronic myeloid leukemia and lymphoma (follicular, large cell, Hodgkin and peripheral T cell). The process of safely performing allogeneic BMT requires the regulated experience of a comprehensive multi-disciplinary team of health care professionals. In principle, the goal is to replace a diseased bone marrow with healthy blood-forming hematopoietic elements from a

fully human leukocyte antigen (HLA)-matched healthy donor. At one time allogeneic BMT was routinely associated with a mortality of greater than 40%. Advances in stem cell acquisition and processing, molecular-level typing of unrelated donors and general supportive care that have reduced infectious complications have collectively improved rates of survival. Essential to success is durable engraftment of donor progenitor cells capable of restoring stable hematopoiesis. In addition to engraftment of hematopoietic progenitors, it is now known that donor immune effector cells (including T-lymphocytes) are required for disease eradication and prevention of relapse^[1-3]. Specific anti-tumor donor lymphocytes engage in an ongoing immune reaction against residual host malignant cells^[4-6]. This graft-versus-tumor (GVT) effect is closely linked to graft-*vs*-host disease (GVHD)^[7-9]. The ability to dissect immune effectors responsible for each process is a element of current BMT research. Recognizing that a successful transplant requires contribution of both donor progenitor and immune effectors has led to substantial changes in the field. These enhancements include: (1) the design of newer less-toxic preparative regimens^[10-13], and (2) expansion of the sources of donor stem/progenitor cell grafts.

Preparative regimens

Myeloablative transplant conditioning has traditionally been used to “create space” for donor progenitor cells and simultaneously kill residual tumor cells. These preparative protocols employ high-dose chemotherapy often in combination with whole-body irradiation. Significant treatment-related toxicities (TRM) restrict the procedure to young, otherwise-fit patients. Shifting the focus and goals of clinical efficacy from stem cell replacement to maintenance of a transplanted donor immune system has enabled the introduction of less-intense preparative regimens. Milder preparative regimens aim to achieve chimeric engraftment of progenitors as well as donor immune effector cells, including T lymphocytes. Reduced-intensity conditioning (RIC) or non-myeloablative allogeneic BMT is currently performed in a growing number of older patients (up to and beyond 70 years of age) diagnosed with a variety of lymphoid and myeloid neoplasms^[13-16]. These hematological malignancies appear to have mixed susceptibility to the GVT effect. Donor immune effector cells impact therapeutic efficacy but also contribute to serious post-transplant side effects, including severe acute and chronic GVHD (aGVHD and cGVHD, respectively). Patient determinants, including remission status, remission duration and disease type, may govern the choice of preparative regimen as well as the type of graft that will contribute to rate of stable engraftment, immune reconstitution, GVL, and GVHD.

Graft sources

Once exclusively obtained by large volume aspiration from the posterior pelvis of a donor, transplantable hematopoietic progenitor cells can now be obtained directly

from peripheral blood as well as from fresh umbilical cord blood. These stem cell products are transported world-wide in a highly regulated manner. Common terminology are used to describe stem cells derived from a bone marrow harvest (Hematopoietic Progenitor Cells - Marrow, HPC-M), from a mobilized apheresis peripheral blood product (Hematopoietic Progenitor Cells-Apheresis, HPC-A), from umbilical cord blood (Hematopoietic Progenitor Cells -Cord, HPC-C), or for donor lymphocyte infusion (DLI) (Therapeutic Cells-T). Each source of hematopoietic progenitors exhibits differences in cellular composition which leads to specific biological properties that may be of therapeutic benefit or risk depending on the transplant recipient, type of transplant to be performed as well as the type and immediate status of the hematological malignancy. For example mobilized peripheral blood products HPC-A often have a higher number of CD34+ stem/progenitor cells but also more CD3+ T lymphocytes (1-log higher). Higher CD34+ progenitor cell counts may improve time to engraftment and be used in a non-myeloablative setting but also appear to increase the risk of cGVHD^[17-19]. Umbilical cord blood (HPC-C) typically contains a much lower absolute CD34+ progenitor cell count leading to significant delays in engraftment (or rejection); however the immature nature of the donor white blood cells from this source may also reduce the risk of GVHD and allow for some degree of HLA mismatch^[20-22].

Understanding the unique properties of each source of hematopoietic cells helps to determine the anticipated performance for a given transplant recipient. Moreover, efforts to improve efficacy and reduce unwanted toxicities are currently under intense investigation. These include efforts to: (1) increase the dose of CD34+ hematopoietic progenitors in umbilical cord transplantation by combining two separate cord products^[23,24] or by performing *ex vivo* CD34+ stem cell expansions^[25]; (2) reduce the number of T-lymphocytes in HPC-A products by *ex vivo* T cell depletion or by *in vivo* administration of anti-thymocyte globulin (ATG)^[26,27]; and (3) improve engraftment kinetics without cGVHD of HPC-M products by administering G-CSF to the donor prior to marrow harvest^[28]. These advances illustrate the growing ability of practitioners to safely manipulate graft sources for maximum clinical benefit.

Identification of a donor

Finding a suitable bone marrow match is based on the HLA system, comprised of genes on chromosome 6. The major histocompatibility complex (MHC) includes two basic classes involved in antigen presentation and subsequent immune activation. MHC class I involves peptide presentation following intracellular digestion, while MHC class II presents extracellular antigens to host T lymphocytes. HLA-A, HLA-B, and HLA-C comprise class I, and HLA-DR, HLA-DQ and HLA-DP are class II. The proteins encoded by HLA define “self” to the host immune system. One set (haplotype) of HLA

genes are maternal and the other paternal. From this, any given sibling, excluding an identical twin, will have only a 25% chance of being fully HLA-matched. While matched related siblings remain the best source of donor material, this approach has several world-wide limitations including a significant reduction of family sizes (fertility rates of 1.5-2.0 per family across Europe and North America), a policy of one child families, as well as the health status and potential co-morbidities of older sibling donors. Moreover, lack of sibling donor availability is predicted to become a much greater issue due to reduced family size. It is estimated that the likelihood of finding a sibling match will decline from 53.7% in 2002, to 37.1% in 2009 and 16.6% in 2024^[29]. Nonetheless, investigation of family members using low-resolution serological typing (antigen level HLA-A,B, C and allele level HLA-DRB1) remains a standard initial evaluation approach.

If a suitable sibling-match cannot be found, a recipient in need of a transplant will require a search for an unrelated HLA-matched donor. Large national marrow donor programs will canvass for potential volunteers, perform HLA typing and maintain data in an ongoing registry. To be eligible volunteer donors must be in good general health and may be asked to undergo bone marrow harvesting under general anesthesia or daily administration of G-CSF (Filgrastim) followed by large volume leukapheresis. Stem cell donors must be screened to exclude active malignancies, transmissible infectious conditions (HIV, Hepatitis, HTLV-1, West Nile virus, Syphilis), hematological disorders (Sickle Cell Disease), and congenital bleeding disorders. A formal donor assessment will include a comprehensive questionnaire, complete medical history, and medical examination. Once screened and considered eligible, the most pertinent factor that predicts transplant success is donor age. Bone marrow recipients from younger donors (*i.e.*, < 30 years of age) demonstrate improved five-year overall and disease-free survival^[30,31]. This survival benefit appears to be the result of lower rates of GVHD when a younger donor is used. A retrospective analysis by the National Marrow Donor Program (NMDP) on over 6900 HLA-matched transplants performed between 1987 to 1999 was conducted to identify unique donor-specific features associated with transplant outcome^[30]. In this analysis use of a donor aged 18 to 30 years correlated with a lower cumulative incidence of grade III/IV acute GVHD ($P = 0.005$) and lower incidence of chronic GVHD at 2 years ($P = 0.02$). Other studies have suggested a higher rate of chronic GVHD in male recipients transplanted from a multiparous female donor or if mobilized progenitor cells are used^[19,30].

Identifying a potential unrelated donor BMT match generally requires high-resolution (HR) HLA typing of both recipient and donor. Studies suggest that employing a molecular (allele level) typing technique can reduce the incidence of severe GVHD and increase survival to levels similar to that seen with a matched-sibling donor^[32,33]. Algorithms exist that combine a serological preliminary

search (antigen level) with latter confirmatory molecular HR analysis (Figure 1). Efforts to decrease time and cost are dependent on clinical urgency and stability of the primary malignancy^[34]. Patients with common alleles and haplotypes have a higher probability of finding a match and generally require fewer pre-screened potential donors to be selected for HR typing (3-5 donors), while those with rare alleles and haplotypes may require as many as 10 or more. High resolution allele level matching for HLA-A, B, C and DRB1 (8/8 match) results in improved survival^[35-37]. Additional typing at HLA-DQB1 (10/10 match) and DPB1 loci, as well as DRB3, 4, 5 can be considered. Single loci mismatches at DQB1 and DPB1 appear to be tolerated better than at A, B, C or DRB1. Although it is necessary to minimize the number of allele mismatches, a single allele 7/8 or 9/10 alteration can still be considered. Single mismatch at B or C may be less of a concern than mismatches at A or DRB1 in patients undergoing HPC-M, but not HPC-A transplantation^[38,39]. Factors such as the recipient diagnosis, CMV status, age, and sex also need consideration^[30].

The ability to perform world-wide searches and identify volunteer donors has dramatically changed the international landscape of BMT. Superior matching as well as enhanced supportive care has improved the overall outcome of matched-unrelated donor (MUD) BMT such that results appear similar to matched-related donor BMT^[32,33,38]. Despite this, national registries face considerable challenges and limitations. Increasing allogeneic transplant indications puts greater pressure on the number of world-wide searches. Donor attrition and maintenance of a donor registry requires ongoing organized drives that reach out to younger volunteers and maintain a large potential pool of active registrants. This may vary from country to country; in the United States it is estimated that 1 out of 44 is registered, Canada 1 in 100, while Germany has a donor ratio of 1 to 17 (calculated # of registrants/total population). Moreover, within any given registry, certain ethnicities are often significantly underrepresented^[40,41]. Ultimately as many as 25% of all patients requiring BMT will never find a donor and either seek alternative treatments or palliation. This pressing unmet medical need has inspired advances in the use of alternative approaches that include the development of umbilical cord blood (UCB) hematopoietic cell transplant and haplo-identical BMT.

Advances in alternative donor hematopoietic cell transplantation

Umbilical cord blood contains hematopoietic progenitor cells that can be used for allogeneic transplant and immunological reconstitution^[42]. Graft composition is a critical element in predicting the short and long-term engraftment performance, rate of rejection, development of GVHD, or ability to provide GVL and prevention of relapse. Potential advantages of pre-stored UCB units include immediate access without donor attrition and an increased availability for ethnic minorities through

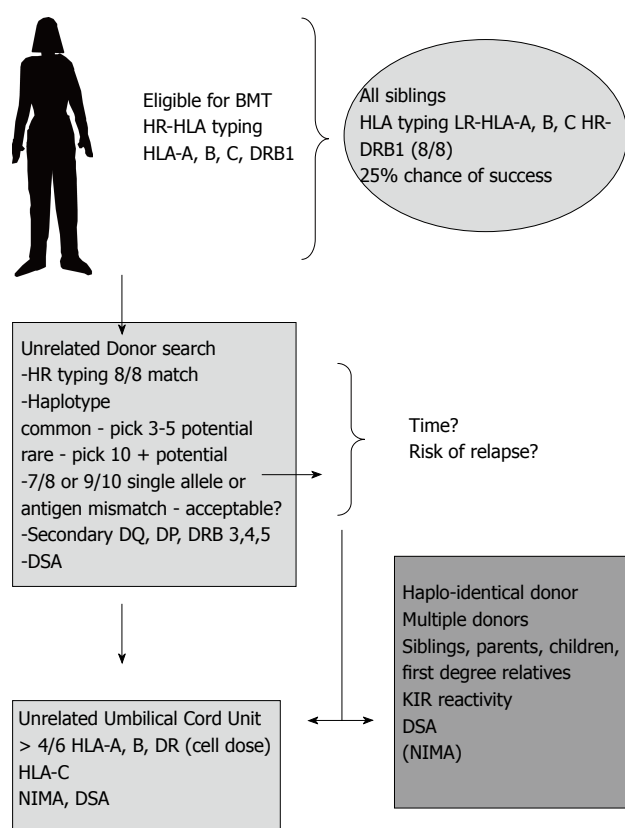


Figure 1 Example of a potential donor search algorithm that aims to expedite finding a suitable stem cell donor. A blend of high and low resolution approaches are employed. In certain situations the likelihood of finding an unrelated donor match is low due to rare alleles and haplotypes. Consideration of a haplo-identical BMT may be considered at an earlier stage. Abbreviations: HR: High resolution (allele level); LR: Low resolution (antigen level); NIMA: Non-inherited maternal antigen; KIR: Killer cell Ig-like receptors; DSA: Donor-specific antibodies; BMT: Bone marrow transplant.

the use of partially matched UCB products. Conversely, donors of unrelated UCB products are not available if future grafts or donor lymphocyte infusions are required. Numerous international cord banks operate under strict standards of testing, storage, and characterization. A minimum target of 3.0×10^7 nucleated cells per recipient weight per unit of cord blood is generally recommended, while flow cytometry-based measurement of CD34+ cells per recipient weight may be more predictive^[42,43]. Most cord products lack sufficient stem cells for most adults or large adolescent recipients. Efforts to accelerate cellular reconstitution following UCB BMT include combining two umbilical cord products (dUCBT) as well as *ex vivo* expansion^[23,25]. When evaluated post-transplant (d 100) typically only one (dominant) cord unit can be identified^[24]. In addition to the risk of graft failure, delayed immune reconstitution and increase in infections or relapse, there are theoretical risks that a potential hematological disease, not yet recognized in the newborn, will be transferred to the BMT recipient.

HLA typing of UCB products requires only low resolution serological testing for HLA-A, B, and molecular typing for DRB1 (6 alleles). Units with matches

of at least 4/6 are potentially acceptable^[44]. Mismatches at DRB1 and C may increase treatment-related mortality. Efforts to safely expand immature progenitors *ex vivo* without increasing differentiation to committed progenitors are moving towards early phase clinical trials^[25].

HAPLO-IDENTICAL BMT IN ADULT PATIENTS

A hematopoietic cell graft (HPC-M or HPC-A) obtained from a family donor that is mismatched at 3/6 loci (HLA-A, B, DRB1) remains a potential for patients who lack a fully matched sibling, 8/8 unrelated donor, or UCB product (patient size, haplotype). Advantages of such a haplo- approach include an expanded potential donor pool that may include parents, siblings, children, and first-degree relatives. Family donors of all ethnicities may be highly motivated, readily available, and willing to donate and be re-mobilized if required. These important theoretical advantages make a compelling case for further development of this approach, especially for patients with high-risk leukemia that are unlikely to maintain a remission during a prolonged unrelated search that may take months. Nonetheless, the ability to safely achieve sustained engraftment of highly HLA-disparate transplanted progenitor cells is a major challenge. Moreover, the risk of lethal aGVHD in the setting of 3/6 HLA disparity is an even greater risk. When GVHD occurs in a fully HLA matched 6/6 sibling transplant it is felt to be the result of minor histocompatibility antigen mismatches, however the nature and extent of immune activation with overt HLA host mismatches will differ in a haplo-identical BMT. Understanding the unique biology, as well as the immediate risk of fatal GVHD, has driven implementation of a variety of relatively effective novel approaches.

Graft-vs-host disease

The essential elements for the development of GVHD include the presence of immunologically competent cells in the hematopoietic cell graft, the presence of transplantation antigens in the host that have not been encountered by the donor, and an inability of the host to destroy the transplanted graft (Billingham's criteria)^[45,46]. Clinical GVHD is generally divided into acute (diffuse maculopapular rash, GI mucosal inflammation, and elevated liver function tests) and chronic GVHD. The diagnosis of chronic GVHD has been recently revised by the National Institutes of Health (NIH) consensus working group report^[47]. Clinical signs involve organs or sites that include skin, nails, mouth eyes, genitalia, GI tract, lung and the musculoskeletal system. Specific abnormalities may be either diagnostic (*i.e.*, poikiloderma, esophageal web, bronchiolitis obliterans) or distinctive (*i.e.*, xerostomia, myositis, keratoconjunctivitis sicca). Diagnosis requires at least one diagnostic category or the presence of least one distinctive manifestation confirmed by biopsy or specialized objective test. While classic acute GVHD occurs within 100 d after transplantation some patients continue

or relapse beyond this time point often during tapered withdrawal of immunosuppressive agents. Similarly, while classic chronic GVHD may occur in the absence of acute GVHD it may also be present along with acute GVHD (overlap syndrome).

The clinical development of the GVHD reaction is complex and involves sequential step-wise immune activation. The primary effector cells are T lymphocytes present in the graft. In the haplo-identical setting, donor T cells may attack disparate non-matched MHC molecules present on the majority of host cells. Donor T cells may be further activated by immunostimulatory cytokines released following tissue damage (gastrointestinal) that results from the preparative regimen^[48,49]. Additional activation may occur at the level of the vascular endothelium, co-stimulatory signals originating from antigen presenting cells, and following release of TNF α and other pro-inflammatory cytokines^[45,46,50]. T cell effector damage appears to be mediated by perforin-based host target cell lysis and Fas-mediated apoptosis^[51,52]. In a standard BMT, treatment options of established GVHD are limited; efforts to prevent GVHD with a combination of a calcineurin inhibitor and methotrexate have proven successful^[53,54]. In this setting about one half of patients will develop significant GVHD requiring additional immunosuppressive therapy (corticosteroids).

With a focus on T-lymphocytes, recent advances have been able to dissect the roles of donor T cell subsets present in the graft^[55]. These include naive T cells, memory T cells, and regulatory T cells. Naive T cells (CD45RA+/CD62L+) may include cells destined to be alloreactive to the host. Memory T cells (CD45RO+/CD62L+/-) include cells that provide protective antimicrobial immunity post-transplant. Regulatory T cells (CD4+/25+/FoxP3) appear to generally dampen other T cell responses and may be useful in attenuating clinical GVHD. Separation of naive T cells responsible for GVHD from donor T cell subsets responsible for GVT remain relatively elusive but are of obvious clinical importance. The ability to customize, harness, or control the fate of these T cell subsets to mitigate GVHD and retain GVT yet provide adequate post-transplant antiviral immunity has considerable clinical potential and is of heightened importance in the setting of high-risk leukemia patients undergoing a haplo-identical BMT. These challenges have led to important advances both in laboratory technologies and clinical application of newer agents.

APPROACHES TO HAPLO-IDENTICAL BMT

Given the potential for development of lethal aGVHD, efforts to entirely eliminate alloreactive donor T cells remain a critical first step. It has been suggested that as few as 3×10^4 T cells per recipient weight are capable of causing clinical GVHD^[56]. Both *in vivo* and *ex vivo* approaches have been developed (Figure 2). *Ex vivo* strate-

gies include immunomagnetic-based positive selection of CD34+ cells or CD3/19 depletion with preservation of NK and gamma-delta T cells^[57,58]. *In vivo* T cell depletion may be accomplished by early administration of post-transplant cyclophosphamide or by aggressive multi-agent anti-GVHD therapies that include anti-thymocyte globulin (ATG), G-CSF, and triple GVHD prophylaxis as well as recent studies using rapamycin (Table 1).

Aversa *et al*^[59-61] in Perugia, Italy, described a series of incremental approaches to haplo-identical BMT from 1993 to 2006. A series of step-wise approaches focused on patients with high-risk acute myeloid (AML) and lymphoblastic leukemia (ALL). The investigators examined: (1) stem cell sources; (2) graft processing technologies; (3) conditioning regimens; and (4) post-transplant administration of G-CSF. Collectively, the investigative team were able to obtain high doses of CD34+ donor progenitor cells ($> 10 \times 10^6$ /kg. recipient weight) that led to a remarkable rate of successful engraftment. Aversa *et al*^[61] and Reisner *et al*^[62] had previously described the ability of purified CD34+ cells to block the action of residual cytotoxic T lymphocytes leading to tolerance^[61]. Moreover when transplanted in very high numbers this “veto effect” could overcome clinical graft rejection by residual host T cells. At the same time newer technologies including CD34-positive selection and use of mobilization agents and peripheral blood progenitor cell collections led to “mega dose” grafts that consistently demonstrated remarkable engraftment of neutrophils and platelets with a low level of graft rejections in a large number of patients. *Ex vivo* T cell depletion was highly effective ($< 0.5 \times 10^5$ CD3+ T cells/kg. recipient wt.) with little or no evidence of significant clinical GVHD even in the absence of prophylaxis. Impressive event-free survival rates of up to 48% were noted in AML patients in first complete remission. Higher rates of relapse were seen in ALL patients. Despite these notable clinical and technological advances, prolonged immune reconstitution (CD4+ T lymphocytes) was problematic and non-relapse mortality in the range of 41%. Infections were mostly cytomegalovirus and fungal in origin. Nonetheless, the ability to use rigorous positive selection of “megadose” CD34+ products and achieve timely multi-lineage engraftment, minimal GVHD, and durable survival in some patients became an important clinical platform for future trials.

Following the work of the Perugia group, Roy and colleagues in Montreal devised a novel strategy to safely “add-back” modified donor lymphocytes to hasten immune recovery and provide anti-viral immunity^[56]. This strategy involved *ex vivo* photo-based depletion of alloreactive T cells derived from a donor-recipient mixed lymphocyte reaction (MLR). Working with a highly potent dibromomorphodamine photosensitizing compound (TH9402) the team demonstrated accumulation of drug in certain cell types including cancer cells and alloreactive T cells. These cells could then be lysed following exposure to a specific wavelength of visible light (514 nm). The mechanism of *ex vivo* cellular lysis was shown

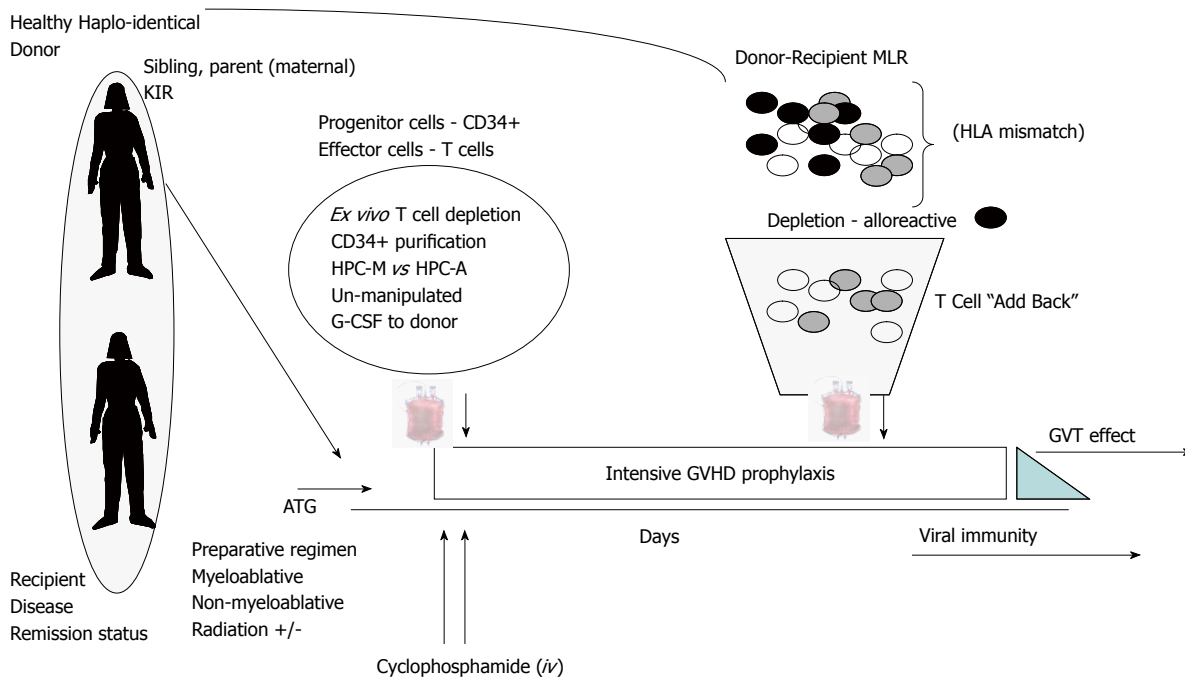


Figure 2 Current approaches to Haplo-identical bone marrow transplant. Recent novel strategies include issues of donor selection, *ex vivo* complete or partial T cell depletion of HPC-M or HPC-A grafts and the use of anti-thymocyte globulin. Additional strategies include in vivo administration of “high dose” cyclophosphamide and intensive multi-agent GVHD prophylaxis. Finally, in an effort to overcome severe prolonged immune suppression the addition of donor T lymphocytes that have been purged of alloreactive T cells may be of benefit. Abbreviations: DSA: Donor-specific antibodies; MLR: Mixed lymphocyte reaction; HLA: Human leukocyte antigen; GVT: Graft-versus tumor; KIR: Killer cell Ig-like receptors; GVHD: Graft-vs-host disease; ATG: Anti-thymocyte globulin.

to involve reactive oxygen species. During an MLR reaction in a haplo-identical setting, donor T lymphocytes respond to donor immune cells and take up TH9402. Non-reactive resting T cells capable of anti-viral immunity do not accumulate the agent and are retained in the infused “add-back” lymphocyte product (ATIR). The characterization of retained cells following photo-depletion differed when HLA-matched or haplo-matched pairs were tested. These experiments demonstrated preservation of CD8+ naïve and effector cells in the matched situation and preservation of naïve and central memory cells of both CD4+ and CD8+ phenotypes when haplo-identical pairs were studied. When tested in a clinical trial, administration of relatively high doses of photo-depleted T cells post-transplant did not increase grade III-IV GVHD. Patients receiving higher doses of cells demonstrated decreased rates of infection and improved overall survival (47.4%) at a median of 4 years^[56]. These encouraging results are now being studied in a multi-institutional phase II setting. A similar approach using anti-CD25 immunotoxin MLR-based purging has been studied in several transplant settings. Similar results indicating successful removal of alloreactive GVHD cells and maintenance of virus-specific T cells have been demonstrated^[63].

Ex vivo graft engineering to eliminate GVHD, yet retain GVT and avoid life-threatening infectious complications, is promising but remains investigational, complex, and costly. Kasamon *et al.*^[64], Fuchs *et al.*^[65], Luznik *et al.*^[66], Brunstein *et al.*^[67] in Baltimore have developed a similar *in vivo* platform using post-transplant cyclophosphamide administered shortly following infusion of an un-

manipulated haplo-identical mismatched marrow graft. Similar to the *ex vivo* MLR approach, this strategy exploits the concept of selective depletion of alloreactive immune cells with preservation of resting non-alloreactive cells. Within a period of 24-48 h post-infusion of mismatched HPC-M, alloreactive T cells will rapidly encounter stimulatory host cells. T cells capable of anti-viral immunity will not respond at this stage. Cyclophosphamide will selectively eliminate reactive cells and limit GVHD. Indeed this approach has proven highly feasible in over 200 patients with a range of advanced hematological malignancies. Using a non-myeloablative regimen engraftment was timely (neutrophils 15 d and platelets 24 d) with graft rejections in the range of 13% (autologous marrow recovery). Rates of acute and chronic GVHD were less than 30%. Post-transplant GVHD prophylaxis included tacrolimus and mycophenolate mofetil. Relapse rates were relatively high in a broad range of advanced stage hematological malignancies and overall survival was in the range of 40%-45%.

A recent publication from Di Bartolomero and colleagues demonstrates the feasibility of performing haplo-identical BMT using an un-manipulated G-CSF-primed approach in patients with high-risk malignancy^[68]. In this series, GVHD prophylaxis was intensive and included ATG, cyclosporine, methotrexate, mycophenolate mofetil, and basiliximab (anti-CD25). Engraftment of neutrophils (21 d) and platelets (28 d) were reasonable. The cumulative incidence of serious acute GVHD was 24% and at 2 years extensive cGVHD was only 6%. The overall 3-year overall survival ranged from 33%-54% (high-risk and

Table 1 Summary of clinical approaches to haplo-identical bone marrow transplant

Ref.	n	Preparative regimen	T-cell depletion/engraftment	GVHD prophylaxis	Acute GVHD	Chronic GVHD	TRM	OS
Perugia, Aversa <i>et al</i> ^[59-61]	255	TBI +/- MA ATG	Yes 84%-96%	none	17%	< 5%	41%	47% ²
Peking, GIAC ^[69,70]	250	MA ATG	No G-BM + G-PB 100%	CsA, MTX MMF	45%	31%	12%-48%	56%-71% AML ¹ 25%-60% ALL ¹
Montreal Bastien <i>et al</i> ^[56]	19	TBI +/- MA ATG	Yes T cell "Add Back" 100%	none	20%	25%	15%	47%
Baltimore Studies ^[64-67]	210	NMA	No 87%	Tacro, MMF PTCyclo	27%	13%	15%	40%-45%
Di Bartolomeo <i>et al</i> ^[68]	88	MA 80% NMA 20% ATG	No G-BM 91%	CsA, MTX MMF Basilixumab	24%	6%	36%	33%-54% ¹

¹Survival range including standard and high-risk groups; ²Survival for patients in complete remission. A variety of approaches have been studied that compare MA-myeloablative to NMA-non myeloablative, T cell depletion of graft, and GVHD prophylaxis. Engraftment rates are high, and GVHD can be attenuated through T cell depletion of the graft or by intensive anti-GVHD prophylaxis, including ATG. GVHD: Graft-vs-host disease; TRM: Treatment related mortality; OS: Overall survival; TBI: Total body irradiation; ATG: Anti-thymocyte globulin; CsA: Cyclosporine A; MTX: Methotrexate; MMF: Mycophenolate mofetil; PTCyclo: Post transplant cyclophosphamide; AML: Acute myeloid leukemia; ALL: Acute lymphoblastic leukemia; Tacro: Tacrolimus.

standard risk). A large series published by Huang *et al*^[69] and Wu *et al*^[70] also described a similar approach to haplo-identical transplantation using un-manipulated cell grafts with escalated post-transplant immunosuppression. The Peking "GIAC" approach was studied in 250 patients and highlighted the effects of administration of G-CSF to the donor for collection of combined HPC-M and HPC-A grafts, intense immunosuppression (cyclosporine, methotrexate, mycophenolate mofetil and G-CSF), and ATG. Engraftment was rapid with neutrophils and platelets engrafting at 12 and 15 d, respectively. Grade III-IV acute GVHD was 45% and any cGVHD was 31%. Relapse in standard risk AML and ALL was 19.4 and 21.2%, in high-risk AML and ALL was 29.4% and 50.8%. Treatment related mortality ranged from 11.9% to 48.5% and was dependent on risk and disease type. Ultimately investigators suggested these results were comparable to results obtained using an HLA-matched sibling donor^[69,70]. In this study investigators administered G-CSF to both the donor and recipient post-transplant. Administration of G-CSF following both autologous and allogeneic BMT has been primarily used to reduce the duration of neutropenia and related complications^[71]. In both settings neutrophil recovery is faster resulting in shorter hospitalization for autologous but not allogeneic BMT. Use of G-CSF in allogeneic transplants is otherwise considered safe; however two retrospective studies have raised concern over a possible increase in GVHD^[72,73]. Still others have suggested that pre-treatment of T-lymphocytes with G-CSF results in an anti-inflammatory (type-2) cytokine profile that attenuates experimental GVHD severity^[74].

Towards the future, Fowler *et al*^[75] at the NIH have recently published a compelling phase 2 study using rapamycin-resistant T cells (2.5×10^7 cells/kg, recipient weight) infused 14 d after hematopoietic cell transplantation for treatment of a variety of refractory hematological malignancies. While that study, on 40 patients of a

wide range of ages (18 of whom remained in sustained complete remission up to 84 mo of follow-up), was done in the context of 6/6 HLA-matched sibling donors, it is conceivable that such an approach using their low-intensity conditioning regimen and this specific immune effector product could be adapted to the haplo-transplant setting. Donor lymphocytes in that study demonstrated a consistent and balanced Th1/Th2 profile; incidence probabilities of aGVHD were 20% and 40% at 100 and 180 d post-transplant, respectively.

The studies described above illustrate a broad range of current and future strategies to advance the field of haplo-identical BMT. *Ex vivo* T cell depletion, selective T cell "add back", *in vivo* T cell depletion, and use of intensive GVHD prophylaxis are being actively improved. Feasibility has now been established with reasonable overall survival in a population of high-risk advanced malignancies who lack a traditional matched donor. Previous limitations of graft rejection and unacceptable rates of serious GVHD have been largely overcome. Efforts to enhance GVT and prevent life-threatening viral and fungal infections remain a current focus. Separation and exploitation of the linkage between GVT and GVHD remain a critical next step. It has also been suggested that alloreactive natural killer cells (NK) may be protective against myeloid leukemia relapse. Given that a patient may have several potential haplo-identical donors, it may be possible to choose a donor with heightened NK alloreactivity^[76,77]. In addition, administration of donor-derived regulatory T cells may attenuate GVHD yet facilitate GVT effectors^[78]. Finally a retrospective analysis of 118 acute leukemia patients undergoing haplo-identical BMT using a parent as a donor suggested improved 5-year EFS when the mother was the donor as compared to the father^[79]. When sibling (non-parent) haplo-identical donors were evaluated, the gender of the donor had no effect on outcome. The presence of donor-specific anti-

bodies (DSA) in the recipient may be evaluated to reduce the risk of graft failure^[80].

CONCLUSION

At present, allogeneic BMT remains the only chance of cure for adults and children with advanced hematological disease. Transplant indications and eligibility are expanding. Outcomes are improving with reduced-intensity conditioning, HR molecular typing of unrelated donors as well as improved general supportive care measures. Most, but not all patients in need of this life-saving procedure will have a suitable sibling, matched unrelated, or UCB donor graft. Haplo-identical transplantation offers hope to those high-risk patients who face limited treatment options. Despite ethnicity, an expanded pool of motivated donors could be immediately available. A wide range of strategies are currently being explored. Previous serious pitfalls, including graft rejection, severe GVHD, and prolonged immune suppression are becoming less problematic as the science of the field advances. Novel experimental utilization of T regulatory cells, alloreactive NK cells, and other T cell subsets (T-Rapa cells, for example) hold great promise in this rapidly emerging and much needed field.

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P- Reviewer: Scatena R, Shao R S- Editor: Wen LL
L- Editor: A E- Editor: Lu YJ



Limbal stem cells: Central concepts of corneal epithelial homeostasis

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Supported by Save Sight Society New Zealand and Auckland Medical Research Foundation

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Received: July 25, 2014 Revised: August 20, 2014

Accepted: August 30, 2014

Published online: September 26, 2014

Abstract

A strong cohort of evidence exists that supports the localisation of corneal stem cells at the limbus. The distinguishing characteristics of limbal cells as stem cells include slow cycling properties, high proliferative potential when required, clonogenicity, absence of differentiation marker expression coupled with positive expression of progenitor markers, multipotency, centripetal migration, requirement for a distinct niche environment and the ability of transplanted limbal cells to regenerate the entire corneal epithelium. The existence of limbal stem cells supports the prevailing theory of corneal homeostasis, known as the XYZ hypothesis where X represents proliferation and stratification of limbal basal cells, Y centripetal migration of basal cells and Z desquamation of superficial cells. To maintain the mass of cornea, the sum of X and Y must equal Z and very elegant cell tracking experiments provide strong evidence in support of this theory. However, several recent stud-

ies have suggested the existence of oligopotent stem cells capable of corneal maintenance outside of the limbus. This review presents a summary of data which led to the current concepts of corneal epithelial homeostasis and discusses areas of controversy surrounding the existence of a secondary stem cell reservoir on the corneal surface

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Key words: Limbal stem cell; Corneal epithelium; XYZ hypothesis; Corneal homeostasis; Corneal wound repair

Core tip: It is a long held belief that stem cells reside only at the limbus. However, there are recent reports that present evidence of corneal repair and maintenance independent of limbal involvement. These findings call to light the possibility of previously undiscovered reservoirs of corneal stem/progenitor cells located at the central and peripheral cornea. A new secondary reservoir of stem cells has a significant clinical implication as new therapeutics for corneal degenerative disorders. This review outlines the historic evidence for limbal stem cells and discusses the role of these putative central and peripheral corneal stems cells in corneal homeostasis.

Yoon JJ, Ismail S, Sherwin T. Limbal stem cells: Central concepts of corneal epithelial homeostasis. *World J Stem Cells* 2014; 6(4): 391-403 Available from: URL: <http://www.wjgnet.com/1948-0210/full/v6/i4/391.htm> DOI: <http://dx.doi.org/10.4252/wjsc.v6.i4.391>

INTRODUCTION

The transparent front surface of the eye, the cornea (Figure 1A) overlies the iris, pupil and anterior chamber. The structures that compose the anterior chamber are

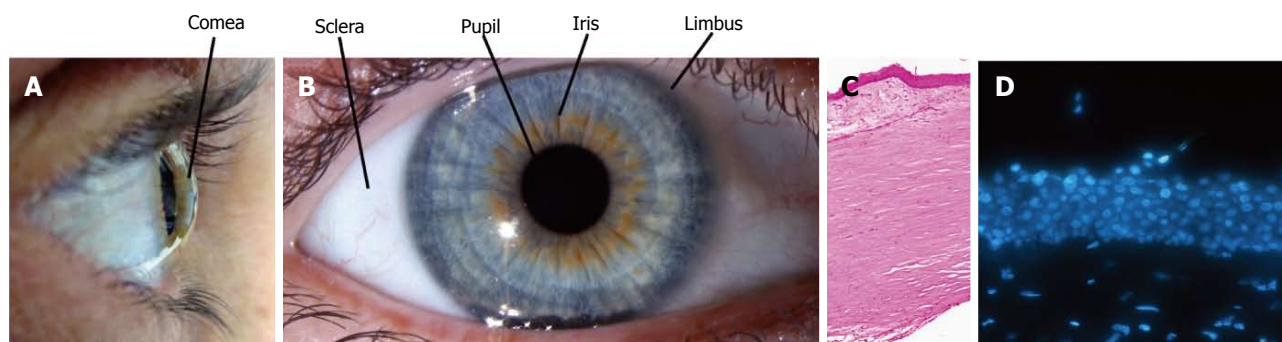


Figure 1 Anatomy of the eye. The cornea (A) comprises the colourless front portion of the eye immediately anterior to the iris and pupil (B). The limbus, located at the corneoscleral junction (B) is the transitional zone where the corneal and conjunctival epithelia merge, is shown in section using Haematoxylin and Eosin stain (C) and is considered a reservoir of stem cells which migrate centripetally to form the 5-7 cell layer corneal epithelium (DAPI fluorescence to highlight cell nuclei in corneal section, D).

surrounded by the white opaque sclera with the tissues meeting at the limbus. Maintenance of corneal integrity is imperative to light entry and refraction onto the correct position on the retina.

The anterior-most ocular surface is composed of corneal and conjunctival epithelia with the limbus at the transition zone between the two (Figure 1B and C). The corneal epithelium undergoes continuous renewal throughout life (Figure 1D). The central dogma of corneal homeostasis states that the mass of the epithelium remains constant so that the rate of cellular addition must equal that of cellular loss^[1]. The predominant theory for corneal homeostasis is the XYZ hypothesis proposed by Thoft *et al.*^[2] in 1983. This theory proposes that the limbus serves as a reservoir of ocular stem cells. Asymmetric division of these stem cells produces a stem-like daughter cell which remains within the limbus and a transient-amplifying cell (TAC) (Figure 2A) which migrates centripetally and anteriorly (Figure 2B). TACs undergo multiple rounds of replication and progressively lose “stemness” (Figure 2C) as they migrate anteriorly and progress to post-mitotic suprabasal wing cells, and then terminally differentiated superficial squamous cells (Figure 2D). The superficial cells are lost from the surface by normal exfoliation (squamation) or traumatic injury (Figure 1E). Therefore anterior migration from cells of the basal epithelium “X” and centripetal migration from the limbus “Y” equals desquamation from the surface “Z”. The entire human corneal epithelium is renewed in 9 to 12 mo^[3].

Whilst the research underpinning the limbus as the main reservoir for corneal epithelial stem cells has been consolidated with sophisticated cell tracking assays, an additional emerging view of the existence of stem cells outside of the limbus is supported by findings from several independent groups. This review analyses the data in support of limbal stem cells (LSCs) and looks at the possibility of a secondary reservoir of stem cells for the corneal epithelium.

LIMBAL EPITHELIAL STEM CELLS: HISTORICAL REVIEW

Studies reporting differences between central corneal and

limbal cells were published as early as the 1940s. These early studies showed increased frequency of mitoses in the basal layer of peripheral cornea using mitotic figure counts and radiated thymidine^[4,5]. Centripetal migration of cells expressing melanin pigment was observed in rabbit as well as human corneas, suggesting the limbus as a source of new cells^[6,7]. Since then, various studies have established the limbus as the location of corneal epithelial stem cells based on a set of unique properties observed within this cell population:

Slow cell turnover rate

DNA label-retention studies have shown the limbus contains cells in a growth-arrested or slow cycling state. Retention of radiated thymidine or 5-bromo-2'-deoxyuridine (BrdU) has been reported in limbal cells of mice cornea *in situ*^[8-10], human limbal explant cultures^[11] and whole cornea organ cultures^[12]. The retention of DNA label was observed for up to nine weeks in these studies. The labelling index, or the percentage of BrdU-retaining cells, was 1%-4% in mice corneas^[9,10,13], and approximately 4% in human limbal explant cultures^[11]. The nuclear label was lost progressively as the labelled cells moved towards the central cornea, indicating increased cell division during centripetal migration^[8].

Slow turnover rate in the limbus has also been demonstrated by resistance to 5-fluorouracil (an anti-metabolite which specifically targets proliferative cells)^[14], cytoplasmic staining for cyclins D, E and A (indicator of a growth-arrested state)^[15] and susceptibility to malignant transformation^[16-18]. The susceptibility to tumour formation is thought to be a property of stem cells as oncogenic mutations are more likely to accumulate in cells with long life span^[19].

Clonogenicity and proliferative potential

Life-long maintenance of any stratified epithelium necessitates a self-renewing pool of stem cells, asymmetric division of precursor cells and a rapid proliferative response upon injury^[20]. Studies have suggested that these attributes are unique to the limbal cell population.

Self-renewal capacity or clonogenicity of limbal cell populations has been shown by their ability to form

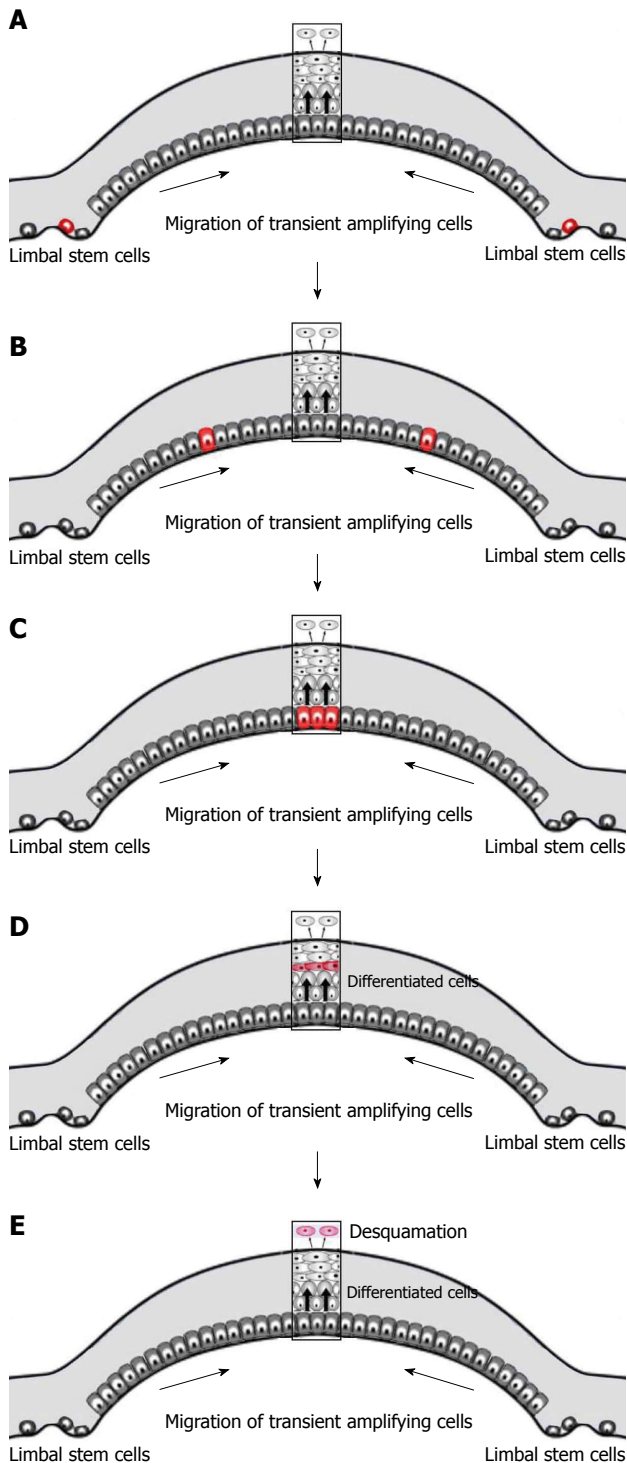


Figure 2 The X, Y, Z hypothesis of corneal maintenance. Limbal stem cells at the peripheral cornea divide and give rise to transient amplifying cells (TACs) (A). These TACs migrate centripetally through the basal epithelium (B) and undergo a limited number of divisions on the central cornea (C). The differentiated daughter cells move anteriorly to replenish the upper layers of the cornea (D) where they are eventually shed from the corneal surface (E). Hence the sum of X (proliferation and anterior migration) and Y (centripetal migration) must equal Z (desquamation of superficial cells) for corneal maintenance. Red cells: Continuum of transient amplifying migrating and/or differentiated cells.

sphere colonies on a 3T3 fibroblast feeder layer^[21]. These authors showed that the holoclone, meroclone and paraclone colony formation system previously identified in

human skin could be translated into spheres derived from human corneal biopsies. The single cell-derived sphere colonies from the limbus (equivalent to holoclones) were capable of undergoing 80 to 100 cell division cycles and could be propagated up to 14 passages before senescence. Single cell isolates from central cornea only formed paraclones (mostly consisting of terminally differentiated cells and capable of 15 cell divisions at maximum) and meroclones (intermediate form between holoclones and paraclones).

Asymmetric cell division has been suggested by uneven distribution of cell fate determinants across the corneal epithelium. Molecules implicated in asymmetric cell division and early cell fate decision, such as Musashi-1^[22], Notch-1^[23], p75^[24], C/EBP δ ^[25] and Δ Np63 α ^[26] have been almost exclusively localised in the mouse and human limbus.

Proliferative potential of limbal cells has been demonstrated by both *in vitro* and *in vivo* studies. Primary human limbal epithelial cell cultures showed high proliferative potential with a mean of 23 population doublings *in vitro*, while central corneal cells could not be propagated^[27]. Explant cultures of human limbal epithelium showed larger outgrowth and higher mitotic rate compared to explants from central epithelia^[28,29]. When transplanted into the flanks of athymic mice, single cell suspensions from limbal cell culture produced cysts which had more organised structure and longer life span than those derived from central corneal cell suspensions^[30]. Furthermore, *in vivo* animal studies have shown that the slow cycling limbal basal cells can rapidly divert to proliferative status upon damage to cornea^[8,13].

Cellular morphology

Morphological differences between limbal and corneal cells have been highlighted using a variety of imaging technologies including synchrotron infrared microspectroscopy^[31], morphometric analysis of DAPI-stained nuclei^[9], transmission electron microscopy^[32,33], *in vivo* confocal microscopy and flow cytometry^[34]. These studies commonly identified cuboidal cells 10 μ m in diameter with a high nucleus-to-cytoplasm ratio in the limbal basal layer. The sparse cytoplasm in these cells appears smooth due to the paucity of organelles and intracellular junctions, another indicator of low metabolic activity and protein turnover. In contrast, basal cells of the central epithelium are more columnar and have a lower nucleus-to-cytoplasm ratio^[31].

Biochemical characteristics

The identification of exclusive biochemical markers of corneal stem cells has been for many years a highly desirable endeavour. A number of putative stem cell markers have been suggested based on the biochemical transition that takes place in the basal cell layer of the corneo-limbal junction^[35-37]. Limbal basal cell layers preferentially express certain structural proteins (vimentin, cytokeratin 14, 15 and 19), cell adhesion molecules (integrin α 6, β 1,

$\beta 4$, P-cadherin and N-cadherin), enzymes (α -enolase, aldehyde dehydrogenase, cytochrome oxidase, Na^+/K^+ -ATPase and carbonic anhydrase), metallothionein, growth factor receptors (KGF-R and NGF-R), cell fate/cycle regulators (notch-1, Musashi-1, $\Delta\text{Np}63\alpha$, p75, Bmi-1 and C/EBP δ) and ABCG2, an ATP-binding cassette transporter protein. ABCG2 has been shown to be responsible for the efflux of the nuclear dye Hoechst 33342, enabling isolation of ABCG2-positive cells using flow cytometry^[38]. This dye efflux property is an established marker of a stem cell in many cell lineages including haematopoietic^[39], neuronal^[40], muscle^[41], and epithelium^[42]. The ABCG2 proteins are thought to protect LSCs from oxidative stress by transporting small regulatory molecules required for their proliferation, differentiation and apoptosis^[43]. ABCG2-positive cells are termed side population (SP) cells, and only a small proportion of limbal basal cells are SP cells. The SP cells have been shown to possess a number of stem cell properties including up-regulation in response to central corneal wounding^[44], small cells with high nucleus-to-cytoplasm ratio, slow cycling, expression of $\Delta\text{Np}63\alpha$ and ABCG2, absence of cytokeratin 3, 12 and involucrin, and increased colony-forming efficiency and growth capacity^[45,46].

As limbal basal cells migrate out of the limbus, their protein expression profile gradually changes. Central corneal epithelium is characterised by the loss of α -enolase and melanin pigmentation and the expression of cytokeratin 3 and 12, connexin 43 and 50, involucrin and *CLED*, a Ca^{2+} -linked protein associated with early epithelial differentiation. The expression of a large amount of metabolic enzymes and proteins in the central corneal cells is thought to contribute to the increase in cell size^[47]. Furthermore, increase in cell size has been correlated with loss of colony-forming efficiency^[48].

Centripetal migration

Centripetal migration of corneal epithelial cells is a well-documented phenomenon^[49,50]. Imaging studies have directly visualised centripetal migration of limbal cells towards the centre of the cornea. One of earliest studies used India ink to mark limbal cells which then migrated centripetally over the wounds of the mice cornea^[51,52]. Centripetal migration was observed in rabbit lamellar keratoplasty model where the host corneal epithelial cells invaded the grafted donor tissue^[53]. Similar results were obtained in the explants of human donor corneal buttons, where all donor corneal epithelial cells were replaced by recipient cells as early as three months post-penetrating keratoplasties^[54]. Both Collinson *et al.*^[55], and Nagasaki *et al.*^[56] used transgenic mice with reporter genes to visualise centripetal migration in normal mice cornea. Interestingly, Matsuda *et al.*^[57] and Srinivasan *et al.*^[58] found that wounds close to the limbus or repeated insult to the central epithelium accelerated the healing rate, the latter implying that rapidly dividing TACs of the periphery have moved to more central areas after the first trauma and respond more quickly to the second.

The chemotactic signal for centripetal migration may be provided in the form of cytokines and/or the difference between the composition of extracellular matrix between the limbus and the cornea^[59]. KGF, a paracrine hormone secreted by stromal cells, has been shown to enhance outgrowth in rabbit limbal explant culture on human amniotic membrane^[60]. While the inflammatory cytokine interleukin-6^[61], fibronectin^[62], and hyaluronan^[63], all of which are highly up-regulated upon injury, have been shown to play a role in drawing rabbit limbal cells towards the wound.

Recently, a very elegant study by Di Girolamo *et al.*^[64] has shown the centripetal movement of cells generated in the limbus using inducible multicolour tagging technology *in vivo*. Furthermore, this study linked the inducible multicolour tagging system with K14, one of the cytokeratin molecules that has been shown to mark an association with limbal stem cells. This study clearly showed that coloured K14 positive cells originated from the basal limbal epithelium and formed narrow corridors of epithelial cells that radiated centripetally onto the corneal surface. These authors do acknowledge that K14 is not an absolute limbal stem cell marker and that they could not exclude the existence of stem cells outside the limbal niche as K14 was targeted because of its limbal location.

Multipotency

Limbal basal cells characteristically lack differentiation markers indicating they are in an undifferentiated state. Several studies however, have implied a high multipotent differentiation potential when appropriate combinations of cellular signalling molecules are encountered: Rabbit limbal epithelial cell sheets transformed into fibroblasts when transplanted onto limbal stroma^[65]; during the culture of human limbal explants, the limbal epithelial cells which invaded into the stroma underwent epithelial-mesenchymal transition^[66]; mouse limbal epithelial cells expressed opsin when transplanted onto mice retina, indicating their potential to differentiate into rod photoreceptors^[67]; and the potential to transdifferentiate to neuronal cells was demonstrated by Zhao *et al.*^[68]. In their study, rat limbal cell isolates maintained in growth factor-driven culture system expressed neuronal progenitors, β -tubulin, nestin and neurofilament. When subject to serum-containing differentiation medium, the limbal cell isolates expressed glial markers such as GFAP and O4. The limbus-derived neuron-like cells not only expressed neuronal markers and neurotransmitter receptors, but also exhibited electrical responses to GABA and kainic acid^[69].

Stem cell niche

A stem cell niche is an anatomically defined area that is thought to provide a variety of intrinsic and extrinsic factors such as the physical protection, survival factors and cytokines and deemed essential to the maintenance of a stem cell population while preventing entry into differentiation^[70,71]. Over the past decade, much progress

has been made in characterising the putative niche in the limbus. The limbal areas are rich in melanin pigments, highly innervated, well-vascularised and have a different array of extracellular matrix components than the central epithelium. Melanocytes or melanin granules within the cytoplasm of progenitor cells are thought to play a role in protection against ultraviolet radiation^[8,72]. Blood-derived growth factors and nutrients provide for the active cell division^[8,73].

The epithelial-stromal interface in the limbus differs from that in the central cornea. Bowman's layer, a densely interwoven collagen sheet lying between the basement membrane of the central corneal epithelium and the stroma, is absent in the limbus^[74]. In the limbus, stroma directly underlies the epithelial basement membrane. The limbal epithelial basement membrane also differs from that of central cornea in its composition^[75-80]. The limbal basement membrane labelled positive for type IV collagen $\alpha 1$ chain, laminin $\alpha 2$, $\beta 1$, $\beta 2$, $\gamma 1$, $\gamma 3$ chains, nidogen, agrin, BM40/SPARC, tenascin-C and thrombospondin-4, whereas central cornea showed positive immunoreactivity to type IV collagen $\alpha 3$ chain, type V collagen, thrombospondin-1 and endostatin. Limbal-specific basement membrane components were co-localised with putative stem cell markers such as ABCG2, p63 and cytokeratin 19, but not with differentiation markers including cytokeratin 3, connexin 43, desmoglein and integrin $\alpha 2$. In addition, the cornea-limbal transitional zone showed strong immunostaining to type XVI collagen, fibrillin-2, tenascin-C/R, vitronectin, bamacan, chondroitin sulfate and versican, and were co-localised with vimentin-positive cell clusters.

To date, four anatomic structures have been proposed as the corneal stem cell niche; Palisades of Vogt, limbal epithelial crypts, limbal crypts and focal stromal projections. The Palisades of Vogt are ridges of epithelium in the limbus that extend centripetally from the bulbar conjunctiva, and are easily visible by slit lamp microscopy, especially in young donors or those with dark skin^[7,81,82]. More recently, Shanmuganathan *et al.*^[83] and Dua *et al.*^[84] identified limbal epithelial crypts located at the interpalisade epithelial rete ridges of the Palisades of Vogt. The limbal epithelial crypts radiate either peripherally into conjunctival stroma or circumferentially into limbal stroma. Shortt *et al.*^[85] proposed two additional niches using *in vivo* confocal microscopy; limbal crypts which are projections of limbal epithelium from the peripheral cornea into the limbal stroma, and focal stromal projections which are finger-like projections of limbal stroma with central blood vessels extending upward into the epithelium. These papillary structures offer physical protection for the deeply seated cells from injuries and shearing forces, and a large surface area that can accommodate increased cell numbers, blood vessels, and other supportive cells such as melanocytes, macrophages and stromal cells. Limbal crypts and focal stromal projections predominantly occur within regions of the cornea normally covered by the eyelids, which is a potential protec-

tive mechanism of these proposed niches^[85]. Some of the putative stem cell features such as expression of ABCG2, p63 and p75, and high nucleus-to-cytoplasm ratio have been identified in the limbal basal cells lining these papillary structures^[24,77]. In patients with limbal stem cell deficiency (LSCD), these four proposed niche structures are absent^[84,85].

Recent studies have identified stromal stem cells which are directly subjacent to limbal basal cells^[86,87]. An arising view of the limbal niche environment is that the limbal basal cells, stromal stem cells and the extracellular matrix molecules function as one unit to maintain the reservoir of ocular stem cells^[88-90]. Human limbal epithelial cells co-cultured with stromal stem cells produced colonies with average diameter five times as large as those obtained with murine 3T3 feeder layer, indicating enhanced proliferation of limbal cells in the presence of stromal stem cells^[91]. Recently, it was shown that limbal epithelial cells actively merge with stromal cells *via* chemokine receptor-mediated signalling in sphere-forming conditions, and this interaction seemed crucial for the maintenance of stem cell phenotype^[92].

Limbal stem cell deficiency

The ability of limbal cells to regenerate corneal epithelium is robust evidence for the existence of stem cells in the limbus. Limbal stem cell deficiency (LSCD) is a complex corneal disorder resulting from functional and/or anatomical loss of limbus due to chemical or thermal burn, radiation, genetic/autoimmune disorders, multiple surgeries, contact lens use, infection or drug use^[93,94]. Signs and symptoms of LSCD include conjunctivalisation, corneal vascularisation, pain, tear, redness, oedema, poor vision and blindness, which are thought to be associated with failure of epithelial regeneration^[95,96]. Similar symptoms and a delayed wound healing response could be reproduced in rabbits by surgically removing the limbus^[95,97]. The degree of loss of limbal tissue has been shown to correlate with the severity of pathology^[98]. Clinical studies have shown that LSCD can be successfully treated with application of limbal cells^[99-102]. Currently the sources of limbal cells are limbal autograft for unilateral LSCD, allogenic limbal graft from living related or cadaveric donors and *ex vivo* expanded limbal cells on transplantable substrate^[93]. The overall success rate of limbal cell transplant is estimated at 76%, ranging from 50% to 100%^[103]. The success rate varies between studies because outcome parameters, *ex vivo* expansion protocol, length of follow-up and aetiology of LSCD are different in each study^[103]. Standard corneal transplants do not appear to provide a cure for patients with LSCD^[104].

LIMBAL STEM CELL CONUNDRUMS

The body of evidence for the presence of stem cells at the limbus is impressive and convincing if largely circumstantial. The final piece of the jigsaw that remains to be revealed is the identification of an absolute stem cell

marker that is definitive of stem cell functionality. Likewise the body of evidence of the origin of epithelial cells at the limbus and their contribution to corneal epithelial homeostasis through the centripetal movement over the corneal surface has been elegantly shown by several research groups in several mammalian systems both *in vitro* and *in vivo*. However, despite this body of evidence, the proof that stem cells of the corneal epithelium reside only at the limbus and nowhere else is lacking and several pieces of knowledge remain unexplained by our current understanding of corneal maintenance by limbal stem cells:

Specificity of putative LSC indicators, criteria and markers

The traditional defining features of stem cells of the corneal epithelium include slow turnover rate, clonogenicity, proliferative potential, characteristic morphology, expression of certain proteins, centripetal migration *in vivo*, multipotency, specialised niche structures and ability to regenerate corneal epithelium. Despite the obvious biochemical changes at the cornea-limbal junction, selection of a consensus LSC marker has not been straightforward because each of these candidate markers has limitations resulting in inevitable ambiguities in separating stem cells from early progenitors^[33,105]. In fact, there is mounting evidence showing that some of the putative markers of LSCs are not unique to the limbal basal cells.

Slow turnover rate has been demonstrated by label retaining studies in animal models. However, there are several pitfalls related to the use of label retention as a marker of stem cells^[106]. The duration of the DNA labelling period was typically less than one week in most label retaining studies^[8-11]. Cells quiescent during the labelling period will not take up DNA label and never be identified by this method. On the other hand, cells that have undergone a few rounds of cell division may still show DNA label albeit at a weaker level. Furthermore, label retention is not an essential property of stem cells as stem cells such as those underlying mammalian intestinal mucosa have short cycle time^[107]. Not all label retaining cells are stem cells and vice versa.

The slow cycling property of the limbal cells has also been inferred from their resistance to 5-fluorouracil and predisposition to cancer. However, cells resistant to 5-fluorouracil are also found in the central epithelium although smaller in number than in the limbus^[14]. Predisposition to cancer is also common in cells at the transitional zone where two types of epithelia unite in non-ocular tissue systems. The endo-ectocervical and oesophagus-stomach junctions are such examples.

Clonogenicity and asymmetric division are not unique properties of the limbal cells. Central corneal cells isolated from various mammalian species including humans have been shown to form clonogenic spheres *in vitro* although the number of spheres formed was smaller than when limbal cells isolates were used^[108,109].

Asymmetric division as a means of self-renewal of

stem cells is a widely accepted concept, but is difficult to show in experimental settings, and therefore it is as yet largely hypothetical due to a lack of compelling evidence. Recent evidence suggests mitotic spindle orientation and direction of asymmetric division are under the influence of specific environmental cues from the limbus rather than intrinsic polarity^[110,111]. Possible environmental cues include growth factors, adhesion molecules and components of basement membrane that are specifically found in the limbus^[112].

In terms of morphological criteria for LSCs, different groups have reported contradictory results. The amount of melanin granules^[8,32,33], prominence of nucleoli and basal membrane invaginations^[9,32,33,73] appear to vary from study to study. The reason for this contradiction is unknown but the lack of clear morphological distinction between stem cells and TACs could be responsible. As yet, TACs cannot be distinguished from true stem cells based on cellular morphology alone.

The expression of the protein markers of the LSCs either occurs in other cell types of the ocular surface, or is subject to change depending on environmental input. Cytokeratin 19, a well-established marker of limbal basal cells is also expressed in conjunctival epithelial cells^[113]. Δ Np63 α was identified in the corneal panni excised from patients with LSCD using western blot^[114]. The free-floating spheres generated from human central corneal cells expressed Δ Np63 α and ABCG2^[109]. ABCG2 was found to be weakly expressed in the central cornea with what appeared to be an increasing gradient of expression towards peripheral cornea and finally the limbus^[109,115].

Furthermore, the link between limbal location and stem cell indicators is further compounded as several studies have indicated that the components of the niche influence the expression of LSC markers. Espana *et al*^[116] transplanted rabbit central corneal or limbal epithelial sheets onto either limbal or corneal stroma, and investigated the expression profile of two differentiation markers, cytokeratin 3 and connexin 43. Regardless of the type of epithelium transplanted, corneal stroma promoted expression of cytokeratin 3 while limbal stroma suppressed it. Expression of connexin 43 and apoptosis only occurred when corneal epithelium was cultured on corneal stroma. Li *et al*^[87] showed that when human limbal epithelial cells were co-cultured with stromal stem cells, p63 α was up-regulated and cytokeratin 12 down-regulated. The opposite expression pattern was observed when corneal fibroblasts were used instead of stromal stem cells. Kurpakus *et al*^[117] showed that bovine conjunctival cells on corneal substrate expressed the differentiation marker cytokeratin 12 only when the basement membrane was left attached to the substrate, suggesting corneal basement membrane may encourage differentiation.

Since there is not one consensus marker for LSCs, a combination of functional, morphological and immunohistochemical markers is perhaps the most useful identifier for LSCs at present. To date, the “SP” property is the only marker that has been aligned with functionality.

ABCG2-positive cells in the limbus exhibited proliferative capacity, label retention and clonogenicity. However, heterogeneity exists even within the limbal SP cells as suggested by the lack of intracellular complexities in 60% to 80% of limbal SP cells^[47].

At the time of writing this article, a newly published study in *Nature* has defined a new gene, ABCB5, as a novel limbal stem cell marker^[118]. The authors have shown ABCB5 positive cells were predominantly BrdU label retaining cells from the limbus and co-localised with Δ Np63 α in both mice and humans. Furthermore, the authors showed that ABCB5 positive cell numbers were reduced in LSC deficient patients and that ABCB5 positive cells isolated from mouse and human corneas had the ability to rescue the cornea in LSC deficient mice in both syngeneic and xenogeneic transplant models. Finally, the paper demonstrated that ABCB5 knockout mice showed disorganised corneal epithelial organisation and reduced wound healing capabilities, although bizarrely the knockout mouse was indistinguishable from wild type littermates by physical examination and contained all anterior and posterior segment components.

This appears to be the first description of a molecular limbal marker with stem cell functionality, and may be the missing jigsaw piece required to define limbal stem cells beyond doubt.

Limbus-independent corneal maintenance

A number of independent studies have challenged the long held belief that the limbus is the sole repository of stem cells in the corneal epithelium. These studies show that wound healing and normal corneal homeostasis can take place in the absence of limbus.

In 1994, Sandvig *et al.*^[119] showed that small lesions made in the rat central corneas did not evoke proliferative responses in the limbus, while medium-sized and large lesions did. This suggests wound healing of small lesions does not require limbal input. Our laboratory developed a “donut” excimer laser ablation model to demonstrate that human corneal epithelial regrowth occurs bi-directionally from both central and peripheral cornea^[115]. In our model, the cell proliferation and migration response to wounding appeared to be as rapid from the central cornea as from the limbus, with central corneal epithelial cells fully capable of corneal epithelial regeneration. When the limbus was also ablated to remove any LSCs, re-growth occurred from the remaining central corneal epithelium and extended right out to the limbus.

Corneal maintenance without limbal input has also been observed by several other researchers. Huang *et al.*^[97] created a rabbit LSCD model by performing 360° cornea-limbal peritomy. After six months, two thirds of the corneas were completely normal while one third showed mild vascularisation. Kawakita *et al.*^[120] blocked communication and migration between the limbus and the cornea by transplanting a stainless steel ring on rabbit peripheral corneas. In their study, the isolated central corneas remained free of epithelial defects for at least

six months. In a mouse LSCD model where the limbus was cauterised, the corneas remained transparent for four months^[108]. In this study, portions of athymic mice limbus were excised and replaced with limbal grafts from β -gal-ROSA26 mice whose cells were β -galactosidase labelled. After four months they observed that β -galactosidase-labelled limbal cells never migrated out of the grafts and hence made no contribution to corneal homeostasis. However, when the eyes with limbal transplants were chemically or physically wounded, the labelled cells rapidly migrated out of the graft, along with unlabelled recipient limbal cells, to create a mosaic in the resulting healed corneal epithelium.

One criticism that these studies commonly face is that their observations may be due to the result of a TAC response as the periods of observation were rather short. If stem cells do exist in the central cornea, one would expect to see long-term corneal maintenance in the animal LSCD models.

Indeed, long-term corneal maintenance in the absence of limbal input has been described in a few case reports. Some patients who had 360° LSCD were found to have normal corneas for up to 12 years^[121]. Also in LSCD patients who received *ex vivo* expanded limbal cell transplants, donor limbal cells that only lasted for 28 wk^[122] or 9 mo^[123] still resulted in the long-term restoration of the central corneal epithelium. What is maintaining the central cornea in these cases? Assuming desquamation of superficial cell layer occurs constantly, there are a few possible scenarios; (1) the amount of limbal stem cells remaining is undetectable but just enough to maintain homeostasis; (2) TACs in the basal cell layer of the central epithelium have an unexpected life span and a greater than previously thought proliferative potential; or (3) a self-renewing pool of precursor cells exist in the central cornea. Two independent groups have proposed the existence of a conceptual type of cell in the central corneal epithelium which is a TAC with more stem cell-like characteristics^[121,124]. Further research efforts are required to explore and clarify these possibilities although a TAC cell with more stem cell-like characteristics sounds uncommonly similar to a stem cell. Thus the question arises - is there a different type of stem cell that exists on the corneal surface that may be activated by different mechanisms, may serve different purposes and may be defined by different markers than the limbal stem cells?

Ex vivo expansion of LSCs on amniotic membrane

A further strong argument against the existence of stem cells in the central cornea is the absence of anatomic niche structure in the central cornea to maintain stemness. However, there is evidence for survival and self-maintenance of LSCs outside of the described limbal niches.

The most frequently used substrate for limbal stem cell expansion is human amniotic membrane, the innermost wall of the placenta consisting of an epithelial monolayer, basement membrane and avascular stroma^[125].

Isolated limbal cells, when cultivated on amniotic membrane, formed stratified epithelium much resembling cornea *in situ* and exhibited limbal stem cell phenotype such as increased expression of Δ Np63, p75, p63, ABCG2, integrin β 1, Pax6, cytokeratin 3 and 19, decreased expression of connexin 43, increased resistance to phorbol ester-induced differentiation^[126], label retention and clonogenicity^[127]. Paulkin *et al.*^[128] analysed corneal buttons from LSCD patients who had previously received limbal cell transplants on amniotic membrane. The regenerated epithelial specimens had normal stratified structures and expressed central corneal markers cytokeratin 3 and 12 but not 19. These techniques provide evidence that limbal stem cells can survive, proliferate and expand outside of their niche which has been previously thought to be necessary for LSC maintenance.

It is not fully understood how an avascular structure like amniotic membrane can maintain the phenotype and metabolic needs of the LSCs^[36,129]. The amniotic basement membrane is thought to promote adhesion, migration and differentiation of limbal epithelial cells, while amniotic stroma provide growth factors and anti-angiogenic and anti-inflammatory cytokines such as KGF, HGF, NGF, TGF- β and bFGF that prevent apoptosis and help maintain the stem cell phenotype.

Cytokine signalling is becoming increasingly recognised as a key component of a niche, regulating stem cell morphology and behaviour^[130]. The Wnt/ β -catenin signalling system has been shown to be responsible for preventing apoptosis of limbal cells *in vitro*^[131]. The authors suggested that as long as survival factors are present, limbal stem cells are likely to survive outside their niche. Indeed, in a mouse model, LSCD was successfully treated with human limbal fibroblast-conditioned culture medium but not with skin fibroblast-conditioned medium, again emphasising the importance of chemical signals produced in the limbus^[132].

There are studies which question the longevity of *ex vivo* expanded limbal epithelial cells. Li *et al.*^[66] showed progressive loss of clonogenicity and proliferative potential of limbal explant cultures on intact amniotic membrane in subsequent passages. The reason for this contradictory result is unknown but slight differences in expansion protocol and donor tissue variability might be responsible.

Furthermore, one study has proposed the existence of compound niches of cells that exist in the limbus of the mouse in unwounded corneas^[133]. However, after wounding these compound niches were able to migrate onto the surface of the cornea and express corneal epithelial cytokeratins while also retaining both features of the compound niche and features of goblet cells. This study serves to illustrate that a niche may not be an immovable structure to which cells attach but may be inherent to the cellular components and therefore able to migrate with those components.

Developmental origin of limbus

Epithelia of skin, gut wall and cornea are outer most coverings of our body and share the same developmental

origin. In all types of epithelia, with the exception of cornea, desquamated cells are replaced with newly generated cells from stem cells located in the basal layer^[8]. Only corneal epithelium is thought to be renewed from a distant repository of stem cells. This is somewhat peculiar in evolutionary sense especially when the directly adjacent conjunctiva is maintained in the same way as any other epithelia^[134].

In fetal eyes, adult LSC markers are found in the basal layer across the cornea^[135,136] and it is unknown how the markers become segregated in the limbus during development. Investigation of limbal organogenesis has raised a possibility that the limbal papillary structures are mere developmental remnants. The limbus does not develop until eyelids open and the ocular surface is exposed to amniotic fluid^[135,136]. The papillary structures of the limbus do not form until post-natal life^[137]. The question remains as to why a microenvironment essential for the support of stem cell maintenance only appears after birth and why stem cells can be maintained on the central cornea prior to birth.

CONCLUSION

A strong body of evidence has accumulated over the past few decades, showing that markers of stemness are exclusively localised at the limbus. Furthermore the centripetal migration of corneal epithelial cells after generation at the limbus has been definitively shown. Therefore, the limbus has been designated as the single repository of stem cells of the corneal epithelium. However, there is mounting evidence showing that the expression of the stem cell markers are largely determined by extrinsic signals provided by the regional microenvironment^[130,138], and the markers themselves do not indicate intrinsic stemness. As shown by the clinical success of LSC transplant on amniotic membrane in LSCD, a niche structure is not an absolute requirement for the survival of ocular stem cells, as long as the right survival signals are provided. The existence of the limbus as the sole repository of corneal epithelial stem cells also does not explain a number of clinical observations which have demonstrated corneal wound healing without limbal input and also does not explain the developmental origin of the limbus.

A vast majority of studies consider central cornea as a lineage-committed, post-mitotic tissue, but some groups have independently suggested a possibility that stem cells exist outside the limbus. Until more definitive data becomes available, the possibility of the existence of progenitor cells outside the limbus should not be excluded as central cornea may provide a new source of stem cells that can serve as a sustainable repository of high quality, evaluated, optimised tissue for the treatment of corneal degenerative disorders.

ACKNOWLEDGMENTS

We would like to thank current and previous members of the laboratory for their input into this manuscript.

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P- Reviewer: Casaroli-Marano RP, Holan V, Jhanji V, Marfe G
S- Editor: Song XX **L- Editor:** A **E- Editor:** Lu YJ



Fetal stem cell transplantation: Past, present, and future

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Supported by JSPS KAKENHI, No. 26460586(TI)

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Received: July 7, 2014 Revised: August 26, 2014

Accepted: August 30, 2014

Published online: September 26, 2014

Core tip: Based on the history of fetal stem cell transplantation since 1928, this article discusses strategies for transplantation, with a focus on donor cells, cell processing, and the therapeutic cell niche, in addition to ethical issues associated with fetal origin. We described the stream line to current clinical trials using fetal and embryonic stem cells based on Clinical. Trials. gov. Finally, we discussed the perspective of fetal stem cell transplantation.

Ishii T, Eto K. Fetal stem cell transplantation: Past, present, and future. *World J Stem Cells* 2014; 6(4): 404-420 Available from: URL: <http://www.wjgnet.com/1948-0210/full/v6/i4/404.htm>
 DOI: <http://dx.doi.org/10.4252/wjsc.v6.i4.404>

Abstract

Since 1928, human fetal tissues and stem cells have been used worldwide to treat various conditions. Although the transplantation of the fetal midbrain substantia nigra and dopaminergic neurons in patients suffering from Parkinson's disease is particularly noteworthy, the history of other types of grafts, such as those of the fetal liver, thymus, and pancreas, should be addressed as there are many lessons to be learnt for future stem cell transplantation. This report describes previous practices and complications that led to current clinical trials of isolated fetal stem cells and embryonic stem (ES) cells. Moreover, strategies for transplantation are considered, with a particular focus on donor cells, cell processing, and the therapeutic cell niche, in addition to ethical issues associated with fetal origin. With the advent of autologous induced pluripotent stem cells and ES cells, clinical dependence on fetal transplantation is expected to gradually decline due to lasting ethical controversies, despite landmark achievements.

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Key words: Fetal tissue; Fetal stem cells; Fetus, Embryonic stem cells; Transplantation; Clinical trials

INTRODUCTION

In 1988, an article reported the hopeful results of a clinical trial in which the fetal mesencephalic substantia nigra was transplanted in patients with Parkinson's disease (PD)^[1]. In the preceding year, 1987, a Chinese team had reported similar findings of fetal tissue transplantation conducted in August 1985^[2]. Following the publication of these reports, similar neural tissue transplantation procedures became widespread. Most notably, a double-blind, sham surgery controlled study of transplantation of fetal dopaminergic neurons in PD patients was reported in 2001^[3], which provided convincing data regarding the efficacy of fetal tissue transplantation for treating this condition. Since then, fetal tissue transplantation has advanced to include the clinical development of isolated fetal cells, particularly neural stem cells in business entities.

Although many review articles have focused on the application of neural tissue and/or cells in fetal tissue transplantation^[4-14], the clinical use of fetal cells is not new or simply confined to the field of neurological field. The rationale of fetal tissue transplantation lies in the potential for fetal cell proliferation and differentiation,

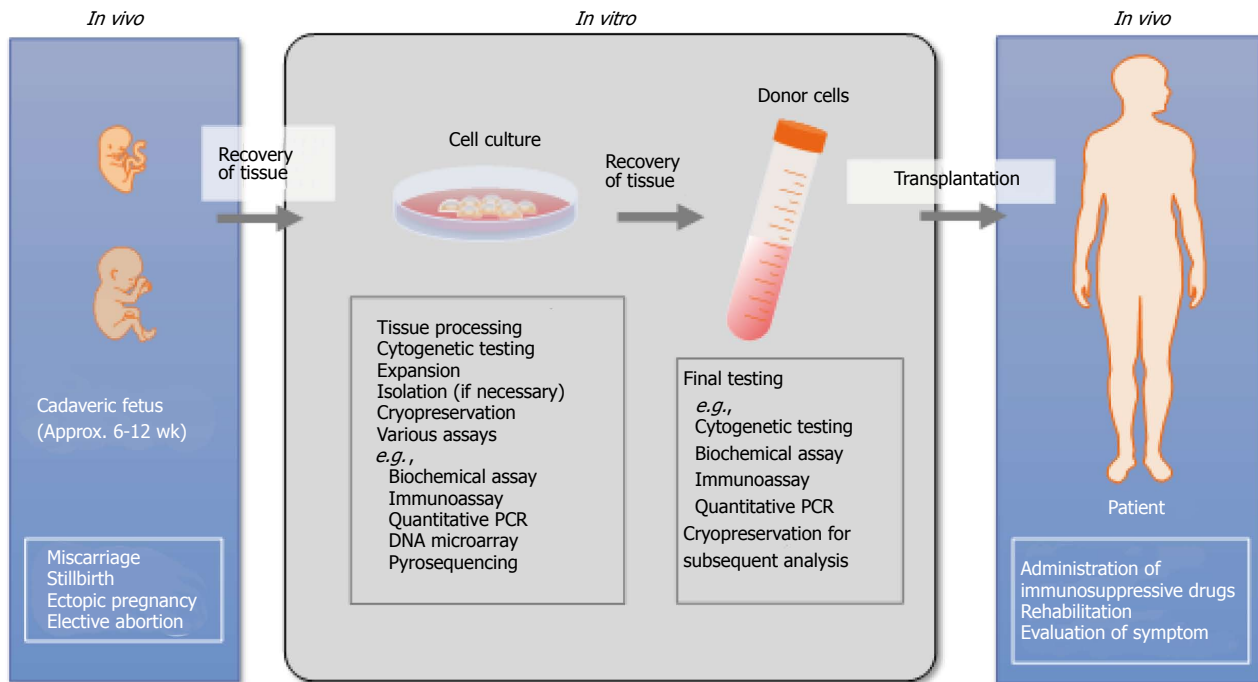


Figure 1 Fetal tissue transplantation procedures. Fetal tissue can be obtained from cadaveric fetuses for medical and non-medical reasons in obstetrics and gynecology hospitals. Procured fetal tissue, which was donated with consent for research, is processed *in vitro*, confirming cell function without contamination and genetic abnormality. After careful examination, donor cells are used for grafts primarily in the form of a cell suspension, which is usually intravenously or intraperitoneally injected or, otherwise, transplanted into predefined implant sites during surgery. Although fetal tissue cells are less likely to be rejected by transplant recipients, immunosuppressive drugs are administered in some cases. PCR: Polymerase chain reaction.

and fetal grafts may be integrated into the host without inducing immune rejection. These features of fetal tissue are well known, as is the established clinical use of transplants derived from cadaveric fetuses in the history of transplantation therapy. For example, early as 1928, a form of fetal tissue transplantation in Italy was documented in a medical journal as a treatment for diabetes mellitus^[15]. Subsequently, the indications for fetal tissue transplantation expanded to other subjects with therapeutic efficacy in conditions other than diabetes. Since the early 1960's, a tremendous number of fetal liver and thymus transplantations have been performed worldwide to treat immunodeficiency and hematological disorders.

In order to gain new perspectives on future clinical application of stem cells, it is worth considering the history of fetal tissue transplantation, taking into account an overview of current fetal stem cell research. In this report, the authors examine the history of fetal tissue transplantation, as well as many associated complications including procuring and processing fetal tissue, selecting appropriate diseases and subjects, developing new transplantation strategies, assessing graft survival and integrity *in vivo*, providing long-term monitoring of patients treated with fetal grafts for adverse events. Moreover, important ramifications of ES cell research are addressed and transplantation strategies are considered from the viewpoint of donor cells, cell processing and the therapeutic cell niche, in addition to ethical concerns. Finally, the authors provide future perspectives on fetal stem cell transplantation.

In this article, the authors offer a discussion of the progression from previous applications of fetal tissue transplantation to current uses of stem cell transplantation. In humans, the product of conception after implantation in the uterine wall through the eighth week of development is referred to as the embryo. From the ninth week to birth, the embryo is called a fetus. The authors largely follow this nomenclature.

FETAL TISSUE TRANSPLANTATION PROCEDURES

Fetal tissue contains a sufficient number of stem cells and progenitor cells for development, making it valuable for some treatments. Namely, fetal tissue cells are easier to culture and proliferate more readily than comparable adult tissue cells^[16-24], with the exception of pancreatic cells^[25,26]. Fetal tissue cells are also less likely to be rejected by transplant recipients, as these cells are less antigenic, expressing HLA-G for immune tolerance during pregnancy^[27]. This fact and the findings of animal experiments suggested a reduced need for an exact tissue match, which is frequently difficult to obtain^[28]. Collectively, the features of fetal tissue cells facilitate engraftment *in vivo* and may provide beneficial effects against diseases difficult to treat.

Fetal tissue can be obtained from cadaveric fetuses following spontaneous abortion, stillbirth, or surgery due to ectopic pregnancy in obstetrics and gynecology hospitals (Figure 1). In addition, such tissue may be derived

from elective abortions. The obtained fetal tissue is ordinarily processed and used for grafts in the form of a cell suspension, which is usually intravenously or intraperitoneally injected or, otherwise, transplanted into predefined implant sites during surgery.

PREVIOUS FETAL TISSUE TRANSPLANTATION PROCEDURES

Early attempts

A bibliographic survey revealed the use of fetal pancreatic transplantation to treat insulin-dependent diabetes mellitus, as well as an attempt to treat human cancer in Italy as early as 1928^[15]. The applied tissues were acquired from three human fetuses. Prior to this period, a diabetic dog experiment was conducted in Canada in 1921, the result of which suggested that injections of insulin, a hormone secreted from the pancreas may be used to treat diabetic patients. The following year, a clinical trial involving a 14-year-old boy with diabetes was performed; the boy recovered from his condition following insulin injections^[29]. This therapeutic achievement was awarded the Nobel Prize in Physiology or Medicine in 1923 and provided a background for the development of fetal pancreatic transplantation in Italy, as the fetal transplants may be used to circumvent the need of repeated insulin injections while offering the potential for curative therapy for diabetes. Nonetheless, this attempt eventually failed, due to a lack of treatment. Meanwhile, the first fetal pancreatic transplantation in the United States was carried out in 1939^[30]. In the clinical setting, pancreatic tissue removed from an aborted fetus was transplanted into a diabetic patient twice, albeit in vain. Subsequently, in 1959, two United States physicians reported the transplantation of fetal tissue derived from six stillborn fetuses into their diabetic mothers^[30]. However, only a transitory reduction in the need for insulin was observed in one case. Although fetal tissues are less likely to be rejected due to their reduced antigenicity, allotransplantation remained difficult until the availability of immunosuppressive drugs, such as azathioprine, in the early 1960's.

In contrast, fetal tissue was frequently used in biomedical research at that time. For instance, fetal kidney cell cultures were applied to produce large quantities of viruses, leading to the development of the polio vaccine, which was awarded the Nobel Prize in Physiology or Medicine in 1954. The application of fetal tissue cultures also contributed to the development of the rubella vaccine.

1960's to mid-1980's

The first bone marrow transplantation to treat fatal leukemia was reported by United States researchers in 1957^[31]. However, the results of marrow transplantation achieved in six patients, after first destroying their marrow with radiation, was disappointing; none of the patients survived beyond 100 d. It was not until the late 1970's when the marrow transplantation consistently resulted in success-

ful outcomes due to tissue matching, thus controlling both infectious complications and graft-*vs*-host disease (GvHD). These experiences in marrow transplantation simultaneously facilitated the development of fetal tissue transplantation, which ultimately became a frequently used therapeutic option in cases where no histocompatible donor was available for marrow transplantation.

In adult humans, hematopoiesis normally occurs in the bone marrow; however, a succession of organs sustains blood cell production during human embryogenesis^[32]. The process of hematopoiesis is initiated in the yolk sac during the third week of development, then subsequently relayed to the liver, thymus, and bone marrow at the 11th week, at which time stabilization of definitive post-natal hematopoiesis begins. Most elective abortions are performed during the first trimester. In this era, clinical availability of fetal liver and thymus tissue has encouraged researchers to performed transplantation to treat hematological disorders and cases of severe immunodeficiency.

In 1958, it was reported that a devastated immune system in rodents was restored by inoculating fetal hematopoietic tissue following lethal total body X-irradiation^[33]. In 1961, a United Kingdom group reported the results of transplantation of fresh or stored fetal liver cells ($1-20 \times 10^9$ /case, gestational age unknown) *via* intravenous injection to treat aplastic anemia, stating that remission was achieved in two of 14 patients (18 mo to 55 years of age)^[34]. Similar findings were subsequently reported from China^[35,36], Hungary^[37], India^[38-41], Italy^[42-44], and United States^[45,46].

In 1975, a United States group reported successful fetal liver transplantation in a male infant (3 mo of age) with adenosine-deaminase (ADA) deficiency, which causes severe combined immunodeficiency (SCID)^[47]. In that case, an 8.5-wk-old embryo was obtained, with permission from a mother undergoing termination of pregnancy and sterilization with hysterectomy. A suspension containing 2.5×10^8 liver cells was injected into the recipient intraperitoneally, who developed immunocompetent T and B cells in an orderly manner until one year after the procedure, when he died of fatal nephrotic disease. Soon after that case, a United States group reported the results of transplantation of fresh fetal liver cells (obtained from 8-, 9-, and 10-wk-old fetuses) in two infants with SCID in 1976^[48]. Although no functional immunological improvements were achieved in the first infant, both clinical and functional immunological improvements were noted in the other patient, who was monitored for 19 mo after transplantation. In that case, the engraftment of fetal cells, as confirmed by chimerism in the recipient's lymphocytes, reversed the patient's immunodeficiency. Similar treatment of ADA-SCID was also reported by a Japanese group in 1985^[49]. In addition, according to a case report published in 1985, a patient with X-linked SCID whose parents and siblings were not suitable HLA-compatible bone marrow donors underwent, embryonic liver cells were transplanted intravenously in 3 stages ($6 \times 10^6 - 9 \times 10^7$)^[50]. Although the procedure resulted in T-cell recon-

stitution in addition to the initiation of immune globulin production, the child died at five months of age due to respiratory failure. In another SCID case reported by a French group in 1979, an infant who received two separate grafts of both hepatic and thymus cells recovered from the same fetus exhibited a partially restored immune system^[51].

Fetal liver transplantation has also been attempted to treat leukemia. In 1982, an Italian group reported the use of fetal liver transplantation in two patients with acute leukemia following the administration of a conditioning regimen consisting of cyclophosphamide and total body irradiation^[52]. Although each patient achieved remission with a hematopoietic recovery, the survival time after transplantation was only 153 and 30 d, respectively. A similar transplantation procedure was subsequently conducted to treat acute myeloid leukemia in India^[53]. In 1986, a Chinese group reported the results of fetal liver transplantation in 10 patients with malignant tumors^[54]. The authors prepared fetal liver cells using 3.5-6-mo-old fetuses and observed 1.8×10^8 - 4×10^{12} fetal liver cells in a fetus over five mo of age, in which most of the cells were are CFU-Cs (granulocyte progenitor cells). These findings suggest that fetal liver transplantation improves the peripheral blood profile and stimulates the production of bone marrow.

In February 1986, a symposium on fetal liver transplantation was held in New-Delhi, India^[55]. A relevant review article critically analyzed progress in the field at that time and reported that over 300 individuals had received fetal liver transplants for a spectrum of disorders, including immunodeficiency, aplastic anemia, leukemia and genetic conditions. Additionally, in a review article published in 1987, a United States researcher, Gale, examined the results of fetal liver transplantation in patients with hematological disorders^[46]. With respect to aplastic anemia, 122 two patients received transplants, with engraftment reported in four patients and GvHD in no cases. Although complete and partial responses were reported in half of the patients, the majority displayed no evidence of engraftment. Meanwhile, 39 patients with leukemia received transplants; transient engraftment was reported in 40% of cases, and two patients developed GvHD. In that report, the survival was extended to more than two years. The relatively high rate of engraftment also suggested the efficacy of pretransplant immune suppression. Therefore, the risk of GvHD appears to be low, despite complete HLA-mismatching.

Regarding thymus transplantation, two cases were reported in 1968, in which fetal thymus tissue was transplanted into neonates suffering from DiGeorge syndrome, which is characterized by the absence or incomplete development of the thymus with varying degrees of T-cell immunodeficiency^[56]. In addition, August *et al*^[50] reported the case of a 21-mo-old male with DiGeorge syndrome who underwent transplantation of thymus fragments derived from a 16-wk-old female fetus. In that case, abnormalities in the patient's lymphocyte function

were promptly ameliorated. Cleveland *et al*^[57] also reported the implantation of three thymus fragments derived from a 13-wk fetus into a 7-mo-old male infant. Although no XX cells were identified in the host, the infant's immunological data and ability to resist infection suggested that his immunological function was reconstituted by the fetal transplants^[57]. Another article reported that the combined transplantation of the fetal thymus and liver resulted in effective immunological reconstitution in a presumed case of DiGeorge syndrome^[58]. Two similar thymus transplantation procedures were performed in Japan^[59,60].

During this period, various cases of fetal tissue transplantation were reported in medical journals. However, the clinical results and patient survival rates were largely dismal. At that time, most fetal tissue transplantations were conducted based on previous experience with bone marrow transplantation in which irradiation-based or chemical conditioning is performed prior to transplantation in order to facilitate post-transplantation engraftment following the administration of immunosuppressive drugs. However, cellular characteristics of fresh or preserved fetal tissue were insufficient in most cases, with total cell count usually being the only parameter reported, while the cell functions was not thoroughly assessed. Moreover, in general, precautions measures to prevent infectious diseases were not taken. For example, fetal tissue donors were not carefully screened, and testing of fetal tissue prior to transplantation was largely insufficient. Despite clinical success in some cases, the use of fetal liver and/or thymus transplantation should have been based on sufficient data from preclinical research using disease model animals, as is common in current stem cell research.

Mid 1980's to early 2000's

Around the mid-1980's, the application of fetal neural transplantation to treat neurological diseases began to receive significant attention. In this era, clinical trials using fetal cerebral tissue were conducted worldwide primarily in patients with Parkinson's disease (PD), a progressive disorder of the central nervous system that affects movement. PD is characterized by the death of dopaminergic neurons, the substantia nigra in the brain for unknown reasons. Langston *et al*^[61] identified a chemical, MPTP (1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine), that selectively damages cells in the substantia nigra, resulting in the development of marked parkinsonism in monkeys and humans, and the injection of MPTP can be used to create an animal model of PD. Preclinical research using such animals has demonstrated that transplanting the fetal substantia nigra significantly improves motion symptoms^[62-65]. Although L-Dopa therapy has been applied to PD since the 1960's, this medication induce troublesome side effects, such hypotension and a variety of abnormal involuntary movements^[66]. Therefore, the transplantation of fetal neural tissue, including dopaminergic neurons, is thought to be an alternative treatment for PD.

In addition to preclinical research using animal dis-

ease models, fetal neural tissue transplantation was performed based on preclinical data, including the impact of cryopreservation^[67], and screening for infection and cytogenetic abnormalities^[68]. Regarding the *in vivo* survival of fetal tissues and cells, Freeman *et al.*^[69] reported the implantation of human mesencephalic dopaminergic neurons in a rat model and suggested that the upper age limit should be postconception (PC) day 56 for suspension grafts and PC day 65 for solid implants.

In September 1986, a Mexican group conducted a renowned clinical trial in which the fetal mesencephalic substantia nigra procured from a 13-wk-old fetus of spontaneous abortion, was transplanted in the caudate nucleus in two PD patients. The cases were subsequently reported in 1988^[1], and the results of monitoring at three months showed a dramatic improvement in symptoms; in particular both rigidity and dyskinesia disappeared^[70]. In the preceding year, 1987, however, a Chinese team had already reported the transplantation of similar fetal tissue in a PD patient in August 1985, the first clinical trial in which brain tissue was transplanted from one human being to another^[2]. In that case, a suspension containing substantia nigra fragments was implanted into the striatal caudate nucleus to which a collateral projection extends from the substantia nigra. The case involved a 54-year-old male patient whose HLA status was determined prior to transplantation, although the fetal HLA status was not tested. The transplanted tissue was obtained from a 5-mo-old fetus, as the authors considered the clinical use of the substantia nigra derived from fetuses of 4.5-5.5 mo of age to be appropriate based on the stage of tissue development at that age. However, this presumption was inaccurate compared to the evidence (in embryos up to nine weeks of age) provided by Freeman *et al.*^[69]. However, the Chinese team reported a reduction in limb tremors and rigidity on the third day after the surgery, with satisfactory control of parkinsonism confirmed after eight months of diagnostic monitoring. Moreover, a United Kingdom group published a case report of fetal tissue transplantation for PD in 1988^[71]. The authors stated that two patients (a 60-year-old female and 41-year-old male) with early and late parkinsonism, respectively, showed immediate improvements in motion symptoms following the administration of a mesencephalic cell suspension (fetal age unknown). These cases, with the exception of the China case, made worldwide headlines, commanding considerable attention from patients and their families. However, all three cases lacked comprehensive, long-term results, including the findings of behavioral, biochemical, psychological, physiological, and motor assessments.

Subsequently, a Swedish group demonstrated that deep brain transplantation of fetal brain tissue could be used to restore local dopamine production and relieve symptoms^[72]. According to their report published in 1990, mesencephalic dopamine neurons derived from embryos of eight to nine weeks of gestation exhibited survival in the recipient. The grafts, which were implanted unilaterally into the putamen *via* stereotactic surgery,

restored dopamine synthesis and storage in the grafted area, as assessed on positron emission tomography with 6-L[18F]fluorodopa. These neurochemical changes resulted in a significant reduction in severe rigidity and bradykinesia, with marked diminution of fluctuations in the patient's condition under optimal medication. Following this achievement, long-term (up to 46 mo) stable improvements and graft integrity were reported in various cases^[73-76]. Stable integration and the persistence of fetal grafts have also been confirmed on functional imaging as well as postmortem analyses^[77-79]. Such clinical results have encouraged many researchers worldwide to apply this therapeutic approach as a treatment for PD. Namely, fetal brain tissue transplantation, which began in China^[2,80], has been attempted in Canada^[81], Cuba^[82], the Czech Republic^[83], France^[84], Mexico^[1], Poland^[85], Slovakia^[86], Spain^[87-89], United States^[2,3,73,77,90,91], and USSR (currently Russia)^[92]. Moreover, the Network of European CNS Transplantation and Restoration (NECTAR) was founded in 1990 to bring together European groups who share the common goal of protecting, repairing and restoring the central nervous system damage resulting from degenerative diseases and/or injury^[93].

Fetal tissue transplantation for PD has also been conducted using fetal adrenal medullary tissue^[1,80] other than the substantia nigra, and several clinical trials have assessed the efficacy of fetal neural transplantation for neurological conditions other than PD. For instance, patients suffering from Huntington's disease (HD) have been evaluated in the United Kingdom^[94]. In the report, cell suspensions of fetal ganglionic eminence were transplanted unilaterally into the striatum in four patients with early to moderate HD, all of whom received immunotherapy with cyclosporin A, azathioprine, and prednisolone for at least six months postoperatively. During the six month post-transplantation period, the only adverse events related to the procedure were associated with the immunotherapy regimen. Magnetic resonance imaging demonstrated the presence of tissue at the implantation site, although no signs of tissue overgrowth were detected. The United Kingdom team concluded that the unilateral transplantation of fetal striatal tissue in patients with HD is safe and feasible. Meanwhile, an Indian group issued a report in which human fetal neuroretinal cells were transplanted in patients with advanced retinitis pigmentosa^[95]. The results of a long-term phase I safety study (12-40 mo) prompted the initiation of phase II trials.

Notably, in 2001, a United States group reported the results of a double-blind, sham surgery controlled-study of transplantation of fetal dopamine neurons in PD patients^[5]. The neural tissues were recovered from 7- to 8-wk-olds embryos, and the tissue cell culture, in which dopamine production was monitored according to homovanillic acid concentration in the medium, was transplanted up to four weeks after recovery. Consequently, a reduction in motor symptoms was observed in the patients 60 years of age or younger, but not in the older

patients. This study provided the first direct evidence that fetal grafts can be used to improve the condition of some PD patients, separate from the placebo effect. Another United States group reported the results of a similar double-blind controlled trial in which, approximately half of the patients treated with solid mesencephalic grafts derived from 6- to 9 wk-old embryos developed dyskinesia, with no significant overall treatment effect^[96]. Moreover, postmortem analyses revealed the subjects who displayed significant improvements had at least 100000 dopaminergic neurons per sides with organotypic reinnervation of the striatum^[97,98]. In these cases, four 6- to 9-wk-old fetuses were required to obtain the requisite number of cells for a graft. Therefore, some research groups have introduced a temporal moratorium on such procedures since 2003 owing to the uncertainty and difficulty in conducting clinical trials^[99].

ETHICAL ISSUES AND POLITICAL RESPONSES

As mentioned above, the results of fetal brain tissue transplantation for PD have received significant attention, making worldwide headlines in the news media since the late 1980's. Such advancements have simultaneously raised profound ethical concerns and objections against the medical use of cadaveric fetal tissue, which is frequently derived from cases of elective abortion. This report briefly addresses this issue and associated political responses. The ethical debate in the United States, which involves anti-abortion movement, led to a moratorium on federal funding (1987-1992) of fetal tissue transplantation research^[100-103]. There are five issues related to fetal tissue transplantation. First, females may be advised or persuaded to undergo induced abortion on the grounds that it may help others by donating fetal tissue. Second, the widespread use of fetal tissue transplantations may result in an increase in the number of abortions. Third, the successful use of fetal tissue may make such procedures more socially acceptable. Fourth, the abortion procedure may be changed based on medical needs. Most notably, the question as to whether rightful informed consent for the use of fetal tissue can be obtained in cases of induced abortion is the most controversial issue. Ideally, the decision to undergo induced abortion should be completely separate from the consent to fetal tissue donation (*i.e.*, the "principle of separation"^[104]).

In the United Kingdom, the Department of Health and Social Security issued a report by their advisory group (the Peel report) regarding the use of fetuses and fetal material for research in 1972. The Department subsequently reviewed their guidelines on the research and use of fetuses and fetal material in the Polkinghorne report in 1989^[105]. The main issue in that report was the consistent application of the "principle of separation"^[104]. In contrast, the British Medical Association (BMA) dissented from the Polkinghorne report in their guidelines on the use of fetal tissue^[106], and United Kingdom sociol-

ogists expressed concerns about the fetal tissue economy from the abortion clinic to the stem cell laboratory^[107, 108]. In 1994, NECTAR considered the ethical issues and published guidelines for the use of human embryonic or fetal tissue for research and clinical use^[109]. In Japan, discussions in the Ministry eventually resulted in the development of guidelines on clinical research using human stem cells, in which the clinical use of fetal cells is intentionally excluded due to potential, profound ethical issues^[110].

Thus, fetal tissue transplantation has raised ethical controversy worldwide. The "principle of separation" suggests fetal tissue can be procured if informed consent is separately obtained from females who underwent spontaneous abortion and still birth in Europe and the United States. It is also suggested that fetal tissues may be obtained if the induced abortion is conducted for a clear medical reason (*e.g.*, ectopic pregnancy). However, if the informed consent is obtained from females who will undergo induced abortion only for a social reason, some would query the validity of the informed consent^[107]. The difficulty in the 'principle of separation' in some cases is likely to lead to the exclusion of fetal cells in stem cell transplantation, as in Japan if future results of fetal tissue transplantation are overhyped.

CHANGES WITHIN THE LAST DECADE

Since 2000, fetal cell transplantation has advanced to the clinical development of isolated fetal stem cells. As mentioned above, there are hundreds of investigator-initiated clinical trials of fetal transplantation in the academic setting. In addition, several companies have developed or are developing fetal stem cell products *via* the use intracerebral or spinal transplantation^[111].

A wide variety of conditions have been assessed using fetal stem cell transplantation. Recently, evaluated conditions can be categorized into six groups: neurological diseases, central nervous system (CNS) injury, heart failure^[112], diabetes^[113], skin wounds^[114], and osteogenesis imperfecta^[115]. Neurological diseases include amyotrophic lateral sclerosis (ALS)^[116-118], cerebral palsy (CP)^[119,120], cerebral atrophy^[121], Huntington's Disease^[122,123], and PD^[124-126]. With respect to CNS injury, spinal cord injury (SCI)^[127] and traumatic brain injury^[128] have been recent topics in the setting of fetal cell transplantation. Some of these reports are described below.

Olfactory ensheathing cells (OECs) are radial glia with a variety of functions. These cells phagocytose axonal debris and dead cells in the olfactory system^[129]. OECs are also known to secrete many neurotrophic factors. A Chinese group, Chen *et al.*^[120] conducted a randomized controlled clinical trial among 33 patients in order to confirm the feasibility of OEC transplantation for treating CP in children and adolescents. In that report, OECs were isolated from aborted human fetal olfactory bulbs, cultured and propagated for two to three weeks and then characterized using immunostaining with Abs against p75. OECs derived from one to two fetuses, representing

two million cells, were transplanted in each patient, and HLA-DR-matching analyses ensured histocompatibility between the donors and recipients. The trial ultimately demonstrated that fetal OEC transplantation is effective for obtaining functional improvements in children and adolescents with CP, without obvious side effects. Another Chinese group, Wu *et al.*^[127] followed patients with complete chronic SCI for an average of 14 mo after OEC transplantation. Consequently, both sensation and spasticity improved moderately, whereas the recovery in locomotion recovery was minimal. In contrast, Piepers and den Berg asserted that there are no benefits from experimental treatment with fetal OECs in patients with ALS^[118]. The authors carried out a prospective study of seven patients who underwent fetal OEC treatment in China^[130], following the subjects for four months to one year after treatment, and found no objective improvements, while the outcome measurements gradually declined in all patients. Two patients experienced severe side effects. Therefore, although careful examination is needed, fetal OEC transplantation is likely to be effective against trauma-induced neurological conditions, but not ALS or the selective degeneration of motor neurons. These findings highlight the significance of selecting appropriate diseases and conditions for each type of stem cell transplantation.

Regarding fetal neural progenitor cells (NPCs) and neural stem cells (NSCs), a Chinese group, Luan *et al.*^[119] performed fetal NPC transplantation in 45 patients with severe CP by injecting NPCs derived from aborted fetal tissue into the lateral ventricle. The NPCs were isolated from aborted human fetal forebrain tissue and likewise propagated. The cells used for transplantation were characterized as nestin-positive and microbe-free with normal karyotype, viability of over 95%, and endotoxin level below 2 EU/mL. After one year, the developmental level for each functional sphere (gross motor, fine motor, and cognition) was significantly higher in the treatment group than in the control group, with no delayed complications. Therefore, both fetal NPC and OEC transplantation appear to be efficacious against CP^[119,120]. A United States group, Grass *et al.*^[117], consequently reported the results of a phase I trial of the intraspinal injection of fetal NSCs in patients with ALS. This study was a first-in-human clinical trial with the goal of assessing the safety and tolerability of introducing stem cells into the spinal cord, in association with the administration of immunosuppressants. Twelve patients received either five unilateral or five bilateral (10 total) injections into the lumbar spinal cord at a dose of 100000 cells per injection. Clinical assessments ranging from six to 18 mo after transplantation demonstrated no evidence of acceleration of disease progression due to the intervention; therefore, the goal of the clinical trial was attained. Hence, ALS may be treated with fetal NSC transplantation, but not fetal OEC transplantation^[117,118].

In addition to the above bibliographic survey, relevant trials were searched on ClinicalTrials.gov in order

to provide an overview of recent clinical trials of fetal transplants (Table 1). Consequently, 11 trials were identified, most of which (7/11) were sponsored by business entities. In addition, fetal neural stem cells were used in most trials (8/11), focusing on ischemic stroke, SCI, age-related macular degeneration, and neurological disorders, including ALS and Pelizaeus-Merzbacher disease (an inherited dysmyelination disorder). Meanwhile, fetal mesencephalic tissue or dopamine neuronal precursor cells were used for transplantation in PD patients in two trials and fetal liver cells were used in one trial. Most of these studies (7/11) were sponsored by private companies, including Stem Cell, Inc. (California, United States), Neuralstem Inc. (Maryland, United States), and ReNeuron Ltd. (United Kingdom). Stem Cell Inc. has developed a neural stem cell product for use in Batten's disease (neuronal ceroid lipofuscinosis) and obtained approval for a new investigational drug (IND) from the FDA, although a phase I trial was terminated due to difficulties in recruiting an adequate number of patients. Instead, the company opted to focus on thoracic SCI, age-related macular degeneration, congenital Pelizaeus-Merzbacher disease for clinical development. Other companies are currently developing neural stem cell products to treat stable ischemic stroke (ReNeuron) as well as ALS and chronic SCI (Neuralstem Inc.).

Among the above companies, Stem Cell, Inc. is the most active developer of fetal neural stem cells. For example, it has generated unique mAbs and isolated neural stem cells derived from fetal brain tissue using cell sorters. The company has identified and enriched CD133⁺ CD24^{-/lo} population cells using their unique mAbs against CD133 and CD24. The transplantation of CD133⁺ sorted/expanded neurosphere cells into the lateral ventricle in newborn NOD-SCID mouse brains has been shown to result in specific engraftment in numerous sites, according to the levels of brain markers. The researchers therefore concluded that human central nervous system (CNS) stem cells can be clonally isolated^[153]. Using CNS stem cells, the company is currently developing stem cell products for use in patients with SCI, macular degeneration, and Pelizaeus-Merzbacher disease. In most cases, de novo neurogenesis is not the goal, but rather the treatment of enzyme deficiencies, as well as remyelination, or the modulation of endogenous repair *via* neoangiogenesis and/or neuroprotection^[131-134]. Moreover, the company has isolated fetal liver progenitor cells and developed a unique co-culture system with endothelial cells in a three-dimensional matrix^[135]. These liver cells are studied for the future application of transplantation therapy and drug discovery assay systems.

Recent fetal stem cell transplantation procedures have used isolated and well-characterized fetal tissue cells designed in a sufficiently rational manner. Clinical trial results also allow researchers to be optimistic about the future of fetal stem cell transplantation. Nevertheless, uncertainties abound in the clinical settings. Amariglio *et al.*^[136] reported an adverse event following NSC trans-

Table 1 Ongoing clinical trials of fetal stem cell transplantation

Clinical Trials.gov	Start (yr)	Sponsor	Status	Title	Interventions	Cell source
NCT 01013194	2007	The Mediterranean Institute	Unknown	Human fetal liver cell Transplantation in chronic liver failure	Human fetal liver cell transplantation	Fetal liver cells derived from fetuses between the 16 th and 26 th week of gestation
NCT 01151124	2010	ReNeuron Limited.	Active, not recruiting	Pilot Investigation of Stem Cells in Stroke	Surgical delivery of a neural stem cell line to the damaged area of the brain	CTX0E03 neural stem cells ¹
NCT 01321333	2011	StemCells, Inc.	Active, not recruiting	Study of HuCNS-SC in patients with thoracic spinal cord injury	Intramedullary spinal cord transplantation of human CNS stem cells	HuCNS-SC cells (Human Central Nervous System Stem Cells) ²
NCT 01348451	2009	Neuralstem Inc.	Active, not recruiting	Human neural stem cell Transplantation for the Treatment of amyotrophic lateral sclerosis	Surgical implantation of human neural stem cells	Human spinal cord derived neural stem cells ³
NCT 01391637	2011	StemCells, Inc.	Active, not recruiting	Long-term follow-up study of human stem cells transplanted in subjects with pelizaeus-merzbacher disease	HuCNS-SC transplantation	HuCNS-SC cells (Human Central Nervous System Stem Cells) ²
NCT 01632527	2012	StemCells, Inc.	Recruiting	Study of HuCNS-SC in age-related macular degeneration	Transplanting HuCNS-SC cells directly into the subretinal space of one eye.	HuCNS-SC cells (Human Central Nervous System Stem Cells) ²
NCT 01640067	2011	Azienda Ospedaliera Santa Maria	Recruiting	Human neural stem cell Transplantation in Amyotrophic Lateral Sclerosis	Surgical microinjection of human neural stem cells	Human foetal neural stem cells suspension
NCT 01730716	2013	Neuralstem Inc.	Enrolling by invitation	Dose escalation and safety study of neural stem cell transplantation for the treatment of amyotrophic lateral sclerosis	Human spinal cord stem cell implantation	Human spinal cord derived neural stem cells ³
NCT 01772810	2014	Neuralstem Inc.	Not yet recruiting	Safety study of human spinal cord-derived neural stem cell transplantation for the treatment of Chronic SCI	Human Spinal Cord-derived Neural Stem Cell Transplantation	Human spinal cord derived neural stem cells ³
NCT 01860794	2013	Bundang CHA Hospital	Recruiting	Evaluation of safety and tolerability of fetal mesencephalic dopamine neuronal precursor cells for Parkinson's disease	Transplantation of fetal mesencephalic dopamine neuronal precursor cells	Fetal mesencephalic dopamine neuronal precursor cells
NCT01898390	2012	University of Cambridge	Enrolling by invitation	TRANSEURO open label transplant study in Parkinson's disease	Neural allo-transplantation with fetal ventral mesencephalic tissue	Fetal ventral mesencephalic tissue

The survey was conducted in ClinicalTrials.gov using key words “fetal + transplantation, or fetus + transplantation”. The status is on June 24, 2014. The description of the table is based on the database. See also the details by entering the identifier No. into the database website. Additional investigation confirmed that the neural stem cells¹ are derived from first trimester human fetal cortical cells, the central nervous system stem cells² from fetal brain tissue, and the spinal cord derived neural stem cells³ from a single eight-week-old fetus.

plantation in a boy with ataxia telangiectasia treated with the intracerebellar and intrathecal injection of human fetal NSCs. Four years after the first injection, the patient was diagnosed with a multifocal brain tumor, a biopsy of which showed a glioneuronal neoplasm. In addition, molecular and cytogenetic studies demonstrated the tumor to be of nonhost origin and microsatellite and HLA analyses revealed that the tumor was derived from at least two donors. This is the first report of a human brain tumor complicating the outcome of NSC therapy. These findings suggest that neuronal stem/progenitor cells may induce gliomagenesis. Therefore, considerable caution is required when implementing NSC transplantation, although clinical trials of NSCs are proceeding worldwide.

RAMIFICATIONS OF EMBRYONIC STEM CELL RESEARCH

The results of previous fetal neural transplantation therapy for PD have indicated that the use of more biologically defined and clinically reliable sources of dopaminergic neurons is required in future clinical trials. For this reason, other stem cell sources are often investigated in parallel with clinical trials of fetal stem cell transplantation.

Pluripotent ES cells are established from preimplantation, not implantation, embryos. ES cells possess self-renewal properties and almost infinitely proliferate in petri dishes. In addition, under appropriate differentiation protocols, ES cells exhibiting pluripotency can be differ-

entiated into any lineages of the ectoderm, mesoderm, or endoderm. Therefore, ES cells can be used to obtain the number of cells required for transplantation therapy for various diseases.

Two reports regarding the establishment of mouse ES cell lines were published in 1981^[137,138]. The first derivation of human ES cell lines was based on knowledge obtained *via* the establishment of non-human primate ES cells, first attained in 1995^[139,140]. It took a considerable amount of time to transition from mouse to human ES cells due to differences in molecular and cellular mechanisms between mice and humans that hampered the technical establishment of the culture method. For instance, human ES cells, unlike their mouse counterparts, do not appear to require leukemia inhibitor factor (LIF) for propagation or the maintenance of pluripotency^[140,141]. Instead, fibroblast growth factor (FGF) signaling has a central role in the self-renewal of human ES cells. It has been previously demonstrated that basic FGF (bFGF) stimulates the clonal growth of human ES cells on fibroblasts in the presence of a commercially available serum replacement^[142]. In addition, while the expression of many of markers is similar in mouse and human ES cells, significant differences are noted in the expression levels of vimentin, β -III tubulin, alpha-fetoprotein, eomesodermin, HEB, ARNT, FoxD3, and the LIF receptor complex LIFR/IL6ST (gp130)^[143]. Furthermore, focused microarray analyses have identified significant differences in cell cycle and apoptosis regulation as well as cytokine expression^[143].

Human ES cells which were first reported in 1998 were established from surplus *in vitro* fertilization (IVF) embryos, a byproduct of assisted reproduction treatment. The creation of embryos for research purposes, which is associated with ethical issues and requires rigorous reviews in many countries even if legally permitted^[144], was not conducted to establish the ES cells. Nonetheless, an ethical debate ensued, as some regard preimplantation embryos to constitute the beginning of human life. Meanwhile, in 2009, the United States FDA approved an IND applied by the Geron Corporation (California, United States)^[111]. The biologics of human ES cell-derived cells was developed in the first clinical trial after the company verified that there were no problems with the cell product regarding the formation of micro-cysts in animal transplants. The approved phase I study was conducted to assess the safety of transplantation of human ES cell-derived oligodendrocyte precursor cells in patients with thoracic spinal cord injury. In that study, the subjects with functionally complete spinal cord injury at the T3 to T10 spinal segments underwent grafting of oligodendrocyte progenitors into the spinal cord at the site of injury under conditions of immunosuppression. Although Geron terminated the study for financial reasons in 2011, another company plans to restart the trial^[145].

Current clinical trials of ES cells (Table 2) include at least eight trials of ES cell-derived cells underway in France, South Korea, United Kingdom, and the United

States. Again, most of these studies are being sponsored by business entities (6/8). Namely, Advanced Cell Technology (ACT), Inc. (Massachusetts, United States) is currently developing ES cell-derived retinal pigment epithelium cells to treat conditions such as age-related macular degeneration and macular dystrophy using an orphan drug status to accelerate clinical trials. In addition, CHA Bio and Diostech (South Korea) is advancing two pipelines similar to that of ACT using the cell product developed by ACT. Pfizer is also currently developing a similar pipeline to that of ACT and CHA Bio and Diostech; however, Pfizer is using a different cell product. Hence, macular generation is the primary condition currently receiving attention with respect to the development of ES cells. The remaining two trials are being sponsored by French and United States universities. UCLA is attempting to initiate a clinical trial in which ACT's cell product applied to treat macular regeneration, while Assistance Publique - Hôpitaux de Paris is recruiting patients to develop a treatment for ischemic heart disease using ES cell-derived CD15⁺ Isl-1⁺ progenitors. All of these trials are open-label, not blind, studies. More recently, the use of autologous ES cells, which reduces the possibility of immune rejection, has recently become realistic based on somatic cell nuclear transfer^[146,147]. Clinical success rates of transplantation using autologous ES cell-derived cells would be expected to increase, although there is a potential ethical issue when procuring oocytes from females.

Another type of pluripotent stem cell, embryonic germ (EG) cells, can be established from cultured human primordial germ cells (PGCs) derived from early embryos. The first establishment of human EG cells from 5-to 9-wk-old embryos obtained as a result of the therapeutic termination of pregnancy, was reported in 1998^[148], followed by other reports^[149]. However, knowledge of human PGCs and EG cells is insufficient, as these cells are difficult to study in the gonadal ridge during the fifth and sixth week of development, with further PGCs often being detected in the gut mesentery, most likely during transit^[149]. To our knowledge, there have been no clinical trials of human EG cells.

FUTURE DIRECTIONS OF STEM CELL TRANSPLANTATION

In the 20th century, clinical issues abounded in the field of fetal tissue transplantation and many lessons were learned from such practices. After reflecting on the history of fetal tissue cell transplantation, this report will now consider the future direction of stem cell transplantation based on issues related to donor cells, cell processing, and therapeutic cell niche.

Donor cells

Earlier fetal tissue cell transplantation procedures required careful screening of maternal donors and testing of fetal tissues in order to prevent infectious diseases as well as match histocompatibility; however, such analyses

Table 2 Ongoing clinical trials of embryonic stem cell-derived cell transplantation

Clinical Trials.gov	Start (yr)	Sponsor	Status	Title	Condition	Intervention	Remarks
NCT01344993	2011	Advanced cell technology	Recruiting	Safety and tolerability of transplantation of MA09-hRPE cells in patients with advanced dry age related macular degeneration	Advanced dry age related macular degeneration	Sub-retinal transplantation of MA09-hRPE	A Phase I / II, open-label, multi-center, prospective study in United States. MA09-hRPE cells are human embryonic stem cell derived retinal Pigmented epithelial cells.
NCT01345006	2011	Advanced cell technology	Recruiting	Transplantation of MA09-hRPE cells in patients with stargardt's macular dystrophy	Stargardt's macular dystrophy	Sub-retinal transplantation of MA09-hRPE	A Phase I / II, open-label, multi-center, prospective study in United States
NCT01469832	2011	Advanced cell technology	Recruiting	Safety and tolerability of transplantation of hESC-RPE cells in patients with stargardt's macular dystrophy	Stargardt's macular dystrophy; fundus flavimaculatus; juvenile macular dystrophy	Sub-retinal transplantation of MA09-hRPE	A Phase I / II, open-label, multi-center, prospective study in the United States
NCT01625559	2012	CHA Bio and diostech	Recruiting	Safety and tolerability of MA09-hRPE cells in patients with stargardt's macular dystrophy	Stargardt's macular dystrophy	Sub-retinal transplantation of MA09-hRPE	A Phase I, open-label, prospective study in Korea
NCT01674829	2012	CHA Bio and diostech	Recruiting	Safety and tolerability of transplantation of MA09-hRPE cells in patients with advanced dry age-related macular degeneration (AMD)	Dry age Related macular degeneration	Sub-retinal transplantation of MA09-hRPE	A Phase I / II a, open-label, single-center, prospective study in Korea
NCT01691261	2014	Pfizer	Not yet recruiting	Implantation of human embryonic stem cell derived retinal pigment epithelium in subjects with acute wet age related macular degeneration and recent rapid vision decline	Age related macular degeneration	Implantation of human embryonic stem cell derived retinal pigment epithelium	Phase 1, open-label, safety and feasibility study in United Kingdom. PF-05206388 is human embryonic stem cell derived retinal pigment epithelium living tissue equivalent.
NCT02057900	2013	Assistance publique - hôpitaux de Paris	Recruiting	Transplantation of human embryonic stem cell-derived progenitors in severe heart failure (ESCORT)	Ischemic heart disease	Human embryonic stem cell-derived CD15+ Isl-1+ progenitors	Phase 1, open-label, feasibility and safety study in France
NCT02122159	2014	University of California, Los Angeles	Not yet recruiting	Research with retinal cells derived from embryonic stem cells for myopic macular degeneration	Myopic macular degeneration	MA09-hRPE cellular therapy	A Phase I / II, Open-label, prospective study to determine the safety and tolerability in United States

The survey was conducted in ClinicalTrials.gov using key words "Embryonic + Stem + Cells". The status of clinical trials listed is confirmed on June 19, 2014. The description of the table is based on the database. See the details by entering the identifier No. into the database website.

were often not conducted sufficiently. In addition, mouse transplantation experiments showed that the immunogenicity of first-trimester human fetal pancreatic grafts (6- and 9-wk-old embryos) is less than that of older, second-trimester human fetal pancreatic grafts^[28]. This reduced immunogenicity is insufficient to completely circumvent the need for immunosuppressive conditioning in the recipient^[150]. Such precautions are now common sense for assuring safety in present-day stem cell transplantation.

The authors emphasize the need for sufficient implementation of cytogenetic testing, such as karyotyping and CGH arrays, in order to attain the therapeutic goal (Figure 1). Fetal tissue can be obtained from cadaveric fetuses following spontaneous abortion, stillbirth, or surgery due to ectopic pregnancy, in addition to elective abortion. Among these types of cells, fetal tissues de-

rived from spontaneous abortion and stillbirth are more likely to induce adverse events after transplantation, and frequent chromosomal or genetic causes of spontaneous abortion and stillbirth are likely to affect the pre- and post-transplantation behavior of donor cells. In addition, genetic changes may occur during cell culture. Therefore, cytogenetic testing is required to confirm the therapeutic validity of stem cells for transplantation. From this viewpoint, fetal tissue derived from cases of elective abortion or ectopic pregnancy is more likely to be an appropriate source for transplantation. However, the use of such cells remains still ethically, and socially controversial, primarily mainly due to the difficulty in consistently applying the "principle of separation" in cases of elective abortion^[100-103,107,108]. For these reasons, the procuring of the required amount of fetal tissue for transplantation is

challenging.

In contrast, adult tissue stem or progenitor cells, or terminally differentiated cells derived from non-fetal, adult tissues are more likely to be candidates for transplantation. In addition, the clinical use of human pluripotent stem cells recently became realistic (Table 2). As mentioned above, ES cells have been established from a more ethical source, surplus IVF embryos^[144]. Compared with adult tissue stem cells, ES cells proliferate more readily *in vitro*, and the directed differentiation of human ES cells can be used to produce a desired lineage, with some types of differentiated cells currently being applied as grafts in clinical trials (Table 2). Furthermore, a far more ethical source, induced pluripotent stem (iPS) cells, which are established from reprogramming the patient's own somatic cells *via* ectopic expression of defined factors, is now available^[151]. Human iPS cells can be likewise differentiated and used for autologous transplantation. Recently, the Japanese Ministry of Health, Labour, and Welfare approved a clinical research application for the use of iPS cell-derived retinal pigment epithelium cells in patients with age-related macular degeneration^[152]. Therefore, with the exception of fetal stem cells, a variety of human pluripotent stem cells are available for study in clinical trials.

Cell processing

A few weeks of culture has frequently been applied to expand fetal cells prior to transplantation^[3,119,120,153]. Close monitoring during cell culture is needed to assess whether the culture changes the cell population and/or function. If a change in cell population is detected, the population intended for use in transplantation must be isolated *via* methods such as a cell sorting^[154], as the presence of a remaining unintentional cell population in the culture may cause side effects. Notably, the effects of intermingled serotonergic neurons in part explain the onset of graft-induced dyskinesia in the setting of fetal neural transplantation^[155]. Such caution should also be applied to cell cultures resulting from the directed differentiation of pluripotent stem cells. In addition, culture additives, such as serum replacement and bFGF, must be carefully tested to avoid contamination with viruses or other microorganisms as well as potential epigenetic effects. Therefore, cell processing requires sufficient optimization in preclinical research.

Again, cell-processing also requires cytogenetic testing to confirm that the absence of karyotype or genetic changes during cell culture. Regarding application of human pluripotent stem cells, there remain still technical obstacles. For example, human ES cells and iPS cells exhibit a progressive tendency to acquire genetic changes during prolonged culture^[156]. In addition, it is necessary to take precautions against genetic instability (in the nucleus and mitochondria) of iPS cells, which may occur regardless of the reprogramming method used^[157]. However, future advances in stem cell research would overcome such obstacles.

Therapeutic cell niche

The selection of appropriate diseases and symptoms largely constitutes successful transplantation therapy, subsequently requiring the systematic consideration of autonomous or non-autonomous cell pathology, the localization of the affected tissue, and the assessment of progressive *vs* chronic disease.

Although only cell transplantation is considered to be efficacious in the setting of autonomous pathology, non-autonomous conditions are more likely to require extrinsic cues (cytokines, growth factors, inflammatory mediators, *etc.*) for proper use in stem cell transplantation. Although the therapeutic intervention requires only NSCs in the two identified pipelines developed for a CNS injury, including SCI (Table 1), the application of extrinsic cues may facilitate graft integration at the site of implantation, thus maximizing the therapeutic efficacy. Hepatocyte growth factor (HGF), a mitogen for mature hepatocytes and mediator of the inflammatory responses to tissue injury, was recently highlighted as a potent neurotrophic factor in the CNS. In addition, the intrathecal administration of human HGF in non-human primates has been demonstrated to have therapeutic efficacy in cases of SCI^[158]. Therefore, combined treatment with HGF and NSCs may improve the outcomes of therapy for SCI.

The localization of affected tissue defines the required number of cells and transplantation methodology. A survey of current clinical trials indicated that macular degeneration is a major subject of current studies using ES cell-derived cells (Table 2). For instance, four cohorts, ranging from 50000 to 200000 MA09-RPE cells, were designed in the NCT01344993 trial. These numbers are relatively small, as the cells are confined to application at the affected site in patients with retinal disease. With respect to fetal neuronal transplantation for PD, significant motion improvements require the integration of at least 100000 dopaminergic neurons into the striatum^[97,98]. However, graft-induced dyskinesia may occur in the setting of cell transplantation in the striatum^[3,96]. The development of a new transplantation procedure to construct dopamine projections from the substantia nigra to the striatum may eliminate the occurrence of dyskinesia.

Presumed pathological changes must be sufficiently considered in patients undergoing stem cell transplantation for progressive diseases. Notably, fetal neural tissue transplantation for in cases of PD has been reported to be efficacious in young and earlier-phase patients, but not old or later-phase patients^[3]. This finding implies that the efficacy of cell transplantation depends on the condition of the recipient. Such indications are represented by a key concept, the therapeutic cell niche, the local environment surrounding the cell graft that makes the graft functional *in vivo*. The therapeutic cell niche may vary based on symptoms depending on the disease.

Currently, researchers are able to differentiate stem cells into the desired lineage *in vitro* to obtain highly specified, isolated differentiated cells. Many pipelines are

sponsored by business entities (Tables 1 and 2). However, current stem cell transplantation procedures may lack firm evidence regarding the therapeutic cell niche *in vivo*. Therefore, it is necessary to provide proof of the therapeutic concept in disease model animals and subsequently confirm the safety and efficacy of the treatment in clinical trials, consistently paying attention to the therapeutic cell niche. Otherwise, similar side effects to the adverse events caused by NSC transplantation^[136] may occur in clinical trials. It is thus vital to continue to take a cautious approach to designing stem cell transplantation protocols for various conditions.

CONCLUSION

This report considered perspectives on fetal stem cell transplantation. To date, hundreds of clinical trials using various types of fetal transplants have been performed worldwide. Although success has been observed in some cases, most cases of fetal tissue or cell transplantation have been hastily implemented, and research groups must share their knowledge and experience. Meanwhile, research communities have learned many important lessons through these experiences and continue to improve transplantation strategies, leading to clinical trials of isolated fetal stem cells and ES cell-derived cells (Tables 1 and 2).

Although there remain still ethical and social issues with respect to the clinical use of fetal tissue, ongoing clinical trials of fetal transplants should proceed as fetal transplantation may be currently the sole benchmark for other types of stem cell transplantation. Indeed, the decade-long moratorium on cell transplantation for PD was recently lifted^[99], and European, United States and Japanese research groups recently formed the Parkinson's Disease Global Force to assess fetal transplant protocols for ES and iPS cell-derived dopaminergic neurons. In this process, essential issues, including those associated with the therapeutic cell niche, donor cells, and cell processing, should be sufficiently considered in order to develop more successful transplantation therapies.

Finally, clinical dependence on fetal transplantation, despite its landmark achievements, is expected to gradually fade in the setting of stem cell research owing to lasting ethical controversies and the advent of autologous iPS cells and ES cells.

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P- Reviewer: Cuevas-Covarrubias SA **S- Editor:** Gong XM

L- Editor: A **E- Editor:** Lu YJ



Early B lymphocyte development: Similarities and differences in human and mouse

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Received: July 25, 2014 Revised: August 29, 2014

Accepted: September 4, 2014

Published online: September 26, 2014

Abstract

B lymphocytes differentiate from hematopoietic stem cells through a series of distinct stages. Early B cell development proceeds in bone marrow until immature B cells migrate out to secondary lymphoid tissues, such as a spleen and lymph nodes, after completion of immunoglobulin heavy and light chain rearrangement. Although the information about the regulation by numerous factors, including signaling molecules, transcription factors, epigenetic changes and the microenvironment, could provide the clinical application, our knowledge on human B lymphopoiesis is limited. However, with great methodological advances, significant progress for understanding B lymphopoiesis both in human and mouse has been made. In this review, we summarize the experimental models for studies about human adult B lymphopoiesis, and the role of microenvironment and signaling molecules, such as cytokines, transforming growth factor- β superfamily, Wnt family and Notch family, with point-by-point comparison between human and mouse.

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Key words: Human B lymphopoiesis; B cell cultures; IL-7; Microenvironment; Wnt signaling

Core tip: There are several species differences between human and mouse, while the mouse studies precede those of human. Recent progresses of experimental techniques have made it possible to understand the biology in human B lymphopoiesis deeply. Various phenotype markers, which can define the distinct developmental stages, and requirement of cytokines are distinguishable. More common issues are observed in the role of signaling molecules, including transforming growth factor- β superfamily, Wnt family, and Notch family, which have been known the high conservation among mammals. The knowledge on niches for human hematopoietic stem cell and B cell development is still limited.

Ichii M, Oritani K, Kanakura Y. Early B lymphocyte development: Similarities and differences in human and mouse. *World J Stem Cells* 2014; 6(4): 421-431 Available from: URL: <http://www.wjgnet.com/1948-0210/full/v6/i4/421.htm> DOI: <http://dx.doi.org/10.4252/wjsc.v6.i4.421>

INTRODUCTION

B lineage cells develop from hematopoietic stem cells (HSCs) in adult bone marrow (BM) through several well-characterized stages before migrating to secondary lymphoid tissues such as a spleen and lymph nodes. Once HSC divides asymmetrically into one stem cell and one differentiating cell, it gives rise to progenitor cells that undergo lineage commitment and the production of specific lineage blood cells starts. Multipotent progenitors (MPP), which lose the reconstituting capacity, differentiate sequentially into lymphoid-committed progenitors, and B lineage-restricted progenitors originate from the lymphoid-primed multipotent/ early lymphoid progenitors (LMPP/ELP), followed by common lymphoid progenitors (CLP), pro-B cells, pre-B cells and immature B cells (Figure 1). Immunoglobulin gene rearrangements

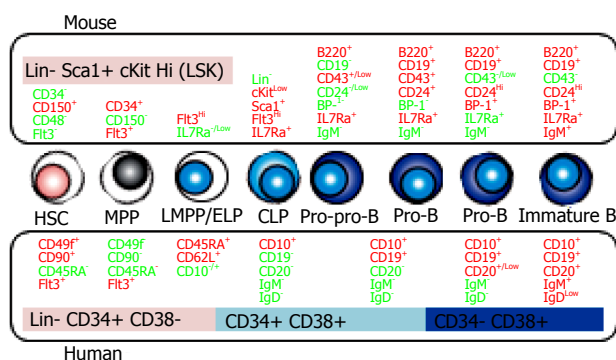


Figure 1 B cell development in bone marrow. B lineage cells are differentiated from hematopoietic stem cells (HSC) through the several steps defined by the distinct surface phenotypes of positive (red) or negative (green) expression. The comparison between mouse (upper side) and human (lower side) is shown. MPP: Multipotent progenitors; LMPP/ELP: Lymphoid-primed multipotent/ early lymphoid progenitors; CLP: Common lymphoid progenitors; IL7Ra: IL-7 receptor alpha.

are required for the process of B lymphopoiesis^[1-3]. The activation of the recombination enzymes, such as recombination-activating gene (RAG)-1, RAG-2 and terminal deoxynucleotidyl transferase, promotes the D-to-J and V-to-DJ rearrangements in the immunoglobulin heavy (IgH) chain locus during the differentiation from CLP to pro-B stage. Signaling through the pre-B-cell antigen receptor (pre-BCR), composed of IgH chains and surrogate light (L) chains, induces VJL rearrangements and allelic excision at IgH chain locus leading the functional BCR expression on immature B cells. This rearrangement machinery is precisely regulated by several transcription factors including PU.1, E2A, early B cell factor (EBF) and Pax5^[2,3]. For example, Pax5 activates the expression of Cd19, Cd79a, Blnk, Igl15 (lamda5) and VpreB1 involving in the pre-BCR signaling. Although it was believed that the fate decision of B cell commitment would occur after becoming CLP, recent studies have shown the lineage skewing begins earlier than previously expected^[3-7]. The expression of lymphoid-lineage priming genes like Satb1 and Ikaros in HSC is recognized^[8,9]. During the differentiation from HSC to CLP, lymphopoiesis proceeds in asynchronous ways. These developmental procedures are regulated by signaling molecules, transcription factors, epigenetic changes and the microenvironment^[6,7,10,11].

It has been known that HSC are extremely heterogeneous. Those can be subdivided to long-term and short-term HSC based on reconstitution time periods in transplantation assays^[12-16]. Recent studies suggest that HSC compartment also contains distinct subtypes with different developmental preferences^[15-18]. Myeloid-biased HSC produce greater numbers of myeloid than lymphoid lineage cells and tend to be quiescent. On the other hand, lymphoid-biased HSC generate more lymphoid cells and have shorter duration of reconstitution than myeloid-biased HSC. In aged mice, which reduce production of B and T cells and diminish function of mature lymphocytes, the number of myeloid-biased HSC increases^[17,19,20]. The distribution of HSC subsets is at least partly respon-

sible for homeostasis of B lymphopoiesis.

The evidences about hematopoietic biology have been accumulated from murine experiments and primary deficiencies in humans. However, recent advances in biological analysis techniques including xenotransplantation model, *in vitro* clonal assays and flow cytometric analysis and sorting made great progress for understanding normal hematopoiesis in human. Mouse and human are obviously different in size, ecology, and lifespan. It has been known that human B lymphopoiesis differs from that in mice with requirement of cytokines and the role of microenvironment. To apply the findings about the regulation of B lymphopoiesis for clinical settings, studies in human are necessary.

In this article, we focus on common and distinct features in human and mouse early B lymphopoiesis. First we discuss the differences of adult B cell development from HSC between these two species. In the late sections, we describe the role of microenvironment in BM including the cellular components and signaling molecules, especially about members of TGF- β superfamily, Wnt family, and Notch family, which have been known the importance in regulating proliferation, differentiation, and survival.

HUMAN AND MOUSE B LYMPHOPOIESIS IN BONE MARROW

Methodological advances in human B lymphopoiesis studies (Figure 2)

As we mentioned above, there are several species differences in B cell lymphopoiesis between human and mouse. The development of human study has been relatively slow with several reasons. The most critical one is the lack of adequate experimental models for evaluating molecular mechanisms *in vivo* and *in vitro*. For murine studies, various *in vitro* assays, such as Whitlock-Witte long-term cultures, cultures of BM cells with or without stromal cell lines, and colony assays for IL-7-responding progenitors are available^[21,22]. However, cultures to generate human B lymphocyte have not been well established. Although murine stromal cell lines can support human B cell development from hematopoietic stem/progenitor cells (HSPC), the species differences make the precise evaluation about some necessary cytokines and interaction with the microenvironment difficult^[23-26]. The establishment of new culture systems reported from our group and others hampered this problem^[27-29]. We established co-culture with human mesenchymal stem cells (MSC) and stromal cell-free culture systems. Our co-culture or stromal cell-free culture systems in the presence of stem cell factor (SCF) and Flt3 ligand (Flt3L) are successfully produced CD10⁺ CD19⁺ B cells within 4 wk from human umbilical cord blood (CB) CD34⁺ CD38⁻ HSC. Surface IgM⁺ immature B cells begin to appear after 4 wk of co-cultures. Although lymphocyte production from adult BM-derived HSC in the stromal cell-free culture is much more difficult than CB cells, both are responsive to granulocyte

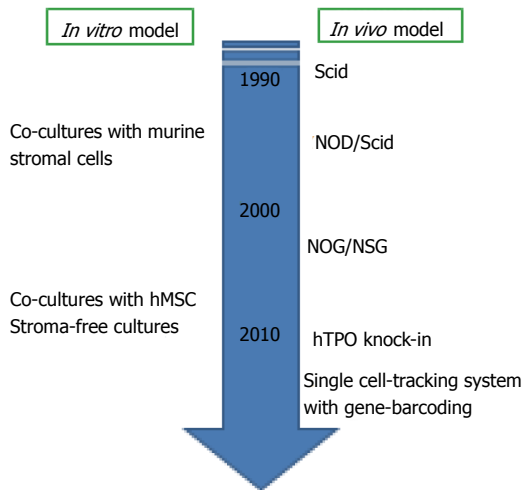


Figure 2 Experimental models for human B lymphopoiesis. Experimental techniques for studying human B lymphopoiesis have incredibly advanced within these two decades. Now several culture systems with human mesenchymal stem cells (hMSC) or without stromal cells are available. For *in vivo* studies, the generation of humanized mice has been developed after the discovery of severe combined immune-deficient mouse (Scid). NOG, nonobese diabetic (NOD)-Scid mouse with truncation in the IL-2 receptor common gamma chain; NSG, NOD-Scid mouse with deletion in the IL-2 receptor common gamma chain; hTPO knock-in, RAG-2^{-/-} NSG mouse with humanization of thrombopoietin.

colony stimulating factor (G-CSF). Our data showed that human MSC can efficiently support commitment and differentiation of human HSC into B lymphocytes, and human does not require the direct interactions with stromal cells for B cell generation.

Concerning about *in vivo* studies, humanized mouse models were established around 1990s with the discovery of the severe combined immune-deficient (Scid) mouse lacking B and T cells^[30,31]. Since then, a variety of xenograft models including nonobese diabetic (NOD)-Scid mice and NOD-Scid with either truncation (NOG) or deletion (NSG) in the IL-2 receptor common gamma chain have been generated to improve the efficiency of human HSC engraftment and long-term reconstitution^[32,33]. With humanized model, we can observe multi-lineage reconstitution from human HSC *in vivo*. Newer generation of transplantation methods are now being developed. To elucidate the role of cytokines which are not cross-reactive, transgenic mice producing human cytokines such as thrombopoietin, IL-3 and GM-CSF, have been generated^[34]. The viral integration site tracking system and the use in combination with massively parallel sequencing make it possible to track human HSC clones in transplanted Scid mice^[35].

Another obstacle to studying human lymphopoiesis is genetic and biological diversity. Human BM samples are all different in age, sex, body size, genetic and epigenetic background and health condition when samples are collected. The development of highly purifying techniques with flow cytometry, single-cell assay methods and gene sequencing would help this problem solved^[36].

Markers of hematopoietic stem cells and B progenitors

HSC is an extremely rare subset. The frequency of HSC

in human BM is only 1 in 10⁶ cells^[37]. In mice, lineage (Lin)⁻/Low Sca-1⁺ c-Kit^{Hi} (LSK) fraction contains multipotent cells such as HSC and MPP^[38]. Using CD34, Flt3, SLAM family markers (CD150, CD48, CD229 and CD244) and Hoechst 33342 efflux, HSPC in LSK cells can be resolved into several subsets with distinct level of reconstituting potential and lineage preference^[12,39-41]. According to c-Kit intensity decline, lymphoid committed cells are differentiated. Kondo *et al.*^[42] defined CLP in mice as Lin⁻ IL-7 receptor alpha (IL-7Ra)⁺ Sca-1⁺ c-Kit^{Low} cells that appear to produce mainly B, T and natural killer (NK) cells. B lineage-restricted progenitors are fractionated based on the developmental stage and surface expression of CD45R/B220, CD19, CD24 (heat-stable antigen), CD43 and BP-1^[43,44]. Mouse lymphopoietic hierarchy with cell surface markers is shown in Figure 1.

In human, HSPC markers are quite different from murine ones (Figure 1). Unlike mice, human HSPC can be enriched with CD34 expression although a very rare subset of HSC are devoid of that^[45-47]. Other phenotypes of HSC are Flt3⁺, CD38⁻ and CD150⁻, in great contrast with the expression on murine one^[48,49]. CD133 helps the isolation of human HSPC and the rare CD34⁻ HSC subset^[46,50]. Recently, Dick and colleagues subdivided human Lin⁻ CD34⁺ HSPC into long-term HSC, short-term HSC/MPP, and 6 lineage progenitor subsets on the basis of expression of the markers CD34, CD38, CD90 (Thy-1), CD49f, CD135 (Flt3), CD45RA, CD10, and CD7^[51,52].

CD10 and CD45RA are often used as human-specific markers of lymphoid progenitors at early stages. Doulatov *et al.*^[51] described CD34⁺ CD38⁻ CD45RA⁺ CD10⁺ fraction as multilymphoid progenitor and CD34⁺ CD38⁺ CD45RA⁺ CD10⁻ fraction as granulocyte and monocyte progenitor. It is known that CD34⁺ CD10⁺ cells have a strong bias toward B cell development with relatively little T or NK cell potential^[53,54]. We previously reported CD34⁺ early lymphocyte progenitors differ in CD10 expression^[53]. CD34⁺ CD10^{Hi} and CD34⁺ CD10^{Low} populations have unique patterns depending on their sources; CB, BM and G-CSF mobilized peripheral blood, and increasing level of CD10 corresponds to expression of B lymphoid related transcription factors and markers, as well as loss of proliferative potential. Recently, Kohn *et al.*^[55] reported that L-selectin (CD62L) is expressed at the earliest stage of lymphoid priming before starting CD10 positive. CD34⁺ CD45RA⁺ CD62L^{Hi} CD10⁻ cells showed lymphoid skewing although they produced both of myeloid and lymphoid cells in transplanted NSG mice. The differentiation potential and gene profiling indicated that CD34⁺ CD45RA⁺ CD62L^{Hi} CD10⁻ cells are placed between HSC and CD34⁺ CD10⁺ lymphoid progenitors.

Requirement of cytokine signaling (Figure 3)

In mice, two cytokines, IL-7 and Flt3L, are known to be essential for adult B lymphopoiesis^[56-58]. The loss of these receptors completely blocks B cell development. The up-regulation of IL-7Ra with Flt3 signaling induces EBF expression in B lineage progenitors, and that al-

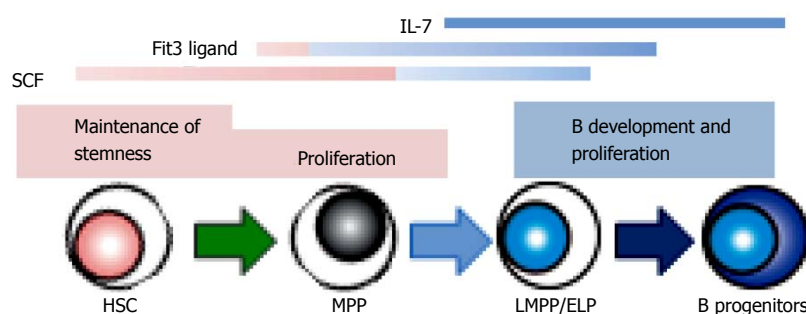


Figure 3 The role of signaling molecules in B lymphopoiesis. Several cytokines, such as stem cell factor (SCF), Flt3 ligand and IL-7, show the various effects depending on the developmental stages. There are several species differences in the role of cytokines between human and mouse. IL-7 is required for adult mouse B lymphopoiesis, but not for that of human. Recent studies indicate that Flt3 signaling plays a crucial role in lymphoid, but not in HSC or myeloid development in mouse, while human Flt3 ligand affects the survival of hematopoietic stem/progenitor cells as well as B cell differentiation. HSC: Hematopoietic stem cells; MPP: Multipotent progenitors; LMPP/ELP: Lymphoid-primed multipotent/early lymphoid progenitors.

lowers differentiation with the consequent expression of B cell-specific genes^[10,11]. Moreover, recent studies have shown that the expression of IL-7Ra denotes the transition from LMPP to CLP, and Flt3L regulates the survival and proliferation of MPP/LMPP with commitment to B lineage fate^[42,59,60]. The combination of crucial cytokines changes during ontogeny. Studies using IL-7 knockout mice showed that only adult but not fetal or neonatal B development is inhibited^[61]. Thymic stromal lymphopoietin (TSLP) regulates IL-7-independent fetal B lymphopoiesis. SCF and chemokines recognized by the CXCR4 receptor also affect the differentiation^[62].

In contrast, IL-7 is not required for human B cell development^[63-66]. Crucial transcription factors including E2A, EBF and Pax5 are expressed during the differentiation from HSC to B lineage progenitors before acquisition of CD19, in the same manner as mouse B lymphopoiesis^[67]. The importance of SCF and CXCL12 for B cell development has been recognized^[68-70]. In human, Flt3L is critical to cell survival and proliferation of HSPC as well as to B lymphopoiesis^[68,69]. Several groups reported that human HSPC could develop B lineage cells independently of IL-7 stimulation, and IL-7 induces little increase of B production in co-cultures^[64]. Moreover, patients with disruption of the human IL-7 receptor spared B lymphopoiesis while development of T and NK cells was severely impaired^[65,66]. Some groups questioned about the interpretation because in these studies fetal materials or murine stromal cells might influence the consequences^[71,72]. However, we found that addition of neutralizing antibody to IL-7 or TSLP has no effect in stromal cell-free cultures we established^[28,29]. In our study, hMSC conditioned medium could support human B lineage generation, indicating the existence of unknown stromal cell-derived factors facilitating B lymphopoiesis. Interestingly, we and others reported that G-CSF promotes human B production from HSC *in vitro*^[23,28]. G-CSF is originally cloned as a glycoprotein which stimulates the production of granulocytes, and now is known the important role in HSC proliferation and mobilization, and bone resorption. For now, nothing has been reported about the influences on early B lymphopoiesis *in vivo* while clinical studies showed a higher proportion of Th2 cells present in peripheral blood cell grafts from G-CSF-stimulated donors and T cell hyporesponsiveness in association with increase of Th2-inducing dendritic cell^[73,74].

There are several possibilities about the mechanism how G-CSF affects B lymphocyte generation *in vitro*. It might have direct effects on cultured cells. Another speculation is that HSC or progenitors with the specific stage or lineage stimulated by G-CSF might regulate B generation indirectly. In B cell cultures, short-term expansion of myeloid cells is observed before emerging B lineage cells^[27,28].

Collectively, the essential key of human B lymphopoiesis is still remained unknown. The recent study with the depth of single-cell mass cytometry and an algorithm analysis of human BM showed the exclusive activation of STAT5, which phosphorylation is known to be induced by IL-7, in early B progenitors^[75]. Using novel technologies, the precise biology could be unveiled in the near future.

ROLE OF MICROENVIRONMENT

In 1978, Schofield proposed the hypothesis that a specialized niche in BM preserves the reconstituting and differentiating ability of HSC, but could not prove that^[76]. It is believed that bone marrow contains specialized niches for differentiation of specific lineage progenitors^[77-79]. With the great advances of gene-modified mice generation and imaging techniques, the anatomical location and cellular components of HSC niches have been elucidated since 2000s, although our understanding is still incomplete and novel analysis tools are needed^[80,81]. In parallel, the roles of molecular and environmental factors in the niches have been extensively studied. Niches make specialized environments, consisting of soluble or surface-bound signaling factors, cell-cell contacts, extracellular matrix (ECM) proteins, and local mechanical environments such as the concentration of oxygen and calcium.

Cellular components

In marrow, there are many types of non-hematopoietic cells including mesenchymal stem/progenitor cells, osteoblastic lineage cells, adipocytes, endothelial cells, reticular cells, pericytes, fibroblasts and nerve cells^[80,81]. The effects of several molecular regulators produced by niche cells, such as chemokines like CXCL12, cytokines (SCF, thrombopoietin, angiopoietin-2, and angiopoietin-like 3), Wnt, Notch, TGF- β and hedgehog signaling, and ECM proteins (osteopontin, decorin, and tenascin C) have been

reported. Based on the concept of HSC niche, the cellular components are supposed to neighbor with HSC, and more importantly, the influence on HSC maintenance should be direct.

Several immunofluorescence imaging studies showed that HSC is consistently located adjacent to the sinusoidal vasculature^[39,82]. In perivascular niches, mesenchymal stem/progenitors which express Nestin, leptin receptor, or fibroblast activation protein (FAP), CXCL12-abundant reticular cells, and endothelial cells are co-localized with HSC and secrete HSC supporting factors like SCF or CXCL12^[39,83-86]. The sympathetic neurons, arteries, macrophages such as osteoclasts and regulatory T cells in the niches affect the frequency, function and localization of HSC^[87-91]. Surrounded by these cells and molecular components, HSC can maintain the capacity of self-renew and multipotent differentiation.

On the other hand, whether the osteoblastic lineage cells at the endosteal surface of the bone, described first as the place where HSC reside, could be the niche is under debate^[82,92]. Although osteoblasts may not be adjacent to HSC, they do have the distinct influences on HSPC through the production of CXCL12 and SCF, and expression of adhesion molecules. It is known that HSPC frequently move out from their own niche^[93,94]. Thirty percent of IL-7Rα⁺ B progenitors are co-localized with bone-lining cells, and acute depletion of them are observed when osteoblastic cells are conditionally deleted^[95]. Interestingly, the deletion of CXCL12 from osteoblasts depletes early lymphoid progenitors, but not HSC or myeloid progenitors^[95,96]. These findings suggest that osteoblasts could be the niches for B lymphopoiesis in endosteal area (Figure 4).

Anatomical location

As well as the identification of cellular components of niches, the anatomical localization of HSC in BM has been the subject of intense researches. The initial studies indicated that HSC might reside in the endosteum, adjacent cortical bones with osteoblasts^[92]. With the great advance in immunostaining methods and understanding HSC characteristics, however, others showed that most of accurate HSC localize adjacent to sinusoid vessels while less than 20% to bone-lining cells^[82,84]. It is consistent that HSC are found in the trabecular regions at metaphysis.

Inside marrow, HSC is mobile when HSC divides or starts to differentiate. Interestingly, it is known that HSC periodically leave and reenter the niches for circulation with circadian oscillation and in response to infection or G-CSF stimulation^[87,91]. *In vivo* time-lapse imaging makes it possible to observe HSC motility and localization of activating HSPC. Another unanswered question is skeletal localization. In human adult, the sternum is active hematopoietic site while long bones are occupied by adipocytes with aging. The three-dimensional, whole-mount confocal immunofluorescence imaging techniques showed the same is true in mice^[89].

Niches in human

In clinical settings, hematopoietic stem cell transplantation offers patients with refractory hematological diseases a curative treatment option. Several types of stem cell sources, CB, BM and G-CSF mobilized peripheral blood are used for the therapy, although differences among sources are still remained unclear^[97]. After transplantation, HSC migrate, localize in niches and start to proliferate and reconstitute all lineage bloods in the recipient BM damaged by the conditioning. A full understanding of the whole process is critical for choosing the adequate strategy of donor sources, conditioning and immunosuppressive therapy before or after transplantation.

Several types of mesenchymal stem/progenitors, osteoblast, and endothelial cells in human have been reported the supportive effects on HSC maintenance or specific lineage differentiation^[27,98,99]. Imaging analysis using bone biopsy specimens to evaluate the actual distance between HSPC and niche component showed that human CD45⁺ CD34⁺ CD38⁻ HSC localize in the trabecular area similar to mice HSC, while CD34⁺ CD38⁺ HPC are dispersed evenly in BM^[99]. HSC in the trabecular area own better HSC functions compared to those in long bone area. There is no information about the niches for human B lymphopoiesis.

SIGNALING MOLECULES

Signaling molecule families like TGF-β, Wnt, Notch and hedgehog are highly conserved in mammals, and control proliferation or cell fate determination during embryonic development and adult homeostasis. In hematopoiesis, the specific ligand-receptor interactions regulate the maintenance of HSC stemness and differentiation through direct and indirect effects via the affected micro-environments.

TGF-β superfamily

The TGF-β superfamily is composed of more than 20 members, including three TGF-βs, bone morphogenetic proteins (BMP), growth and differentiation factors (GDF), Activins, and Nodal. TGF-β signaling regulates HSC quiescence, and is reduced in aged HSC^[88,100]. The activation is restricted although many cells can produce TGF-β ligands and express the receptors. For HSC maintenance, the latent type of ligand is produced from the HSC microenvironment and activated by the nonmyelinating Schwann cells ensheathing sympathetic nerves in contact with HSC^[88]. We and others reported the effects of TGF-β signaling for mouse and human B lymphopoiesis^[27,101-103]. We showed that both Activin A and TGF-β1 inhibit generation of B cells from CB CD34⁺ cells in cultures. The receptors are expressed by not only CD34⁺ HSPC but also CD34⁺ CD10⁺ cells, and we observed the same effects of the signaling when the inhibitor was added at the later periods of the co-cultures. These findings indicate TGF-β superfamily might affect early B lymphocyte progenitors. Transition into IgM⁺ immature

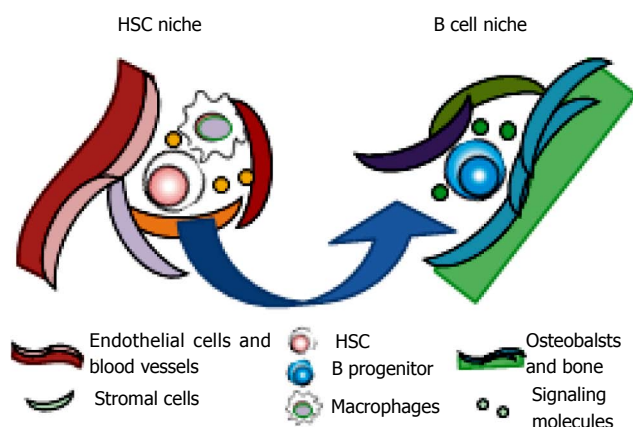


Figure 4 Motility of hematopoietic stem and progenitor cells in bone marrow. Hematopoietic stem and progenitor cells reside in their own specialized niches where they could preserve the reconstituting and/or differentiating ability. The cellular components and anatomical localization make specialized environments, consisting of soluble and surface-bound signaling molecules, cell-cell contacts, extracellular matrix proteins, and local mechanical environments. The niches for stem cell maintenance and differentiation are distinct. It is believed that hematopoietic stem cells (HSC) self-renew in their niches adjacent to the sinusoidal vasculature with mesenchymal progenitors, endothelial cells, sympathetic neurons, arteries, and macrophages such as osteoclasts. Once the differentiating daughter cell is generated after asymmetrical division of HSC, it moves to the favorable space for undergoing specific lineage commitment. For B lymphopoiesis, progenitors are co-localized with bone-lining osteoblasts in endosteal area.

B-cells was not influenced by the TGF- β superfamily in our culture systems.

Wnt family

Wnt is a large family of glycoproteins. Canonical pathway used by Wnt3a has been most studied in hematopoiesis^[104]. After Wnt3a binds Frizzled receptor, this canonical signaling stabilizes intracellular β -catenin by inhibition of GSK-3 β , and then β -catenin translocates to the nucleus and interacts with transcription factors. The role of Wnt for HSC maintenance has been a debatable issue. Constitutively active β -catenin blocks their differentiation, but induces exhaustion by shifting HSC cell-cycling status, although some of conditional deletion of β -catenin mouse have no abnormality in hematopoiesis^[105,106]. However, now it is known that these discrepant results from studies using gain or loss of functions reflect the sensitivity to the dosage^[107]. Wnt5a associated with noncanonical pathway also regulates HSC maintenance and differentiation^[108-110]. Recent studies showed that noncanonical signaling is balanced with canonical signaling under inflammatory and aging condition^[109].

Our and other groups reported the inhibitory effects on B lymphopoiesis^[108,110,111]. Wnt3a, using canonical pathway, inhibits B and pDC but not cDC development, and Wnt5a promotes B lymphopoiesis *in vitro*. The observations about canonical Wnt signaling can translate from mouse to human^[111]. It is known that Wnt ligands and receptors are expressed in both of hematopoietic tissues and the niche cells, and Wnt3a regulates mesenchymal lineage differentiation^[111,112]. While hematopoietic

cells themselves are Wnt responsive, we showed that the regulation of niches by Wnt3a mediates the effects. Specifically, Wnt3a strongly induces the production of ECM protein, decorin, which inhibits B lymphopoiesis and retains the HSC phenotype, from stromal cells. Decorin is a small leucine-rich proteoglycan secreted by MSC, and regulates TGF- β signaling, although the detailed mechanisms have not been elucidated^[111,113]. Collectively, the findings suggest that Wnt signaling is important for maintaining not only hematopoiesis but also the niches.

Notch family and hedgehog family

The ligands of Notch signaling are membrane bound proteins, and the function depends on the type of ligand, such as Delta-like and Jagged, and responsive receptors, Notch 1-3. Notch is essential for early T lymphopoiesis, and B lymphopoiesis is suppressed by the interactions between Delta-like and Notch1 to avoid B cell generation in thymus^[114]. The precise role in adult HSC at physiological levels is still controversial. Loss of the function in HSC did not show any influences for reconstitution and differentiation in mice, while *in vitro* expansion of HSC is promoted by the signaling^[115,116]. The same is true in human^[117]. The two recent studies published in 2013 emphasized the importance of Notch signaling in the interaction between human HSC and the microenvironment. Human CD146⁺ perivascular cells maintain stemness of HSC via Notch activation^[98]. Bhatia and colleagues showed that in the trabecular bone area where HSC can hold the regenerative and self-renewing capacity, 3-fold greater of proportion of mesenchymal cells express Jagged-1 compared to those in long bone area^[99]. More recently, it is reported that Notch signaling in HSC stimulated after the activating mutation of β -catenin in mouse osteoblasts induces the leukemogenesis^[118]. The mutation induces Jagged-1 expression in osteoblast leading the Notch activation in HSC, and the inhibition of Notch signaling prevents the onset of leukemia. According to this study, 38% of patients with acute myeloid leukemia or myelodysplastic syndromes showed increased β -catenin in osteoblasts and increased Notch signaling in hematopoietic cells. The cooperation between Wnt and Notch is also reported in HSC maintenance^[119]. Further study about the role of Notch signaling is warranted.

Although hedgehog signaling is also important for the development, stem cell maintenance, and tumorigenesis in various organs, the detailed effects on hematopoiesis have remained unclear. In mice, hedgehog signaling in HSC is not required for hematopoiesis although several studies showed the effects on cell-cycle and differentiation of HSC^[120,121]. The activation of hedgehog signaling in stromal cells promotes B lymphopoiesis and HSC expansion^[122]. Both of cell-extrinsic and cell-autonomous effects might be critical.

CONCLUSION

B lineage commitment starts at the early stage of HSC in

asynchronous ways. The fate decision and development are affected by the microenvironmental factors including cellular niche components and signaling molecules. In this review, we described the common and different features in early B lymphopoiesis between human and mouse. The surface phenotypes on human HSC and B progenitors and requirement of cytokines are distinct while many effects of signaling molecules are consistent with mice.

It is known that immune system can be harmed by malignant disease, chronic inflammation and normal aging. Many studies concerning impairments in cellular and humoral immunity have focused on regulation of mature lymphocyte function. Recent studies, however, revealed that the earliest stage of B lymphopoiesis plays an important role in the immune decline. Understanding the precise mechanism in human and mouse BM, and the assessment of species variations with novel technologies would make the potential applications to cancer immunotherapy and the discovery of novel treatment for autoimmune diseases possible.

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P- Reviewer: Borrione P, Regueiro JR, Song J
S- Editor: Song XX **L- Editor:** A **E- Editor:** Lu YJ



Training stem cells for treatment of malignant brain tumors

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Supported by The CHOC Children's Foundation, CHOC Neuroscience Institute, CHOC Research Institute, The Austin Ford Tribute and Keck Foundation; by The United States National Institutes of Health, 1R01CA164509-01; and The United States National Science Foundation, CHE-1213161

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Received: May 28, 2014 Revised: August 9, 2014

Accepted: August 30, 2014

Published online: September 26, 2014

tients. Here, we perform a literature review to identify the problems in the field. Given the lack of efficacy of most stem cell-based agents used in the treatment of malignant brain tumors, we found that stem cell distribution (*i.e.*, only a fraction of stem cells applied capable of targeting tumors) are among the limiting factors. We provide guidelines for potential improvements in stem cell distribution. Specifically, we use an engineered tissue graft platform that replicates the *in vivo* microenvironment, and provide our data to validate that this culture platform is viable for producing stem cells that have better stem cell distribution than with the Petri dish culture system.

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Key words: Stem cells; Malignant brain tumors; Engineered tissue graft; Organotypic slice model

Core tip: Neural stem cells can target malignant brain tumors in preclinical models; however, clinical trials show dismal efficacy. We reviewed the literature and found that only a small fraction of applied stem cells can move toward tumors while the majority of stem cells cannot reach the target tumor. To fill in the gap in stem cell technology, we propose a solution to train stem cells in a native tissue environment, allowing them to move through tissue barriers and arrive at the target tumor.

Li SC, Kabeer MH, Vu LT, Keschrumrus V, Yin HZ, Dethlefs BA, Zhong JF, Weiss JH, Loudon WG. Training stem cells for treatment of malignant brain tumors. *World J Stem Cells* 2014; 6(4): 432-440 Available from: URL: <http://www.wjg-net.com/1948-0210/full/v6/i4/432.htm> DOI: <http://dx.doi.org/10.4252/wjsc.v6.i4.432>

Abstract

The treatment of malignant brain tumors remains a challenge. Stem cell technology has been applied in the treatment of brain tumors largely because of the ability of some stem cells to infiltrate into regions within the brain where tumor cells migrate as shown in preclinical studies. However, not all of these efforts can translate in the effective treatment that improves the quality of life for pa-

INTRODUCTION

Malignant brain tumors are devastating to patients

Billions of dollars have been spent since United States

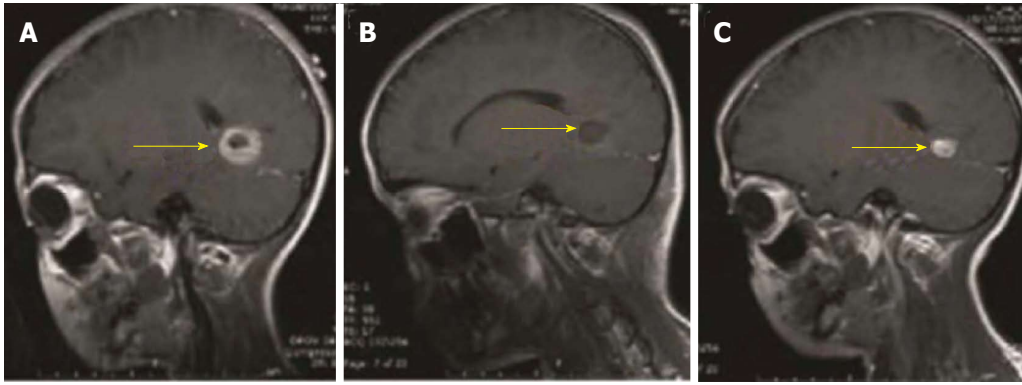


Figure 1 Magnetic resonance imaging graphs illustrate the presence, removal, and reappearance of a glioblastoma patient (yellow arrow: tumor mass). A: Pre-operation, visualizing the presence of the tumor; B: Post-surgery, visualizing disappearance of the tumor; C: 3-mo post-surgery, visualizing the reappearance of the tumor.

President Richard Nixon declared the “war on cancer.” Understanding the molecular biology of cancer led to gain better survival in certain cancers, such as childhood leukemia. However, survival in solid tumors has not improved since the 1970s. New studies revealed that unexpected factors such as intratumoral heterogeneity^[1] and clonal evolution force us to realize that classical therapies cannot fully address the tumor subclonal switch mechanism that allow tumors to escape therapy^[2]. This includes chemotherapy drug temozolomide-driven evolution of recurrent glioma^[3] into a restricted subclonal cell population of drug-resistance^[4]. Ineffective cancer treatment results in mortality and economic burden: one-third of 2007 healthcare dollars (total: \$686 billion) was spent on 1.4 million cancer patients in the United States^[5-7]. Some pediatric malignant brain tumor patient costs \$67887, which is 200 times as much as a demographical control, \$277^[5,8]. It is devastating, considering that the fortunate survivors suffer cognitive changes, cognitive deficiency that challenges the quality of life of both patients and their care givers^[9].

LIMITATION OF CURRENT STANDARD TREATMENT

Cancer treatment is largely unsuccessful due to current blindfolded anti-cancer strategic and tactical issues in the fight. Surgical resection allows glioma patients survive the traumatic attack; however, surgery alone cannot clear the residual infiltrative glioma. Malignant brain tumors disseminate widely to distant regions of normally functioning tissues^[10]. Thus, surgery in conjunction with chemotherapy and radiation therapy still cannot eradicate residual tumors^[11,12].

ADVERSE SIDE EFFECTS OF STANDARD THERAPIES

Chemotherapy and radiation therapy do not strictly discriminate tumor cells from normal cells, resulting in

adverse effects. Survivors of current standard brain tumor treatment show neurological, cognitive, endocrine sequelae, and metabolic side effects^[11,13-20]. These side effects result from the cumulative effects of pre-treatment injury caused by the growing tumor, the adverse impact of surgery and from adjuvant therapeutics (chemotherapy and radiation therapy)^[21]. The surgical removal of the initial tumor followed with adjuvants (radiation plus chemotherapy) may awaken the dormant clones of the primary tumor and these cells then grow to form a secondary tumor (Figure 1) as the dormant cells go through switch-board signaling to become dominate clones of cancer^[2]. These glioma residues grow back, leading to recurrent incurable and metastatic cancer. Adjuvant therapies (Local radiotherapy, chemical sensitizers, gene therapy) did not provide any survival advantage in clinical trials.

GENETIC PROFILING

Genetic profiling shows the potential genetic risk factors for patients and a way to predict how a patient may react with a given tumor treatment. Across 12 tumor types in 2928 out of 3277 patients, The Cancer Genome Atlas Network (TCGA) analyzed 10281 somatic alterations^[22]. This TCGA data set predicts patient survival when applying therapies useful in one cancer type to other cancer types. This molecular profile-based prediction of therapeutic efficacy may imply a new classification system different from the previous organ-based tumor classification system^[23].

For example, the analysis of somatic mutations in glioblastoma multiforme (GBM)^[24] helped establish Pro-neural, Neural, Classical, and Mesenchymal subtypes^[24]. Each subtype, with its own molecular stratification (*PDGFRA*, *IDH1*, *EGFR*, and *NF1* gene), can exhibit specific drug targets that minimize adverse effects and enhance efficacy. Another study shows that recurrent H3F3A mutations are further characterized into six methylation patterns^[25]. The methylation patterns help design epigenetic-pattern-specific targeted therapies^[25]. Molecular changes in *BRAF*, *RAF1*, *FGFR1*, *MYB*, *MYBL1*, *H3F3A*,

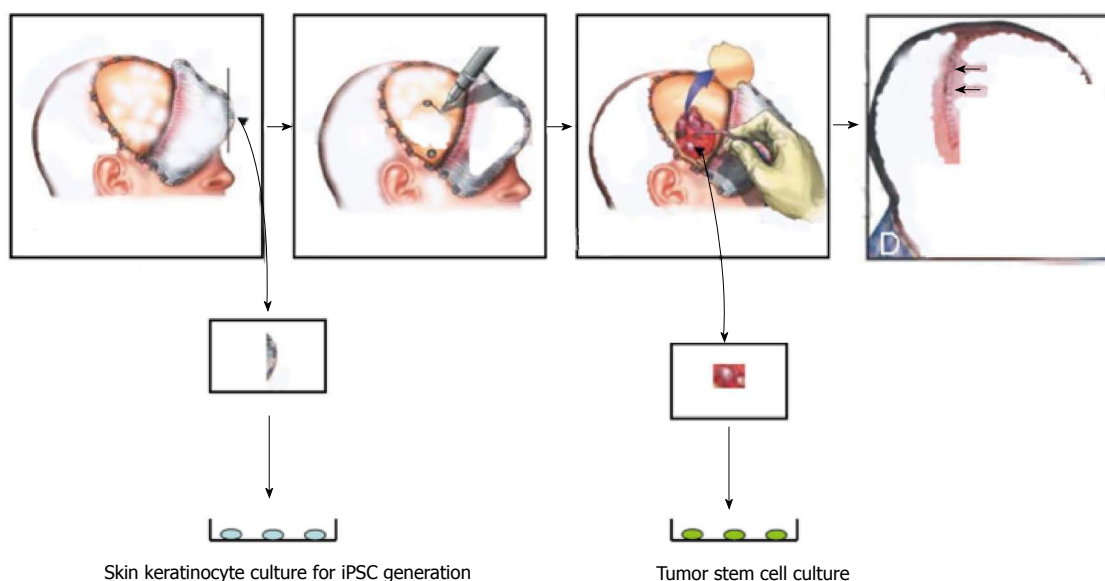


Figure 2 Personalized treatment of brain tumors by using autologous stem cells (induced pluripotent stem cells) through the induced pluripotent stem cells strategy for treating brain tumors. During surgery, a piece of skin is obtained to generate induced pluripotent stem cells (iPSCs) while tumor cells are processed to obtain tumor stem cells (TSCs). The iPSCs are used to take therapy specific to autologous TSCs.

and ATRX were identified in 151 low-grade gliomas (LGGs)^[26]. Another study defined recurrent activating mutations in FGFR1, PTPN11, and NTRK2 genes in LGGs^[27]. The mutations imply some targeted therapies, *e.g.*, specific inhibitors against FGFR1 autophosphorylation can block MAPK/ERK/PI3K, preventing cancer cells from proliferating.

These mutations can help focus targeted therapies for patients. Temozolomide (TMZ) and radiation, increase survival for patients with Classical or Mesenchymal subtypes but not with Proneural subtype^[24]. However, chemotherapy can activate chemoresistant cancer cells. TMZ drives a subset of endogenous cells out of their quiescent subventricular zone to develop to a new tumor^[4]. Evidence shows that TP53, ATRX, SMARCA4, and BRAF mutations in the initial tumor but were undetected at recurrence, suggesting new mutations occur upon drug-driven tumor evolution. TMZ-activated RB (retinoblastoma) and Akt-mTOR (mammalian target of rapamycin) mutations led to recurrent tumors^[3]. New strategy to address these therapy-driven detrimental effects in a real-time manner is needed.

EMERGING THERAPIES

Neural stem cells (NSCs) possess the tumor-tracking capacity as shown in preclinical models^[28]. NSCs modulate the brain tumor microenvironment^[29-32]. Other candidate stem cells include HSCs^[33], BM-MSC^[34], and induced pluripotent stem cells (iPSC)^[35]. Because iPSC technology enables autologous transplantation allowing immune compatibility with a host immune system (Figure 2), iPSCs are proposed for replacement therapy in certain diseases^[36]. However, potential immune rejection of these autologous iPSCs remains to be tested in clinical trials^[37].

These stem cells could be engineered as delivery vehicles for therapeutic agents^[38] such as antibody^[39], oncolytic adenoviral virotherapy^[40], and prodrug therapy^[41]. NSCs inhibit glioma proliferation *in vivo* and *in vitro*^[42]. Intracranial tumors activate endogenous NSCs to migrate towards neoplastic target lesions^[43,44].

Evidence shows that BM-MSCs work in the same fashion as NSCs^[34]. MSCs exhibit tropism towards gliomas^[45-47]. MSCs locally produce IFN- β that suppresses cancer cells^[48].

CLINICAL TRIALS SHOW DISCREPANCIES

Serving to reconstitute hematopoietic and immune function, some stem cells act as a salvage therapy for surgery, radiation therapy, and high dose chemotherapy. For example, patients rely on autologous hematopoietic stem cell transplantation to replenish immune capacity against recurrent cancer after surgery and chemotherapy^[49]. Currently, 240 studies on “stem cell therapy of cancer” exist in mostly Phases I / II clinical trials (See <http://clinicaltrials.gov>, accessed on August 22, 2014) using HSCs and BM-MSCs. Interestingly, genetically modified NSCs orchestrate flucytosine and leucovorin calcium in treating gliomas [ClinicalTrials.gov identifier NCT02015819 (2014)]. The genetically modified NSCs carry the gene for *Escherichia coli* (*E. coli*) flucytosine that sensitizes cancer to chemotherapy while leucovorin calcium helps stop cancer cells from dividing. The project of ClinicalTrials.gov Identifier NCT01540175 aimed at replenishing an immune system (T cell, B cell, and NK cell compartment) on autologous transplant to the baseline values, representing an innovation that is expected to replace

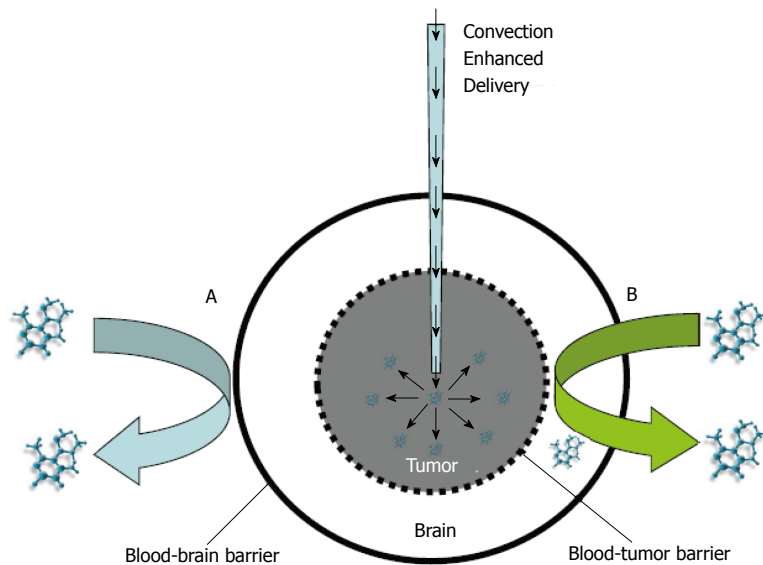


Figure 3 Convection enhanced delivery of therapy to overcome two barriers of brain tumors. A: Systemic delivery of drugs blocked from entry into the brain by the blood brain barrier; B: Drug delivery inhibited by the brain-tumor barrier. This convection enhanced delivery can be used to deliver neural stem cells locally onto a tumor.

the conventional HSC transplantation. Phase I trials using tumor dendritic vaccines evaluated the side effects of vaccine therapy on recurrent GBM (ClinicalTrials.gov Identifier: NCT00890032 - tumor cells/dendritic cells; ClinicalTrials.gov Identifier: NCT01171469-tumor stem cells; assessed on August 22, 2014), a potential that a real-time anti-cancer system is established *in vivo* to monitor cancer growth. These everlasting vaccines are expected to set up an immune response to stop cancer.

Discrepancies of efficacy occurred in all these clinical trials and efforts have been made to explain what roadblocks are in the way for achieving consistent efficacy. Roadblocks for stem cells to reach the site of the tumor include the blood brain barrier (BBB) and the brain tumor barrier (BTB) (Figure 3). Most intravenously administered NSCs cannot cross BBB and BTB but only a few do^[7]. These roadblocks must be removed to clear that path for success of stem cell therapy for cancer^[7]. Specifically, we need to cultivate potentiated stem cells to be potent to tranverse these roadblocks.

THE NEED TO FIND WAYS OF IMPROVING THE POTENCY OF STEM CELLS

What qualities for stem cells could allow therapeutic effectiveness? The ideal stem cells should provide: (1) long-distance inter-organ autopilot traveling to surgically inaccessible tumors, ideally when administrated by peripheral intravenous injection; (2) accuracy in eliminating tumors without adversely affecting normal organs; (3) capability of suppressing primary and metastatic tumor; and (4) memory so that recurrence never occurs.

Components of an inter-organ movable vehicle for targeting cancer

(1) The therapeutic agent shows the maximum anti-cancer efficacy with the minimum adverse effect; (2) The vehicle should protect the therapeutic agent for its potency

and specificity; and (3) The vehicle possesses the ability to home in on targets.

Stem cell therapy provides the essential components of such a defined therapeutic agent, as fellows.

The therapeutic agent: Therapeutic benefits of stem cells include (1) regenerative action; (2) neuroprotective modulation; and (3) immune regulation. The BM-MSCT transplantation induces survival and proliferation of host neurons through secreting BDNF, β -NGF, and adhesion molecules^[50]. Stem cells can serve as a “Trojan Horse” for transplantation of cancer drugs^[50,51].

The autopilot vehicle: NSCs can detect a target (homing) *via* chemokines produced by tumors (Figure 4, Li *et al.*^[7] 2008), the capacity like a self-driving vehicle. Following this chemokine gradient, NSCs can move through tissue barriers such as the blood brain barrier and brain tumor barrier (Figure 3) to reach their target tissue. We need to determine the therapeutic window of stem cell development, the window of stem cell development that is capable for targeting tumors^[52]. If stem cells develop outside of a window period, thereby lose the ability of migrating toward tumors because their migration-required molecules are down regulated^[53].

Delivery system: Stem cell delivery for cancer remains to be defined. For brain tumors, we can use a stereotactic injection for a specific brain region. Mooney and colleagues show that NSCs can facilitate the tumor-selective distribution of nanoparticles, a drug-loading system that is promising in cancer therapy^[54]. We can apply CED (convection-enhanced delivery) to deliver stem cells across the blood-brain barrier and the brain-tumor barrier (Figure 3). We need further to track down stem cell migration *in vivo* by using a real-time tracking system as we discussed previously^[55], a way that can address possible adverse effects.

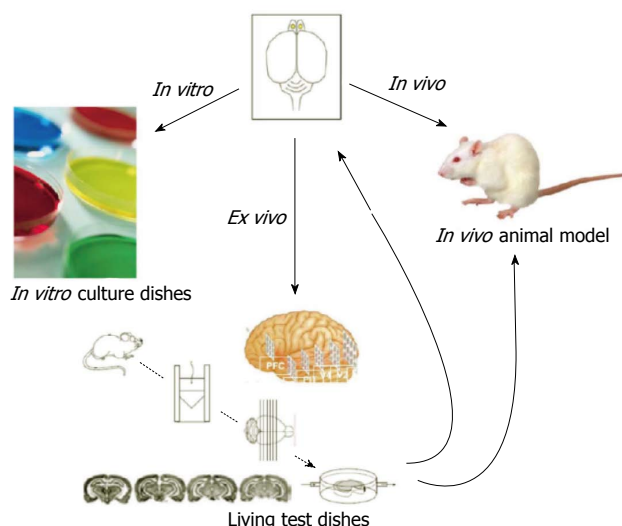


Figure 4 Three ways to drug testing: *In vitro* Petri dishes, *in vivo* animal model and *ex vivo* engineered tissue graft. An engineered tissue graft has an intrinsic character of native brain environment.

A PROBLEM IN STEM CELL TRANSPLANTATION AND ITS SOLUTION

Only marginal effects can be observed in stem cell therapy despite exciting potency shown in some animal models^[52]. In fact, it is a game of number wrestling between good stem cells and tumor cells^[52,53]. Current stem cell experiments in mouse models involve transplantation of millions of stem cells, with only some migrating toward tumors, a few surviving at the tumor site, and rare engraftment^[52,53]. The rest of the non-migratory stem cells are detrimental to a recipient, because these can induce the formation of heterogeneous tumor and inflammation. Thus, we must enable stem cells to pass certain uniform quality control standard so that they can fulfill their designed purpose of targeting brain tumors.

TRAIN STEM CELLS IN AN ORGAN-SPECIFIC MICROENVIRONMENT

The low number of stem cells capable of migrating toward tumors derived from Petri dish culture system as shown in preclinical and clinical studies may result from the following differences: (1) the source of stem cells; (2) methods of stem cell culture; (3) differentiation status (percentage of differentiated cells); (4) the age of the stem cells in culture; and (5) the nature of a tumor^[34].

We found that culture matrix makes a difference in stem cell characteristics. NSCs behave differently in coated Petri culture plates (Figure 5). NSCs show much more neurite growth on Matrigel-coated Petri polystyrene plates than on other adhesion molecule-coated plates (Collagen I, Collagen IV, or Laminine). Nevertheless, none of these adhesion molecules can generate uniform populations of stem cells. We have designed an engineered tissue graft model as a universal training platform

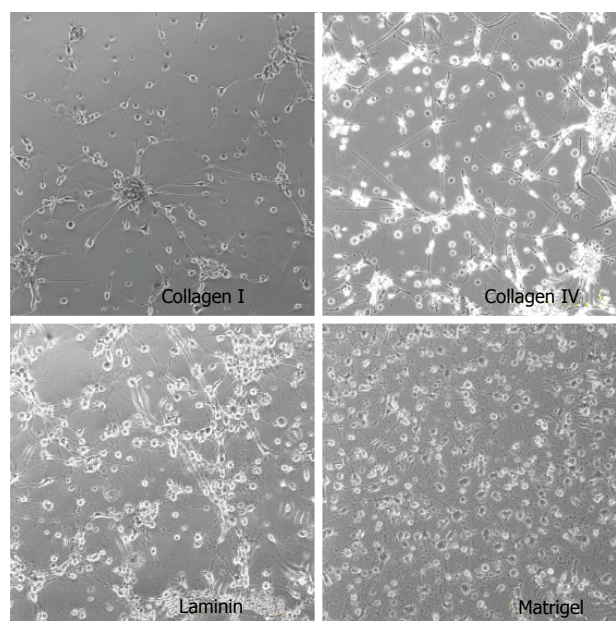


Figure 5 Stem cells cultured on Petri dishes coated with different matrix, showing non-physiologically relevant morphology with a few neurite growth.



Figure 6 An engineered brain tumor tissue graft in culture, showing tumor lesions (black dots) that attract stem cells to engraft.

to address the issue of heterogeneity of cultured stem cells^[7]. An engineered tissue graft (ETG) provides a native organ microenvironment closer to an *in vivo* model and very different from an *in vitro* Petri dish (polystyrene plates) system (Figure 4)^[56]. This ETG model can be generated from patient specific brain tumor specimens for autologous characterization of therapeutic *in vivo-like* trials of a new drug (Figure 6). This ETG material was made according to our patented technology - an ETG generated by seeding brain tumor stem cells onto slice cultures of patients' pathological brain tissue harvested during tumor resection - which preserved the pathological micro-environment^[52].

Such a culture platform can train stem cells to fulfill the purpose of targeting brain tumor cells as they help generate uniform neurite formation in culture that is essential for brain-tumor-targeted migration (Figure 7). These ETG-based matrix produced cells express molecu-

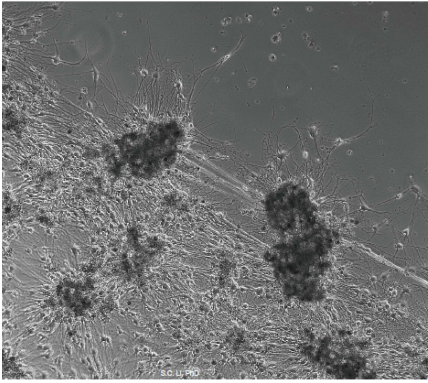


Figure 7 Neural stem cells cultured on the engineered tissue graft showing abundant neurite formation and neuronal morphology.

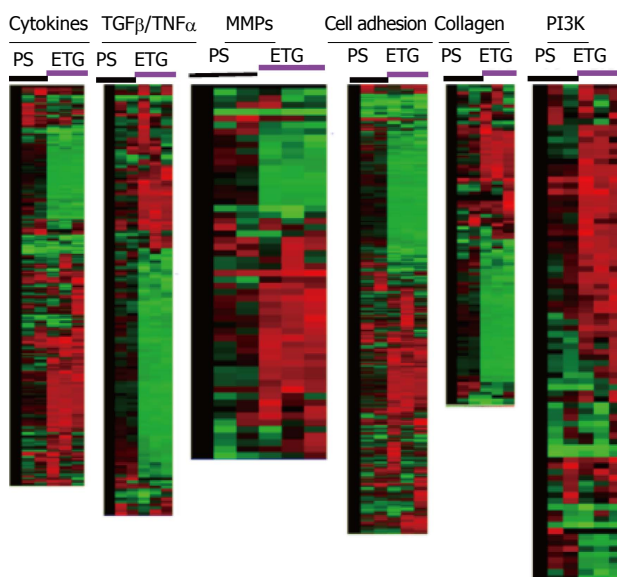


Figure 8 Data analysis of Affymetrix Gene Chip arrays for pediatric derived brain tumor stem cells grown on engineered tissue graftmatrix-like surface or polystyrene dish. The cells were grown on engineered tissue graft (ETG) matrix-like surface or polystyrene dish (PS) for 7 d for gene chip array analysis showing gene clusters on different functional group of signaling pathways. Notice that red color represents the highest expression, green color for medium expression, and black for lowest expression. MMPs: Matrix-remodeling matrix metalloproteinases; TNF: Tumor necrosis factors; TGF: Transforming growth factor.

lar markers different from cells cultured on polystyrene plates (PS) as shown in gene arrays (Figure 8). We can obtain a morphologically uniform population of stem cells in an ETG microenvironment (Figure 9). Optimizing the chemokine responsiveness (chemokine receptors expressed by stem cells) and upregulating matrix-remodeling matrix metalloproteinases (MMPs) are essential: Both chemokine receptor and MMPs are well expressed in cells with ETG but not with Petri dish culture system^[7]. Additionally, to overcome the problem of immune response, we have designed autologous iPSCs (induced pluripotent stem cells) for certain patient tumors (Figure 3), a dual system that can mutually promote each other for better efficacy. These trained stem cells can act as an autopilot

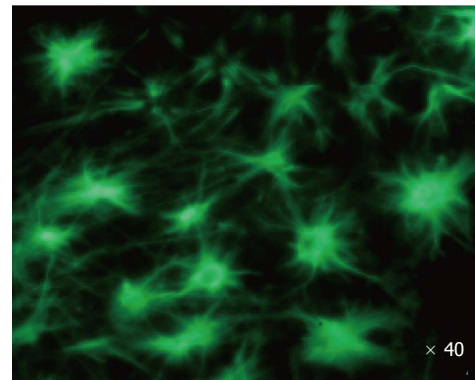
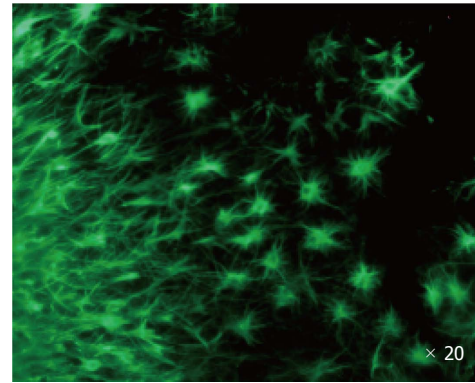


Figure 9 An engineered tissue graft is used as the designer matrix to train stem cells to target a specific tumor as shown for production of a morphologically homogeneous population of stem cells.

vehicle that is self-driven to its target (Li *et al.*^[7], 2008, Figure 5). This ETG can be engineered to mimic the *in vivo* fluidic microenvironment with the continuous flow of physicochemical buffer, the microfluidic system that can be coupled with real-time imaging for analysis of cell development as the quality control (QC) as detailed in a recent report^[57]. In the future, a QC system should be implemented for the structural and functional characterization of stem cell production before using for transplantation. This ETG could be scaled for automatic studies.

CONCLUSION

Stem cell therapies of brain tumors are being investigated preclinically; however, little efficacy has been found in clinical trials. We reviewed the literature and found that heterogeneous stem cell populations were made using artificial matrices, a roadblock to achieve consistent efficacy. We provide an ETG as a uniform platform to train stem cells for attacking tumor cells, which may address the discrepancies of current clinical trials.

ACKNOWLEDGEMENTS

We thank Maria Minon, MD; Saul Puszkun, PhD; Michael P Lisanti, MD-PhD; Richard G Pestell, MD-PhD; Joan S Brugge, PhD; Robert A Koch, PhD; Philip H Schwartz, PhD; for their support and enthusiasm.

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P- Reviewer: Lichtor T, Mueller WC **S- Editor:** Ji FF
L- Editor: A **E- Editor:** Lu YJ



Ovarian cancer stem cells: Can targeted therapy lead to improved progression-free survival?

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Received: June 4, 2014 Revised: July 22, 2014

Accepted: August 30, 2014

Published online: September 26, 2014

Abstract

Despite significant effort and research funds, epithelial ovarian cancer remains a very deadly disease. There are no effective screening methods that discover early stage disease; the majority of patients are diagnosed with advanced disease. Treatment modalities consist primarily of radical debulking surgery followed by taxane and platinum-based chemotherapy. Newer therapies including limited targeted agents and intraperitoneal delivery of chemotherapeutic drugs have improved disease-free intervals, but failed to yield long-lasting cures in most patients. Chemotherapeutic resistance, particularly in the recurrent setting, plagues the disease. Targeting the pathways and mechanisms behind the development of chemoresistance in ovarian cancer could lead to significant improvement in patient outcomes. In many malignancies, including blood and other solid tumors, there is a subgroup of tumor cells, separate from the bulk population, called cancer stem cells (CSCs). These CSCs are thought to be the cause of metastasis, recurrence and resistance. However, to

date, ovarian CSCs have been difficult to identify, isolate, and target. It is felt by many investigators that finding a putative ovarian CSC and a chemotherapeutic agent to target it could be the key to a cure for this deadly disease. This review will focus on recent advances in this arena and discuss some of the controversies surrounding the concept.

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Key words: Epithelial ovarian cancer; Cancer stem cells; Chemoresistance; Targeted therapy; Chemotherapy; Recurrent ovarian cancer

Core tip: Ovarian cancer stem cells (CSCs) are difficult to isolate, identify, and target. However, they are often thought to be the source of development of chemoresistance. Finding a therapeutic target in ovarian CSCs and identifying the mechanisms associated with the development of chemoresistance may lead to a long-lasting cure for patients with epithelial ovarian cancer.

Walters Haygood CL, Arend RC, Straughn JM, Buchsbaum DJ. Ovarian cancer stem cells: Can targeted therapy lead to improved progression-free survival? *World J Stem Cells* 2014; 6(4): 441-447 Available from: URL: <http://www.wjgnet.com/1948-0210/full/v6/i4/441.htm> DOI: <http://dx.doi.org/10.4252/wjsc.v6.i4.441>

INTRODUCTION

It is estimated that over 14000 women in the United States will die with ovarian cancer and more than 22000 women will be newly diagnosed with the disease in 2013^[1]. Women with early stage disease often have vague symptoms such as bloating, back pain, and fatigue leaving most women undiagnosed until later stages of the disease. Standard treatment of ovarian cancer consists of surgical resection of disease followed by taxane and plati-

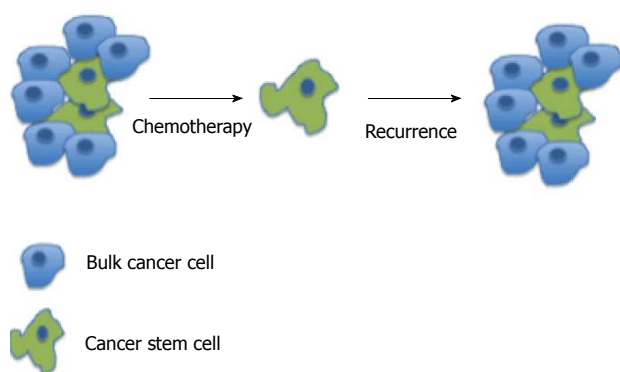


Figure 1 Death of bulk cancer cells by chemotherapy, but not cancer stem cells leads to recurrence.

num-based chemotherapy which yields a partial response rate of greater than 80% and a complete response rate of 40%-60% in patients with advanced disease^[2]. Although initial response rates are promising, the recurrence rate is approximately 70% and five-year survival is 45% in patients with advanced disease^[3]. While it appears that the majority of ovarian cancer cells are initially chemosensitive as evidenced by the high initial chemotherapy response rates, the high recurrence rates suggest development of chemoresistance. Some believe that a population of cells are not killed by chemotherapy, or they repopulate after exposure to chemotherapeutic agents. These cells have been called ovarian cancer stem cells (CSCs).

CSCS

It has been theorized that CSCs exist in certain malignancies, particularly the blood cancers and basal-like breast cancer. For the blood cancers, identifying CSCs has been in progress since the first stem cells were identified^[4]. In acute myeloid leukemia, CSCs have been proven to be an immature abnormally differentiated cells that have the ability to self-renew^[5]. It is felt by some investigators that these CSCs exist to promote tumor growth and metastasize to other organs. They have an increased tumorigenicity and differentiating capacity compared to other cells. The majority of solid tumor cells, may not have a differentiation capacity or the ability to develop chemoresistance but offer support to angiogenesis or signaling pathways. The CSCs (progenitor cells) are typically a small portion of the tumor and give rise to differentiated progeny that comprise the bulk of tumors (Figure 1), and are capable of unlimited growth^[6,7]. CSC markers have been shown to be upregulated in cells growing in tumorspheres compared to single cells suggesting that CSCs are enriched in this population. In ovarian cancer, this spheroid form of tumor cells is thought to be involved in the dissemination of cancer in the peritoneal cavity. This suggests that CSCs are involved in metastasis intra-abdominally. CSCs are generally thought to have the ability to self-renew, differentiate, and metastasize to form secondary and tertiary tumors^[8]. It has been shown that primary treatment with chemotherapeutic agents

results in increased drug-resistant CSCs and this leads to recurrence^[9]. Unlike some of the blood cancers which have known normal stem cells, there is no known normal ovarian stem cell^[6]. This obviously complicates the identification of specific ovarian CSCs. The majority of evidence in favor of ovarian CSCs exists from the identification of markers of "stemness" as identified in other malignancies. Still, many researchers are investigating the existence of specific ovarian CSCs.

OVARIAN CSCS

The isolation of ovarian CSCs is fraught with difficulty, like that of many other solid tumors. For isolation to occur, a single-cell suspension must be made from a solid tumor while sustaining viability. While there may be a large volume of tumor or ascites, the actual CSCs are a rare population of that tumor; unlike blood tumors, there is no specific marker for an ovarian CSC. The first model for this process was described by Bapat *et al*^[10] in 2005. They collected ascites from a patient sample and were able to develop 19 immortalized tumor sphere-forming clones. Two of these were passaged into nude mice and grew into tumors that closely resembled the parental tumor. A single transformed clone was able to be isolated that demonstrated increased aggressiveness from the parent tumor. This experiment was some of the first evidence to show heterogeneous growth properties of tumor cell subpopulations in ovarian cancer. Also, these tumor cells demonstrated the ability to self-renew by continuing to form tumors even after serial transplantation.

CSC MARKERS

There is no specific ovarian CSC marker and researchers have relied on markers of "stemness" identified from other malignancies. Some of these proteins used as CSC markers include CD44, CD133, CD117, ALDH1A1, and EpCAM (Table 1). There are many other proteins that have been used as markers of "stemness" but are not as well defined in ovarian cancer. Discovered as a marker for breast development and breast carcinoma, CD44 is a hyaluronate receptor^[11] that is involved in cell-cell and cell-matrix interactions and ultimately affects cellular growth, differentiation, and motility^[12,13]. Zhang *et al*^[14] found that CD44+/CD117+ cells had increased chemoresistance to taxane and platinum-based chemotherapy as well as the ability to self-propagate. Similarly, Alvero and colleagues showed that CD44+ cells were enriched in ovarian cancer patient ascites and once isolated and xenografted gave rise to tumor with both CD44+ and CD44- cells suggesting they can differentiate and self-renew^[15]. Orian-Rosseau described various strategies to target the CD44 receptor, which included binding to hyaluronic acid and osteopontin, a protein involved in interleukin production and overexpressed in ovarian cancer, as well as contributing to receptor tyrosine kinase activation^[16].

CD133 is a transmembrane glycoprotein that is expressed in normal hematopoietic and epithelial stem

Table 1 Cancer stem cell markers and significance

Cancer stem cell marker	Expression	Significance
CD44	Hyaluronate receptor	Cell growth, differentiation, motility, increased chemoresistance, self-propagation
CD133	Transmembrane glycoprotein	Increased tumor formation, increased chemoresistance, regeneration of original tumor cells
CD117	Tyrosine kinase receptor	Cell signaling, apoptosis, cell differentiation, proliferation, cell adhesion
ALDH1A1	Cell protector from aldehydes	Regeneration of tumor cells, chemoresistance
EpCAM (CD326)	Transmembrane glycoprotein	Cell adhesion, cell proliferation, tumor formation, epithelial to mesenchymal transition
CD24	Transmembrane glycoprotein	Cell adhesion, aggressive phenotype, metastasis

cells, and has also been described as a CSC marker in solid tumors. Ferrandina *et al.*^[17] showed that the amount of CD133 positive cells was higher in ovarian carcinoma than in normal ovarian tissue. In 2009, Baba and colleagues reported the ability of CD133+ cancer cells to generate both CD133+ and CD133- cells, similar to what Alvero had seen with CD44+ cell spore^[18]. CD133 has also been shown to be involved in increased tumor formation, increased chemoresistance, and the ability to recapitulate the original heterogeneous tumor^[19].

CD117, also known as c-kit or stem cell growth factor receptor, is a proto-oncogene encoded by the KIT gene. It is a type of tyrosine kinase receptor involved in cell signal transduction. It has been shown to be involved in apoptosis, cell differentiation, proliferation, and cell adhesion^[20]. CD117 was shown by Kusumbe *et al.*^[21] to have high expression in ovarian cancer cells. Interestingly, cells expressing CD117 appear to be highly tumorigenic as it only takes approximately 10^3 cells to be able to self-renew, differentiate, and regenerate tumor in mouse models^[22]. The Wnt/ β -catenin pathway which has been implicated in the development of chemoresistance is activated by CD117^[23].

ALDH1A1 is a member of the ALDH group of proteins, which contains 19 enzymes that function as cell protectors from carcinogenic aldehydes^[24]. Landen *et al.*^[25] declared it a putative CSC marker and showed its association with chemoresistance in ovarian carcinoma. Cells that are double positive for CD133 and ALDH1A1 have a greater ability to develop tumors in mouse models as compared to CD133+/ALDH1A1 - or ALDH1A1 +/CD133 - cells^[26]. Recently, Shank *et al.*^[27], showed that metformin decreased the population of ALDH+ cells in ovarian cancer cell lines as well as decreased the formation of tumor spheres in patient tumors. *In vivo*, they also presented that metformin would restrict the growth of whole tumor cell line xenografts^[27].

EpCAM (CD326) is a transmembrane glycoprotein involved in cell adhesion. EpCAM has been shown to have oncogenic signaling properties which result in cell proliferation and tumor formation^[28]. Higher expression of EpCAM has also been seen in metastatic ovarian tumors^[29] and it is involved in epithelial to mesenchymal transition leading to metastasis^[30].

Another glycoprotein identified as an ovarian CSC is CD24 which is a cell membrane glycoprotein involved in cell adhesion. In 2005, the movement of CD24 from the cell membrane to the cytoplasm in borderline ovarian tu-

mors was associated with microinvasion and omental implants as well as shorter survival time in adenocarcinoma of the ovary^[31]. Moulla *et al.*^[32] also demonstrated that the transition from membrane to cytoplasmic CD24 expression was associated with a more aggressive phenotype in borderline tumors.

CLINICAL SIGNIFICANCE

While it is interesting to utilize proteins to identify CSCs in various tissues, the clinical significance of these markers is still being determined. In 2012, Meng and colleagues reported on CD44+/CD24- cells in ovarian cancer cell line studies and patient ascites samples. Ovarian cancer cell line studies confirmed that increased numbers of CD44+ cells increased chemoresistance. Patient ascites samples with > 25% CD44+ cells had significantly decreased median progression-free survival (6 mo *vs* 18 mo, $P = 0.01$) as well as propensity to recur (83% *vs* 14%, $P = 0.003$)^[33]. Zhang and colleagues studied 400 ovarian cancer tissue samples for CD133 positivity. They found associations between CD133+ and higher grade ovarian tumors, advanced stage disease, and decreased response to chemotherapy. They also found that CD133+ tumors are associated with decreased overall survival ($P = 0.007$) and shorter disease free interval ($P < 0.001$)^[34]. In a study by Chau *et al.*^[23], they evaluated 3 patient samples in a xenograft mouse model and it was found that there was increased chemoresistance in patients with CD117+ tumor cells. In 65 ovarian cancer patients with advanced stage disease, greater than 20% of ALDH1A1+ cells correlated with decreased progression-free survival (6 mo *vs* 14 mo, $P = 0.035$)^[25]. Recently, Zhu *et al.*^[35] reported on overexpression of CD24 in epithelial ovarian cancer and found that it was an independent variable associated with a low survival rate, increased metastasis, and decreased survival time.

Recent studies have indicated an enriched population of CSCs in ovarian cancer patients with recurrent carcinoma as compared to patients with primary cancer. Rizzo *et al.*^[36] noted an increased percentage of side population cells (generally accepted to be CSCs) in the ascites of patients with first recurrence after platinum-based chemotherapy as compared to ascites of chemo-naïve patients. Steg *et al.*^[37] compared 45 matched primary and recurrent ovarian cancer patient samples for expression of stem cell markers including ALDH1A1, CD44, and CD133. Primary samples showed low densities of the markers,

but samples collected after primary therapy showed higher densities of ALDH1A1, CD44, and CD133 due to the death of the non-stem cells. Stem cell markers were also examined in this study and 14% of recurrent tumors showed overexpression of these markers compared to primary tumors.

TARGETING OF OVARIAN CSCS

Stem cell markers have been implicated in chemoresistance and recurrence of ovarian cancer; therefore, it is reasonable to evaluate agents that could target these cells. CD44 has been studied with phase I trials in head and neck cancer *via* an antibody drug conjugate, BIWI 1^[38]. There have also been several monoclonal antibodies designed to target CD44 in squamous cell cancers which could be extrapolated to adenocarcinomas^[39]. CD44+ cells have been targeted in an intraperitoneal (IP) mouse model with cisplatin via a conjugate of hyaluronic acid and cisplatin which was then internalized more efficiently than CD44+ cells in ovarian cancer cell lines (A2780 and OV2008). Li and Howell^[40] also demonstrated decreased growth in IP inoculated A2780 ovarian cancer cells treated with a hyaluronic acid-cisplatin conjugate when compared to free cisplatin. A hyaluronic acid-paclitaxel (HA-TXL) conjugate to target CD44+ cancer cells has also been studied in an IP mouse model with ovarian cancer cell lines (SKOV3ip1 or HeyA8) and showed significantly reduced tumor weights and nodules^[41]. Similarly, CD133 has been targeted by IP administration of an anti-CD133 targeted toxin (dCD133KDEL), in an ovarian cancer cell line (NIH:OVCAR5-luc) in a mouse model, which resulted in significant decrease in progression of CD133 expressing tumors^[42].

Noguera *et al.*^[43] evaluated imatinib mesylate, a CD117 specific inhibitor, in low grade recurrent platinum resistant tumors of the ovary in a single site phase II trial. Thirteen patients were enrolled and 48% of those had c-kit positive tumors. Eleven patients were eligible for evaluation of response, and though well-tolerated, no antitumor activity was seen in these low-grade tumors^[43]. An anti-EpCAM monoclonal antibody, catumaxomab, was evaluated in a phase II/III trial in 258 patients with malignant ascites from epithelial cancer, half of which were ovarian carcinomas. When compared to paracentesis alone for treatment of ascites, addition of catumaxomab increased the median time to next paracentesis (11 d *vs* 77 d, $P < 0.0001$). Patients who received catumaxomab also had decreased signs and symptoms of ascites. The safety profile was acceptable^[44]. Catumaxomab was evaluated in conjunction with steroid premedication (Catumaxomab Safety Phase IIIb Study with Intraperitoneal Infusion in Patients with Malignant Ascites Due to Epithelial Cancer) as well as in retreatment with IP therapy (SECIMAS), but results from these studies have not yet been posted (www.clinicaltrials.gov). It is also being evaluated in combination with cytotoxic chemotherapy in a phase II trial [ENGOT-ov8]^[45].

Another method of targeting CSCs is to target their signaling pathways, which include Notch, Wnt/ β -catenin, TGF- β , and Hedgehog pathways. McAuliffe and colleagues demonstrated this concept with the Notch pathway and platinum resistant ovarian cancer^[46]. In particular they looked at Notch3, and showed that it was overexpressed in ovarian CSCs and was correlated with increased platinum resistance. A pan Notch inhibitor, gamma-secretase inhibitor (GSI), when used in combination with cisplatin, had a synergistic cytotoxic effect, and led to decreased numbers of CSCs (12.8% side population cells in the control, 2.31% with Notch inhibitor alone, and 0.81% with GSI and cisplatin). A Notch ligand, Jagged 1, was targeted in taxane-resistant ovarian cancer cell lines by Steg *et al.*^[47]. They showed that targeting Jagged1 induced chemosensitivity to docetaxel *in vivo* and reduced tumor weights. They implicated the Hedgehog pathway in these experiments with Jagged1 by showing that rather than the chemoresistance being mediated by MDR1 as expected, it was GLI2, a Hedgehog downstream marker, that was downregulated. Another study with Jagged1 found that inhibition of the Wnt/ β -catenin signaling pathway reduced its expression^[48]. Wnt/ β -catenin pathways have previously been demonstrated to produce self-renewal in ovarian cancer and appear to be a driving force behind ovarian cancer progression^[49]. The Hedgehog signaling pathway has been implicated in the growth regulation of spheroid-forming cells in ovarian cancer. This was demonstrated by Ray *et al.*^[50], in four ovarian cancer cell lines (ES2, TOV112D, OV90, and SKOV3) where spheroid volume was increased up to 46-fold with Hedgehog agonists. Cyclopamine, a Hedgehog inhibitor, was used to prevent further growth of spheroid-forming cells in these cell lines and showed up to a 10-fold reduction in growth in ES2 cells^[50]. Multiple groups are actively working to target these signaling pathways in hopes of altering ovarian cancer chemoresistance and recurrence.

CONTROVERSIES

Although there is growing evidence that ovarian CSCs are relevant, there are still many who debate the existence of these cells. At the forefront of this debate, remains the fact that a specific ovarian CSC marker has not been identified. None of the markers discovered are exclusively found in ovarian cancer cells. CD133 is recognized as the putative CSC marker for many human solid tumors, however, signaling pathways that regulate its behavior remain unknown^[51]. Some studies presented in this review may be showing that CSCs are more “tumorigenic” based on ability of preferential or improved grafting. It will give much more credence to the argument if some of the pathways or markers being targeted show significant clinical results.

FUTURE DIRECTIONS

If progression and development of chemoresistance is due to the ovarian CSCs, then specific therapy for CSCs

must be developed. In ovarian cancer, the use of monoclonal antibodies to many surface markers for CSCs has proven of some potential value. The most utilized monoclonal antibody, bevacizumab, an anti-vascular endothelial growth factor agent, has been shown to improve progression-free survival in advanced ovarian cancer^[52]. Recently, CSCs have been implicated in the hypoxic environment that bevacizumab creates, but this relationship has not yet been well defined^[53]. In addition to those mentioned previously, the anti-CD44 antibody, A3D8 was shown to produce significant apoptosis and arrest of cell cycle in the S phase for the SKOV3 ovarian cancer cell line by Du *et al*^[54] and may represent a therapeutic option. Patients taking metformin for diabetes have previously been reported to have improved survival and some groups postulate that this relationship is due to the downregulation of CSC growth. A phase II trial is currently underway to evaluate this relationship (NCT01579812) (www.clinicaltrials.gov). There are over 3000 results when searching for clinical trials related to CSCs on Clinicaltrials.gov. While the majority of these are not specific for ovarian cancer, many are for breast cancer or other solid tumors, which have traditionally led to findings applicable to ovarian cancer.

CONCLUSION

It appears that ovarian CSCs are involved in chemoresistance and likely contribute to an overall poor prognosis in ovarian cancer patients. Researchers continue to study the role of ovarian CSCs and develop targeting agents for specific identification and therapeutic treatment. Clinical trials are ongoing for agents targeting ovarian CSCs and data from these trials will be important to determine future research directions aimed at improving survival in women with ovarian cancer.

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P- Reviewer: Iavazzo CR, Gardner Mutch D

S- Editor: Song XX **L- Editor:** A **E- Editor:** Lu YJ



Kallikrein-kinin in stem cell therapy

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Author contributions: Chao J reviewed the literature, conceived the paper and wrote the paper; Bledsoe G reviewed the literature, conceived the paper and wrote the paper; Chao L reviewed the literature, conceived the paper and wrote the paper.

Supported by National Institutes of Health, No. HL118516, HL29397 and HL44083

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Received: July 22, 2014 Revised: August 27, 2014

Accepted: August 30, 2014

Published online: September 26, 2014

Abstract

The tissue kallikrein-kinin system exerts a wide spectrum of biological activities in the cardiovascular, renal and central nervous systems. Tissue kallikrein-kinin modulates the proliferation, viability, mobility and functional activity of certain stem cell populations, namely mesenchymal stem cells (MSCs), endothelial progenitor cells (EPCs), mononuclear cell subsets and neural stem cells. Stimulation of these stem cells by tissue kallikrein-kinin may lead to protection against renal, cardiovascular and neural damage by inhibiting apoptosis, inflammation, fibrosis and oxidative stress and promoting neovascularization. Moreover, MSCs and EPCs genetically modified with tissue kallikrein are resistant to hypoxia- and oxidative stress-induced apoptosis, and offer enhanced protective actions in animal models of heart and kidney injury and hindlimb ischemia. In addition, activation of the plasma kallikrein-kinin system promotes EPC recruitment to the inflamed synovium of arthritic rats. Conversely, cleaved high molecular weight kininogen, a product of plasma kallikrein, reduces the viability and vasculogenic activity of EPCs. Therefore, kallikrein-kinin provides a new approach in enhancing the efficacy of stem cell therapy for human diseases.

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Key words: Tissue kallikrein; Plasma kallikrein; Kinin; Mesenchymal stem cells; Endothelial progenitor cells; Neural stem cells; Heart; Kidney; Brain

Core tip: Tissue kallikrein-kinin exerts beneficial actions in the cardiovascular, renal and central nervous systems. Recent studies demonstrated that genetic modification of mesenchymal stem cells (MSCs) and endothelial progenitor cells (EPCs) by tissue kallikrein provides enhanced protection against renal ischemia/reperfusion, lupus nephritis, myocardial infarction and hindlimb ischemia. Tissue kallikrein stimulates the proliferation, viability, migration and functional activity of cultured MSCs, EPCs and neural stem cells. Moreover, plasma kallikrein-kinin augments EPC mobility and function in arthritis, whereas the cleaved kininogen product of plasma kallikrein inhibits EPC viability and tube formation. Thus, kallikrein-kinin may enhance the efficacy of stem cell therapy for human diseases.

Chao J, Bledsoe G, Chao L. Kallikrein-kinin in stem cell therapy. *World J Stem Cells* 2014; 6(4): 448-457 Available from: URL: <http://www.wjgnet.com/1948-0210/full/v6/i4/448.htm> DOI: <http://dx.doi.org/10.4252/wjsc.v6.i4.448>

INTRODUCTION

Tissue kallikrein (*KLK1*) and plasma kallikrein (*KLKB1*) are serine proteinases encoded by distinct genes, and thus differ in molecular weight, amino acid sequence and immunogenicity^[1-3]. Human tissue kallikrein cleaves low molecular weight (LMW) kininogen to produce Lys-bradykinin (Lys-BK), which is subsequently converted to BK by aminopeptidase^[2]. Plasma kallikrein processes high molecular weight (HMW) kininogen substrate to form BK^[2]. Both kinin peptides bind to the kinin B2 receptor to elicit a diverse array of biological effects^[2-5], including enhancing stem cell function (Figure 1). The kinin B2 re-

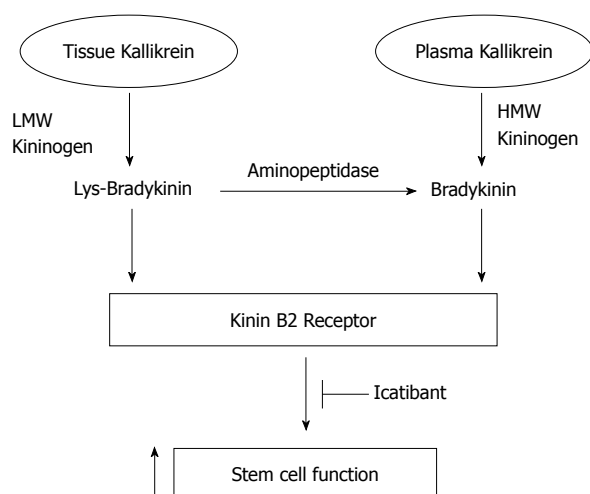


Figure 1 Tissue kallikrein-kinin and plasma kallikrein-kinin systems on stem cell function. LMW: Low molecular weight; HMW: High molecular weight.

ceptor is constitutively expressed with a wide tissue distribution, but can be blocked by the specific antagonist icatibant (Hoe140)^[5]. Kinin metabolites of kininase I, such as des-Arg⁹-BK and des-Arg¹⁰-Lys-BK, bind to the kinin B1 receptor, which is expressed at very low levels under normal conditions but is induced by inflammation^[5]. The tissue kallikrein-kinin system triggers a broad spectrum of biological activities, including stimulation of angiogenesis and reduction of hypertension, cardiac and renal damage, ischemic stroke, restenosis, diabetes and skin wound injury^[6]. Plasma kallikrein circulates in the blood as a proenzyme and, upon its activation, functions to produce BK to increase vascular permeability and stimulate vasodilation and inflammation^[7,8]. Activated plasma kallikrein also initiates the intrinsic pathway of coagulation and the fibrinolytic system^[7,8]. In this review, we discuss the involvement of tissue kallikrein, plasma kallikrein and kinin peptides in promoting the mobility and functional capacity of stem cells, which may lead to enhanced protection against organ injury in human diseases.

TISSUE KALLIKREIN-KININ IN RENAL INJURY

Tissue kallikrein was first discovered in human urine as a hypotensive substance^[9]. Urinary (tissue) kallikrein excretion is significantly reduced in patients with mild kidney disease and severe renal failure^[10,11]. Tissue kallikrein gene transfer or protein infusion in hypertensive Dahl salt-sensitive (DSS) rats has been observed to decrease kidney injury, improve renal function, and stimulate nitric oxide (NO) generation *via* the kinin B2 receptor^[12-15]. Moreover, tissue kallikrein or kinin administration not only attenuated but also reversed renal inflammation, apoptosis and fibrosis in conjunction with reduced oxidative stress and increased NO production in hypertensive DSS and deoxycorticosterone acetate (DOCA)-salt rats^[15-18]. The renal protective effects of tissue kallikrein in DSS rats

were abolished by icatibant, indicating a kinin B2 receptor-mediated event^[15]. Conversely, endogenous tissue kallikrein depletion in DOCA-salt rats augmented renal injury, inflammation and fibrosis in association with increased expression of pro-inflammatory and pro-fibrotic genes, oxidative stress, and reduced NO levels^[19]. Moreover, double knockout of the kinin B1 and B2 receptors in mice demonstrated that these receptors protect against ischemia/reperfusion (I/R)-induced renal damage, apoptosis and mortality^[20]. In a unilateral ureteral obstruction model, interstitial collagen content in the kidney was increased in kinin B2 receptor deficient mice, whereas transgenic rats expressing human tissue kallikrein displayed reduced renal fibrosis^[21]. Therefore, endogenous tissue kallikrein-kinin *via* kinin B2 receptor signaling can prevent and reverse renal injury by inhibiting oxidative stress, apoptosis, inflammation and fibrosis.

TISSUE KALLIKREIN-KININ IN CARDIAC INJURY

Tissue kallikrein-kinin components have been localized in the heart and blood vessels, indicating their involvement in cardiovascular function^[22-24]. Indeed, both tissue kallikrein and kinin B2 receptor knockout mice develop dilated cardiomyopathy, and mice with kinin B2 receptor genetic ablation exhibit cardiac fibrosis^[25,26]. However, expression of tissue kallikrein in transgenic rats reduces isoproterenol-induced cardiac hypertrophy and fibrosis^[27]. Likewise, tissue kallikrein gene delivery protects against cardiac remodeling as well as neovascularization in spontaneously hypertensive rats (SHR) and salt- and pressure-induced hypertensive rats^[28-31]. Tissue kallikrein infusion or gene transfer also improved impaired cardiac function and reduced heart remodeling, apoptosis and inflammation in animal models of myocardial infarction (MI), myocardial I/R and streptozotocin-induced diabetes^[32-36]. The cardioprotective effects of tissue kallikrein on apoptosis and inflammation were blocked by icatibant and a NO synthase (NOS) inhibitor, indicating a kinin B2 receptor-NO-mediated event^[35,36]. Furthermore, tissue kallikrein gene delivery to the peri-infarct myocardium increased cardiac progenitor cell (CPC) levels and promoted cardiac neovascularization and function in rats with post-MI heart failure^[37]. Although tissue kallikrein increased CPC density, their levels were low compared to other cardiac cells. Thus, the regenerative capacity of CPCs in the adult heart appears to be limited and requires further investigation. Taken together, tissue kallikrein-kinin elicits cardiac protection by inhibiting apoptosis, inflammation and myocardial remodeling, and increasing angiogenesis through kinin B2 receptor-NO signaling.

TISSUE KALLIKREIN-KININ IN VASCULAR INJURY

Endothelial cell loss leads to vascular dysfunction and

vascular-related diseases. Tissue kallikrein levels in the circulation are significantly higher in patients with coronary artery disease (CAD) compared to non-CAD patients, and increase with disease severity, from moderate CAD to multi-vessel CAD with acute obstruction^[38]. This suggests that circulating tissue kallikrein levels may be used as a predictive tool to assess the presence and extent of CAD. Tissue kallikrein gene transfer into rat left common carotid artery after balloon angioplasty was shown to cause a marked reduction in neointima formation at the injured vessel, and this effect was mediated by a kinin B2 receptor-NO pathway^[39,40]. In addition, endothelium-dependent relaxation was improved in tissue kallikrein transgenic rats with diabetic cardiomyopathy, but significantly reduced in kinin B1 and B2 receptor knockout mice in association with a decrease in NO production^[41,42]. Moreover, kinin B2 receptor-deficient mice exhibit myocardial capillary rarefaction^[43]. Conversely, tissue kallikrein gene delivery promoted neovascularization and attenuated cardiac remodeling in animal models of hypertension and MI^[31,32]. Tissue kallikrein is capable of accelerating spontaneous angiogenesis in a mouse model of hindlimb ischemia by activating Akt and endothelial NOS (eNOS) signaling pathways^[44,45]. Tissue kallikrein also enhanced the migration and tube formation of cultured endothelial cells, but these effects were blocked by icatibant, constitutively active glycogen synthase kinase (GSK)-3 β , vascular endothelial growth factor (VEGF) antibody and VEGF receptor inhibitor^[46]. Furthermore, kinin stimulated the proliferation and capillary tube formation of endothelial cells *via* transactivation of VEGF receptor-2 through the kinin B2 receptor^[47,48]. These findings indicate that tissue kallikrein-kinin attenuates vascular injury by preventing neointima formation and promoting angiogenesis through Akt-eNOS and Akt-GSK-3 β -VEGF mediated signaling pathways.

TISSUE KALLIKREIN-KININ IN ISCHEMIC STROKE

The time window for treatment of stroke patients is limited, as the clinically accepted treatment regimen with tissue plasminogen activator (tPA) requires initiation within 3 h of symptom onset^[49]. Tissue kallikrein has a superior advantage over tPA with a wide time window after stroke. In a double-blinded clinical trial, human tissue kallikrein was shown to be effective in the treatment of patients with acute brain infarction when infused within 48 h of established stroke^[50]. These findings indicate that tissue kallikrein therapy is a promising regimen in the treatment of ischemic stroke in humans. Moreover, tissue kallikrein-kinin therapy has been shown to be an effective approach in the treatment of stroke-induced brain injury in animal models^[51,52]. Neuroprotective effects were observed upon local injection of the human tissue kallikrein gene into rat brain immediately after cerebral I/R injury, or by systemic delivery of the tissue kallikrein gene at 8 h after ischemic stroke onset^[51,52].

Tissue kallikrein administration reduced I/R-induced cerebral infarction and promoted the survival and migration of glial cells from penumbra to the ischemic core up to two weeks^[51,52]. Tissue kallikrein also decreased I/R-induced apoptosis of neuronal cells and inhibited inflammatory cell accumulation in the ischemic brain, but these effects were blocked by icatibant^[52]. Furthermore, tissue kallikrein gene transfer enhanced neurogenesis and angiogenesis in rats after cerebral I/R^[52]. Tissue kallikrein's effects occurred in association with increased NO levels and reduced oxidative stress *via* activation of the kinin B2 receptor^[51,52]. In contrast, ischemic brain injury is exacerbated in kinin B2 receptor knockout mice^[53]. Thus, tissue kallikrein-kinin therapy may serve as a valuable approach in the treatment of stroke-induced brain injury, especially if treatment is delayed.

MESENCHYMAL STEM CELLS IN RENAL AND CARDIAC DISEASES

Mesenchymal stem cells (MSCs) are heterogeneous, multi-potent stromal cells that possess non-immunogenic and immunosuppressive properties^[54]. MSCs have been documented to reside in bone marrow, adipose tissue, umbilical cord blood, placenta, amniotic fluid and amniotic membrane^[55]. MSCs can be characterized by three main criteria: (1) adherent to plastic culture dishes; (2) expression of the cell surface markers CD73, CD90, CD105, and CD271; and (3) differentiation into lineages of osteoblasts, adipocytes, and chondroblasts *in vitro*^[55]. MSCs have the ability to migrate to sites of organ injury and participate in tissue repair by exerting paracrine actions to produce therapeutic effects, such as neovascularization and organ regeneration^[54,56-59]. Clinical trials using human bone marrow-derived MSCs are currently underway to treat diseases such as renal, cardiovascular, and cerebrovascular disorders (<http://clinicaltrials.gov>). Efficacy can be maximized by pre-treatment of MSCs with drugs, cytokines, and growth factors, and by genetically modifying MSCs^[60]. Indeed, enhancing stem cell therapy by genetic modification has been shown provide advanced benefits in the treatment of various diseases^[61]. For example, MSCs genetically modified with hepatocyte growth factor or VEGF ameliorated I/R- or cisplatin-induced renal damage, inflammation and apoptosis^[62,63]. Moreover, modification of MSCs with the anti-apoptotic *Akt* gene or the anti-oxidant heme oxygenase-1 gene was observed to augment ischemic cardiac function and stem cell viability, and decrease ventricular remodeling and apoptosis compared to control MSCs^[64,65]. Thus, modification of MSCs with a gene that suppresses inflammation, apoptosis and oxidative stress would be highly desirable in the treatment of renal and cardiovascular dysfunction. Tissue kallikrein fits this profile, and MSCs modified with tissue kallikrein have been shown to exert enhanced protective actions in the heart and kidney as well as *in vitro*^[66,67].

STUDIES OF CULTURED MSCs MODIFIED WITH TISSUE KALLIKREIN GENE

Bone marrow-derived rat MSCs transduced with adenovirus harboring the human tissue kallikrein gene (*TK-MSCs*) secrete tissue kallikrein along with elevated VEGF levels in culture medium^[66,67]. *TK-MSCs* were also found to be more resistant to hypoxia- and H₂O₂-induced apoptosis, and exhibited less caspase-3 activity compared to control MSCs. In addition, *TK-MSC* conditioned medium stimulated the proliferation, migration and tube formation of cultured human endothelial cells, most likely *via* VEGF^[67]. In cultured cardiomyocytes, conditioned medium from *TK-MSCs* suppressed hypoxia-induced apoptosis and caspase-3 activity, and increased Akt phosphorylation^[67]. Moreover, human MSCs possess kinin B2 receptors, as kinin stimulation increased intracellular calcium levels in MSCs, but this effect was blocked by icatibant^[68]. This suggests that *TK-MSCs* exert their effects *via* autocrine and paracrine mechanisms. Furthermore, these results demonstrate that culture medium of MSCs genetically modified with the tissue kallikrein gene promotes the function, migration and viability of cultured endothelial and cardiac cells.

TISSUE KALLIKREIN-MODIFIED MSCs PROVIDE ENHANCED PROTECTION IN KIDNEY INJURY

Acute renal failure is a common disease with high morbidity and mortality^[69]. In kidney transplants, ischemia can lead to long-term renal dysfunction^[69,70]. However, implantation of bone marrow-derived MSCs after acute I/R resulted in renal function and morphological recovery, implicating the high therapeutic potential of MSCs in healing damaged kidney^[56,71]. Indeed, *TK-MSC* administration in rats subjected to I/R injury was shown to be protective against kidney damage^[66]. After systemic injection of *TK-MSCs*, human tissue kallikrein expression was identified in rat glomeruli. Rats receiving *TK-MSCs* exhibited an improvement in renal function after I/R. *TK-MSC* implantation in the kidney also markedly reduced tubular injury, renal cell apoptosis, and interstitial inflammatory cell accumulation. The protective effects of *TK-MSCs* occurred in conjunction with decreased myeloperoxidase activity, superoxide formation, and pro-inflammatory gene expression. Therefore, MSCs incorporating the human tissue kallikrein gene have advanced benefits in protection against ischemia-induced renal injury by suppression of oxidative stress, apoptosis and inflammation.

TISSUE KALLIKREIN-MODIFIED MSCs IN LUPUS NEPHRITIS PROTECTION

Tissue kallikrein has been identified as a lupus nephritis-

susceptibility gene and is associated with anti-glomerular basement membrane (GBM) antibody-induced nephritis^[72,73]. *TK-MSCs* were shown to exert beneficial effects in mice receiving anti-GBM antibody injection and in a murine model of lupus nephritis by suppressing inflammation and oxidative stress^[74]. *TK-MSC* administration to mice subjected to anti-GBM antibody injection resulted in the expression of human tissue kallikrein in the kidney as well as a significant reduction in proteinuria, blood urea nitrogen levels and renal pathology, compared to mice injected with control MSCs. Similarly, *TK-MSC* implantation in lupus-prone bicongenic mice improved kidney function and attenuated renal inflammatory cell infiltration and apoptosis in conjunction with reduced expression of numerous inflammatory cytokines and apoptotic factors in both kidney and serum. These novel findings indicate that tissue kallikrein-modified MSCs may serve as a targeted therapeutic agent in lupus nephritis.

TISSUE KALLIKREIN-MODIFIED MSCs IN CARDIAC PROTECTION

Chronic heart failure induced by MI leads to a loss of cardiac tissue and impairs left ventricular function^[58]. MSCs are a promising strategy for the repair and regeneration of heart cells as well as the restoration of cardiac function after an ischemic insult. However, a major limitation to the efficacy of stem cell therapy is the poor viability of implanted cells. Thus, genetic modification of MSCs to promote their viability may further aid in the treatment of cardiac damage. Cell culture studies showed that *TK-MSCs* display decreased apoptosis induced by hypoxia or oxidative stress^[66,67]. In rats with acute and chronic MI, myocardial injection of *TK-MSCs* resulted in enhanced cardiac protection compared to control MSC treatment^[67]. One day after MI, rats receiving *TK-MSC* administration were shown to have improved cardiac function and decreased apoptosis, inflammatory cell accumulation, and expression of pro-inflammatory genes. At two weeks after MI, *TK-MSC* implantation enhanced cardiac function, decreased infarct size, and attenuated cardiac hypertrophy and fibrosis. Furthermore, *TK-MSC* injection increased capillary and arteriole density in the peri-infarct area. These results indicate that *TK-MSC* treatment after acute and chronic MI provides significant protection against heart damage by promoting neovascularization and preventing apoptosis and inflammation.

ENDOTHELIAL PROGENITOR CELLS IN CARDIOVASCULAR DISEASES

Endothelial injury is a critical factor for complications associated with cardiovascular disease^[75]. Endothelial progenitor cells (EPCs) are a continuous endogenous source of replenishment for damaged vessels, and thus serve to maintain vascular integrity in response to endothelial injury^[75,76]. Bone marrow-derived EPCs are considered to be adult stem cells due to their participation in postnatal

angiogenesis^[77]. EPCs contribute to vasculogenesis by incorporating into the vasculature, thereby implicating their therapeutic potential in endothelial repair^[78]. Decreased numbers of circulating EPCs have been observed in patients with hypertension, chronic renal failure, CAD, and rheumatoid arthritis^[78-81]. Moreover, EPCs isolated from patients with hypertension and CAD displayed an impaired migratory response^[79]. However, the correlation of circulating EPC number and outcome of stroke patients is inconsistent. Lower EPC numbers were found to be associated with acute ischemic stroke^[82], whereas higher EPC levels were reported in hemorrhagic stroke patients^[83]. Reduced EPC numbers may be attributed not only to defective mobility and proliferation, but also to accelerated apoptosis or senescence. Therefore, augmented viability and mobilization of EPCs from bone marrow may be an alternative means to promote vascular repair. Furthermore, EPCs may serve as a vehicle for gene transfer approaches in the treatment of cardiovascular diseases. The tissue kallikrein-kinin system has been shown to be involved in cardiovascular remodeling, vascular function and angiogenesis^[6], making tissue kallikrein an ideal candidate for EPC genetic modification.

KININ B2 RECEPTOR ACTIVATION PROMOTES EPC RECRUITMENT

Healthy human subjects express high levels of kinin B2 receptor in CD133⁺CD34⁺ peripheral blood-mononuclear cell (PB-MNC) subsets and EPCs; kinin B1 receptor expression, however, is barely detectable in these cells^[84]. Kinin administration exerted a potent chemoattractant activity on EPCs *via* a kinin B2 receptor-phosphoinositide 3-kinase (PI3K)-eNOS-mediated mechanism. The role of the kinin B2 receptor in kinin-induced migration was verified using EPCs derived from kinin B2 receptor knockout mice. Kinin-responsive human PB-MNCs exhibited a pronounced pro-angiogenic activity, whereas EPCs from kinin B2 receptor-deficient mice were unable to sufficiently stimulate neovascularization in a mouse model of hindlimb ischemia. In addition, circulating CD133⁺CD34⁺ progenitor cells from patients with acute MI or stable angina expressed low levels of kinin B2 receptor, which corresponded to diminished migratory capacity toward kinin. Moreover, human circulating CD34⁺CXCR4⁺ MNCs expressing high levels of kinin B2 receptor adhered to cultured endothelial cells upon kinin treatment, and these kinin-stimulated mononuclear subsets were recruited to injured arterial wall *in vivo via* the kinin B2 receptor^[85]. Conversely, CD34⁺CXCR4⁺ MNCs from CAD patients exhibited low kinin B2 receptor expression levels. Furthermore, kinin administration had no effect on cellular recruitment upon icatibant treatment or in monocytes with low kinin B2 receptor expression. These studies indicate a novel mechanism of kinin B2 receptor activation in endothelial repair through recruitment of circulating EPCs and MNC subsets.

TISSUE KALLIKREIN-MODIFIED EPCs ENHANCE CARDIAC PROTECTION BY PROMOTING EPC MOBILIZATION AND FUNCTION

Tissue kallikrein was recently demonstrated to promote vasculogenesis and improve cardiac function after MI by enhancing peripheral EPC functional capacity^[86,87]. Human tissue kallikrein gene delivery significantly increased the number of circulating CD34⁺Flk-1⁺ EPCs as well as the growth of capillaries and arterioles in the peri-infarct myocardium in a mouse model of MI^[86]. In cultured EPCs, tissue kallikrein treatment stimulated cell migration and tube formation, and decreased hypoxia-induced apoptosis^[86]. Tissue kallikrein's effects were blocked by icatibant and a PI3K inhibitor, indicating a kinin B2 receptor-Akt signaling event. Moreover, adenovirus-mediated transduction of cultured EPCs with tissue kallikrein (TK-EPCs) resulted in the secretion of tissue kallikrein and VEGF into culture medium^[86,87]. TK-EPCs were also resistant to oxidative stress- and hypoxia-induced apoptosis in association with increased Akt phosphorylation and decreased caspase activity. Furthermore, mice receiving intra-myocardial injection of TK-EPCs after MI exhibited advanced protection against ischemic damage, as indicated by improved cardiac function and reduced infarct size^[87]. TK-EPC engraftment significantly decreased cardiomyocyte apoptosis and increased the retention of transplanted EPCs in the myocardium. The effects of TK-EPC administration were accompanied by increased capillary and arteriole density in the infarct border zone. These results show that implantation of tissue kallikrein-modified EPCs in the heart augments protection against cardiac injury by reducing apoptosis and promoting angiogenesis.

TISSUE KALLIKREIN-MODIFIED EPCs INDUCE ANGIOGENESIS IN THE ISCHEMIC HINDLIMB

Tissue kallikrein's pro-angiogenic activity has been clearly established^[6,44,45,86], and genetic modification of EPCs with tissue kallikrein was shown to promote neovascularization and cardiac function in an MI mouse model^[87]. Moreover, the effect of TK-EPC administration on spontaneous angiogenesis was identified in a rat model of hindlimb ischemia^[88]. Compared to control EPCs, TK-EPC injection *via* the caudal vein markedly increased muscular capillary density, blood flow and myofiber number at 7, 14 and 21 d after femoral artery ligation. The angiogenic effect of TK-EPCs correlated with elevated expression of eNOS and integrin α 3 on the surface of EPCs. Moreover, cultured TK-EPCs exhibited higher proliferative, migratory and adhesive activity than control EPCs^[88]. Inhibition of integrin α 3 blocked TK-EPC

Table 1 Enhanced protection by stem cells genetically modified with tissue kallikrein

	Apoptosis	Inflammation	Oxidative stress	Tissue remodeling	Angiogenesis
TK-MSCs					
Renal I/R ^[66]	↓	↓	↓	--	--
Lupus nephritis ^[74]	↓	↓	↓	--	--
MI ^[67]	↓	↓	--	↓	↑
TK-EPCs					
MI ^[87]	↓	--	↓	--	↑
Limb ischemia ^[88]	--	--	--	--	↑

TK-MSCs: Tissue kallikrein-modified mesenchymal stem cells; I/R: Ischemia/reperfusion; MI: Myocardial infarction; TK-EPCs: Tissue kallikrein-modified endothelial progenitor cells.

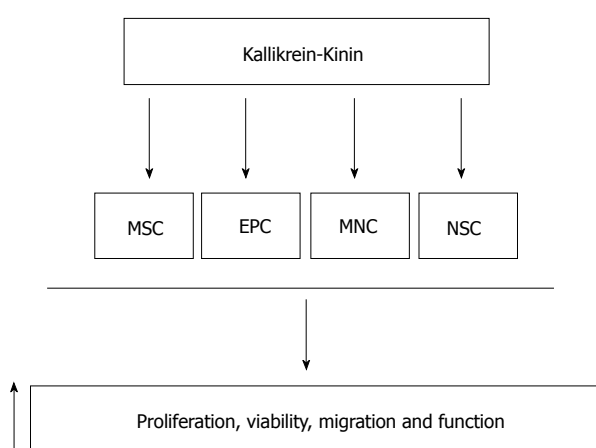


Figure 2 Kallikrein-kinin enhances the proliferation, viability, migration and function of stem cells. MSC: Mesenchymal stem cell; EPC: Endothelial progenitor cell; MNC: Mononuclear cell; NSC: Neural stem cell.

migration and adhesion, but had no effect on the proliferative activity of TK-EPCs. This suggests that EPCs genetically modified with tissue kallikrein enhance neovascularization and blood perfusion recovery after hindlimb ischemia.

TISSUE KALLIKREIN STIMULATES NEURAL STEM CELL GROWTH

Tissue kallikrein-kinin treatment has been shown to be effective in preventing stroke-induced ischemic brain injury by promoting neurogenesis and angiogenesis in animal models and cultured cells^[51,52]. In addition, tissue kallikrein was observed to stimulate the growth of rat neural stem cells independent of kinin formation, as icatibant had no effect on tissue kallikrein's actions^[89,90]. However, tissue kallikrein did not induce the differentiation of neural stem cells to neurons or glial cells^[90]. The proliferation of neural stem cells by tissue kallikrein is quite specific, with no detectable effect on other cell types, such as glial, pheochromocytoma, pituitary tumor, and cervical cancer cells^[90]. Thus, stimulation of neural stem cell proliferation by tissue kallikrein administration may lead to the generation of new neurons in the ischemic brain. Importantly, this stimulating effect of tissue kallikrein on neural stem cells may have significant value in the treatment of isch-

emic stroke.

PLASMA KALLIKREIN-KININ SYSTEM IN RECRUITMENT OF EPCs TO INFLAMED SYNOVIUM

Plasma kallikrein has been demonstrated to play a role in the pathogenesis of arthritis^[91,92]. As kinins are known to promote EPC mobilization and functional activity^[84,85], the involvement of the plasma kallikrein-kinin system in EPC mobilization was examined in a Lewis rat model of arthritis^[93]. The Lewis rat strain possesses a mutation in HMW kininogen (HK), resulting in accelerated HK cleavage and increased susceptibility to chronic inflammation^[94]. In arthritic Lewis rats, EPCs were recruited to the synovium at the acute phase of arthritis, and then differentiated into endothelial cells to form new blood vessels^[93]. Inhibition of plasma kallikrein by a specific inhibitor or anti-plasma kallikrein antibody dramatically suppressed synovial recruitment of EPCs and the proliferation of synovial cells. Moreover, EPCs isolated from bone marrow of Lewis rats were observed to have higher expression levels of kinin B2 receptor compared to control rat lung microvessel endothelial cells^[93]. In addition, kinin stimulated EPC migration and up-regulated expression of the homing receptor CXCR4 *in vitro* via the kinin B2 receptor. These results demonstrate a potential role of plasma kallikrein-kinin, *via* a kinin B2 receptor-dependent mechanism, in the recruitment of EPCs to inflamed synovium in arthritis.

CLEAVED HIGH MOLECULAR WEIGHT KININOGEN INHIBITS EPC FUNCTION

Cleaved HMW kininogen (HKa), a product of plasma kallikrein, has been shown to reduce the angiogenic function of endothelial cells as well as to stimulate their apoptosis^[95,96]. In cultured EPCs, HKa significantly inhibited VEGF-mediated tube formation and cellular differentiation into capillary-like networks^[97]. VEGF stimulated the secretion and activation of matrix metalloproteinase-2 (MMP-2), but not MMP-9, in the conditioned medium of EPCs. Inhibition or gene knockdown of MMP-2 indicated that this enzyme is required for EPC vasculo-

genesis. Although HKa prevented the conversion of pro-MMP-2 to MMP-2, it had no effect on MMP-2 activity. Furthermore, HKa was demonstrated to accelerate EPC senescence by increasing oxidative stress, leading to activation of the p38MAPK-p16^{INK4a} signaling cascade^[98]. These results indicate that HKa inhibits the vasculogenic capacity of EPCs by suppressing MMP-2 activation and promoting EPC senescence *via* oxidative stress-p38MAPK signaling, thus providing a link between the plasma kallikrein product HKa and EPC function.

CONCLUSION

The tissue kallikrein-kinin system plays an important role in the cardiovascular, renal and central nervous systems by inhibiting apoptosis, inflammation, fibrosis and oxidative stress. Tissue kallikrein-kinin may also enhance stem cell number and function. Indeed, tissue kallikrein-kinin increases the mobility, viability and functional capacity of stem cells, such as MSCs, EPCs, and MNC subsets, leading to protection against multi-organ injury and stimulating neovascularization. Tissue kallikrein may also exert a protective effect against cerebral ischemic damage in stroke patients by promoting neural stem cell growth. Moreover, studies showed that tissue kallikrein-modified MSC or EPC engraftment into injured tissues provided advanced protection against vascular and organ damage (Table 1). Thus, transplantation of tissue kallikrein-modified stem cells may be used for the treatment of patients with renal, cardiovascular, and cerebrovascular diseases. Furthermore, plasma kallikrein-kinin was observed to enhance EPC mobility and functional capacity in arthritis, while the cleaved kininogen product HKa inhibited EPC tube formation and viability. Collectively, these studies show that kallikrein-kinin stimulates the proliferation, viability, migration and function of various types of stem cells (Figure 2), and implicate the potential role of kallikrein-kinin in stem cell-based therapy for numerous human diseases.

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P-Reviewer: Miloso M, Pimentel-Coelho PM, Pochynyuk O, Yu J **S-Editor:** Tian YL **L-Editor:** A **E-Editor:** Lu YJ



Renal stem cell reprogramming: Prospects in regenerative medicine

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Author contributions: Morales EE and Wingert RA both designed the content of the manuscript, drafted and revised the manuscript, and approved the final version for publication.

Supported by National Institutes of Health, No. DP2 OD008470, R01 DK100237; Start-up funds from the University of Notre Dame and College of Science; and a generous donation for stem cell research to the University of Notre Dame by Elizabeth and Michael Gallagher on behalf of the Gallagher family

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Received: July 26, 2014 Revised: August 21, 2014

Accepted: August 30, 2014

Published online: September 26, 2014

Abstract

Stem cell therapy is a promising future enterprise for renal replacement in patients with acute and chronic kidney disease, conditions which affect millions worldwide and currently require patients to undergo lifelong medical treatments through dialysis and/or organ transplant. Reprogramming differentiated renal cells harvested from the patient back into a pluripotent state would decrease the risk of tissue rejection and provide a virtually unlimited supply of cells for regenerative medicine treatments, making it an exciting area of current research in nephrology. Among the major hurdles that need to be overcome before stem cell therapy for the kidney can be applied in a clinical setting are ensuring the fidelity and relative safety of the reprogrammed cells, as well as achieving feasible efficiency in the reprogramming processes that are utilized. Further, improved knowledge about the genetic control of renal lineage development is vital to identifying predictable and efficient reprogramming approaches, such as the expression of key modulators or the regulation of gene

activity through small molecule mimetics. Here, we discuss several recent advances in induced pluripotent stem cell technologies. We also explore strategies that have been successful in renal progenitor generation, and explore what these methods might mean for the development of cell-based regenerative therapies for kidney disease.

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Key words: Kidney; Regeneration; Induced pluripotent stem cell; Reprogramming; Differentiation; Stem cell; Renal progenitor

Core tip: The identification of regenerative therapies to treat kidney disease is an exciting but challenging area of ongoing scientific investigation. Cellular reprogramming may provide a tractable means to replace damaged renal tissue, and current researchers have pursued a number of innovative ways to produce renal cell types. Here we explore the issues confronting several reprogramming technologies, recent advances in reprogramming renal cells, and discuss areas of future scrutiny that are needed to help develop cell-based therapies for various kidney disease conditions.

Morales EE, Wingert RA. Renal stem cell reprogramming: Prospects in regenerative medicine. *World J Stem Cells* 2014; 6(4): 458-466 Available from: URL: <http://www.wjgnet.com/1948-0210/full/v6/i4/458.htm> DOI: <http://dx.doi.org/10.4252/wjsc.v6.i4.458>

INTRODUCTION: KIDNEY DISEASES AND THE NOTION OF THERAPEUTIC USES OF INDUCED PLURIPOTENT CELLS FOR RENAL REPLACEMENT THERAPY

Kidney organs perform essential physiological roles in

excretion and homeostasis^[1]. Kidney diseases can arise during development, juvenile, or adult life. Types of renal disease include acute kidney injury (AKI), which is the abrupt loss of renal function that can often become permanent, and chronic kidney disease (CKD), the progressive loss of renal function that culminates in organ failure known as end stage renal disease (ESRD)^[1,2]. The need for new treatments for kidney disease, the 8th leading cause of death in the United States^[3], is a growing concern in the medical field. For example, there are approximately 31 million people in the United States diagnosed with CKD^[4]. Unfortunately, kidney diseases are a global health problem as well, and have continued to increase in incidence in correlation with the rise in aged populations and escalation in conditions like diabetes that often negatively impact renal health^[5-8]. At present, kidney disease treatments deal with symptom management through the renal replacement therapies of dialysis or organ transplant. However, formulating therapies that repair kidney structure and restore compromised functionality is of the utmost importance considering the limited numbers of viable kidneys available for transplant, as well as the complications that can arise in organ recipients^[9-13]. To identify innovative ways to combat kidney diseases, numerous research groups have focused their energies on the identification of adult renal stem cells^[14-17]. However, this has remained a controversial topic despite the multitude of studies performed to date^[14-17]. In addition to the search for endogenous renal stem cells, the study of renal lineage specification during kidney organogenesis has been pursued—knowledge which can be applied toward the development of cell-based therapies for the purpose of kidney regeneration that would not necessitate the employment of adult stem cells.

One such cell-based alternative is the use of induced pluripotent stem cells (iPS) derived from the patient's own tissue. iPS cells can be used to study development and cell differentiation without the need for embryonic stem (ES) cell lines, whose cell source carries with it a surplus of ethical concerns, and can provide a resource to help researchers with disease modeling and drug development^[9]. Using iPS cells from the patient's renal tissue can serve to circumvent the need for a kidney transplant and avoid the use of lifelong immunosuppressant drug treatments. Thus, the notion of iPS-based regenerative medicine has many appealing benefits if the paramount challenges associated with realization of such cell-based therapies can be overcome. Utilizing integrating viral vectors containing the “Yamanaka factors” to reprogram cells has shown substantial success in generating iPS cells (approximately 0.1%), but the fact that these viral vectors integrate into the genome (sometimes in large copy number) has been a serious cause for debate as to their toxicity and their relative capability to be used in a clinical setting. Researchers have also investigated other avenues such as the use of non-integrating vectors so as to make the iPS cells safer to use in cell therapies, but with limited success, as evidenced by the very low induction rates and relative efficiency of the reprogramming method

(approximately 0.001%). Making safer and more controllable iPS cells is an integral part of developing cell-based therapies for the treatment of diseases and injuries. For example, Abad *et al.*^[18] shows evidence of how uncontrolled reprogramming can affect the body in the form of teratomas developing in multiple organs of transgenic mice transiently expressing the four “Yamanaka factors”. Other alternatives to the use of reprogramming factors are also being investigated, such as the use of microRNAs (miRNAs) to generate iPS cells. This method shows much promise, even though the cells' behavior *in vivo* still has to be controlled (approximately 10% efficiency reported in previous studies)^[19].

For the purposes of treating kidney disease, researchers have been assessing different ways of obtaining renal progenitor cells, and one such way involves partial reprogramming of differentiated renal cells into a renal progenitor state. Experimental evidence has supported the notion that the more closely related the start and end cells types are, the more efficient the reprogramming process will be. Although the method proved to be better than most at producing reprogrammed cells (approximately 0.875%)^[20], the overall amount of progenitors produced is still not cost-effective enough to be of applicable merit for therapeutic purposes. Another drawback to this partial reprogramming method is the thorough screening process that has to be applied in order to find the adequate combination of genes that will successfully reprogram the kidney cells into a progenitor-like state, which would be both time-consuming and costly. A method of obtaining renal progenitors that has received significant attention is the directed differentiation of iPS cells. Typically done with growth factors (which are rather expensive), exciting recent reports have now suggested that certain low-cost chemical compounds can be used to achieve the same goal of directing iPS cells towards a specific renal cell lineage with an approximate 90% conversion rate in one week. Although still dependent on the production of iPS cells, directed differentiation into renal progenitors is still a promising method that can be applied in tandem with a more optimized, efficient, and safer reprogramming protocols. In the following sections we further discuss these and other recent advances, as well as their general impact in the medical field.

REPROGRAMMING METHODS: REVERSE ENGINEERING TO OBTAIN STEM AND OTHER PROGENITOR CELLS FROM DIFFERENTIATED CELLS

Current therapies directed towards the treatment of kidney disease focus on symptom management instead of treating and hopefully curing the overall condition, and because of this researchers are working on alternatives that may now aid in the restoration of normal kidney function. As aforementioned, one alternative to current methods is the use of reprogrammed cell-based therapies

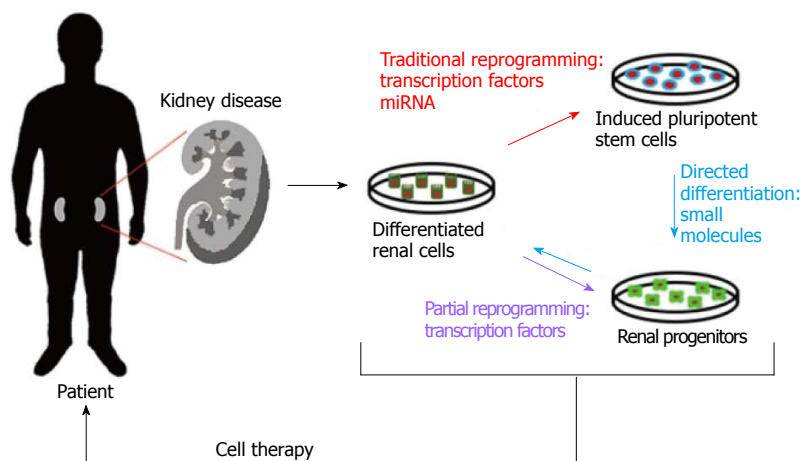


Figure 1 Renal cell reprogramming methods. (Red) Traditional reprogramming involving the use of transcription factors or miRNAs to generate pluripotent stem cells; (Purple) partial reprogramming with transcription factors to obtain multipotent progenitors; (Blue) directed differentiation into cells with a specific phenotype by treating induced pluripotent stem cells with small molecules. These newly reprogrammed/differentiated cells can then be used therapeutically to replace lost cell types within the injured kidney.

in order to restore damaged or diseased kidneys. Two of the most prominent reprogramming strategies currently being used involve either the conversion of different sources of stem cells into renal progenitors, or the reprogramming of differentiated renal cell populations into a more pluripotent state (Figure 1).

Traditional cell reprogramming involves the overexpression of developmental genes in differentiated adult cells in order to induce an earlier developmental and pluripotent phenotype. The typical factors that are overexpressed for cell reprogramming, discovered by Takahashi *et al.*^[22] and Yamanaka *et al.*^[22] back in 2006, are OCT4, SOX2, c-MYC, and KLF4 (now deemed “Yamanaka factors”), these factors are typically transfected into cells through the use of lentiviral vectors, which insert these exogenous genes into the host genome. At first, a cocktail of four viral vectors, each one containing one of the previously mentioned “Yamanaka factors” was introduced into the cell in order to promote a change in cell phenotype. However, these techniques lacked efficiency due to many non-specific genomic integrations, as well as the heterogeneous population that resulted from the process (some cells were only partially reprogrammed because not all of the vectors integrated)^[21,22]. In terms of kidney disease, producing iPS cells from cells of renal origin would contribute greatly to the development of cell therapies and treatments as they would be predicted to integrate more readily into the diseased kidney due to their conserved epigenetic memory^[23].

Interestingly, Zhou *et al.*^[24,25] were able to generate iPS cells from human exfoliated renal epithelial cells found in urine, something that can be collected without the need for surgical intervention, which would help in the development of therapies for kidney disease due to their epigenetic memory of renal origin. Using a cocktail of four different retroviruses containing the four “Yamanaka factors” they were able to create iPS cells (in about 16-25 d after transduction) from the previously mentioned cell source (cultured for about a week from 13 different test subjects, which means that the complete protocol would last about a month to produce iPS cells) with varying degrees of reprogramming efficiency (0.01%-4.0%) and

ability to differentiate, something that is to be expected when you use multiple integration vectors, mainly because the researcher cannot assess if the cells have incorporated all of the four reprogramming factors, or if the integrated vector copy number is low enough for the reprogrammed cells to be viable for therapeutic purposes (something that was not investigated in this study). Although a reprogramming efficiency of 4.0% is relatively high compared to other studies, the fact that there is great variation between the iPS cells produced (evidenced by the varying degrees of reprogramming efficiencies between the different donors, and that not a lot of the iPS cells produced could differentiate into other cells types) greatly diminishes their clinical applications, and provides further evidence that utilizing multiple integration vectors to reprogram cells is not an effective method for producing iPS cells for therapeutic purposes.

Researchers have tried to address this issue by trying create a better method of reprogramming that could decrease the number of genomic integrations, and assure that all of the factors necessary for reprogramming are being expressed within the cell. Sommer *et al.*^[26] managed to do just this by creating a single lentiviral vector containing all of the “Yamanaka factors” which is able to convert mouse postnatal fibroblasts into iPS cells. Not only were they able to ensure that all four transcription factors were integrated into the cell’s genome, but they were able to reduce the amount of genomic integrations to a mean of about 1.5-2.8 proviral copies^[26]. Compared to the multiple vector approach, this single vector method has an increased efficiency of 0.5%, which is about 50 times more efficient (relative efficiency of the multiple vector method is about 0.01%-0.05%), but this number might vary between cell types due to the many unique properties found in the various tissues throughout the body. Fortunately iPS cells have already been created from cell sources from cells of distinct embryonic origins (endoderm, mesoderm, ectoderm). This will benefit the development of regeneration cell therapies as iPS cells derived from the affected or injured organ will work more effectively in cell therapies that intend to regenerate the particular organ of interest, because of the genome-

wide epigenetic memory of the differentiated adult cell that is to be reprogrammed^[27].

Working under the previously stated premise (specifically in the case of renal disease), Wang *et al.*^[23] were able to generate iPS cells from mouse renal tubular epithelial cells (RTECs) using a single lentiviral vector containing the previously mentioned “Yamanaka factors” in about 21 d. The cells produced in this study were relatively indistinguishable from mouse ES cells, as confirmed by morphological, immunocytochemical, genetic expression, and karyotype analysis^[23]. Not only did these cells adopt an ES-like morphology and were able to express undifferentiated ES cell markers such as fibroblast growth factor 4 (FGF-4) and NANOG, but they were also able to differentiate into cell types of all three germ layers, as evidenced by the presence of AFP, desmin, and nestin (endoderm, mesoderm, and ectoderm markers respectively) in embryoid bodies formed from said cells^[23]. The cells also exhibited a normal 40XY karyotype and once reprogrammed, the viral transgenes were largely silenced which is necessary if there is any chance of applying this method in clinical applications, mainly to avoid problems during differentiation that might result in tumor development; the relative efficiency of this method, however, leaves something to be desired (0.1%)^[23].

Another reprogramming strategy that researchers have pursued is partial reprogramming of cells into a more multipotent phenotype that can produce cell lineages of a specific organ structure, which in the case of Hendry *et al.*^[20] would be embryonic nephron progenitors (NPs). The efficiency of the reprogramming process is correlated to the lineage relationship between the start and end cell types, in other words, the more closely related the start and end cells types are, the more efficient the reprogramming process^[20]. Hendry and colleagues investigated this premise by trying to generate NPs from HK2 cells line (human kidney cell line; adult proximal tubule cells)^[20]. Through combinatorial screening of 15 different transcription factors associated with the specification of the nephron progenitor phenotype they were able to identify 6 (*SIX1*, *SIX2*, *OSR1*, *EYA1*, *HOXA11*, *SNAIL2*) genes that would recapitulate the network of genes associated with the cap mesenchyme (CM)^[20]. Each factor was packed into individual lentiviral constructs accompanied by green fluorescent protein (GFP) to identify successfully infected cells, and successful reprogramming events were defined by significant morphological changes as well as robust expression of *SIX2* and Cbp/P300-interacting transactivator 1 (CITED1) protein, CM-specific markers^[20]. Reprogrammed cells showed upregulation of *Matrix metalloproteinase 9* and *2* (*MMP9* and *MMP2*), epithelial-to-mesenchymal transition (EMT) markers, as well as repressed expression of epithelial cadherin (*E-CADHERIN*), which suggests the occurrence of an EMT event within these cells^[20].

Further evidence of the cells’ conversion into nephron progenitors can be seen in a recombination assay that was developed to test the induced NPs’ potential in *ex*

vivo organoid cultures^[28,29]. They found that the induced progenitors were able to integrate with the endogenous NP field, and failed to integrate into the uretic bud compartments (a cellular population that the CM does not make). The overall efficiency of this partial reprogramming process is about 0.875%, which is substantially better than many of the techniques discussed so far (most likely due to the close relation between adult proximal tubule cells and NPs), however, the use of multiple lentiviral constructs makes the use of these cells quite toxic; therefore integrating all of these factors into a single construct might increase the efficiency of the reprogramming quite drastically, as well as their potential for use in therapies.

TANGENTIAL AND NON-INTEGRATION METHODS OF REPROGRAMMING

The use of integrating viral vectors has become quite widespread in the field of cell reprogramming, but because of various concerns that have arisen during their use (interruption of the cell’s genome and/or the risk spontaneous reactivation of the viral genome that might lead to tumor formation) researchers are actively looking for different alternatives so as to decrease the risk of using reprogrammed cells in the treatments of diseases such as end-stage renal disease (ESRD). Nightingale *et al.*^[30] (2006) were able to produce a non-integrating lentiviral vector that was able to transiently express GFP in about 90% of cultured human T lymphoid cells for approximately 20 d, which speaks to the potential of non-integrating vectors^[30]. In 2008, Stadtfeld *et al.*^[31] were able to generate mouse iPS cells from fibroblasts and liver cells by using non-integrating adenoviruses that transiently expressed the four “Yamanaka factors”. The cells were showed distinct characteristics of pluripotency such as the expression of endogenous pluripotency genes, demethylation of *Oct4* and *Nanog* promoters, and the ability to produce teratomas *in vivo* and contribute to all three germ layers^[31]. Even though the infection efficiency of the adenoviral vectors was relatively high (50%-60% for quadruple infected cells), the overall reprogramming efficiency this non-integrating method was between 0.0001% to 0.001% (significantly lower than integrating viral methods; 0.1% on average)^[31], something that is probably due to the rapid dilution of the adenoviruses during cell division which results in the cells not being exposed to the reprogramming factors for an adequate amount of time so as to induce a successful change in phenotype.

Another example of iPS cells created by non-integrating vectors can be seen in Guarino *et al.*^[32]. Yu and colleagues were able to create human iPS cells by utilizing three modified episomal vectors containing different combinations of six reprogramming factors (*OCT4*, *SOX2*, *NANOG*, *LIN28*, *c-MYC*, and *KLF4*) and the SV40 large T gene (*SV40LT*) to counteract the toxic effects of *c-MYC* expression, a cis-acting oriP element and an Epstein-Barr nuclear antigen 1 (EBNA1) gene^[32]. The

latter of these elements provided the vector with the ability to be transfected without the need for viral packaging and to be stably replicated outside of the chromosome^[32]. The factors packaged inside the vectors were linked by the internal ribosome entry site 2 (IRES2), and this was done in order to increase reprogramming efficiency by coexpressing them^[32]. Utilizing these vectors researchers were able to make iPS cells that exhibited typical ES cell colony morphology and gene expression profile, and they were able to produce teratomas *in vivo* that contained differentiated derivatives of all three germ layers^[32]. Subclones of the reprogrammed cells showed no signs of the vector or transgene sequences other than the change in phenotype, which is an incredible accomplishment, the reprogramming efficiency of the method however, is rather low (about three to six colonies per 10⁶ input cells)^[32].

Although non-integrating vectors are a good alternative in order to produce safer iPS cells for use in treatments, they are not very cost-efficient considering that these methods and vectors produce very low amounts of reprogrammed cells. Another alternate method that has seen a lot of attention in recent years is the use of miRNAs instead of exogenous transcription factors as a means of reprogramming^[19]. Wang *et al*^[19] used a lentiviral vector containing *miR302/367*, a unique cluster of miRNAs that is highly expressed in EM cells, in order to produce iPS cells from human embryonic kidney (HEK) 293T cells and found that these reprogrammed cells generated ES-like colonies, showed increased expression of ES cell markers (SOX2, KLF4, c-MYC, OCT4, LIN28 and NANOG), could form embryoid bodies, and could differentiate into germ-like cells *in vitro* and *in vivo*. So as to improve the differentiation potential of the miRNA-induced iPS cells researchers cultured the HEK293T cells in serum-free media, as well as in the presence of two small molecules: vitamin C and fibroblast growth factor (bFGF) so as to better shape the morphology of the reprogrammed cells^[19]. Although the overall efficiency of the reprogramming method described is yet to be determined, previous reprogramming studies with miRNAs have demonstrated this type of approach to be more efficient than the standard reprogramming factor methods (10% *vs* 0.1%, respectively)^[33], making this type of method a promising candidate for further studies.

FORWARD THINKING: OBTAINING RENAL PROGENITORS USING LOW-COST AND EFFICACIOUS SMALL MOLECULES

In the endeavor to create renal progenitors, controlled differentiation of iPS cells has become a good alternative to partial reprogramming of differentiated cells. One particular technique that stands out is the use of small molecules in order to induce a more renal-specific pluripotent state. Lam *et al*^[34] created an intermediate mesoderm (IM)-specific differentiation platform around

the small molecule CHIR99021, a glycogen synthase kinase-3 β inhibitor (CHIR). This inhibitor manages to recapitulate mesendoderm formation during development in human pluripotent stem cells (hPSCs), as evidenced by the compounds ability to produce cell lineages that transiently expressed various primitive streak genes such as *BRACHY*, *MIXL1*, *FOXA2*, *EOMES*, and *GSC*^[34]. The transient expression pattern of these genes in the CHIR-treated cells during a 72 h period is also consistent with that found in cells during the course of gastrulation, which means that CHIR99021 imitates normal developmental mimetics^[34].

Utilizing this compound researchers were able to screen various exogenous factors in order to determine the minimum requirements needed promote differentiation of these CHIR-induced mesendoderm-like cells toward IM^[34]. They reported that fibroblast growth factor-2 (FGF2) in combination with retinoic acid (RA) was able to induce IM differentiation in the mesendoderm-like cells. This conclusion was drawn from the fact that the treated cells were both *PAX2* and *LHX1* positive, two markers for which coexpression in the same domain has only been described in the developing kidney and dorsal spinal cord. Further evidence that these *PAX2*⁺*LHX1*⁺ cells were directed to an IM state and that they could produce IM-derived cell populations and tissues came in the form of tubule structures (with primary cilia) expressing proximal tubular markers once the exogenous FGF2 and RA were removed from the culture media. One of the many differentiated kidney markers whose expression was evaluated in the *PAX2*⁺*LHX1*⁺ cells was *SIX2*, a multipotent nephron progenitor cell marker. This nephron progenitor population composes what is known as the CM and these give rise to nearly all epithelial cell types in the nephron tubule, with the exception of those from the collecting duct. Lam *et al*^[34] were able to use the double positive IM-like cells in order to screen different growth factors so as to identify the conditions that promote and sustain a *SIX2*⁺ cell population, and they were able to determine that the addition of FGF9 and Activin A could do just this, as well as induce the expression of other CM markers such as *SALL1* and *WT1*. Although researchers were able to effectively produce IM-like cells that are able to differentiate into subsequent renal cell populations, the need for exogenous growth factors is still an issue due to that fact that these very same growth factors are incredibly expensive, and therefore not very cost-effective to use in clinical applications.

Araoka *et al*^[35] on the other hand, utilized a combination consisting of only small molecules, as opposed to small molecules and growth factors, in order reach the same goal. In this particular strategy the mesendoderm stage is skipped altogether and the hPSCs are differentiated directly into an IM state. Using high throughput chemical screening they were able to identify two compounds that increased induction of *Odd-skipped related 1* (*Osr1*), a transcriptional regulator that is expressed in the embryonic day 7.5 IM until kidney organogenesis and

therefore a good marker to utilize in order to identify IM cells. The two compounds identified were AM580 and TTNPB, RA receptor antagonists (RAR) that induce differentiation of hPSCs into OSR1⁺ IM cells with relatively high efficiency (> 60% and > 50% respectively) when compared to positive controls. To further optimize OSR1 induction researchers combined each RAR with CHIR, which resulted in an increased induction rate of around 80% in only 5 d utilizing only two chemicals in a serum – free environment.

As mentioned before, one of the main differences between the methods used Araoka *et al.*^[35] and Lam *et al.*^[34] is that the former can skip the mesendoderm stage altogether. This was demonstrated when researchers analyzed mesendoderm markers (*BRACHYURY*, *GOOSECOID*, and *MIXL1*) in the small molecule-treated hPSCs, and found that the induction rate for *BRACHYURY*⁺ cells was around 6%, and that expression levels for said markers were very low in cells produced from the small molecule method when compared to cells produced with CHIR and growth factor activin A. The ability of these IM-like cells to produce the various IM-derivative cell types was also evaluated, and after additional days of differentiation researchers found that these induced IM cells did in fact produce cells expressing marker genes for various IM-derivative cell types such as *FOXD1*, *SALL4*, *GATA4*, among others. These cells also had the ability to give rise to the derivative cell types *in vivo*, as well as renal tubule-like structures positive for renal tubule markers such as *Lotus tetragonolobus* lectin (LTL), E-CADHERIN, and laminin *in vitro*.

Both of these studies^[34,35] provide evidence that utilizing small molecules in order to produce renal progenitors for cell therapies is a viable option in the field of regenerative medicine, and the various benefits that this type of method provides makes it a good alternative to explore. Utilizing these chemical compounds is not only less costly, but more efficient in terms of number of cells converted to the desired phenotype (even though the reprogramming efficiencies for the template iPS cells were not stated in either one of the studies). Unfortunately there is still some variability between studies that needs to be addressed before any progress can be made on any viable therapeutic solution.

Both of the methods described above are highly efficient for IM differentiation of hPSCs in terms of the time the procedure takes, the markers analyzed, and the compounds used^[34,35]. Lam *et al.*^[34] utilizes a method that has both chemical compounds and growth factors, but only takes 3 d to produce IM cells. Araoka *et al.*^[35] on the other hand only use chemical compounds, but take about two more days in order to reach the same goal. In terms of markers utilized the former uses a combination of LHX1 and PAX2 (a pair of markers that, as stated previously, are only found in the developing kidney and dorsal spinal cord), while the latter uses an engineered *OSR1-GFP* human iPS cell line to verify if the cells have reached an IM state, a gene that is also expressed in the

lateral plate mesoderm and can therefore provide some heterogeneity to the sample that might alter the results of future studies.

CONCLUSION

Recent progress in knowledge about cellular reprogramming has rapidly advanced prospects for the development of regenerative therapies for the medical treatment of many conditions, among them being kidney diseases, making this a very exciting time in the field of nephrology. Here, we discussed a number of research studies in the field of stem cell reprogramming. We explored how such methods have been utilized to reprogram renal lineages, and thus might be used to develop therapies to treat kidney disease. Additionally, iPS cells can be used for disease modeling to identify targeted therapeutics for heritable conditions^[36]. Moving forward, there are a number of complex issues to further resolve about the therapeutic application of iPS cells for disease treatment, and most assuredly other issues yet to be identified, which apply both to the kidney and other organs within the body (Figure 2).

Issues involved in the therapeutic application of reprogrammed cells include the number and type of cells needed, along with the identification of an appropriate delivery system for the condition to be treated. Currently, there are various ongoing clinical trials in the United States that are using stem cells to treat a wide range of conditions such as age-related macular degeneration to polycystic kidney disease^[37]. The amount of cells utilized by these studies can fluctuate between the stem cells type and the way they are used (50000-200000 human embryonic stem cells in retinal cell transplants and 2×10^6 mesenchymal stem cells (MSCs)/kg of patient's weight in kidney disease treatments^[37,38]), but even so the amount of pluripotent cells produced by the methods mentioned in this review are still relatively low when compared to the amount used in the before-mentioned trial therapies.

Other issues that still need to be addressed are cell quality (can you isolate healthy renal cells to reprogram as opposed to the diseased ones?) and downstream processing, a problem because, due to ethical reasons, many of the pluripotency tests that are usually performed on reprogrammed cells can't be done with human iPS cells, which might create some heterogeneity within the human iPS cell lines. Also, we have barely scratched the surface of how epigenetic memory affects iPS cell differentiation patterns. All of these concerns still need to be investigated before adequate therapies can be developed (Figure 2).

Although there remains a sizable amount of work to be done in order to optimize the efficiency of these methods, they still represent a promising alternative to current therapies, mainly because they have the potential to provide the affected patient with the means to regain kidney function without the need for a kidney transplant or dialysis. It would be interesting to see how these

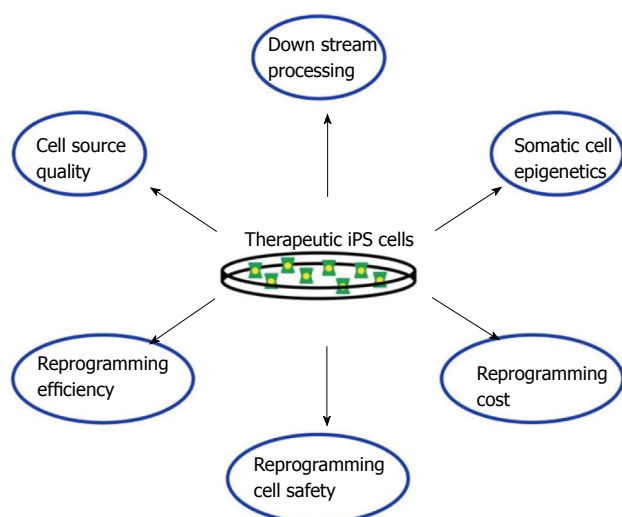


Figure 2 Challenges faced when developing induced pluripotent stem cell-based therapies. Many issues still have to be overcome before any effective cell therapies can be used.

methods would be affected if they were done with other animal models, such as in the zebrafish, an organism that has the capacity to regenerate renal tissue^[39-41], and what type of information can be learned from animal models about how reprogramming methods can be optimized or the nature of renal progenitors^[42]. As more insights continue to be gathered about the genetic mechanisms of renal lineage development and regeneration in various vertebrate models, as represented for example by recent reports in the zebrafish^[43-45], frog^[46], and mouse^[47], crucial information may be elucidated about potent methods to regulate renal reprogramming or even to promote pathways of endogenous cell regeneration in the damaged kidney.

Moving forward, there may be significant challenges for cell-based therapies posed by the microenvironment in the damaged kidney—termed by some as the “seed and soil” dilemma. Namely, the importance of an appropriate microenvironment, or niche, the so-called “soil”, is essential for the prosperity and normal growth of the stem cells, or “seeds”, to be administered in a putative treatment^[48]. The complexity of renal anatomy and composition alone may pose significant hurdles to cell-based therapies, and can be further complicated if the environment due to the disease state is refractory to the success of the regenerative therapy. In sum, altering the microenvironment to facilitate success of the cellular therapy is likely to be vital.

One promising avenue is the utilization of other stem cells, *e.g.*, MSCs, which have been shown in a number of contexts to stimulate a local, if not organismal, humoral environment that facilitates regeneration^[37,38]. The kidney is in fact among such organs whose status can be improved by MSCs in some disease settings^[49]. In animal models of AKI, administration of MSCs has provided renoprotective effects^[50-53]. Notably, a limitation that has been recognized is the inability of MSCs to mediate im-

provements in chronic renal disease states^[54]. These observations indicate that much remains to be learned about how to facilitate cell-based therapies with approaches that address the complex variables associated with any given disease state. Thus, it is imperative that future research is performed to better understand the relationships and physiological impacts of disease states within organisms. Nevertheless, the progress in stem cell biology to date continues to fuel enthusiasm that methods like reprogramming can be harnessed to improve quality of life and relieve suffering in the decades to come.

ACKNOWLEDGEMENTS

We thank the staffs of the Notre Dame Department of Biological Sciences and Office of Research for their support, and thank the Center for Zebrafish Research at Notre Dame for their outstanding dedication in the care and welfare of our zebrafish colony. Finally, we thank our research lab for their comments, discussions, and insights about this work, and our families for their constant love and support.

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P- Reviewer: Coopman K, Yorioka N **S- Editor:** Song XX
L- Editor: A **E- Editor:** Lu YJ



Human induced pluripotent stem cells: A new source for brown and white adipocytes

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Author contributions: Hafner AL and Dani C solely contributed to this review.

Supported by Fondation ARC and ANRS

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Received: July 22, 2014 Revised: August 25, 2014

Accepted: August 30, 2014

Published online: September 26, 2014

ate white and brown adipocytes and we discussed their therapeutic capacities for obesity and lipodystrophy diseases. Then, we described the main approaches to derive human induced pluripotent stem cells-mesenchymal stem cells (hiPSC-MSCs). Finally, we underlined the low adipogenic capacity of hiPSC-MSCs compared to adult-MSCs and proposed several hypothesis to explain this feature.

Hafner AL, Dani C. Human induced pluripotent stem cells: A new source for brown and white adipocytes. *World J Stem Cells* 2014; 6(4): 467-472 Available from: URL: <http://www.wjnet.com/1948-0210/full/v6/i4/467.htm> DOI: <http://dx.doi.org/10.4252/wjsc.v6.i4.467>

Abstract

Mesenchymal stem cells (MSCs) derived from human induced pluripotent stem cells (hiPSCs) provide a novel source for generating adipocytes, thus opening new avenues for fundamental research and clinical medicine. We present the adipogenic potential of hiPSCs and the various methods to derive hiPSC-MSCs. We discuss the main characteristic of hiPSC-MSCs, which is their low adipogenic capacity as compared to adult-MSCs. Finally, we propose several hypotheses to explanation this feature, underlying a potential critical role of the micro-environment. We favour the hypothesis that the range of factors or culture conditions required to induce adipocyte differentiation of MSCs derived from adult tissues and from embryonic-like cells could differ.

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Key words: Human induced pluripotent stem cells; Mesenchymal stem cells; Brown adipocytes; White adipocytes; Obesity; Lipodystrophy

Core tip: In this mini-review, we summarized the potential of human induced pluripotent stem cells to gener-

INTRODUCTION

Mesenchymal stem cells (MSCs) derived from human induced pluripotent stem cells (hiPSCs) provide a novel source for generating adipocytes. hiPSC-MSCs satisfy the minimal criteria for defining MSCs proposed by the International Society for Cellular Therapy, *i.e.*, express certain cell surface markers, adhere to tissue culture plastics and exhibit differentiation towards osteogenic, chondrogenic and adipogenic lineages^[1]. However, hiPSC-MSCs have also been referred to as MSC-like, mesenchymal progenitors or fibroblast-like cells by different authors, including us, because hiPSC-MSCs are not identical to MSCs isolated from adult tissues, as we will discuss. For simplicity, we use the general term MSCs in this review. We present hiPSCs as a unique source of adipocytes, thus opening new avenues for fundamental research and clinical medicine. We also underline the low adipogenic capacity of hiPSC-MSCs compared to adult-MSCs, based on our expertise regarding the study of human MSCs isolated from iPSCs and adipose tissue.

In mammals, two adipose tissue functional types

coexist, *i.e.*, brown and white adipose tissues, which are both involved in the energy balance while having opposite functions. White adipose tissue (WAT) is mainly involved in energy storage and mobilization in the form of triglycerides. In contrast, brown adipose tissue (BAT) burns fat and is specialized in energy expenditure. BAT is a key thermogenic organ since brown adipocytes convert nutrients into heat by uncoupling respiration from ATP synthesis. BAT implants were recently shown to improve the metabolic conditions in obese mice and to restore normoglycemia and glucose tolerance in streptozotocin-induced diabetic mice^[2]. These recent findings offer promising prospects for basic research and clinical medicine-increasing energy expenditure *via* recruitment of brown adipocyte progenitors could be a valuable therapeutic approach to counteract obesity and its associated metabolic complications. However, BAT represents a minor fraction of the adipose tissue in humans, is found throughout the body and disappears from most areas with age, persisting only around deeper organs^[3]. Human BAT is hard to isolate in this regard, so an alternative cellular source is required to generate brown adipocytes.

Congenital or acquired lipodystrophies result from the loss, degeneration or misdistribution of white adipose tissue, leading to diabetes, severe defects in lipid homeostasis and ectopic fat accumulation. Transplantation of white adipose tissue in a lipoatrophy mouse model has been shown to improve the metabolic parameters^[4]. Therefore, isolation of hiPSCs-MSCs differentiating into brown and white adipocytes could provide an unlimited source of adipocytes for autologous cell-based therapy to treat obesity and lipodystrophy diseases.

Differentiation of iPSCs into MSCs, and then into adipocytes, also offers a unique opportunity to determine adipocyte properties according to their embryonic origins, but this issue has yet to be investigated in humans. Indeed, individual white adipose tissue depots are not equivalent, as functional differences have been reported in mice and humans^[5]. These distinct properties of individual adipose tissue depots could play a role in obesity-related complications and might explain why the spread of only certain depots is associated with severe metabolic disorders. It has also been observed that the different fat depots are not altered in a similar manner in genetic- and drug-induced lipodystrophies^[6]. These regional differences were conserved during *in vitro* propagation and adipocyte differentiation of MSCs, strongly suggesting that MSCs from different fat depots are indeed inherently different^[7]. Recent published data, including ours, suggested that MSC embryonic origins could play a role in these intrinsic differences. Surprisingly little is known about the developmental origin of adipocytes in rodents, and nothing is known in humans. Gesta *et al.*^[8] compared gene expression profiles of intra-abdominal and sub-cutaneous adipose tissues in mice and found major differences in the expression of several genes involved in embryonic development and pattern specification. We recently demonstrated that subsets of adipocytes have mesodermic

and neuroectodermic origins depending on the location of adipose tissue depots in mouse and quail^[9,10]. These data are in full agreement with the demonstration of Takashima *et al.*^[11] that MSCs originate both from mesoderm and neuroectoderm. Interestingly, the potential of neuro- and meso-derived MSCs to differentiate and participate in tissue repair differs depending on the embryotic origin^[12].

DIFFERENTIATION OF HUMAN-INDUCED PLURIPOTENT STEM CELLS INTO ADIPOCYTES

Differentiation of hiPSCs offers a unique opportunity to purify human MSCs for the purpose of generating brown and white adipocytes, as well as adipocytes of different embryonic origins from the same patients' somatic cells. Following the pioneer work of Yamanaka's group on the generation of iPSCs by reprogramming human fibroblasts^[13], the capacity of hiPSCs to generate functional adipocytes was first reported by Nakao's group. The authors showed that hiPSCs have an adipogenic potential comparable to human embryonic stem cells. Interestingly, hiPSC-adipocytes can maintain their functional properties for several weeks after transplantation into nude mice^[14,15]. These data revealed that hiPSC-adipocytes could potentially be used to correct metabolic parameters in patients. In these experiments, differentiated hiPSCs, but not MSCs, were transplanted into mice. Indeed, hiPSC differentiated cultures are enriched with adipocytes after adipogenic induction, but also contain several other cell types that are undesirable for transplantation, including immature neural cells and undifferentiated iPSCs that can form teratomas several weeks after transplantation. As indicated by Noguchi and colleagues, transplantation of mature adipocytes alone results in graft loss that could be improved by transplanting adipocyte progenitors^[15]. Therefore, purification of hiPSC-MSCs with a high adipogenic capacity is required prior to an hiPSC-based therapeutic approach. Nishio *et al.*^[16] developed a procedure to generate functional brown adipocytes at a high frequency from hiPSC using a hematopoietic cocktail to induce hiPSC differentiation. Remarkably, hiPSC-brown adipocytes were able to improve glucose tolerance after transplantation in mice. This report established a link between brown adipocytes and hematopoietic cells, and indicated that hiPSCs could potentially be used to generate brown adipocytes with therapeutic properties. As recently reported, we designed a procedure to derive MSCs with a capacity to differentiate into both brown and white adipocytes^[17]. In this latter study, the use of small molecules during hiPSC differentiation revealed that TGF β and retinoic acid pathways regulate the generation of MSC subtypes having a brown or white adipogenic potential, respectively. Differentiation of hiPSCs offers the opportunity to characterize the earliest steps of adipogenesis and identify signalling pathways regulating brown and white fat cell lineages. However, as discussed below,

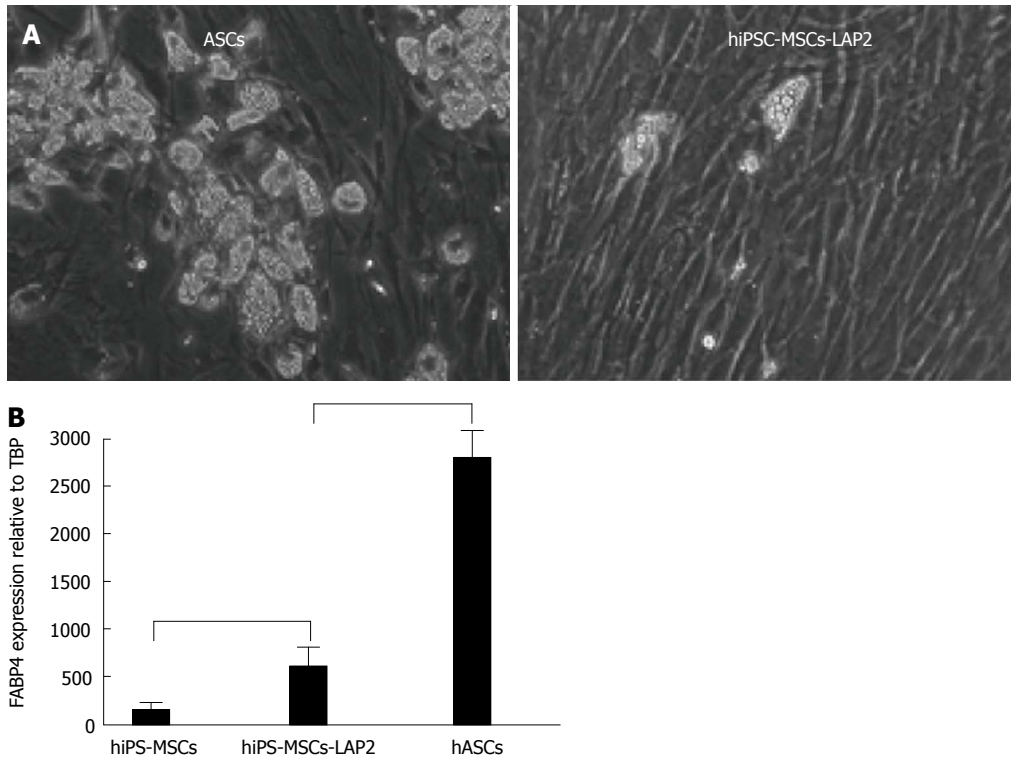


Figure 1 Adipogenic capacities of mesenchymal stem cells derived from human induced pluripotent stem cells and from human adipose tissue (adipose-derive stem cells). A: Microphotographic images showing adipocytes generated from hiPSC-MSCs transduced with C/EBP β LAP (left) and from adult ASCs (right); B: Adipocyte differentiation potentials quantified via FABP4 adipogenic gene expression. MSCs: Mesenchymal stem cells; hiPSCs: Human induced pluripotent stem cells; ASCs: Adipose-derive stem cells.

hiPSC-derived MSCs have a low potential to differentiate into adipocytes compared to hMSCs derived from adult adipose tissue.

ADIPOGENIC CAPACITY OF hiPSC-DERIVED MSCS

Chen *et al.*^[18] first underlined the limited capacity of hiPSC-MSCs to undergo adipocyte differentiation, a feature that has often been observed by authors but not always highlighted. Ahfeldt *et al.*^[19] were able to generate pure brown or white adipocytes from hiPSCs, but only following genetic modification of MSCs. These artificially programmed hMSCs are of great interest from a therapeutic standpoint, but cannot be applied to investigate ontogenesis of human adipocytes. The fact that MSCs must be transduced with adipogenesis master genes clearly illustrates the low adipogenic potential of hiPSC-derived MSCs. This low adipogenic potential is illustrated in Figure 1. As shown, transduction of hiPSC-MSCs with a vector expressing an adipogenic gene, such as C/EBP-LAP, dramatically enhanced the MSC adipogenic potential, as monitored with the FABP4 adipogenic marker, but still at a lower level compared to MSCs derived from adipose tissue, also named adipose-derive stem cells (ASCs). Interestingly, some authors claim that the low differentiation capacity is limited to adipogenesis since hiPSC- and hESC-MSCs are able to differentiate

towards chondrogenic and osteogenic lineages at high levels^[20-22]. MSCs are abundant in adipose tissue, can be easily harvested using liposuction and have a considerable expansion potential *ex vivo*, particularly when isolated from young donors^[23]. Adipose tissue derived MSCs are currently being investigated in autologous transplantations to improve revascularization and tissue perfusion in ischemic limbs^[24-26]. Results so far suggest that the efficiency of adipose tissue-MSCs in regenerative medicine could rely more on their cytokine secretory functions and potential use as immunomodulators than on their differentiation potential^[27]. The therapeutic potential of MSCs derived from hiPSCs and from human adipose tissue should therefore now be carefully compared.

In recent years, various groups have successfully derived MSCs from hESC and hiPSC using a range of methods. There are two main approaches to differentiate pluripotent stem cells into MSCs (Figure 2).

One strategy involves embryoid body (EB) formation. In this approach, suspension cultures allow pluripotent stem cells to form 3-dimensional structures called EB (Figure 2A). This step models the *in vitro* embryonic development with the commitment of cells into the three primary germ layers. The duration of this stage may range from 7 to 14 d. EBs are then seeded onto culture dishes and, after a proliferation step, outgrowth cells are maintained in a mesenchymal culture medium. Subsequently, adherent cells display a fibroblast-like morphology and acquire specific MSC markers after se-

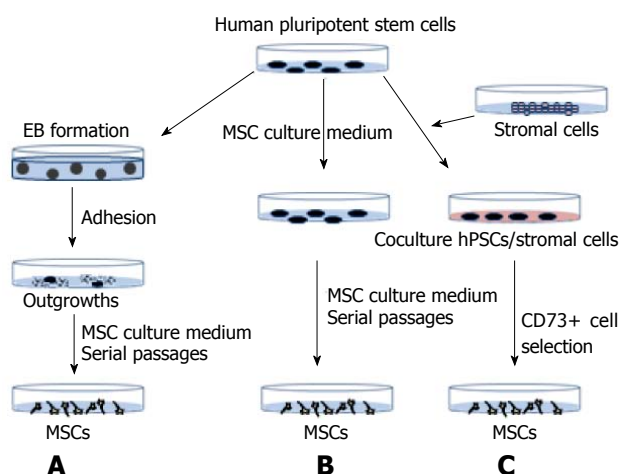


Figure 2 Mesenchymal stem cell derivation strategies. MSCs were derived from hiPSCs following (A) or not (B) embryoid body formation. Alternatively, hiPSCs were maintained in co-culture with murine stromal cells, and then MSCs were derived following selection of CD73-positive cells (C). MSCs: Mesenchymal stem cells; hiPSCs: Human induced pluripotent stem cells.

rial passages^[28,29]. An alternative strategy involves direct differentiation of pluripotent stem cells without the EB step (Figure 2B). Pluripotent stem cells are dissociated into single-cell suspensions and then maintained in mesenchymal medium for several weeks. The resulting cell cultures are enriched for MSCs through serial cell passaging^[30]. Another version of this protocol relies on the spontaneous differentiation of pluripotent stem cells into MSCs^[31]. Different research groups have developed additional steps to improve this method. For instance, before single-cell suspension, hiPSCs are committed towards MSC differentiation *via* treatment with small molecules, such as inhibitors of the TGF β pathway^[18]. Using small molecules with the EB method has been shown to promote the generation of two MSC subtypes having a selective potential towards brown and white adipocyte lineages^[17]. Growing hiPSCs on a fibrillar-collagen matrix has also been shown to improve their differentiation into MSCs^[32]. Coculture of pluripotent stem cells with murine stromal cells, followed by the selection of CD73-positive cells^[33], is another way to derive MSCs (Figure 2C). Interestingly, MSCs from mesoderm or from neuroectoderm origins can be derived depending on the stromal cells used^[34,35]. Finally, MSCs derived from different hiPSC lines generated from different donors, or from the same hiPSC clone using different derivation approaches, have the same adipogenic features^[30,31]. MSC adipogenic characteristics are therefore not dependent on the derivation method used.

Several hypotheses could be put forward to explain the low hiPSC-MSC adipogenic capacity compared to adult MSCs. Interestingly, the low differentiation potential is not restricted to hiPSC-MSCs since MSCs derived from human embryonic stem cells (hESCs) display the same feature, thus ruling out the possibility that the low hiPSC-MSC adipogenic capacity could be due to the reprogramming process or to an epigenetic mechanism. The fact

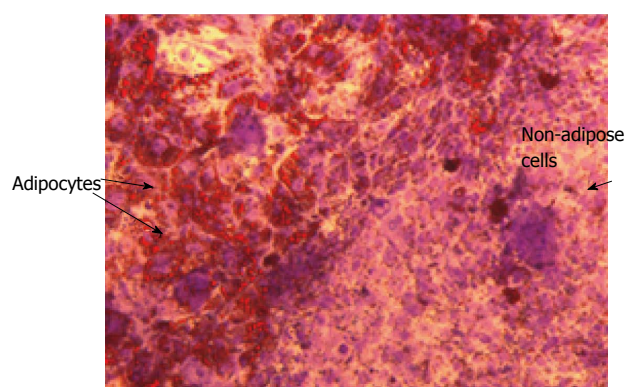


Figure 3 A niche for adipocytes when mesenchymal stem cells are maintained in the human induced pluripotent stem cells environment. Differentiated hiPSCs stained with Oil Red O, a specific stain for lipid droplets, to reveal adipocytes, and with violet crystal, a non specific stain, to reveal other cells. hiPSCs: Human induced pluripotent stem cells.

that hiPSCs generated from adipose-derived stem cells or from neural stem cells displayed a similar adipogenic capacity is in agreement with this hypothesis^[17]. One possibility is that the criteria to identify hiPSC-MSCs and adult MSCs could differ. For instance, individual cell surface markers used to characterize MSCs are also expressed by non-MSCs such as by fibroblasts^[36]. Therefore, it could be important to select hiPSC-MSCs based on the co-expression of several mesenchymal markers and not on CD73 expression only. We favour the hypothesis that the range of factors or culture conditions required to induce adipocyte differentiation of MSCs derived from adult tissues and from embryonic-like cells could differ. The low hiPSC-MSC adipogenic capacity is a reminiscence of an earlier observation reported by Han *et al.*^[37]. The authors observed that epididymal adipose tissue, which undergoes postnatal development in mouse, is composed of multipotent progenitor cells that meet the MSC criteria but lack an adipogenic capacity *in vitro*. In contrast to cells derived from other fat pads, epididymal fat cells require a three-dimensional culture conditions and a different micro-environment to undergo differentiation. These results underline that the micro-environment has a critical role in differentiation but could differ for adult and embryonic-like cells. We have observed that hiPSCs can generate nice adipocyte colonies when MSCs are not derived but are maintained in an iPSC environment. As shown in Figure 3, adipocytes are close to non-adipose cells in hiPSC-differentiated cultures. As isolated MSCs have been found to have a low adipogenic capacity, we propose that these non-adipogenic cells are required for full differentiation of MSCs into adipocytes. The identification of these non-adipogenic cells and factors that they secrete could be of a great interest.

CONCLUSION

The potential of hiPSCs to generate MSCs having an adipogenic capacity represents a powerful cellular model for studying brown and white adipocyte ontogenesis and

comparing the properties of adipocytes derived from mesoderm or neuroectoderm. This also provides a basis for investigating factors involved in the recruitment of MSCs having different potentials in normal and pathological contexts.

From a clinical standpoint, many issues have to be resolved before using hiPSC-MSCs in cell-based therapeutic for obesity and lipodystrophy diseases. However, the differentiation of iPS cells towards the adipogenic lineage offers a unique opportunity to purify white and brown adipocytes from patients, which could lead to the development of autologous transplantation procedures to treat obese and lipodystrophic patients. It would be essential to determine the factors underlying the low adipogenic capacity of hiPSC-MSCs. They are functionally distinct from adult hMSCs and the challenge will be to determine the cellular and molecular events necessary to prime hiPSCs towards the adipogenic lineage at a high level.

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P- Reviewer: Chen LY, Zhang Q S- Editor: Song XX

L- Editor: A E- Editor: Lu YJ



Identification and targeting leukemia stem cells: The path to the cure for acute myeloid leukemia

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Author contributions: Zhou J and Chng WJ all reviewed the literature and wrote the manuscript; both authors approved the final version of the manuscript.

Supported by National Research Foundation Singapore and the Singapore Ministry of Education under its Research Centres of Excellence initiative, NMRC Clinician-Scientist IRG Grant CNIG11nov38 and NMRC Clinician Scientist Investigator award

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Received: July 22, 2014 Revised: August 22, 2014

Accepted: August 30, 2014

Published online: September 26, 2014

HSC. In this review, we will first provide a historical overview of the discovery of LSC, followed by a summary of identification and separation of LSC by either cell surface markers or functional assays. Next, the review will focus on the current, various strategies for eradicating LSC. Finally, we will highlight future directions and challenges ahead of our ultimate goal for the cure of AML by targeting LSC.

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Key words: Acute myeloid leukemia; Leukemia stem cell; Immunotherapy; Cancer stem cell; Cell therapy

Core tip: Acute Myeloid Leukemia (AML) remains an incurable disease in most of cases. Leukemia stem cells (LSC) are a subpopulation of leukemic cells responsible for the continued proliferation and propagation of bulk leukemic cells. Growing evidence support the notion that LSCs are the root source of disease relapse and treatment resistance. Here we review the literature on historical overview of the discovery of LSC, identification and separation of LSC and strategies of targeting LSC as a potential cure for AML.

Abstract

Accumulating evidence support the notion that acute myeloid leukemia (AML) is organized in a hierarchical system, originating from a special proportion of leukemia stem cells (LSC). Similar to their normal counterpart, hematopoietic stem cells (HSC), LSC possess self-renewal capacity and are responsible for the continued growth and proliferation of the bulk of leukemia cells in the blood and bone marrow. It is believed that LSC are also the root cause for the treatment failure and relapse of AML because LSC are often resistant to chemotherapy. In the past decade, we have made significant advancement in identification and understanding the molecular biology of LSC, but it remains a daunting task to specifically targeting LSC, while sparing normal

Zhou J, Chng WJ. Identification and targeting leukemia stem cells: The path to the cure for acute myeloid leukemia. *World J Stem Cells* 2014; 6(4): 473-484 Available from: URL: <http://www.wjgnet.com/1948-0210/full/v6/i4/473.htm> DOI: <http://dx.doi.org/10.4252/wjsc.v6.i4.473>

INTRODUCTION

Acute myeloid leukemia (AML) remains a hefty challenge for hematologists and oncologists. There are approximate 18800 new cases diagnosed with AML each year in United States alone, but estimated death cases is as high as 10000, ranking AML as the 6th highest cancer-related

death in male population (Cancer Facts and Figures 2014, American Cancer Society). AML is a group of morphologically, genetically and epigenetically heterogeneous disorders characterized by the accumulation of differentiation-arrested abnormal hematopoietic progenitor cells in the bone marrow and blood. The complexity of AML is further complicated by the existence of a spectrum of functionally diverse leukemic and preleukemic clones. Recent strides in massively parallel sequencing technology and powerful bioinformatic tools enable us to gain a deep and panoramic insight of AML genome and epigenome at unprecedented level. Elegant studies tracking clonal evolution from diagnosis to relapse revealed the greater clonal heterogeneity in AML than we previously estimated^[1-3]. Some clones either founding clone (major clone) or subclones (minor clone) at diagnosis, can survive chemotherapy. These survival clones may gain a small number of cooperating mutations, eventually leading to a relapse^[1-3]. For example, a subclone within the founding clones containing somatic mutations in some well-characterized pivot genes such as *DNMT3A*, *FLT3*, *NPM1*, *etc.*, can develop into dominant clone after acquiring additional mutations in *ETV6* and *MYO18B*. The mutations in these pivot genes are recurrent in AML^[1].

From the identification of chromosomal translocation in the 1970s, leukemia has been a prime and pioneering paradigm for the breakthrough discoveries in cancer genetics and the development of novel therapeutics^[4]. For example, the demonstration of the presence of leukemia stem cells (LSC) has preceded the discovery of the first cancer stem cells (CSC) in solid tumor (breast cancer) by almost 10 years^[5]. LSC, or leukemia initiating cells (LIC), are a subpopulation of cells that acquire self-renewal function and sustain the disease. AML LSC is the not only the first identified CSC, but also the best characterized CSC. It has become increasingly apparent that AML LSCs are generally insensitive to the conventional chemotherapy. They reside in the bone marrow micro-environment and are poised to propagate, leading to the treatment failure and relapse. This suggests that the LSC subpopulation is the culprit for the poor outcome of AML patients and selectively targeting LSC will be a important strategy towards curing AML.

IDENTIFICATION OF LSC-CELL SURFACE MARKERS IN COMBINATION WITH FUNCTION ASSAYS

CD34+CD38-: the beginning of LSC hunting

Pioneer studies from John Dick's group in 1990s firmly established the AML LSC model, that AML is a hierarchical disease which is initiated and sustained by a rare subset of LSC. Only the subset of immature CD34+CD38-leukemia cells is capable of not only initiating leukemia in sublethally irradiated immunodeficiency mice, but also transplantable in second and third generation mice. In contrast, the fraction of more mature CD34+CD38+

leukemia blasts failed to imitate disease under the same condition. The estimated frequency of LSC in the CD34+CD38- cells is one in one million, thus LSC represent a very rare of unique population of leukemia cells sharing the similar cell surface marker as normal immature hematopoietic cells. Importantly, several clinically observatory studies demonstrated that high frequency of CD34+CD38- cells, but not total CD34+ cells, amongst blast cells at diagnosis correlates with poor survival in both adult and pediatric AML patients^[6,7]. More recently, gene expression profiles generated from this rare subset of CD34+CD38- cells support their clinical impact that high expression of LSC signature predicts worse outcome^[8-11].

However, recently findings derived from newly generated NOD/ShiLtSz-*scid*/IL2R γ^{null} (NSG) and NOD/ShiJic-*scid*/IL2R γ^{null} (NOG) mice, the most immunodeficient strains, cast new light on the origin of LSC. These two strains of mice don't express the IL-2 receptor common gamma chain, which allow more efficient engraftments of human hematopoietic cells than SCID or NOD/SCID mice in previous studies. Using these more immunosuppressive mice as hosts, CD34+CD38+ cells from some primary AML can induce transplantable disease, indicating CD34+CD38+ cells have LSC activity too^[12,13]. Works from Bonnet's laboratory unveiled the possibly confounding factor that the anti-CD38 antibody used for separation of primary AML cells has significant inhibitory effect on engraftment of leukemia cells^[13]. Taken together, these studies suggest LSC might co-exist in CD34+CD38- and CD34+CD38+ subpopulation.

Cell surface markers differentially expressed between LSC and normal HSC

Because LSC and HSC sharing similar CD34+CD38-surface immunophenotype, the search of cell surface markers unique to LSC (ideal circumstances) or at least differentially expressed has attracted intensive enthusiasm in hematology and oncology field. Such makers will provide excellent therapeutic windows for specifically targeting LSC, while sparing normal HSC. Such therapies are expected to be much tolerable for AML patients.

CD90

CD90, also known as Thy-1, is a small glycosylphosphatidylinositol (GPI)-anchored protein (25-37 kDa) regulating multiple signaling cascades which control cellular survival, proliferation, adhesion and response to cytokines^[14]. One of the early studies reported that the majority of AML blasts did not express CD90 and CD34+CD90-cells were capable of maintaining the disease *in vitro* and *in vivo* as demonstrated by production of leukemic clonogenic cells (CFU) and engraftments in nonobese diabetic severe combined immune deficient (NOD/SCID) mice, respectively^[15]. However, independent study to validate CD90 as a possible LSC marker is scarce in the literature. In contrast, CD90 expression was detected

at high frequency of a group of high-risk AML, such as secondary AML (40%) and elderly > 60 years AML (24%) patients^[16]. Univariate analysis revealed that CD90 expression was an independent prognostic factor for a shorter survival^[16]. This finding appears to contradict to the proposal of CD34+CD90- fraction is the source of LSCs because it is generally believed that abundant level of LSC markers is associated with poor survival. Interestingly, CD90 has been identified as marker of cancer stem cell (CSC) of hepatocellular carcinoma^[17], esophageal cancer^[18] and high-grade gliomas^[19].

CD96

CD96 (also known as TACTILE), a type I membrane protein, belongs to the immunoglobulin superfamily. CD96 plays a role in the antigen presentation of immune response the adhesive interactions of activated T and NK cells. CD96 is expressed on the majority of CD34+CD38- AML cells and vice versa^[20]. In contrast, CD96 is weakly expressed in cells in the normal HSC-enriched population [Lin(-)CD34(+)-CD38(-)CD90(+)]. Significant level of engraftment is only achieved in mice implanted with CD96+ AML cells, but not CD96- AML cells^[20]. From a therapeutic point view, this LSC marker offers a few new avenues for treatment of AML disease. Firstly, CD96 specific monoclonal antibody can be used to selectively eradicate AML-LSCs before autologous stem cell transplantation^[21]. Secondly, Fc-engineered mini-antibodies directed against CD96 shows enhanced antibody-dependent cell-mediated cytotoxicity (ADCC) activity of affinity and the highest cytolytic potential^[22].

CD123

CD123 is also known as interleukin 3 receptor, alpha (IL-3R α). IL3R is a heterodimeric cytokine receptor comprised of the alpha unit and beta unit, which is activated by the ligand binding and necessary of IL-3 activity^[23]. IL-3 is one of the prominent cytokines that controls proliferation, growth and differentiation of hematopoietic cells^[24]. Compared to all other cell surface antigens as potential LSC markers, the studies on CD123 have been investigated into much more details and targeting CD123 is now in clinical trials^[23].

Jordan and colleagues^[25] first reported that CD123 was aberrantly expressed on CD34+CD38- cells from AML patients, but not detectable on CD34+CD38- cells from healthy controls. Moreover, purified CD34+CD123+ cells from AML patients were capable of establishing and propagating leukemia disease in NOD/SCID mice^[25]. This result functionally validated CD123 as a LSC marker. A following-up study from the same group further revealed that NF κ B activity was constitutively activated in the CD123+ LSCs, but not CD123+ normal HSC, providing a molecular difference between these two cell entities^[26]. Higher level of spontaneous signal transducer and activator of transcription 5 (STAT5) activity is an-

other factor contributing to the proliferative advantage and resistance to apoptosis of AML blasts with elevated CD123^[27]. It is well documented that enhanced STAT pathway activity confers drug resistance in AML^[28], possibly through two distinct mechanisms: upregulation of anti-apoptotic survivin (*BIRC5*), Bcl-xL (*BCL2L1*) genes and ATP-binding cassette (*ABC*) family genes, which encode multidrug-resistance (MDR) transport proteins.

The utility of CD123 as a LSC marker has been convincingly confirmed by many other studies^[29,30]. A flow cytometric analysis of CD123 expression of diagnostic blasts from 111 *de novo* AML patients younger than 65 years old shows the presence of more than 1% population of CD34(+)-CD38(low/-)-CD123(+) cells adversely affected the disease-free-survival and over-all survival^[30]. Notably, not only the percentage of CD123+ cells, but also the expression level of CD123+ predicts clinical outcome. Patients whose AML blasts have higher CD123 expression have a lower complete remission (CR) rate and shorter survival duration than those showing normal CD123 expression level^[27]. In AML arising from Fanconi anemia (FA) background, only CD123+ cells achieve significant level of engraftment and cause leukemia in a "humanized" FA xenotransplant model^[29].

Other studies have depicted what other molecules are co-expressed with CD123 in AML-LSCs. High CD123 AML cells often exhibit elevated level of receptor tyrosine kinases (RTKs) such as FLT3 (Fms-Related Tyrosine Kinase 3), c-Kit^[31], N-cadherin and Tie2 (Tunica Interna Endothelial Cell Kinase)^[32]. Both FLT3 and c-Kit are important RTKs for the survival of hematopoietic stem/progenitor cells. N-Cadherin and Tie2 play a pivotal role in regulation of interaction between LSCs and their niche in the bone marrow microenvironment. These findings reinforce the role of CD123 as a LSC marker because these co-expression molecules provide CD123+ cells survival advantages and sanctuary in their niche environments.

Antibody therapy specifically targeting CD123 has been advanced to clinical development over a short 5-year period since the first report of *in vivo* preclinical study^[33]. Anti-CD123 monoclonal antibody 7G3 has been shown to completely inhibit bone marrow engraftment by ex vivo treatment and partially impede bone marrow engraftment in a pre-established disease model in mice. CSL360, a recombinant chimeric IgG1 mAb derived from 7G3, was evaluated in phase I clinical trial against AML. The preliminary results showed that anti-CD123 mAb therapy with CSL360 is safe and tolerable and biological effects have been observed (ClinicalTrials.gov Identifier: NCT00401739). A humanized, affinity-matured version of anti-CD123 antibody, CSL362, was developed through engineering the Fc-domain for increased affinity for human CD16 (Fc γ RIIIa) on (natural killer) NK cells. CSL362 exhibits greater ADCC against both bulks of AML blasts and CD34+CD38-CD123+ LSCs^[34]. Currently, CSL362 is under phase I clinical trials in patients with CD123+ AML in complete remis-

sion (CR) or CR with incomplete platelet recovery at high risk for early relapse (Clinical Trials.gov identifier: NCT01632852). Novel molecules targeting both CD123 and CD33 have been shown to have stronger anti-AML effect than mono-targeting agents *in vitro*^[35]. It will be interesting to test these dual-targeting or triple-targeting molecules in animal studies or even in human clinical trials against LSC.

Adoptive T cell therapy is an alternatively attractive approach for the treatment of cancer utilizing chimeric antigen receptors (CARs)^[36]. The third generation of CARs consist of an extracellular antigen-binding domain and three or more intracellular signaling domains^[36]. CD123 chimeric antigen receptor (CAR) redirected T cells/cytokine-induced killer (CIK) cells show robust activity against CD123+ cell lines, primary AML cells and mouse xenograft models transplanted with patient AML cells^[37-39]. One important advantage of this approach lies on the observation that relapsed or refractory AML cells which often are chemotherapy-resistant are still vulnerable to CD123 CAR T cell therapy^[37]. However, depletion of normal human myelopoiesis caused by CD123 CAR T cells as a potential side effect should be taken account when planning a clinical trial^[38].

Taken together, novel immunotherapy approaches such as improved variants of anti-CD123 monoclonal Ab and CD123 CAR T cell therapy hold great promising for AML treatment.

CD47

CD47 (also known as Integrin-associated protein, IAP) is one of the unique member of the Ig superfamily, consisting of a V-type Ig-like extracellular domain at its N-terminus, five hydrophobic membrane-spanning segments and a variably spliced (3-36 amino acids) cytoplasmic tail at its C-terminus^[40]. CD47 is a receptor for the C-terminal cell binding domain of thrombospondin-1 (TSP-1) and a ligand for the extracellular region of signal-regulatory protein alpha (SIRPα)^[41]. CD47 is ubiquitously expressed on human cells and involved in many fundamental cellular processes including immune and angiogenic responses^[40].

Majeti and co-workers first discovered higher expression of CD47 on AML LSC compared to their normal counterparts, HSC and multipotent progenitor cells (MPP), by flow cytometer and microarray gene expression analysis^[42,43]. The association between increased CD47 expression with worse outcome has been validated in 3 independent, large clinical cohorts with total 664 AML patients. Moreover, increased CD47 expression remains a prognostic factor for poor event-free survival and over-all survival in multivariable analysis considering age, FLT3-ITD status^[42]. SIRPα serves as inhibitory receptor expressed on phagocytic cells such as macrophages and dendritic cells. It was previously reported CD47 expressed on red blood cells (RBC) as a marker of self and interaction of CD47 and SIRPα on phagocytic

cells delivered a “do not eat me” message, limiting clearance of circulating RBC by the means of phagocytosis^[44]. Similarly, upregulation of CD47 on AML LSCs prevents themselves from the attack of phagocytic cells through the interaction of CD47 with its inhibitory ligand SIRPα. This conclusion is supported by several lines of evidence. Firstly, human AML cell line with low endogenous CD47 level fails to engraft in immunodeficient mice, while ectopic expression of mouse CD47 in this cell line improves engraftment^[45]. In an inducible and controlled expression of CD47 *in vitro* and *in vivo* models, it has shown that the level of CD47 expression negatively correlates the percentage of phagocytosis by the macrophages^[45]. Secondly, transgenic mice expressing SIRPα variants with differential ability to bind human CD47 demonstrates that the engraftment of AML LSCs depends on the interaction of CD47 with SIRPα and AML LSCs are eliminated by macrophage-mediated phagocytosis in the absence of SIRPα signaling. In addition, pharmacological disruption of CD47-SIRPα binding by SIRPα-Fc fusion protein augments phagocytosis of AML cells by both mouse and human macrophages and damages engraftment of CD34+CD38- AML LSCs in mice^[46]. Thirdly, AML patients with high SIRPα mRNA expression on AML blasts have poor survival and inhibition of SIRPα signaling lead to reduced cell proliferation and enhanced apoptosis of AML cells^[47]. Based on the aforementioned evidence generated from *in vitro* experiments, *in vivo* mouse model and clinical data, we believe elevation of CD47 expression in AML LSCs appears to enable them to evade host immune surveillance.

A few anti-CD47 monoclonal antibodies have been tested *in vitro* and animal models. Two antibodies that block CD47/SIRPα interaction induce phagocytosis of AML cells *in vitro* and *in vivo* and eradicate LSCs in xenograft mouse and isogenic mouse leukemia models, while an anti-CD47 antibody that does not disrupt CD47 binding to SIRPα fails to promote phagocytosis of AML cells^[42]. The other promising strategy to target this interaction is to use soluble SIRPα-Fc fusion proteins to neutralize CD47^[46]. Treatment of SIRPα-Fc fusion proteins leads to activate macrophages mediated phagocytosis, resulting in potent anti-AML effect and clearance of LSCs^[46].

Taken together, these evidences indicate that delivering a “do not eat” signal to phagocytic cells is a prime consequence of CD47/SIRPα interaction, which suppresses phagocytosis. Disruption of this interaction would successfully initiate innate immune response to eliminate LSCs through macrophage phagocytosis.

CD44

CD44 belongs to a family of transmembrane glycoproteins that act primarily as a receptor for hyaluronan acid (HA), but it also binds to other receptors including osteopontin, collagens, matrix metalloproteinases (MMPs), *etc.*^[48]. Hyaluronan is one of the major components of

the extracellular matrix^[49]. The major function of CD44 is to regulate cell-cell adhesion and cell-matrix interaction through binding to HA and other receptors^[49]. Specifically, the roles of CD44 in haematopoiesis include cell migration, proliferation, differentiation, survival and bone marrow homing of hematopoietic stem/progenitor cells^[50].

It has been long recognized that CD44 is expressed in normal and leukemic CD34+ early hematopoietic cells and empowers them to seek intramedullary or extramedullary sanctuary^[51]. It has been postulated that such protective ability resulted from CD44 interaction with various cellular receptors and matrix components allows small numbers of leukemic cells to survive from the attack of cytotoxic chemotherapy^[52].

Detection of CD44 and coexpression of CD123 (abovementioned) on CD34+CD38- AML cells indicates the CD44 is a potential candidate of LSC marker^[53]. Jin *et al.*^[54] first comprehensively characterized CD44 as a critical regulator of AML LSCs in a few mouse models. Treatment with H90, a monoclonal antibody targeting CD44, significantly prolonged survival of NOD/SCID mice transplanted with CD34+CD38- AML LSCs and reduced the number of LSCs in mouse bone marrow as compared to control IgG treatment. Furthermore, in a secondary transplantation experiment, leukemic cells obtained from H90 treated mice (primary mice) failed to engraft into the secondary receipt mice. However, in the parallel experiment, leukemic cells harvested from primary mice treated with control IgG initiated robust engraftment in the secondary receipt mice^[54]. The power of eliminating LSCs by anti-CD44 monoclonal antibody treatment could be explained by three different mechanisms by which targeting CD44 induces leukemic cell differentiation^[54-58], inhibits cell cycle progression and cell proliferation^[58,59] and impedes LSCs homing to bone marrow niches^[54]. Collectively, these data conclusively demonstrate that CD44 is functional important for LSCs.

CD32 OR CD25

CD32 is a member of a family of immunoglobulin Fc receptors, expressed on macrophages, neutrophils and nature killer cells^[60]. CD32 binds to the Fc region of immunoglobulins gamma (Igγ) and executes phagocytosis and clearing of immune complexes^[60]. CD25 is also known as interleukin 2 (IL2) receptor alpha (IL2RA)^[61]. IL-2 cytokine regulates cell proliferation, differentiation, survival and apoptosis^[62].

CD32 and CD25 were discovered to be overexpressed on quiescent and chemotherapy-resistant human AML LSCs by microarray study of LSCs *vs* normal HSCs. Normal CD34+CD38-CD133+ HSCs are negative for CD32 or CD25 expression^[63]. In xenotransplantation experiments with sorted human AML cells injected into immunodeficient mice, CD32+CD34+CD38- or CD25+CD34+CD38- cells were capable of engraftment and inducing AML. On the contrary, no engraft-

ment was detected in mice inoculated with CD32-CD34+CD38- or CD25-CD34+CD38- cells^[63]. The CD32+CD34+CD38- or CD25+CD34+CD38- cells not only survived after treating the mice with cytosine arabinoside (Ara-C), but also initiated *in vivo* AML when injected into the secondary receipt mice in a serial transplantation model^[63].

CLL-1

C-type lectin-like molecule-1 (CLL-1) is a member of type II transmembrane receptor family containing C-type lectin/C-type lectin-like domain (CTL/CTLD). CLL-1 was initially identified as a novel surface marker of AML cells through phage display technology combined with flow cytometry^[64]. Further studies revealed that CLL-1 was expressed on CD34+CD38- cells in 87% of AML patients, but was not expressed in normal HSCs^[65]. Successful engraftment was observed in all 3 NOD/SCID mice transplanted with CD34+CLL-1+ AML cells^[65]. The same group also reported that side population (SP) cells isolated from AML samples which were highly enriched for LSCs also expressed CLL-1^[66].

A series of monoclonal antibodies against CLL-1 was developed and two lead antibodies were chosen based on their high affinity and potent cytotoxic activity^[67]. These antibodies induced dose-dependent complement-dependent cytotoxicity and antibody-dependent cellular cytotoxicity against AML cell lines, primary AML patient cells and xenograft mice implanted with HL-60 AML cells. However, the possibility of targeting LSCs was not assessed in this study^[67]. It would be of interesting to further evaluate the impact of these anti-CLL-1 antibodies on LSCs in animal experiments. Nanomicelles decorated with CLL1-targeting peptides can specifically binds to CD34+CLL-1+ primary AML cells and delivered chemodrug daunorubicin directly to target cells^[68]. Importantly, these nanomicelles did not bind to normal CD34+ cells, so it was not expected to harm normal hematopoiesis^[68]. The challenge of *in vivo* delivery of nanomicelles remains a concern.

TIM-3

T cell immunoglobulin-3 (TIM-3) belongs to the mucin domain-containing molecule (Tim) superfamily and is a member of the T cell Ig^[69]. TIM-3 is expressed on CD4+ Th1, CD8+ T cytotoxic 1 (Tc1) cells, monocytes/macrophages, dendritic cells and mast cells^[70]. TIM-3 plays an important role in T cell response and regulation of innate immunity^[69,70].

TIM-3 was found to be expressed on CD34+CD38- fraction of AML cells except FAB M3 subtype (acute promyelocytic leukemia, APL) but absent on normal CD34+CD38- HSCs through comparative analysis of transcriptome of these two populations^[71]. TIM-3 expression was significantly higher in a distinct subtype of AML with core binding factor (CBF) translocation or

CEBP α mutation^[72]. This association was a bit puzzling because AML patients with CBF and CEBP α abnormalities often have favourable prognosis^[73]. Reconstitution of AML in immunodeficient mice was established only when TIM-3+ AML cells were transplanted, but not TIM-3- AML cells. Treatment of mice injected with human primary AML cells with an anti-TIM-3 monoclonal antibody, ATIK2a, effectively blocked reconstitution of AML. Importantly, human CD45+ AML cells harvested from the primary recipient mice treated with ATIK2a lose the ability to initiate AML retransplanted into secondary recipient mice^[71]. Normal HSCs were not damaged by ATIK2a treatment because normal HSCs appear to reside in TIM-3- population^[71,72]. These data suggest that TIM-3 could serve as a useful marker to distinguish LSCs from HSCs and monoclonal antibody against TIM-3 holds promise to eradicate LSCs.

Aldehyde dehydrogenase

Aldehyde dehydrogenase (*ALDH*) gene superfamily consists of 19 functional genes and three pseudogenes. ALDH oxidise a wide range of endogenous and exogenous aldehyde substrates, thus detoxifying large portion of adverse aldehydes to the cells. ALDH is highly expressed in primitive stem cells from several tissue origins, including bone marrow and intestine^[74]. HSCs have high level of ALDH activity^[75] and can be distinguished using a fluorescent aldehyde, dansyl aminoacetaldehyde (DAAA) in conjunction with FACS analysis^[74,76].

Since LSCs share some functional similarity with HSCs, researchers soon started to investigate the role of ALDH in AML LSCs. In total, 3 distinct patterns of ALDH activity were documented. In the first pattern, the subpopulation of AML cells with high ALDH activity was rare, which was similar to the pattern seen in normal core blood. In the second pattern, the frequency of cells with ALDH activity was more frequent and their side scatter profiles were higher than normal stem/progenitor cells. No fraction of cells with high ALDH activity was present in the third pattern^[77]. Xenograft transplantation experiments demonstrated that ALDH+ cells were enriched for LSCs and engrafted better than ALDH- cells^[77,78]. From a clinical point of view, higher ALDH activity is associated with dismal prognosis, drug resistance and relapse^[78-80].

SMALL MOLECULE INHIBITORS

TARGETING LSCS

Parthenolide and analogs

Dimethylamino-parthenolide (DMAPT), modified analog of parthenolide (PTL) which is a major active component of herbal medicine Feverfew, possesses improved pharmacologic properties and is orally bioavailable^[81,82]. DMAPT and PTL preferentially kill AML leukemia stem/progenitor cells through mechanisms involved in inhibition of NF κ B pathway, induction of tumor suppressor p53 and reactive oxygen species (ROS) produc-

tion^[81,82]. DMAPT shows potent *in vivo* biological activity in spontaneous canine acute leukemia and mouse xenotransplantation models^[82]. DMAPT is a novel compound that is specifically target LSCs and now is being evaluated in a phase 1-2 “first in man” in clinical trial in AML in Cardiff University, United Kingdom.

Epigenetic inhibitors

AR-42 (OSU-HDAC42), a novel histone deacetylase inhibitor (HDACi), inhibits NF κ B activity and HSP90 interaction with its various client proteins, leading to robust and selective apoptosis of AML LSCs^[83]. Currently, AR-42 is being tested in advanced or relapsed multiple myeloma (MM), chronic lymphocytic leukemia (CLL), or lymphoma in clinical trials (ClinicalTrials.gov Identifier: NCT01129193).

BRD4 (Bromodomain-containing protein 4) was identified as a promising anti-AML target in a whole-genome RNAi screening^[84,85]. BRD4 is a chromatin “reader” that recognizes and binds acetylated histones. JQ1 is a novel small molecule inhibitor that competes with BRD4 to bind acetyl-lysine recognition motifs^[86]. JQ1 can induce apoptosis in CD34+CD38- and CD34-CD38+ stem- and progenitor cells from both *de novo* AML and refractory AML patients^[87].

3-Deazaneplanocin A (DZNep), is a newly discovered S-adenosyl-methionine-dependent methyltransferase inhibitor^[88]. DZNep inhibits EZH2, disrupts polycomb-repressive complex 2 (PRC2), and preferentially induces apoptosis in cancer cells^[88]. We and another group showed that DZNep promoted cell death in CD34+CD38- AML cells, but not normal CD34+ progenitor cells^[89,90].

Apoptosis pathway modulators

ABT-737, a BCL-2 homology domain 3 mimetic inhibitor, have been shown to target Lin-/Sca-1(+)/c-Kit(+) primitive cells, and progenitor population in a myelodysplastic syndrome (MDS)-AML transgenic mouse model^[91].

Using reversed-phase protein array, Carter BZ and colleagues^[92] found that CD34+CD38- AML stem/progenitor cells expressed increased caspase 8 and increased ratio of cIAP (Baculoviral IAP Repeat Containing 2, BIRC2) to SMAC (second mitochondrial-derived activator of caspases) compared to bulk AML cells. Birinapant is a novel bivalent SMAC mimetic with high affinity for IAP proteins. Treatment with birinapant induced apoptosis of AML stem/progenitor cells involving in activation of DR (death receptor)/caspase-8 complex. In human AML xenograft mouse model, diseased mice treated with birinapant or in combination with 5-azacytidine (5-Aza), decitabine (DAC), survived significantly longer than mice administrated with vehicle control^[92].

Kinase inhibitors

Rapamycin is the first generation of mTOR (mammalian target of rapamycin), a downstream target of

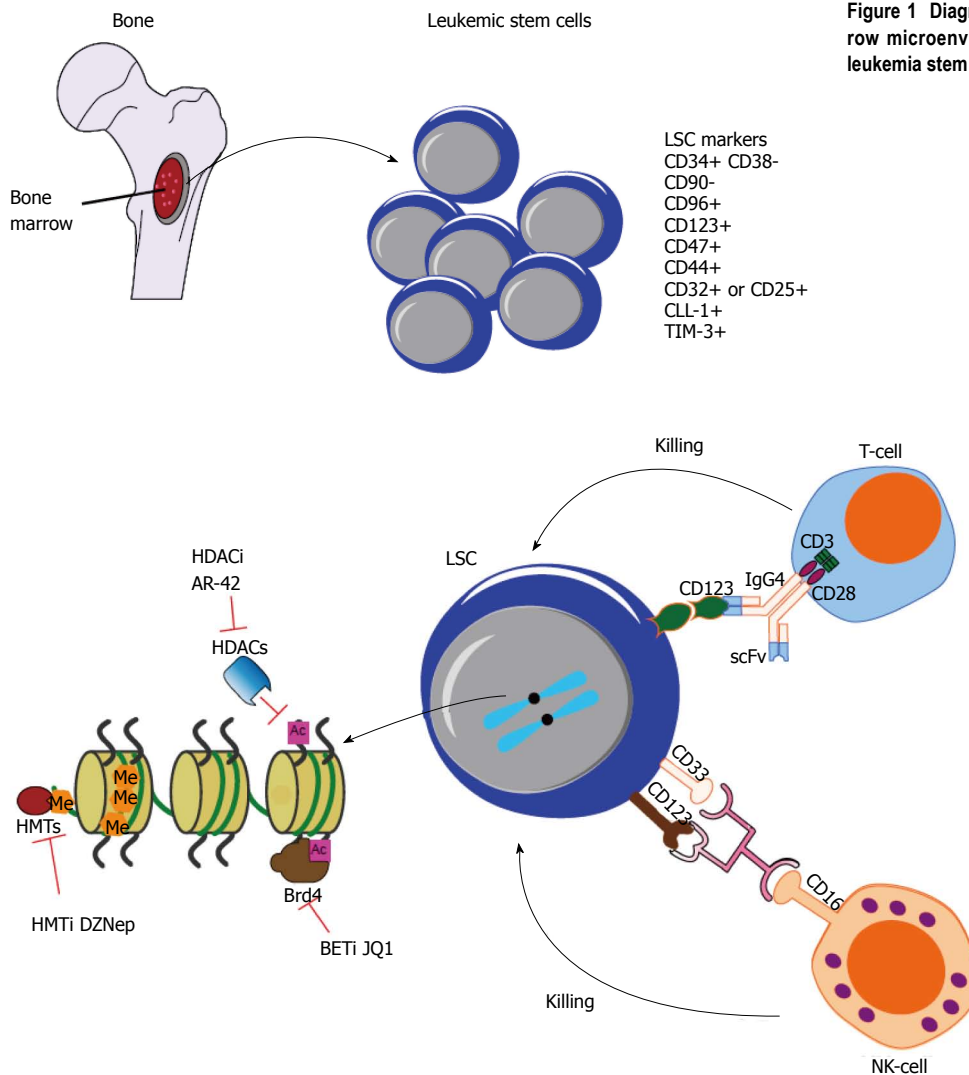


Figure 2 This illustration shows combination therapies aiming to achieve maximal and synergistic anti-leukemia stem cells effect. HDACi: Histone deacetylase inhibitor; HMTi: Histone methyltransferase inhibitor; BETi: Bromodomain and Extra-Terminal inhibitor; Brd4: Bromodomain-containing protein 4; Ac: acetylation; Me: methylation; NK-cell: Natural killer-cell.

phosphatidylinositol 3-kinase (PI3K)-Akt pathway, inhibitor^[93]. Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) negatively regulates PI3K-AKT-mTOR activity. Tissue specific deletion of PTEN in hematopoietic cells led the mice to develop AML and acute lymphoid leukemia (ALL) and all mice succumbed to disease rapidly in one month^[94]. One out of 81 Flk-2-Sca-1+Lin-c-Kit+CD48- (enriched for LSCs) from PTEN null AML mice was able to initiate AML in serial transplantation experiments^[94]. A search of “ClinicalTrials.gov” database on 10 July 2014 identified a total of 40 clinical trials that test Rapamycin or its analogs, Temsirolimus (CCI-779) or Everolimus (RAD001), in AML either by alone or in combination with chemotherapy or kinase inhibitors or transplantation. However, it appears that the evaluation of the effect of mTOR inhibitors against LSCs is not included in these trials.

Dasatinib is a multiple kinase inhibitors targeting Abl, Src family and c-Kit. Dos Santos *et al.*^[95] reported that

combination of dasatinib and daunorubicin enhanced the eradication of AML LSCs in mouse xenotransplantation model through increasing p53 activity^[95].

Hematopoietic Cell Kinase (HCK) belongs to the Src family of tyrosine kinases. HCK is mostly expressed in hematopoietic cells, particularly phagocytes. HCK was reported by Saito Y, et al. to overexpress on quiescent, chemotherapy-resistant LSCs compared to normal HSCs^[63]. The same group performed integrated, multiple platform analysis to uncover RK-20449, a pyrrolo-pyrimidine derivative as a potent inhibitor of these LSCs *in vitro* and *in vivo*^[96].

CONCLUSION

The advance in high-throughput and whole genome techniques in conjunction with the development of more immunocompromised mouse strains helps deepen and broaden our understanding of LSCs, the enigmatic frac-

tion of leukemic cells which is the origin of the disease. From the single pattern of CD34+CD38- as phenotypic hallmark for LSCs, a long-list of additional cell surface antigens such as CD123, CD47, CD44, CLL-1, CD96, CD90, CD32, CD25, and TIM-3, has been identified to separate LSCs from normal HSCs (Figure 1). From the notion that LSC is extremely rare, it is now clear that the frequency of LSC among AML patients is highly heterogeneous, ranging from very low to frequent. From the concept that LSCs only reside in CD34+CD38- subpopulation, emerging study reveals that CD34+CD38+ fraction also harbours LSCs. From the idea that one patient only has one population of LSCs, we now understand that some patients may have more than one populations of LSCs.

Along the advance in our understanding of LSC, a growing list of strategies for targeting LSC has been proposed and some of these agents as summarized above have advanced into clinical trials. Currently, monoclonal antibodies targeting CD123 or their related immunoconjugate therapy or CD123 CAR T cell therapy appear to be the front runner leading the way to eliminate LSC and eventually cure AML. The second gold mine for the discovery of drug targets is how LSCs employ “epigenetic machinery” to program or reprogram themselves because epigenetic changes are reversible and epigenetic enzymes are often targetable. The first generation of some of these small molecule inhibitors such as DZNep, JQ1, already showed potent effect in killing LSCs. We shall witness the second generation of these compounds or novel small molecule inhibitors with favourable pharmacological profiles and safety profiles entering clinical trials in the next few years.

However, the real impact on clinical management of AML is far less promising than the remarkable response observed in *ex vivo* cell culture models or xenotransplanted mouse experiments as reported in numerous “sophisticated” studies. In our opinion, although many surface antigens have been identified to be aberrantly expressed on LSCs, it is probably impossible for any single monoclonal antibody targeting one of these surface antigens to eradicate LSCs, given such heterogeneity and dynamics of LSC properties in AML patients. Synergistic therapies in combination with immunotherapy, cell therapy and epigenetic drugs may provide a better opportunity to achieve our ultimate goal of targeting LSCs and curing AML (Figure 2). By using CD123 target as an example, it is hoped that combination of CD123 CAR T cells which bind to CD123 on the surface of LSC or mono- or dual-targeting antibody with small molecule inhibitors targeting epigenetic machinery, such as Brd4 inhibitor or HMTi or HDACi, will be effective for the treatment of AML.

ACKNOWLEDGEMENTS

The authors thank Mr Ching Ying Qing for his excellent illustration of Figures 1 and 2. Due to space limit, some of important works in this field were not cited and we

sincerely apologize to those authors whose important studies were not summarized.

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P- Reviewer: Chen SS, Fukuda S, Krimerk DB

S- Editor: Tian YL **L- Editor:** A **E- Editor:** Lu YJ



Sox2 transcription network acts as a molecular switch to regulate properties of neural stem cells

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Supported by The Nagasaki ken Medical Association.

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Received: July 25, 2014 Revised: August 29, 2014

Accepted: August 30, 2014

Published online: September 26, 2014

Abstract

Neural stem cells (NSCs) contribute to ontogeny by producing neurons at the appropriate time and location. Neurogenesis from NSCs is also involved in various biological functions in adults. Thus, NSCs continue to exert their effects throughout the lifespan of the organism. The mechanism regulating the core functional properties of NSCs is governed by intra- and extracellular signals. Among the transcription factors that serve as molecular switches, Sox2 is considered a key factor in NSCs. Sox2 forms a core network with partner factors, thereby functioning as a molecular switch. This review discusses how the network of Sox2 partner and target genes illustrates the molecular characteristics of the mechanism underlying the self-renewal and multipotency of NSCs.

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Key words: Neural stem cells; Self-renewal; Multipotency; Sox2; Transcriptional network

Core tip: Neural stem cells (NSCs) are cells that are capable of both self-renewal and multipotency. In these two processes, the transcription factor Sox2 serves as a switch for the central molecular mechanism. Sox2

forms complexes with its partner factors to perform its transcription-related functions. This partner switching presumably serves as an important key to the intrinsic functions of NSCs. A detailed understanding of these molecular mechanisms will advance our understanding of basic neuroscience and increase the feasibility of employing cell reprogramming technology in regenerative medicine.

Shimozaki K. Sox2 transcription network acts as a molecular switch to regulate properties of neural stem cells. *World J Stem Cells* 2014; 6(4): 485-490 Available from: URL: <http://www.wjgnet.com/1948-0210/full/v6/i4/485.htm> DOI: <http://dx.doi.org/10.4252/wjsc.v6.i4.485>

INTRODUCTION

Neural stem cells (NSCs) are cells that are capable of self-renewal and maintaining multipotency^[1,2]. NSCs differentiate into neurons, astrocytes, and oligodendrocytes. The cellular origin of mouse NSCs dates back to the initial stage of ontogeny. A blastocyst generates the primitive ectoderm, further differentiating into the neuroectoderm, which serves as a source of primitive NSCs^[3,4]. The neuroectoderm then develops and differentiates into the neuroepithelium^[3,4]. Primitive NSCs exhibit self-renewal with a rather limited multipotency^[5]. On embryonic day 11.5 (E11.5) in the murine fetal period, differentiation into neurons dominates while differentiation into the astrocyte lineage is suppressed by DNA methylation. Then at E14.5, NSCs begin to produce neurons and astrocytes^[6-8]. After birth, NSCs manifest their ability to produce oligodendrocytes^[8]. NSCs also actively undergo repeated self-renewal in the region of the central nervous system after birth to generate neurons, astrocytes, and oligodendrocytes in a region-dependent manner to build the brain as an organ. It was previously believed that neurons

do not regenerate once the brain organogenesis is complete in an adult organism. However, the study^[9-13] revised this dogma, and it is now known that neurogenesis takes place even in the adult brain. This process has been best studied in the subventricular zone (SVZ) of the lateral ventricle and the subgranular zone (SGZ) lining the hippocampal region, where NSCs are located and produce new nerve cells through self-renewal^[1,14-20]. Understanding the molecular biology underlying the capacity of NSCs to exhibit self-renewal and multipotency is expected to stimulate our exploration of basic neuroscience and lead to practical applications in regenerative medicine, allowing lost neurons to be regenerated as desired. Although some NSCs can be cultured from body tissues as the monolayer^[21-23], there are technical challenges as well as issues of productivity and quality related to the practical use of such cultured NSCs in regenerative medicine. However, a new technology was recently developed to reprogram somatic cells through the gene transfer. Using this technology, combinational transfection of the *Oct4*, *Sox2*, *Klf4*, and *c-Myc* genes into various cells can establish a type of multipotent stem cells, called induced pluripotent stem (iPS) cells^[24,25]. By changing the culture conditions under which iPS cells are established, we can artificially induce differentiation into NSCs^[26]. This technology has also been utilized to develop induced neuronal (iN) cells, which directly induce differentiation into neurons^[27]. iN cells are obtained by transfecting the *Ascl1*, *Brn2*, and *Myt1l* genes into fibroblasts. The gene cluster serving as the switch to precisely regulate cell fate mainly includes transcription factors. One key factor that plays an important role in NSCs is the transcription factor Sox2. Transcription factors bind to response regions in the genome to initiate or terminate the expression of target genes. Concomitantly, they interact with a group of chromatin-regulating factors other than transcription factors to perform various regulatory functions. In this review, I focus on the transcription regulatory network centered around Sox2 to shed light on the molecular regulatory mechanism underlying the biology of NSCs.

NEURAL STEM CELLS AND SOX2

Sox2 belongs to the *Sry* gene family and contains a DNA-binding domain referred to as a high-mobility group (HMG) domain, which is highly conserved across the family. To date, more than 20 genes have been identified in the *Sox* gene family^[28,29]. Sox2 is a maternal factor that is specifically expressed in the inner cell mass (ICM) and primitive ectoderm^[30]. Sox2 expression is widely observed among the cells within the neural tube at early stages of neurodevelopment^[31]. Its expression is subsequently localized to the ventricular layer in the neuronal cortex, where NSCs and their precursor cells are present after the mid-fetal period. During this period, Sox2 is not expressed in layers where terminally differentiated neurons are present^[32]. In the adult brain, NSCs are localized to the SVZ of the lateral ventricle and the SGZ lining the hip-

poampal region, where they undergo self-renewal and perform neurogenesis^[1,14]. All of such self-renewing cells express Sox2. Sox2 plays an important role in maintaining the functions of NSCs^[32-35]. It has been reported that SoxB1 family members, Sox1 and Sox3, which show high sequence homology to Sox2, exhibit similar functions^[36]. Sox2 functions as a maternal factor in pre-implantation embryos^[30]. Zygotic knock-down of Sox2 using a specific siRNA resulted in an incomplete trophectoderm (TE) in fertilized embryos, which failed to progress beyond the morula stage^[30]. Sox2 expression is detected in both the ICM and TE, and its expression becomes restricted to the ICM^[29]. During embryogenesis, the ICM becomes the embryo, and the TE forms the placenta. A high level of *Sox2* gene expression has been confirmed in the neuroectoderm that gives rise to NSCs^[31]. During embryogenesis, Sox2 promotes neuroectoderm cell fate by suppressing the mesodermal cell fate^[37]. Moreover, Sox2 plays important roles in the differentiation of the central nervous system and peripheral nervous system during embryogenesis by controlling the proliferation and differentiation of neural stem/progenitor cells^[32]. Sox2 deficiency is embryonically lethal in mice because the fetus fails to form embryonic stem (ES) cells from the ICM or produce trophoblast stem cells^[30,38]. Sox2 conditional knock out (KO) mice have been reported to undergo neurodegeneration leading to dysfunctional neuronal differentiation in the adult brain^[35,39]. Various research approaches have been employed to demonstrate that Sox2 expression is localized to NSCs and that its function is essential for these cells.

SOX2 AND ITS PARTNERS

Sox2 collaborates with other transcription factors^[40,41]. In ES cells and NSCs, Sox2 regulates the self-renewal mechanism and suppresses differentiation in a dosage-sensitive manner^[42,43]. Sox2 and a POU factor known as Oct4 form a specific partnership to coordinately regulate the mechanism that maintains undifferentiated ES cells^[44,45]. The target genes of this partnership include *Nanog*, *Ulf1*, and *Fgf4*^[4]. Sox family members form partner complexes with POU factors, but the partnership assumes various forms depending on the cell type^[41]. In NSCs, Sox2 interacts with POU factors such as Pax6, Brn1, and Brn2, where Pax6 forms complexes with Sox2 to regulate the differentiation of cells of the optic nerve and lens^[46-49]. Pax6 is coexpressed in Sox2-positive cells and reportedly regulates the self-renewal and neurogenesis of NSCs in the hippocampus in the adult brain^[50]. The expression of Nestin, a marker for NSCs, is coordinately regulated by Sox2 and POU factors^[47,51]. Sox2 and the partner code of Brn1 and Brn2 bind to the regulatory region of the Nestin and Sox2 genes to perform an important function in the regulation of gene expression^[47,51-53]. Furthermore, Sox2 can bind to Prx1 (MHox1/Prrx1) and function as its partner^[54]. Because Prx1 and Sox2 are coexpressed in certain cells in the NSC region, they are expected to

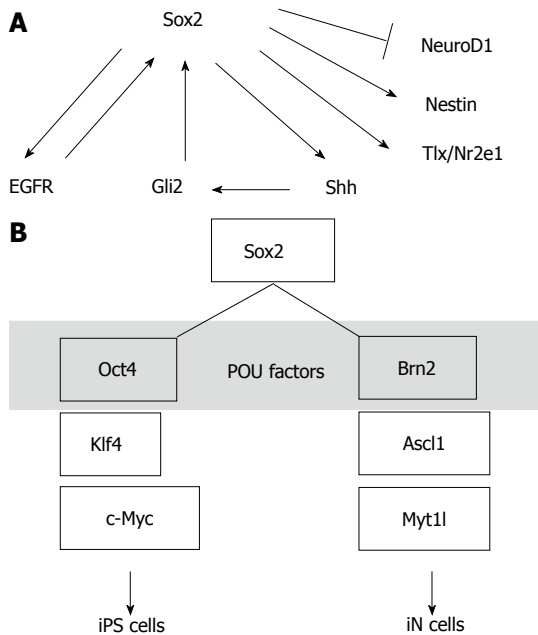


Figure 1 Diagrams of the Sox2 transcription network and reprogramming. A: Examples from the Sox2 transcription network. Sox2 activates *Egfr* transcription, and EGFR signaling activates Sox2 transcription. Sox2 also activates *Shh* transcription, and the *Shh* signaling downstream factor *Gli2* activates Sox2 transcription. Sox2 activates the *Nestin* and *Tlx/Nr2e1* genes but represses *NeuroD1* transcription; B: Sox2 and POU factors are assumed to function as a core-partner unit in gene-induced cell reprogramming. iPS: Induced pluripotent stem; iN: Induced neuronal.

coordinately activate the target genes, and they have been suggested to be involved in the regulatory mechanism that maintains the undifferentiated state of NSCs.

SOX2 TARGET GENES AND STEM CELL FUNCTION

Sox2 is a transcription factor, and many reports have been published describing analyses of its target genes. Sox2 regulates the expression of its target gene called *Sonic hedgehog* (*Shh*) to regulate NSCs in the hippocampus^[39,55,56]. *Shh* is a humoral factor that transmits outside signals from outside into the cell via its receptor Patched, and induces Smo/Gli signal activation^[57-59]. Another transcription factor, *Gli2*, is a downstream target of *Shh* and regulates *Sox2* gene expression^[60]. Therefore, these factors may constitute a positive feedback loop. Additionally, Notch and the epidermal growth factor receptor (EGFR) pathway regulate the number of NSCs and their self-renewal^[61]. EGF stimulation can turn neural progenitors into multipotent NSCs through the receptor, EGFR^[62]. Whereas EGFR signaling increases *Sox2* expression, Sox2 enhances *Egfr* expression, which suggests a positive feedback mechanism^[63] (Figure 1A). The nuclear receptor, *Tlx* (*Nr2e1*), is an essential factor in the mechanism that maintains undifferentiated NSCs^[64-66]. A possible negative feedback model of *Tlx* gene expression has been reported, in which Sox2 binds to *Tlx* to regulate its transcription^[67]. Based on these findings, it is conceivable that the

Sox2-centered feedback loop mechanism involving Sox2 target genes serves as an important system for the self-renewal mechanisms of NSCs.

It was recently reported that the crosstalk between Sox2 and Wnt signaling regulates the switching during the differentiation of NSCs to neurons^[68]. Sox2 and Tcf act as molecular switches thus interacting with the overlap sequence^[68], and this process, in turn, regulates *NeuroD1* expression^[68]. Although the mechanisms underlying the molecular switching of numerous genes are being increasingly revealed, it remains unknown how such mechanisms activate differentiation switches at the appropriate times and locations in response to intra- and extracellular changes, while suppressing the expression of genes other than those involved in neuronal differentiation.

STEM CELL REPROGRAMMING AND THE SOX2 GENE NETWORK

Combined transfection of the *Oct4*, *Sox2*, *Klf4*, and *c-Myc* genes transforms somatic cells into pluripotent stem cells^[24,25]. In this process, the transcriptional network is switched on to generate multipotent stem cells. It is likely that the partnership between Sox2 and Oct4 functions as the core switch^[4]. The addition of *Klf4* to the partner complexes presumably allows for multidimensional regulation of various modes of switching. In the multipotency induction process, the use of serum-free culture medium with EGF actively induces the formation of NSCs^[26]. Conversely, induction of the iN cell phenotype is conducted using a cell engineering technology that directly transdifferentiates somatic cells into neurons^[27]. Forced expression of the *Ascl1*, *Brn2*, and *Myt1l* genes can induce neuronal differentiation. However, this method is not intended for the maintenance of NSCs. *Brn2* is a partner factor of Sox2^[51,53]. When Sox2 is added to the group of iN-factors and cells are cultured in EGF- or bFGF-containing medium, combinations other than Oct4, *Klf4*, and *c-Myc* may be able to produce artificial NSCs. Moreover, based on the concept of the Sox2 partner code^[41], the establishment of neuronal subtype-specific NSCs also seems possible, using combinations of Pax6 and Prx1 or other POU factors (Figure 1B).

CONCLUSION

I have reviewed the link between the molecular mechanisms at work in NSCs and properties of stem cells, with a focus on the network involving the Sox2-centered partner code and its target genes. The localized expression of Sox2 in NSCs/neural progenitors enhances its molecular specificity. By forming complexes with its partner factors, Sox2 exerts its transcriptional-regulation function. The partner factors involved vary depending on the molecular context of the stem cell lineage. Sox2 target genes include molecular switches controlling the *NeuroD1* gene (which is capable of inducing neuronal differentiation) as well as the feedback loop with the factors involved in self-renew-

al such as members of the EGFR signaling pathway. By manipulating Sox2 and its partner factors, researchers can now artificially induce differentiation into pluripotent, or multipotent stem cells and into neurons. Nevertheless, many questions remain unanswered regarding the Sox2-based self-renewal mechanism and the regulatory mechanism underlying multipotency. Further research using conditional KO mice is needed to explore functions of Sox2, its partner factors, and chromatin-regulating factors that interact with Sox2 and its partner factors as well as to identify the entire panel of Sox2 target genes.

ACKNOWLEDGEMENTS

I thank the members of the Center for Frontier Life Science for helpful discussions. Crimson Interactive Pvt. Ltd. (Ulatu) and NPG Language Editing are acknowledged for their assistance in translating and editing the manuscript.

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P- Reviewer: Abdelalim EM, Fukuda T, Lu F
S- Editor: Song XX **L- Editor:** A **E- Editor:** Lu YJ



New advances in the mesenchymal stem cells therapy against skin flaps necrosis

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Received: July 16, 2014 Revised: August 6, 2014

Accepted: August 30, 2014

Published online: September 26, 2014

pears to hold substantial promise in the treatment against skin flaps necrosis. This review involved four out of the top 10 innovations of the 20th century and four out of the 10 most important, current innovations. We hope that these contents could help you to pick up the new advances in the MSCs therapy against skin flaps necrosis.

Zhang FG, Tang XF. New advances in the mesenchymal stem cells therapy against skin flaps necrosis. *World J Stem Cells* 2014; 6(4): 491-496 Available from: URL: <http://www.wjgnet.com/1948-0210/full/v6/i4/491.htm> DOI: <http://dx.doi.org/10.4252/wjsc.v6.i4.491>

Abstract

Mesenchymal stem cells (MSCs), multipotential cells that reside within the bone marrow, can be induced to differentiate into various cells, such as osteoblasts, adipocytes, chondrocytes, vascular endothelial progenitor cells, and other cell types. MSCs are being widely studied as potential cell therapy agents due to their angiogenic properties, which have been well established by *in vitro* and *in vivo* researches. Within this context, MSCs therapy appears to hold substantial promise, particularly in the treatment of conditions involving skin grafts, pedicle flaps, as well as free flaps described in literatures. The purpose of this review is to report the new advances and mechanisms underlying MSCs therapy against skin flaps necrosis.

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Key words: Mesenchymal stem cells; Skin flaps; Endothelial progenitor cells

Core tip: Mesenchymal stem cells (MSCs) therapy ap-

INTRODUCTION

According to the report by Hultman *et al*^[1] from the American Council of Academic Plastic Surgeons and the Southeastern Society of Plastic and Reconstructive Surgeons, four out of the top 10 innovations of the 20th century were myocutaneous flaps, microsurgery, skin grafts, and transplantation, and four out of the 10 most important, current innovations are hand/face transplantation, fat grafting, stem cells, and perforator flaps. So these separately important contributions may lead to such a promising prospect by the combination of two or three or even more of them.

Mesenchymal stem cells (MSCs) mainly include widely applied bone marrow MSCs (BMSCs), adipose tissue-derived SCs (ADSCs), and Human umbilical cord matrix stem cells (HUCMSCs). Circulating BMSCs homed to perivascular sites in critically ischemic tissue, exhibited paracrine function and augmented microhemodynamics. These effects were mediated through arteriogenesis and angiogenesis, which contributed to vascular regeneration^[2]. MSCs are relatively easy to isolate and expand in culture and therefore have potency as a therapeutic tool in ischemic disease and in transplantation. The current

dilemma is that MSCs may not have a long lifespan after administration^[3,4]. A rapid disappearance of MSCs raises the question of how MSCs therapy might work. It is possible that partial administered MSCs escape from death and migrate to sites of injury and inflammation and that MSCs are able to rapidly pass on their effect to other cells that subsequently mediate tissue repair or immunomodulation. The dramatically decreased quantity of MSCs will absolutely affect their angiogenic and immunomodulatory function. Fortunately some authors have suggested that the combination of MSCs and gene therapy might generate a synergistic effect on stem cells therapy against skin flaps necrosis^[5,6].

This review is willing to elicit the stem cells treatment of conditions involving skin grafts, pedicle flaps, as well as free flaps and stem cells immunomodulation in skin flaps therapy based on the published data.

MSCS THERAPY AGAINST FREE FLAPS NECROSIS

For majority of surgeons, one of the most amazing medical miracles is the total or partial human face transplantation. Dubernard *et al*^[7] reported the encouraging outcomes 18 mo after the first human partial face transplantation which was performed on November 27, 2005. Then human face transplantation was successively reported.

Some free flaps combined with or without MSCs therapy already have clinical applications, but, for some cases they are lack of appropriate research models, and, for the remains the mechanisms are still disputed. A novel murine free flap model of acute hindlimb ischemia-reperfusion combined with Laser-Doppler Flowmetry, quantitative immunohistochemistry and immunofluorescence detection is maybe a suitable and reproducible experimental procedure of translational research that allows *in vivo* investigation of diverse molecular and cellular mechanisms^[8]. Some authors considered the patient body as an ideal bioreactor to induce vascularisation in large volumes of grafted tissues. But, for volumes limited by the lack of vascularisation, engineering a bone free flap for maxillofacial reconstruction still exists technical restrictions^[9].

It's good news for some patients who had undergone ablative tumor surgery, radiochemotherapy and primary reconstruction to receive the secondary reconstruction of the mandible by the prefabricated bony radial forearm flaps consisting of iliac crest and radial forearm flaps. And the iliac bone graft might be replaced with scaffold seeded with stem cells for further reduction of donor site morbidity^[10]. And the fact that MSCs combine with growth factors therapy is extremely promising. MSCs transduced by stromal cell-derived factor-1 α (SDF-1 α) definitely augmented ischemic free flaps survival, which was initially reported by us previously^[5]. Although the free flaps are versatile, they are deserted sometimes. For example, ADSCs enhance the survival of fat grafted into

the face, and a microfat graft with simultaneous ADSCs injection may be utilized to treat Parry-Romberg disease without the need for microvascular free flap transfer by Koh *et al*^[11], which has demonstrated the promising prospect by the combination of two current innovations.

MSCS THERAPY AGAINST PEDICLE FLAPS NECROSIS

At the very beginning, flap anti-necrosis therapy might be performed without directly division and culture of MSCs. For example, an intramedullary muscle flap could improve the functional results of joints reconstructed with partially demineralized and lyophilized osteochondral allografts by providing both vascularity and an increased population of MSCs capable of responding to bone morphogenetic proteins^[12]. By far the major pathway of cranial defects repair induced by implantation of demineralized bone matrix is by the direct induction of resident MSCs to osteoblasts and by the direct formation of bone through upgrading osteocalcin and Collagen type I mRNA^[13].

A series of endogenous growth factors and chemokines may do great contribution to flaps survival. A positive correlation existed between MVD and the high expression of SDF-1 and Chemokine receptor type 4 (CXCR4) following hyperbaric oxygen treatment in promoting neovascularization, which might be explained by the upregulation of SDF-1 and CXCR4 expression in the skin flaps of rats^[14]. Human umbilical cord mesenchymal stem cells (HUCMSCs) could improve the survival of ischemic skin flaps by promoting vascularization, which might be attributed to the increased expression of basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF)^[15]. Hypoxia preconditioned BMSCs or ADSCs transplantation improved ultra-long random skin flaps survival *via* promoting angiogenesis by upgrading VEGF^[16,17]. ADSCs could enhance the survival of random-patterned skin flaps in streptozotocin-induced diabetic mice *via* elevated expression of hypoxia-inducible factor-1 α ^[18], be capable of promoting flap prefabrication by VEGF-A^[19], and prevent ischemia-reperfusion injury, mainly by regulating the growth factors, such as VEGF, bFGF and transforming growth factor-beta (TGF- β)^[20]. However, the angiogenic effect by ADSCs is still controversial. Although the mean survival area ratios in the ADSCs treatment group and in the BMSCs treatment group had no significant difference, higher levels of bFGF and VEGF were found in the BMSCs transplantation group^[21]. In addition, ADSCs also had a significantly angiogenic response^[22], better immune compatibility and potential for enhancing the blood supply. These together suggest that both ADSCs and BMSCs have proangiogenic effect, but their promoting angiogenesis mechanisms may be quite different.

A large sum of exogenous stem cells and growth factors are utilized to promote flaps survival. An optimal delivery route should have been screened through a lot

of researches. There are mainly five methods to deliver MSCs: (1) intravenous injection; (2) subcutaneous injection; (3) intramuscular injection; (4) application with collagen sponge seeding; and (5) application with fibrin glue seeding. Hu *et al.*^[23], as many did, suggested that intravascular delivery of BMSCs increased wound healing and promoted flap survival following ischemia-reperfusion injury of cutaneous tissue flaps. Lee *et al.*^[24] suggested that the collagen sponge method delivered ASCs most effectively within the flaps and increased flap vascularity. VEGF and MSCs had synergetic effect when they were used together^[6], as we suggested the synergetic effect by SDF-1 α and MSCs co-application^[5], which could help rebuilding the blood circulation of the ischemic region in random flaps.

Recent advances about the flap anti-necrosis therapy should not neglect the prefabricated flaps and/or tissue engineering flaps. The prefabricated groin flaps with skin substitutes provided a useful vehicle for the implantation of MSCs to serve as an autologous microvascular bioscaffold^[25]. Poly(L-lactic-co-glycolic acid) or poly(ϵ -caprolactone) scaffolds seeded with co-cultured chondrocytes and BMSCs, were wrapped in a pedicle muscle flaps^[26].

MSCS THERAPY AGAINST SKIN GRAFT NECROSIS

Autologous transplantation of BMSCs was a promising therapeutic strategy for prevention of skin-graft contraction^[27]. A typical combined graft consisting of a free full-thickness skin graft and cultured autologous fibroblast-like BMSCs was effectively implanted and healed on the facial soft tissue defect^[28]. Some scholars suggested that the autologous ADSCs transplantation increased full-thickness skin graft survival and showed promise for use in skin graft surgery. This might be both due to *in situ* differentiation of ADSCs into endothelial cells and increased secretion by ADSCs of growth factors, such as VEGF and TGF- β 3 that enhanced angiogenesis^[29].

The most vital role of skin graft may not only be to repair a defect, but also to study the immunomodulatory mechanisms. Human MSCs have immunomodulatory properties. They inhibited lymphocyte (especially, T-cell) proliferation to mitogens and alloantigens *in vitro* and prolonged skin graft survival *in vivo*^[30,31]. MSCs increased interleukin (IL)-2 and soluble IL-2 receptor in MSCs and lymphocyte co-cultures and antibodies against IL-10 further suppressed proliferation, that is to say, MSCs induced suppression was a complex mechanism affecting IL-2 and IL-10 signaling and might function differently, depending on T-cell stimuli^[30]. Alloreactivity was marked by pronounced CD45+ T-cell infiltration consisting of CD4+ and CD8+ T cells and increased skin graft IFN- γ expression which was significantly inhibited by both BMSCs and ADSCs^[32].

Human MSCs and their stromal cell antigen 1 Stro-1 positive [Stro-1(+)] subgroup possess immunosuppres-

sive properties. Stro-1(+) MSCs induced greater prolongation of skin graft in mice than unsorted MSCs^[33]. Transplantation of allogeneic bone marrow-derived flk-1+Sca-1- MSCs led to stable mixed hematopoietic chimerism, permanent donor-specific immunotolerance in allogeneic host and long-term allogeneic skin graft acceptance^[34]. The co-infusion of MSCs with unmodified donor bone marrow limited the toxicity of allogeneic bone marrow transplantation, treated graft *vs* host disease (GVHD), enhanced mixed chimerism and improved vascularized skin graft survival^[35]. The high level of TNF- α also demonstrated a possible immunogenic role for donor (allogeneic) MSCs against skin allograft rejection^[36]. Lee *et al.*^[37] suggested that ADSCs and their secretome had the potential to induce immunologic tolerance in full-thickness skin allotransplantation model. Moreover, the immunosuppressive properties of ADSCs were mediated by the ADSCs secretome. However, these chimerism induced tolerance theories were still disputed, for example, as Carrier *et al.*^[38] suggested, microchimerism did not lead to the induction of a high degree tolerance after utero transplantation but instead lead to the development of alloreactivity to donor cells.

Furthermore, infusion of MSCs exosomes enhanced the survival of allogeneic skin graft in mice and increased Tregs to help MSCs to show their immunosuppressive characters^[39]. In addition, infusion of ADSCs dramatically increased skin allograft survival by inhibiting the Th-17 pathogenic immune response and enhancing the protective Treg immune response^[40]. However, this viewpoint might be controversial. Co-administration of allogeneic hematopoietic stem cells and third-party myeloid progenitor (MP) transplantation simultaneously with placement of a MP-matched skin graft demonstrated that the organ donor matched Treg was not essential for tolerance but MP did^[41].

Donor specific immune tolerance could be effectively induced by intra-bone marrow-bone marrow transplantation combined with BMSCs treatment without any additional cytoreductive recipient treatment, which provided a promising allograft transplantation strategy whenever the donor bone marrow was available^[42]. Moreover, third-party BMSCs transplantation could prolong skin graft survival time by inhibiting T lymphocyte activation and proliferation^[43]. Third-party MSCs were able to suppress allo-specific antibody production *in vitro*, and they might rescue patients with life-threatening GVHD *in vivo*^[44]. Likewise, the donor haematopoietic stem cells had the capacity to reduce the risk of GVHD^[45]. A split-thickness skin graft from the donor was accepted, however, a third-party graft was rapidly rejected without the help of the third-party MSCs^[46]. And all dogs received donor bone marrow at the time of vascularized composite allograft (VCA) transplantation were tolerant to their donor skin graft and promptly rejected the third-party skin grafts. These data demonstrated that donor-specific tolerance to all components of the VCA could be established through simultaneous allogeneic hematopoietic third-party stem

cells transplantation^[47].

For some extreme situations, such as diabetic and radiation-induced tissue defects, stem cells therapy shows their unique advantages. Autologous ADSCs transplantation could enhance skin graft survival in diabetic rats through differentiation, vasculogenesis, and secretion of growth factors, such as VEGF and TGF- β 3. This might represent a novel therapeutic approach in skin graft surgery for diabetic wounds^[48]. MSCs combined with plastic surgery or skin graft therapy may be a promising therapeutic approach for improving radiation-induced skin and muscle damages^[49].

Recently, tissue engineered skin graft develops rapidly when taking micro-environment into consideration. Chitosan-modified poly(3-hydroxybutyrate-co-3-hydroxyvalerate) scaffold loaded with HUCMSCs or unrestricted somatic stem cells could significantly contribute to full-thickness skin defects repair and be potentially used in the tissue engineering^[50,51]. Laser microporous porcine acellular dermal matrix, which provided a “cell niche-like” micro-environment for the migration and differentiation of the BMSCs population, could induce exogenous differentiation of BMSCs *in vivo* and achieve the reconstruction of skin appendages, when combining with the split-thickness skin graft^[52].

CONCLUSION

Thanks to all published data, we have to acknowledge that we yet know little about how MSCs therapy against skin flaps necrosis works. Further studies aimed at exploring angiogenic signaling pathway after administration and optimal treatment approach will shine light on effective MSCs therapy against skin flaps necrosis.

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P- Reviewer: Kashani IR, Liu L **S- Editor:** Gong XM

L- Editor: A **E- Editor:** Lu YJ



Ability of bone graft substitutes to support the osteoprogenitor cells: An *in-vitro* study

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Author contributions: All the authors solely contributed to this paper.

Supported by Educational grant by Smith and Nephew

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Received: May 20, 2014 Revised: August 20, 2014

Accepted: August 30, 2014

Published online: September 26, 2014

Abstract

AIM: To compare seven commercially available bone graft substitutes (BGS) in terms of these properties and without using any additional biological growth factors.

METHODS: Porcine osteoprogenitor cells were loaded on seven commercially available BGS and allowed to proliferate for one week followed by osteogenic induction. Staining for live/dead cells as well as scanning electron microscopy (SEM) was carried out to determine viability and cellular binding. Further outcome measures included alkaline phosphatase (ALP) assays with normalisation for DNA content to quantify osteogenic potential. Negative and positive control experiments were carried out in parallel to validate the results.

RESULTS: Live/dead and SEM imaging showed higher viability and attachment with β -tricalcium phosphate

(β -TCP) than with other BGS ($P < 0.05$). The average ALP activity in nmol/mL (normalised value for DNA content in nmol/ μ g DNA) per sample was 657.58 (132.03) for β -TCP, 36.22 (unable to normalise) for calcium sulphate, 19.93 (11.39) for the Hydroxyapatite/Tricalcium Phosphate composite, 14.79 (18.53) for polygraft, 13.98 (8.15) for the highly porous β -Tricalcium Phosphate, 5.56 (10.0) for polymers, and 3.82 (3.8) for Hydroxyapatite.

CONCLUSION: Under the above experimental conditions, β -TCP was able to maintain better the viability of osteoprogenitor cells and allow proliferation and differentiation ($P < 0.05$).

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Key words: Bone graft; Bone graft substitute; Osteoprogenitor cells; Fracture healing; Bone

Core tip: Various commercially available bone graft substitutes (BGS) exist today and are used for the restoration of bone defects resulting from traumatic injury, tumor resection and congenital or degenerative diseases. Such BGS should pose osteoinductive and osteoconductive properties and support cell response to the osteogenic signalling. This study evaluated seven commercially available BGS in terms of osteoprogenitor cell adherence, proliferation and osteogenic differentiation. β -tricalcium phosphate was found to have the most favourable effect on cell viability and allow for their subsequent proliferation and differentiation.

Dahabreh Z, Panteli M, Pountos I, Howard M, Campbell P, Giannoudis PV. Ability of bone graft substitutes to support the osteoprogenitor cells: An *in-vitro* study. *World J Stem Cells* 2014; 6(4): 497-504 Available from: URL: <http://www.wjgnet.com/1948-0210/full/v6/i4/497.htm> DOI: <http://dx.doi.org/10.4252/wjsc.v6.i4.497>

INTRODUCTION

Bone graft materials are routinely used to fill bony defects and provide structural support stimulating the bone healing process^[1,2]. Autologous bone grafting is currently the gold standard graft material which contains all the requisite osteogenic, osteoinductive and osteoconductive properties^[3,4]. However, the available size, shape and quantity of autologous bone graft is limited and considerable morbidity is associated with its harvest^[5-7]. Due to these limitations, the development of alternative approaches resulted in several allogeneic and synthetic materials to be commercially available for clinical use. Concerns suggesting unfavourable osteogenic properties, host immune reactions and risk of pathogen transmission have been raised^[8,9]. However, synthetic bone graft substitutes (BGS) offer potentially limitless supply, have no risk of disease transmission or an immunogenic response, and offers optimum osteoconductive properties^[1,10,11]. The ideal BGS should provide a suitable environment for tissue development. It should favour cell attachment, growth and differentiation, bone growth, *in vivo* vascularisation, osteointegration with host bone, and the gradual replacement of the scaffold by newly formed bone^[12].

Combining a biological element such as mesenchymal stem cells or osteoblasts with BGS is believed to enhance some of these characteristics and may improve the bone healing process^[12-17]. Therefore, experimental models where such bone graft materials were loaded with either bone marrow aspirates or even culture expanded osteoprogenitor cells have been previously explored by several authors^[18,19]. Osteoprogenitor cells are relatively rare in bone marrow aspirates, estimated at 0.01%-0.001% of nucleated cells in the bone marrow, hence it is of paramount importance the graft material to allow the adherence, proliferation and differentiation of these cells^[12,15-17,20,21]. Therefore, the aim of this study was to compare seven commercially available BGS in terms of these properties and without using any additional biological growth factors. An observation of macroscopic properties of the BGS was also carried out.

MATERIALS AND METHODS

BGS

Seven commercially available BGS were included in the study (Table 1). Polyglycolic acid (PGA), poly (Lactide-co-Glycolide) [PLGA], β -tricalcium phosphate (β -TCP), calcium sulphate (CS) and bovine DBM granules were supplied by Smith and Nephew, Inc. (Memphis, TN, United States). Highly porous (90%) β -TCP morsels were supplied by Orthovita® (Malvern, PA, United States), hydroxyapatite/tricalcium phosphate (HA/TCP) composite by Zimmer® (Swindon, United Kingdom), hydroxyapatite (HA) by Interpore (Irvine, CA, United States) and Polygraft® (PG) by Osteobiologics, Inc. (San Antonio, TX, United States).

Cell isolation, cultivation and expansion

Cell isolation and culture expansion was performed according to previously described methods^[22-25]. Nucleated cells were isolated from the cancellous bone of the greater trochanter of fresh porcine bone obtained from the local abattoir on the day of sampling. Using porcine bone enabled us to obtain a fresh population of cells thus simulating a clinical situation where an autologous population of cells would be harvested and concentrated intra-operatively before being added to BGS. Using porcine cells also avoids all the limitations of human tissue handling. The femur was detached from the hip joint and all the muscle, cartilage, tendons and any other tissue that could contaminate the sample was removed. The proximal femur was dissected using a coping saw to expose cancellous bone, which was removed with a borer under sterile conditions and collected into phosphate buffered saline (PBS) solution. This was further crushed into smaller chips using scissors. The sample was then agitated on an orbital plate shaker at 37 °C in a 5% CO₂ humidified atmosphere for 30 min. After agitation samples were filtered through a 70 μ cell strainer to remove debris. Samples were then centrifuged at 1000 rpm for 5 min at room temperature and the cellular pellet re-suspended in minimum essential medium eagle with alpha modification, known as alpha-MEM (Sigma Aldrich®) containing 15% (v/v) foetal calf serum, 50 IU/mL Penicillin, 50 IU/mL Streptomycin, 2 mmol/L L-glutamine, 1% non-essential amino acids (Sigma Aldrich®) to obtain a concentration of 2.5×10^5 nucleated cells/mL.

Mesenchymal osteoprogenitor cells for the positive control were initially isolated from porcine femora using the same technique and then were culture expanded in T-175 flasks containing alpha-MEM. Cells were released from their culture using a 0.05% trypsin-EDTA. The cells were then centrifuged at 1000 rpm for 5 min at room temperature and then re-suspended in media to obtain concentrations of 2.5×10^5 cells/mL.

Cellular loading of BGS

Sterile, non-tissue culture, 24-well plates (Becton Dickinson Labware Europe, France) were used. Each well was filled with 0.5 cm³ of BGS utilising a standardised measuring beaker which has a maximum volume of 0.5 cm³. A fixed volume of BGS (0.5 cm³) rather than a fixed weight was used in order to simulate the clinical situation where the volume of BGS required would be dependent on the size of defect to be filled, irrespective of such parameters as surface area, surface geometry or weight of the BGS.

β -TCP, Calcium Sulphate, DBM, Highly porous β -TCP, HA/TCP composite, HA and Polygraft were used in granular form. Polyglycolic acid (PGA) and poly (Lactide-co-Glycolide) [PLGA] were used in soft solid form.

Experimental wells ($n = 6$) received 1.5 mL (2.5×10^5 cells/mL) of freshly isolated cells (freshly isolated group).

Table 1 Composition and characteristics of the bone graft substitutes used in this study

BGS	Micropore size	Macropore size	Resorption
β-TCP	< 5 μm	55% inter-granular	9-12 mo
CS	NA	55% inter-granular	1-3 mo
Highly porous (90% porosity) β-TCP	1-100 μm	100-1000 μm	4 mo
HA 60%/β-TCP 40% (HA/TCP)	< 5 μm	400-600 μm	
HA	-	280-770 μm	yr
PG ¹	250 μm	75%	4-8 mo
PLGA	-	-	1-6 mo

¹Polygraft [2.5% poly (D,L-lactide-co-glycolide), 10%CS, 12.5% polyglycolide]. BGS: Bone graft substitutes; β-TCP: β-tricalcium phosphate; CS: Calcium sulphate; HA: Hydroxyapatite; PLGA: Poly (Lactide-co-Glycolide); PG: Polygraft.

Positive control wells ($n = 6$) received 1.5 mL (2.5×10^5 cells/mL) of culture expanded cells (culture expanded group). The third group of wells ($n = 6$) received 1.5 mL of media only (negative control group). Plates were continuously agitated for 24 h to enhance uniform exposure of BGS to cells. The medium was changed after the first 48 h to remove non-adherent cells. Subsequently, the medium was replaced three times a week. During the first week, the medium used was alpha-MEM. For the two weeks that followed, the medium was replaced with an osteogenic medium containing 3 mmol/L beta-glycerophosphate, 1×10^{-8} M dexamethasone, and 50 μg/mL ascorbic acid (Sigma Aldrich®) in addition to the same constituents of alpha-MEM. Cells and BGS were left in culture for a total of 21 d at 37 °C in a 5% CO₂ humidified atmosphere. Outcome measures were performed after the 21st day.

Outcome measures

Equal amounts of BGS were collected from all 6 wells of each group to constitute a total volume of 0.5 cm³ for each of the groups. One half (0.25 cm³) was utilised for analysis by staining for live/dead cells. The other half was utilised for analysis by SEM. Further outcome measures included ALP (an early marker for the osteoblastic differentiation of osteoprogenitor cells^[26,27]) assays with normalisation for DNA content. Similar analysis was performed on the negative and the positive (culture expanded) control groups. The prevalence of potential osteoblastic progenitors in cancellous bone may be estimated by counting colony-forming units (CFU), which express ALP^[28]. Previous CFU assays using similar porcine cells in our laboratories have shown that 2.5×10^5 cells/mL was the optimum working concentration^[29]. Furthermore *in-vitro* analysis in our institution of similarly isolated populations of mononuclear cells showed evidence that they do possess osteogenic properties (alizarin red staining to confirm mineralisation)^[29]. However, such analysis was not performed on the specimens used in this study in order to avoid compromising the quantitative analyses of ALP activity and DNA content. Furthermore, alizarin red staining would have strongly stained the mineral in most BGSs such that cellular contribution towards ALP activity would be masked.

Staining and microscopy

Staining for viable cells was carried out using a live/dead assay according to manufacturer's instructions (20 μmol/L ethidium bromide and 5 μmol/L calcein in 10 mL of PBS, prepared fresh from stock (Molecular Probes). Samples were wrapped in foil and were incubated for at least 30 min at 37 °C. After removal of the live-dead stain, 1 mL of PBS was added to each sample and images were captured by fluorescent microscopy, (Excitation wavelength of 488 nm for Green and 568 nm for Red).

Biochemical analysis

Alkaline phosphatase activity was assessed by detecting the conversion of p-nitrophenyl phosphate to p-nitrophenol (Sigma Aldrich®, Dorset, United Kingdom, N3129-5G, 124K5371). Deoxyribonucleic acid (DNA) content was estimated using the Quant-iT™ Picogreen® dsDNA reagent (Molecular Probes®, Invitrogen®, Oregon, USA). Cell lysis was achieved by washing the BGS with 100 μL of 0.1% Triton X-100/0.2 mol/L carbonate buffer followed by three cycles of freeze-thawing. Each cycle involved immersion of the plates in liquid nitrogen followed by incubation at 37 °C. Samples were then loaded into a 96-well plates in duplicates (50 μL per well). The plates were incubated at 37 °C for one hour. The absorbance was then measured at 405 nm after 20, 40 and 60 min. For DNA assays, the fluorescence was measured at excitation 485 nm and emission 538 nm. The ALP activity of cultures was normalised with respect to DNA content (nmol/μg).

Macroscopic observations

Pertinent observations of handling properties and stability of the BGS in medium were recorded.

Statistical analysis

Statistical analysis was performed using SPSS version 18.0 for Windows. Normality was confirmed using the Shapiro-Wilk test and equality of variance between groups was confirmed using Levene's test. Paired *t*-test was used to test the significance of the difference between freshly isolated and culture expanded groups of the same BGS. ANOVA analysis was carried out between the various

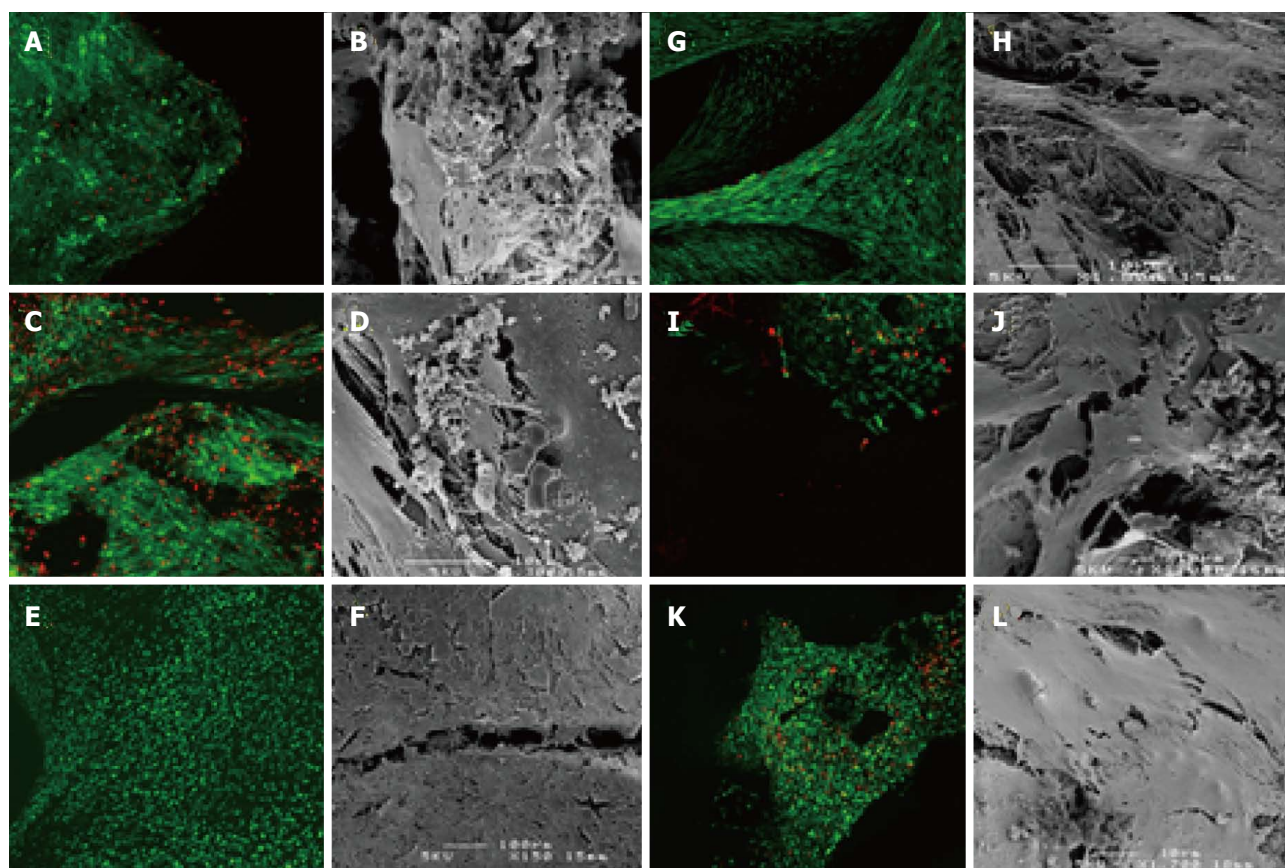


Figure 1 Live (Green)/Dead (Red) staining and scanning electron microscopy images of some bone graft substitutes (magnification is 100 unless otherwise stated). A: Highly porous β -tricalcium phosphate (β -TCP), (Freshly Isolated group); B: Highly porous β -TCP (Freshly Isolated group); C: Highly porous β -TCP (Culture expanded group); D: Highly porous β -TCP (Culture expanded group); E: β -TCP (Freshly Isolated group); F: β -TCP (Freshly Isolated group); G: β -TCP (Culture expanded group); H: β -TCP (Culture expanded group); I: Hydroxyapatite (Freshly Isolated group) - (Magnification $\times 200$); J: Hydroxyapatite (Freshly Isolated group); K: Hydroxyapatite (Culture expanded group); L: Hydroxyapatite (Culture expanded group).

groups of BGS. Normalised ALP activity/DNA content for each BGS was represented on a bar chart in descending order from right to left for the freshly isolated groups. Significance was assumed at the $P < 0.05$ level.

RESULTS

Staining and microscopy

The assay for cell viability (Figure 1) revealed that most BGS support the viability and proliferation of mesenchymal cells. SEM showed cellular attachment to the BGS and in some cases matrix deposition. Typically, both live/dead staining as well as SEM showed more cells and more matrix deposition in the culture expanded group as compared to the freshly isolated group (Figures 1 and 2). In some samples it was easier to see the cells on live/dead staining than on SEM's. Compared to the amount of green staining, there appears to be a small to moderate (Figure 1C and K) proportion of red staining (non-viable cells) in all BGS in both the freshly isolated and the culture expanded groups. SEM was more difficult to perform on BGS that had a patchy colonisation of cells (*e.g.*, HA).

Biochemical analysis

A gradual increase in absorbance with time was recog-

nised in samples exhibiting active ALP enzymatic activity. Measured ALP levels at 60 min were compared for samples and plotted in ascending order for the freshly isolated group after subtracting the values obtained from the negative control group. Significantly highest levels were recorded for β -TCP ($P < 0.05$). It was noticed that in the culture expanded group, three BGS's had very high ALP activity (PLGA, HA/TCP composite, and β -TCP). Overall, ALP activity was higher in the culture expanded group when compared to the freshly isolated group for the same BGS ($P < 0.05$). Complete dissolution of PGA occurred at the end of the three-week culture period and no ALP activity was recorded. The fluid phase obtained after cell lysis of the DBM samples was very turbid and neither ALP absorbance nor DNA content analysis using the fluorescent plate reader could be performed using the above method. Therefore, PGA and DBM were excluded from final analysis. After subtracting the values obtained from the negative control group, the DNA content in BGS ranged from zero to $4.98 \mu\text{g/mL}$ and from 1.46 to $9.98 \mu\text{g/mL}$ in the freshly isolated and the culture expanded groups respectively. For each BGS, DNA content in the culture group was higher than that in the freshly isolated group. In the freshly isolated group, highest levels ($P < 0.05$) were seen for β -TCP. In the culture expanded

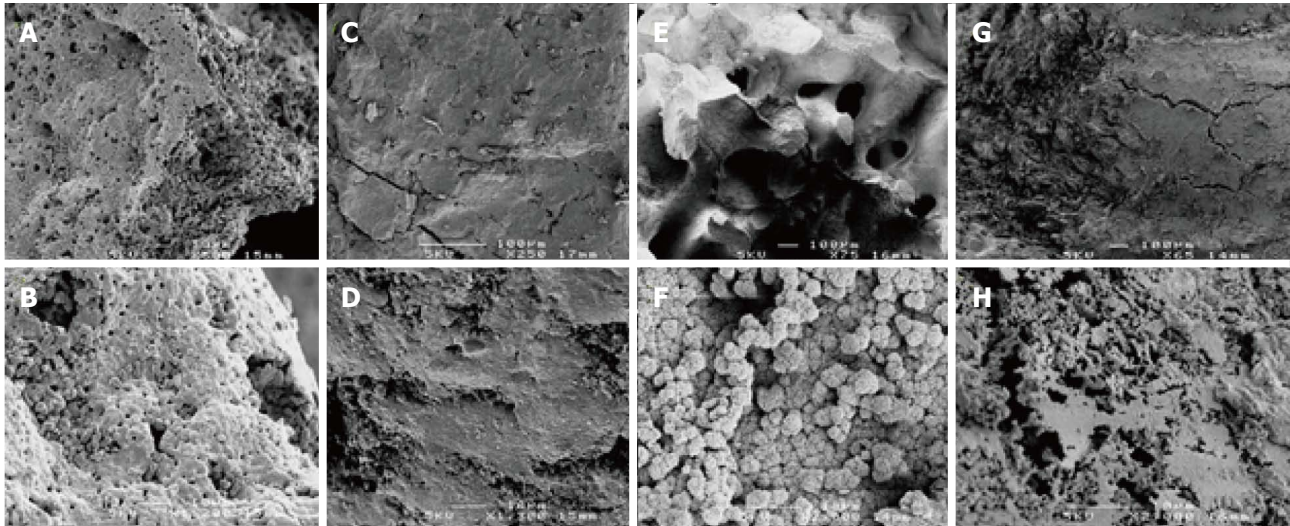


Figure 2 Scanning electron microscopy images of some bone graft substitutes in the negative control group. A, B: Highly porous β -tricalcium phosphate (β -TCP) - (Negative control group); C, D: β -TCP (Negative control group); E, F: Hydroxyapatite (Negative control group); G, H: Calcium sulphate (Negative control group).

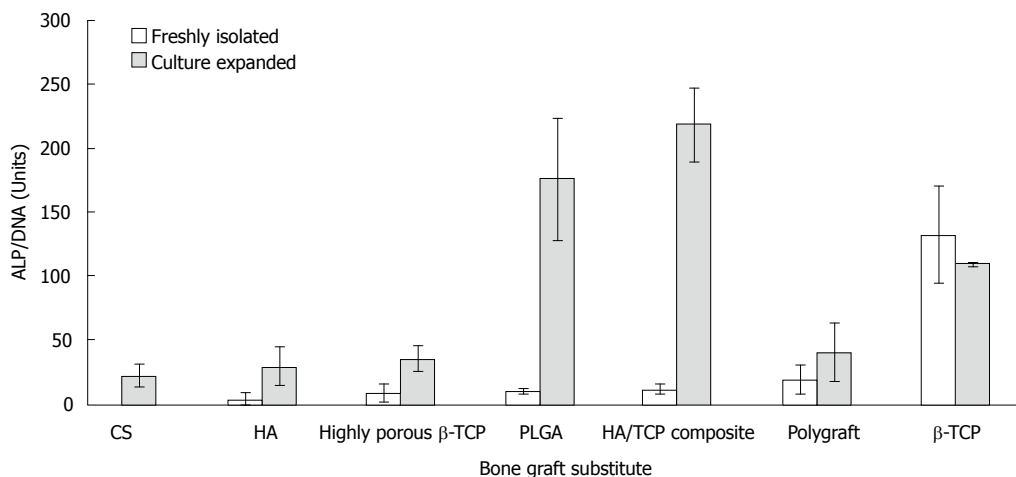


Figure 3 Normalisation of alkaline phosphatase activity to DNA content (mean \pm SD). In descending order from right to left for the freshly isolated group. β -TCP: β -tricalcium phosphate; CS: Calcium sulphate; HA: Hydroxyapatite; PLGA: Poly (Lactide-co-Glycolide); PG: Polygraft.

group, highest levels of DNA content ($\mu\text{g/mL}$) were detected with β -TCP (9.98), PLGA (5.32), HA/TCP (4.75), highly porous TCP (4.61), and CS (2.53). When the ALP activity was normalised for DNA content, β -TCP was again the BGS with the highest ALP activity (132.03 Units). As would be expected, the culture expanded group for all samples (except β -TCP) showed higher values for normalised ALP activity than their freshly isolated counterparts (Figure 3).

Macroscopic observations

Polymer BGS (PGA, PLGA) were too friable in a wet environment. PGA (which has been excluded from final analysis) completely disintegrated while PLGA lost its strength towards the end of the culture period in the freshly isolated group. Calcium sulphate started to disintegrate towards the end of the culturing process. Interparticulate bonds were formed in some samples mainly

in the culture expanded group (β -TCP > HA/TCP composite > polygraft).

DISCUSSION

This study demonstrates that a fresh population of cells extracted from porcine cancellous bone has the ability to attach to commercially available BGS with simple seeding techniques and after a period of incubation to proliferate and undergo osteogenic differentiation. Adding a concentrated or enriched population of bone marrow derived osteoprogenitor cells to an osteoconductive BGS is believed to enhance bone healing^[12,14-16,30,31]. The cells in this study were concentrated by filtration and centrifugation. In a clinical situation, the volume of BGS required would depend on the bony defect size irrespective of such parameters as BGS surface area, surface geometry or weight. Therefore, our standard for comparison of BGS

was a fixed volume rather than a fixed weight. Modified seeding techniques were not used thus simulating simple mixing of cells with BGS that would occur in the clinical situation.

Despite the small starting number of osteoprogenitor cells in the population of nucleated cells in the freshly isolated group, we believe this was compensated for by allowing the cells to proliferate for one week in medium before the environment became osteogenic. An assumption supported by DNA content analysis on the freshly isolated and the culture expanded groups.

Collectively, ALP activity was higher in the culture expanded group either due to higher cell numbers; or due to removal of dead cells during cell culture and passage leaving a healthy population prior to seeding. The ability of β -TCP to attach fresh cells that produced the highest ALP activity indicates that it has the ability to either retain a high proportion of cells initially, or to enable attached cells to proliferate into considerable numbers before differentiation. Qualitative imaging demonstrated high cellularity with β -TCP as well as other BGS (Figure 1). Other BGS that supported cellular proliferation in the culture expanded group (HA/TCP composite, PLGA, highly porous β -TCP) did not show correlating high levels of ALP in the freshly isolated group. Furthermore, even when DNA content suggested a high amount of freshly isolated cells attached to BGS (*e.g.*, HA/TCP composite, highly porous β -TCP, HA), ALP activity was not impressive. Experiments using culture expanded cells may not be representative of how freshly isolated cells behave towards a certain BGS. Normalisation of ALP activity for DNA content may simplify comparison, but it may not reflect the magnitude of ALP activity or the amount of cells investigated. For instance, β -TCP showed higher ratios of ALP activity for DNA content in the freshly isolated group than in the culture expanded group, although the absolute DNA content and ALP activity were both higher in the culture expanded group. In contrast, Polygraft showed a reasonable ALP to DNA ratio although fewer cells seemed to attach to the BGS. Absolute ALP activity and DNA content values remain useful in conjunction with normalised values.

The chosen BGS were commercially available, biocompatible, non-immunogenic products with no pathogen transmission risk (excluding DBM). BGS that are used in cement form^[32] were excluded as they lack the ability to support the viability of cells. Other factors influencing the performance of a cellular-BGS composite include the mechanical properties, surface area, surface chemistry, surface texture, pore size, pore geometry, three-dimensional architecture, and in-vivo degradation properties of the BGS. Finally, the number and concentration of cells transplanted into a given site will have a profound influence on the biologic microenvironment. The biologic environment will have a critical balance between the local metabolic demand of transplanted cells and the capacity for nutrients and oxygen to diffuse into or out of the site through the BGS. This relationship be-

tween cells and matrix or BGS structure presents a wide range of variables that need consideration when trying to optimise cellular-BGS composites for bone grafting applications^[16]. However, little is known regarding the efficacy of different BGS as means for selective attachment and delivery of osteoprogenitor cells.

We can only speculate about the factors responsible for the performance of β -TCP. Its mechanical properties would not have influenced the outcome since our system was not subjected to mechanical stresses. Macroporosity provides a space in which bone in growth occurs by osteoconduction. Small pores (1-100 μ m) are less available for bone ingrowth but may enhance fluid flow and diffusion, thus improving the metabolic environment within the matrix. It has been suggested that optimal macroporosity for cell infiltration has a range of 150-500 μ m^[33-35]. Larger pores support deeper penetration of new tissues, but optimal pore size for ingrowth deeper than 3-4 mm into the scaffold has not been studied systematically. This is relevant to current clinical practice of filling large bone defects with granular BGS, since the spaces between packed particles are generally significantly larger than the stated microstructure or pore size of most BGS granules^[36]. The β -TCP granules used in our study are six-armed granules which interlock to provide 55% porosity, allowing for cell and nutrient infiltration. They are clinically indicated for filling non load-bearing defects of 4-5 cm. This unique geometry and inter-granular porosity may enhance cellular attachment, proliferation and extracellular matrix deposition. Tissue connections were indeed observed between individual granules of β -TCP [Figure 1G].

Other characteristics of BGS, such as, surface chemistry, topography, roughness, wettability and surface energy were not compared in this study. Proteins and lipids can coat BGS and act as bio-mediators of the cellular responses to BGS. Surface characteristics of BGS may play a role in preferential interaction with certain proteins or adhesion molecules leading to better cell adhesion and subsequent proliferation^[10,36,37]. We do not have data regarding the surface characteristics of the β -TCP granules and can only assume that they have been favoured by the osteoprogenitor cells for adhesion and proliferation.

A limitation of the study was the disintegration of polymer BGS (PGA, PLGA), and calcium sulphate. Adding an earlier time point to the analysis such as at day 14 would have affected the study of other BGS and would have been too early to show significant cellular proliferation and differentiation as previous experiments in our institution have shown. This may explain the un-recordable reading of DNA content in the freshly isolated CS group, which then precluded normalising ALP activity to DNA content. It may be that such BGS are not appropriate for this type of study or even for use in a clinical situation where some structural integrity is required. Despite the fact that the BGSs group was rather heterogeneous in terms of composition and porosity this study highlights their efficacy in terms of cellular attachment and viability

and differentiation within the graft. Therefore, it could be hypothesized that such structural differences could contribute to the results presented in the herein study. Future research to identify a property responsible for these results together with additional assays to demonstrate specific osteoblast functions like osteocalcin, bone sialoprotein or osteopontin as well as *in-vivo* data would shed more light in this area.

We believe that under the *in-vitro* conditions described in this paper, β -TCP was able to favourably maintain the viability of osteoprogenitor cells and allow for their subsequent proliferation and differentiation. Further work needs to be carried out to understand the effect that BGS have on osteoprogenitor cells and to assess the optimum ratio of cell number/concentration to BGS volume. The introduction of biomaterial technologies enhanced with growth factors, genes and cells are certain to have far-reaching effects on the way that musculoskeletal conditions are managed in the future.

COMMENTS

Background

Commercially available bone graft substitutes (BGS) are routinely used in the clinical practice. The ideal BGS should provide a suitable environment for tissue development. It should favour cell attachment, growth and differentiation, bone growth, *in vivo* vascularisation, osteointegration with host bone, and the gradual replacement of the scaffold by newly formed bone.

Research frontiers

The authors of this study have analysed the efficacy of seven commercially available BGS to support the adherence, proliferation and differentiation of osteoprogenitor cells loaded within the graft material *in-vitro*.

Innovations and breakthroughs

Under the *in-vitro* conditions described in this paper, β -tricalcium phosphate (β -TCP) was able to favourably maintain the viability of osteoprogenitor cells and allow for their subsequent proliferation and differentiation.

Applications

β -TCP showed the most favourable results in terms of MSCs adhesion, proliferation and osteogenic differentiation. These results warrant further animal studies to determine the grafts behaviour in *in-vivo*.

Terminology

BGS consist of several types and encompass various materials, material sources, and origins (natural or synthetic). Osteoprogenitor stromal cells are undifferentiated cells found in bone but also in a variety of other tissues. Under specific signals (example: trauma) they give rise to those cells that form mesenchymal tissues, including bone and cartilage.

Peer review

This manuscript describes, in a clearly written style, a series of experiments *in vivo* attempting to define which, out of a panel of commercially available materials used as bone graft substitutes, performs best with respect to colonization by osteogenic cells and expression of a differentiation marker (alkaline phosphatase). The study was carried out in a system which minimizes the relevance of mechanical factors on the outcome, and care was taken to normalize alkaline phosphatase activity relative to the number of cells in the same, as estimated from the DNA content.

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P- Reviewer: Kan L, Pedro XE, Yao CL **S- Editor:** Song XX
L- Editor: A **E- Editor:** Lu YJ



Transplantation of stem cells from human exfoliated deciduous teeth for bone regeneration in the dog mandibular defect

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Received: April 9, 2014 Revised: June 5, 2014

Accepted: July 25, 2014

Published online: September 26, 2014

Abstract

AIM: To investigate the effect of stem cells from human exfoliated deciduous teeth (SHED) transplanted for bone regeneration in the dog mandibular defect.

METHODS: In this prospective comparative study, SHEDs had been isolated 5 years ago from human exfoliated deciduous teeth. The undifferentiated stem cells were seeded into mandibular bone through-and-through defects of 4 dogs. Similar defects in control group were filled with cell-free collagen scaffold. After

12 wk, biopsies were taken and morphometric analysis was performed. The percentage of new bone formation and foreign body reaction were measured in each case. The data were subject to statistical analysis using the Mann-Whitney *U* and Kruskalwalis statistical tests. Differences at $P < 0.05$ was considered as significant level.

RESULTS: There were no significant differences between control and SHED-seeded groups in connective tissue ($P = 0.248$), woven bone ($P = 0.248$) and compact bone ($P = 0.082$). There were not any side effects in transplanted SHED group such as teratoma or malignancy and abnormalities in this period.

CONCLUSION: SHEDs which had been isolated and characterized 5 years ago and stored with cryopreservation banking were capable of proliferation and osteogenesis after 5 years, and no immune response was observed after three months of seeded SHEDs.

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Key words: Bone regeneration; Isolation; Stem cells from human exfoliated deciduous teeth

Core tip: Stem cells from human exfoliated deciduous teeth (SHED) exist in the living pulp remnants of exfoliated deciduous teeth. The aim of this study was to investigate the effect of SHED transplanted for bone regeneration in the dog mandibular defect. In this study we found that SHEDs which had been isolated and characterized 5 years ago and stored with cryopreservation banking were capable of proliferation and osteogenesis after 5 years, and no immune response was observed after three months of seeded SHEDs.

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Transplantation of stem cells from human exfoliated deciduous teeth for bone regeneration in the dog mandibular defect. *World J Stem Cells* 2014; 6(4): 505-510 Available from: URL: <http://www.wjgnet.com/1948-0210/full/v6/i4/505.htm> DOI: <http://dx.doi.org/10.4252/wjsc.v6.i4.505>

INTRODUCTION

Most of the head and neck lesions are caused by maxillofacial tumor surgeries, infection, trauma, and congenital skeletal deformities. Reconstruction of these defects is one of the most difficult and complex parts in maxillofacial surgeries. Autogenous tissue and alloplastic materials have been used for the treatment of these defects, each of which has its own disadvantages such as bleeding, nerve injuries, esthetic problems, pain, infection and loss of tissue function^[1,2].

Tissue engineering is a fundamental science that can be used as a solution for autograft and allograft tissue in reconstruction surgeries. To the best of our knowledge, the tissue engineering approach requires three key elements: stem cell, scaffold and growth factor. Using this method the individual cell will be isolated and cultured and then can be implanted or injected directly to the damaged tissue, and surgeons can consider tissue engineering to reconstruct the tissue defect without any severe complications^[3,4].

Stem cells have been isolated and characterized from a variety of sources such as bone marrow^[5], adipose tissue^[6], hair follicles^[7], synovial membrane^[8], skeletal muscle^[9], dental pulp^[10], *etc.* The types of isolated stem cells are very important due to the capacity of proliferation and differentiation rate of each tissue. It has been demonstrated that stem cells from human exfoliated deciduous teeth (SHED) can be easily isolated and expanded in the culture medium^[11]. Moreover, Miura *et al.*^[12] showed that SHED is capable of extensive proliferation and multi-potential differentiation. They also discussed that deciduous teeth can be considered as an ideal source of stem cells to induce bone regeneration.

In-vitro studies have shown that there is no immune reaction and tissue rejection of SHED, which lead to non-immunosuppressive therapy^[13-15]. de Mendonça Costa *et al.*^[16] studied the capacity of human dental pulp stem cells isolated to reconstruct large-sized cranial bone defects in non-immunosuppressed rats. The results of their study showed that using these cells can induce osteogenesis without any graft rejection.

Recently *in-vivo* studies have shown that using human dental pulp stem cells will not lead to any tissue rejection^[16,17]. The aim of the present study was to investigate the effect of transplantation of SHED for bone regeneration in the dog mandibular defect.

MATERIALS AND METHODS

Ethical approval

This study was approved by the Regional Bioethics

Committee of Isfahan Province (191086) and was conducted in accordance with the ethical principles and standards for the conduct of human and animal biological rhythm^[18].

Study population

Four male dogs (mixed breed, Iranian) between 15-25 kg were included in this prospective experimental study. The animals were accommodated in the animal house at 22°C-24°C with 55%-70% humidity, light cycle of 12 h, air renewal 15 times/h with the same diet. Also, during the study the animals were monitored for general appearance, activity, exertion, and weight. Exclusion criteria included undesirable changes in vital signs, physical examination and any visible swelling of lymph nodes in the head and neck area that disqualify subjects from inclusion in the study.

SHED isolation and cultivation

Normal exfoliated human deciduous teeth were collected from 6- to 9-year-old children under approved guidelines which were set by Nourbakhsh *et al.*^[19]. Based on this protocol, we used SHED which had been isolated and characterized their markers 5 years ago and stored with cryopreservation banking in which cells were preserved in liquid nitrogen vapor (Royan institute, Isfahan, Iran.) at a temperature of less than -150 °C. After defrosting the isolated cells, they were transferred to the flask containing medium consisting of Dulbecco modified eagle medium (sigma, St.Louis, United States) enriched with fetal bovine serum 10% (FBS; Dainippon pharmaceutical, Osaka, Japan) and penicillin-streptomycin 0.5% (Gibco-BRL, Life Technologies, MD, United States) (Figure 1).

The SHEDs were expanded through 3 passages (Figure 2), for each passage the medium was removed, irrigation was performed with phosphate buffered saline (Gibco, Grand Island, NY, United States) Trypsin-EDTA (Gibco, Grand Island, NY, United States) was added for 3 min and then neutralized with medium consisting of Dulbecco modified eagle medium enriched with fetal bovine serum 10% and penicillin-streptomycin 0.5%. After centrifuging (1400 rpm, 10 min), the fluid on top was discarded and the remainder of the suspension was transferred to a new flask.

Non-adherent cells were removed from the culture by washing with PBS. The adherent cells were expanded as monolayer culture in the medium consisting of Dulbecco modified eagle medium enriched with fetal bovine serum 10% and penicillin-streptomycin 0.5% at 5% Co2 and at the temperature of 37 °C.

The obtained stem cells were counted and trypsinized with Trypsin-EDTA (Gibco, Grand Island, NY, United States). About 10⁶ cells were suspended in a little amount of medium (100 µL)^[14,16]. The suspension was transferred to a 9 mm × 5 mm cylindrical collatamp (syntacoll, GmbH, Germany) scaffold with a sampler and incubated for 2 h. After that the medium consisting of Dulbecco modified eagle medium enriched with fetal bovine serum 10% and penicillin-streptomycin 0.5% was added and

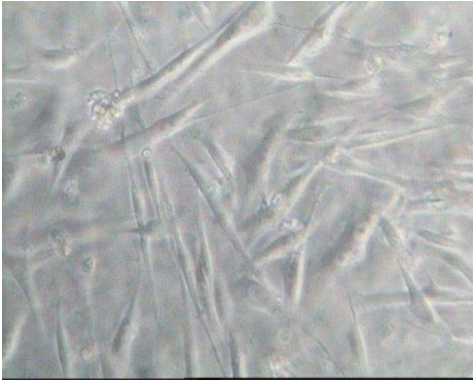


Figure 1 Colony derived from stem cells from human exfoliated deciduous teeth at the first passage.

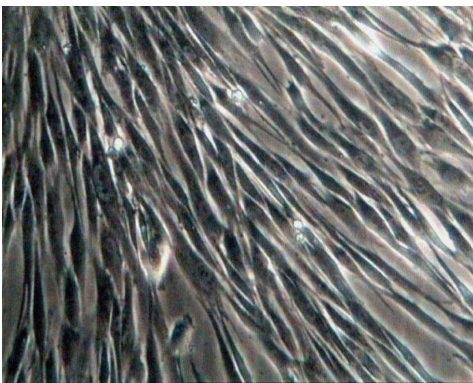


Figure 2 Stem cells from human exfoliated deciduous teeth at the third passage.

incubated again for 48 h before implanting in mandibular bone defects.

Site preparation and cell implantation

First each dog was firstly sedated by 0.02 mg/kg Acepromazine (Aveco Co; Inc, Fort Dodge, LA, United States) and sub-cutaneous 0.05 mg/kg Atropine sulfate (Darou Pakhsh pharmaceutical, Tehran, Iran). Then, each dog was anesthetized with an intramuscular injection of ketamine 10% (Alfasan, Woerden, Holland). Animals were also intubated by anesthesiologist. The mandible was shaved and the skin surface was disinfected with povidone iodine solution (Aida chemie co, Mashad, Iran) before the operation. The mandible bone was exposed through a skin incision of approximately 5 cm. Layered dissection was performed through the mandibular bone and full thickness through-and-through bony defects were created on each side of the inferior mandibular border by trephine bur (Meisinger, Dusseldorf, Germany) that were 9 mm in diameter (Figure 3).

One defect was filled with scaffold plus SHED and the other one was only filled with scaffold to serve as a control group. The periosteum was closed with resorbable 4/0 suture (Vicryl, Johnson Somerville, NJ, United States) and non-resorbable 4/0 suture (SURG1PRO Polypropylene Monofilament, Richmond, VA, United

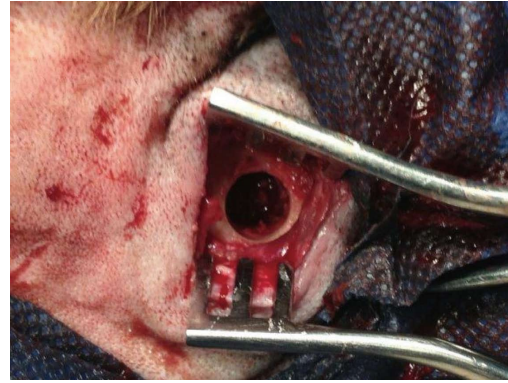


Figure 3 Through-and-through defects caused by trephine bur in the dog mandible.

States) for the skin. All animals recovered from anesthesia without complications. Postoperative medications included tramadol (Darou Pakhsh pharmaceutical, Tehran, Iran) 2 mg/d for 3 d and antibiotic Penicillin 6.3.3 (Jaber Ebne Hayyan Pharmaceutical Co., Tehran, Iran) for 6 d, intramuscularly.

Morphologic and histologic evaluation

After 12 wk, biopsies were taken by a larger trephine bur (Meisinger, Dusseldorf, Germany). The animals were not sacrificed at the end of the experiments and that only biopsy specimens were taken. Samples were cut horizontally from the middle by surgical saw (Stryker Instruments, Kalamazoo, MI, United States), the specimens were fixed in formaldehyde 10% (Sina chemical industrial, Tehran, Iran) buffer solution at pH 7.0 and were treated with 10% formic acid decalcifying solution (Kimia Tehran Acid, Tehran, Iran) for two weeks. Samples were dehydrated with alcohols and embedded in paraffin. For macroscopic evaluation, the features were analyzed by Image Analysis software (IHMMMA, Ver. 1, Sbm. Iran) which is able to segment the input image into predefined regions (three regions were chosen) based on the closeness of colors in each region. First, images were taken from samples cross section and bone formation area and original bone defects were defined using color codes with Image Analysis Software. In order to determine the area of bone formation we used the image segmentation in our analysis, which is the process of partitioning a digital image into multiple segments (super pixels). The goal of segmentation is to simplify and/or change the representation of an image into something that is more meaningful and easier to analyze. Image segmentation is typically used to locate objects and boundaries (lines, curves, *etc.*) in images. More precisely, image segmentation is the process of assigning a label to every pixel in an image such that pixels with the same label share certain visual characteristics. Then, percentage of each area was provided by the software (Figure 4). Light intensity was constant for all samples. The software automatically calculates the percent of different tissues and reports the area ratio of each region to the total, quantitatively. The specimens

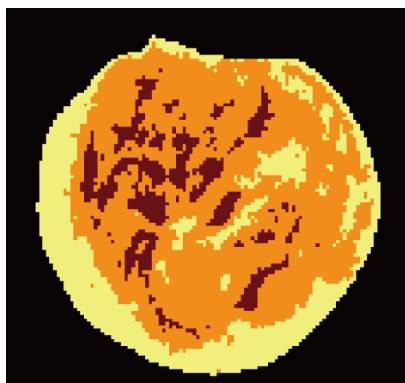


Figure 4 Percent of area 1 with brown color (connective tissue), area 2 with orange color (woven bone) and area 3 with yellow color (compact bone) in sample 3 from control group by image analysis software.

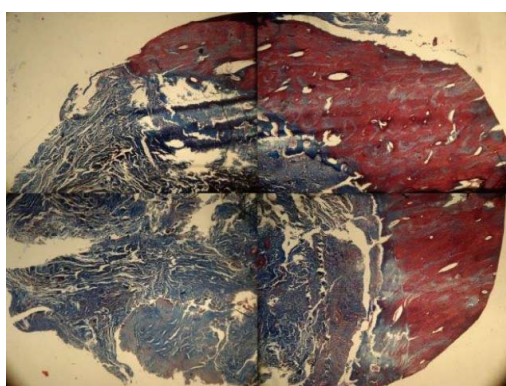


Figure 5 Histological view of regenerated bone (Masson's trichrome stain, $\times 40$).

were stained with hematoxylin and eosin (sigma) for the new bone tissue regeneration and Masson's trichrome (sigma) for detecting tumors arising from fibroblasts (Figure 5).

The histological evaluation was performed at $40 \times$ magnified optical microscope (Olympus BX 51-Olympus co, Tokyo, Japan).

In histological evaluation, the presence of macrophages, monocytes, and giant cells was considered as foreign body reaction. The presence of neutrophils was considered as acute inflammation and presence of mononuclear cells like lymphocytes and monocytes was considered as chronic inflammation^[20]. Pathologists were blinded to the graft material for each sample.

Statistical analysis

All data were expressed as Median (Range) and were analyzed by SPSS version 16 (SPSS Inc., Chicago, United States). Data were analyzed using non parametric test such as Mann-whitney *U* and Kruskalwalis statistical tests. $P < 0.05$ was considered as a significant test.

RESULTS

The histological results did not show any foreign body



Figure 6 From left to right: buccal, middle and lingual sites of specimen in stem cells from human exfoliated deciduous teeth seeded groups.

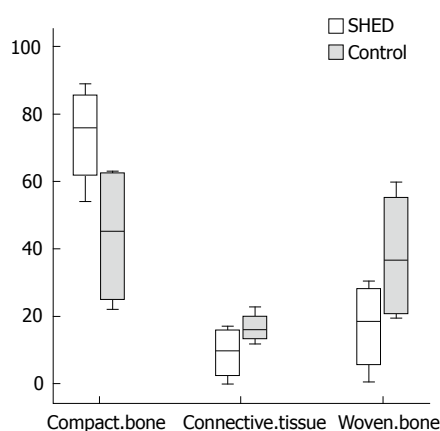


Figure 7 Mean of percentage in compact bone, connective tissue and woven bone in stem cells from human exfoliated deciduous teeth-seeded and control group.

reaction or severe inflammation in each of the specimens. Bone tissue type in both control and SHED-seeded group was similar. There were not any side effects owing to transplanted SHED such as teratoma or malignancy. In control and SHED-seeded groups the new bone formation was observed in both lingual and floor parts of the defect, that was compact bone. The middle part revealed newly formed lamellar and woven bones with limited connective tissue and in lateral cortex of mandible the defect site was restored with the connective tissue (Figure 6).

The median (range) of osteogenesis rate of the area 1 (connective tissue), area 2 (woven bone) and area 3 (compact bone) in the SHED-seeded group were 16.1 (10.66), 36.59 (40.36) and 45.23 (41.07), respectively (Figure 7). There were no significant differences between control and SHED-seeded groups in area1 ($P = 0.248$) area 2 ($P = 0.248$) and area 3 ($P = 0.082$) There were not any side effects in transplanted SHED group such as teratoma or malignancy and abnormalities in this period.

DISCUSSION

The results of the present study showed that bone regen-

eration in the segmental defects of the dog mandible can not be enhanced by the presence of cultivated SHEDs. In the study by Schliephake *et al.*^[21] human bone cells isolated and seeded in three different types of scaffolds and stem cell seeded scaffolds were implanted in rat mandibular defects and after 6 wk the presence of human cells was assessed. The results of their study showed no increase in the amount of early bone formation.

de Mendonça Costa *et al.*^[16] conducted a research in which the stem cells from human deciduous teeth were isolated and implanted in the cranial bone defect of rat. They reported bone regeneration without any immune reaction. Kerkis *et al.*^[22] transplanted the human immature dental pulp stem cells to dog's retriever muscular dystrophy locally and systemically, reporting no immune reaction. It has been assumed that SHEDs transplantation have immunosuppressive activity which is an important item in the field of regenerative medicine^[13,14]. Also, Yamaza *et al.*^[15] investigated the immunomodulatory properties of SHEDs and reported that SHEDs has significant effects on inhibiting T helper cells *in vitro*. Also they reported that SHED transplantation is capable of effectively reversing Systemic Lupus Erythematosus-associated disorders in mice.

The results of the present study showed no immune reaction after three months of implanted cell in the dog mandibular bony defect, so the results of our study are in agreement with the de Mendonça Costa *et al.*^[16] and Kerkis *et al.*^[22] studies. Therefore it can be concluded that the time of isolation had no effect on the immunomodulatory properties of SHEDs.

The bone regeneration was evaluated after three months^[23], because after three months we were able to evaluate the osteogenesis rate without missing any variation, immune reaction and inflammatory process. After this period, histological results did not show any foreign body reaction or severe inflammation in each of the specimens.

Miura *et al.*^[12] and Seo *et al.*^[17] studies showed that SHEDs have the ability to produce lamellar bone. Therefore, the results of this study are similar to Miura *et al.*^[12] and Seo *et al.*^[17] studies.

SHEDs have extensive proliferation and differentiation, which make them a critical source of stem cells for the regeneration and repair of craniofacial defects, tooth loss and bone regeneration^[24]. SHED cells may also be beneficial for the treatment of neurodegenerative diseases and the repair of motoneurons following stroke or injury^[12]. There are many advantages for SHEDs banking; such as no immune reaction and tissue rejection of the cells, and no immunosuppressive therapy^[24].

These cells can be best utilized for the patients including children. The cost and technical simplicity of this procedure makes it an ideal source. In the present study we used human exfoliated deciduous teeth, which had been isolated 5 years ago. We found that SHEDs were still capable of proliferation and differentiation.

In this study the defect created was small, but results

were promising. Hence, further studies should be done with larger defects using the same methods for more confirmation. The use of stem cells to repair bone defects is not perfect. The biggest disadvantage of using this method is the possibility of uncontrolled cell growth and tumor formation. Therefore further studies should be done to evaluate the risk and prognosis of this therapeutic method. Furthermore more studies with longer follow up should be done to evaluate the efficacy of this method. The advantage of this study compared to other studies was the use of fewer stem cells in the repair of bone defects that showed good results.

SHEDs which had been isolated and characterized 5 years ago and stored with cryopreservation banking were capable of proliferation and osteogenesis after 5 years, and no immune response was observed after three months of seeded SHEDs.

ACKNOWLEDGMENTS

The authors would like to thank the staff of Torabinejad Dental Research Center for their cooperation in all stages of this research.

COMMENTS

Background

Stem cells from human exfoliated deciduous teeth (SHED) exist in the living pulp remnants of exfoliated deciduous teeth. They are a heterogeneous population with a fibroblastic morphology that has clonogenic capacity and the ability to form adherent colony clusters with extensive proliferating capacity. The aim of this study was to investigate the effect of SHED transplanted for bone regeneration in the dog mandibular defect.

Research frontiers

Tissue engineering is a fundamental science that can be used as a solution for autograft and allograft tissue in reconstruction surgeries. Using this method the individual cell will be isolated and cultured and then can be implanted or injected directly to the damaged tissue, and surgeons can consider tissue engineering to reconstruct the tissue defect without any severe complications. SHED can be easily isolated and expanded, SHEDs are capable of extensive proliferation and multi-potential differentiation, also deciduous teeth can be considered as an ideal source of stem cells to induce bone regeneration.

Innovations and breakthroughs

In-vitro studies have shown that there is no immune reaction and tissue rejection of SHED, which lead to non-immunosuppressive therapy. Researchers studied the capacity of human dental pulp stem cells isolated to reconstruct large-sized cranial bone defects in non-immunosuppressed rats. The results of their study showed that using these cells can induce osteogenesis without any graft rejection. Recently *in-vivo* studies have shown that using human dental pulp stem cells will not lead to any tissue rejection.

Applications

The present study enhanced the reconstruction of facial skeleton using direct bone regeneration to avoid the need for autogenous bone grafts. The results of the present study showed that bone regeneration in the segmental defects of the dog mandible can be enhanced by the presence of cultivated SHEDs. These cells can be best utilized for the patients including children. The cost and technical simplicity of this procedure makes it an ideal source. In the present study we used human exfoliated deciduous teeth, which had been isolated 5 years ago. The authors found that SHEDs were still capable of proliferation and differentiation.

Terminology

Stem cell: Cell that upon division replaces its own numbers and also gives rise to cells that differentiate further into one or more specialized types; SHED: Stem cells from human exfoliated deciduous teeth exist in the living pulp rem-

nants of exfoliated deciduous teeth; Tissue engineering: Use of a combination of cells, engineering and materials methods, and suitable biochemical and physio-chemical factors to improve or replace biological functions; Scaffold: A temporary structure for holding materials during the repair, Regrowing bone requires a scaffold that is stiff, long-lasting and safe.

Peer review

This is an interesting and outstanding article. Methods are appropriate. Results are clearly presented. Discussion is interesting.

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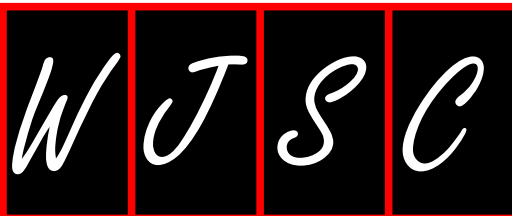
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World Journal of Stem Cells

ISSN

ISSN 1948-0210 (online)

Launch date

December 31, 2009

Frequency

Quarterly

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- 4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMID: 2516377 DOI: 10.1161/01.HYP.0000035706.28494.09]

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- 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]

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- 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]

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- 9 Outreach: Bringing HIV-positive individuals into care. *HRS-A Careaction* 2002; 1-6 [PMID: 12154804]

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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

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- 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

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- 12 **Breedlove GK**, Schorfeide AM. Adolescent pregnancy. 2nd ed. Wiczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

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- 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

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- 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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Write as mean \pm SD or mean \pm SE.

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