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REVIEW

High dose chemotherapy with stem cell support in the treatment of testicular cancer

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Abstract

Testicular germ cell cancer (TGCC) is rare form of malignant disease that occurs mostly in young man between age 15 and 40. The worldwide incidence of TGCC is 1.5 per 100000 man with the highest rates in North Europe. After discovery of cisplatin cure rates of TGCC are very favorable between 90%-95% and unlike most solid tumors, cure rate for metastatic TGCC is around 80%. Metastatic TGCC is usually treated with 3-4 cycles of bleomycin, etoposide, cisplatinum chemotherapy with or without retroperitoneal surgery and cure rates with this approach are between 41% in poor risk group and 92% in good risk group of patients. Cure rates are lower in relapsed and refractory patients and many of them will die from the disease if not cured with first line chemotherapy. High dose chemotherapy (HDCT) approach was used for the first time during the 1980s. Progress in hematology allowed the possibility to keep autologous haematopoietic stem cells alive *ex-vivo* at very low temperatures and use them to repopulate the bone marrow after sub-lethal dose of intesive myeloablative chemotherapy. Despite the fact that there is no positive randomized study to prove HDCT concept, cure rates in relapsed TGCC are higher after high dose therapy then in historical controls in studies with conventional treatment. Here we review clinical studies in HDCT for TGCC, possibilities of mobilising sufficient number of stem cells and future directions in the treatment of this disease.

Key words: High dose chemotherapy; Germ-cell cancer; Stem cell transplantation; Plerixafor

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Core tip: High dose chemotherapy with autologous haematopoietic stem cell transplantation is effective option in treating relapsed metastatic germ-cell cancer. We reviewed this topic in regard of clinical studies, optimal mobilising and conditioning regimens, with special review on plerixafor in this indication. We also analysed riska adapted approach in those patients and future directions in field.

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INTRODUCTION

Testicular germ cell cancer (TGCC) is a rare form of malignant disease that occurs mostly in young man between age 15 and 40. The worldwide incidence of TGCC is 1.5 per 100000 man with the highest rates in Northern Europe[1]. One half of all TGCC are seminomas and other half are non-seminomas. The majority of TGCC arise from the gonads while around 5% arise from extragonadal sites in the body's mid-line: retroperitoneum, mediastinum or brain[2]. After discovery of cisplatin TGCC cure rates have become very favorable ranging between 90%-95% and unlike most solid tumors, cure rate for metastatic TGCC is around 80%[1,2]. Metastatic TGCC is usually treated with 3-4 cycles of bleomycin, etoposide, cisplatinum (BEP) chemotherapy with or without retroperitoneal surgery and cure rates with this approach are between 41% in poor risk group and 92% in favourable risk group of patients[3] (Table 1). Cure rates are lower in relapsed and refractory patients and many of them will die from the disease if not cured with first line chemotherapy[3]. High dose chemotherapy (HDCT) approach was first used during the 1980s. Progress in hematology allowed the possibility to keep autologous haematopoietic stem cells alive *ex-vivo* in very low temperatures and using them to repopulate the bone marrow after sub-lethal dose of intensive myeloablative chemotherapy[4].

RATIONALE FOR HIGH DOSE CHEMOTHERAPY

Resistance to chemotherapy is a major problem in the treatment of patients with malignant diseases. Large number of studies are directed towards finding and overcoming resistance mechanisms. One of the simplest and most logical way is to increase the dose of cytotoxic drugs[5]. The evidence that higher doses of cytotoxic drug kill more malignant cells has been well known for decades. Back in 1964, Skipper *et al*[6] demonstrated that the curve showing the dose dependency of the treatments with cytotoxic drugs is very steep, indicating that even a small increase of the cytotoxic drug dose will kill more malignant cells. Also, this curve for cytotoxic drugs, unlike the curves from other drugs, has no plateau, which means that a constant increase of the dose of cytotoxic drugs leads to a steady increase in numbers of destroyed malignant cells[6]. During the 1980s, Frei *et al*[7] showed a dose-dependent killing of malignant cells in AKR and L1210 cell lines. Frei *et al*[8] demonstrated the same on MCF7 breast cancer cells treated with alkylating agents BCNU, melphalan and nitrogen mustard. However, it was not possible to administer the 5 to 10-fold higher dose of chemotherapy *in vivo* due to the high toxicity and virtually lethal toxicity on bone marrow. Therefore, the researchers started to scrutinize bone marrow transplantation as a method for overcoming this high toxicity after chemotherapy[9]. Afterwards, numerous studies of high dose chemotherapy and autologous stem cell transplantation in a large number of solid tumor cases were completed, however, this form of treatment

Table 1 Prognostic criteria for metastatic germ cell tumors[3]

	Good prognosis group	Intermediate prognosis group	Poor prognosis group
Seminoma	90% of cases 5 yr PFS 82% 5 yr OS 86% All of the following criteria: Any primary site No non-pulmonary visceral metastases Normal AFP Any hCG Any LDH	10% of cases 5 yr PFS 67% 5 yr OS 72% Any of the following criteria: Any primary site Non-pulmonary visceral metastases Normal AFP Any hCG Any LDH	-
Non-seminoma	56% of cases 5 yr PFS 89% 5 yr OS 92% All of the following criteria: Testis/ retroperitoneal primary Non non-pulmonary visceral metastases AFP < 1000 ng/mL hCG < 5000 IU/L (1000 ng/mL) LDH < 1.5 × ULN	28% of cases 5 yr PFS 75% 5 yr OS 80% Testis/ retroperitoneal primary Non non-pulmonary visceral metastases AFP 1000-10000 ng/mL hCG 5000-50000 IU/L (1000 ng/mL) LDH 1.5-10 × ULN	28% of cases 5 yr PFS 75% 5 yr OS 80% Any of the following criteria: Mediastinal primary Non-pulmonary visceral metastases AFP > 10000 ng/mL hCG > 50000 IU/L (1000 ng/mL) LDH > 10 × ULN

AFP: Alpha-fetoprotein; hCG: Human chorionic gonadotrophin; LDH: Lactate dehydrogenase; PFS: Progression free survival; OS: Overall survival.

has remained standard practice only for TGCC[10-13].

CLINICAL TRIALS WITH HDCT TGCC

As already mentioned, the majority of patients with metastatic TGCC are cured with standard chemotherapy: 3-4 cycles of BEP protocols[3]. However, in patients with a poor prognosis, cure rate is below 50%. In these patients, and in patients with relapsed testicular cancer, unsatisfactory performance standard chemotherapy has directed researchers to search for new forms of treatment. The rationale of using high dose chemotherapy in chemo-sensitive cancer lead on investigators to start clinical trials with high dose chemotherapy and stem cell support[3,14].

HDCT AS THE INITIAL TREATMENT IN PATIENTS WITH POOR PROGNOSIS

The initial studies of high dose chemotherapy for patients with poor prognosis in the first line setting were completed in the nineties. Motzer *et al*[15,16] from Memorial Sloan-Kettering Cancer Center in phase Ⅱ studies demonstrated slightly better response to HDCT compared to a historical control with the standard dose chemotherapy (SDCT). In a study from 1993, 15 of 27 patients (56%) achieved a complete remission, 46% were free of disease, and 57% alive after a median of 31.2-mo follow-up[15]. In another study by the same authors 30 patients were treated, 16 with etoposide, ifosfamide, cisplatin (VIP) chemotherapy, while 14 patients after VIP therapy, received HDCT, combination carboplatin, etoposide, cyclophosphamide (CEC). Patients selected for HDCT included those in whom tumour markers did not normalise after two cycles of chemotherapy. After a median follow-up of 30 mo, 15 (50%) patients remained progression-free. Patients treated with marker-dependent, early-intervention HDCT experienced longer survival[16]. Bokemeyer *et al*[17] published in 1999 a match-paired multivariate analysis which compared the outcomes of patients with poor prognosis metastatic TGCC treated sequentially with standard VIP protocol and HDCT in a multicentric study including patients from German group studies and patients treated in two studies from Indiana University, with BEP or VIP conventional chemotherapy. High dose chemotherapy group included 147 patients, while 309 patients were in the SDCT group. Patients treated with HDCT had a longer progression free survival (PFS) 75% *vs* 59% (P = 0.0056) and a longer overall survival (OS) 82% *vs* 71% (P = 0.0184)[17]. After that, Schmoll *et al*[18] from German Testicular Cancer Study Group (GTCSG) published a phase Ⅰ/Ⅱa study where they treated poor prognosis TGCC patients with a VIP-escalated protocol. After one cycle of standard VIP protocol, they applied dose escalated VIP with autologous stem cell transplantation, three to four cycles. Five-year PFS in this group of patients was 68%, which is longer than the historical control with SDCT. After the advent of paclitaxel, and proven effectiveness of this drug in cisplatin-resistant TGCC, GTCSG announced the study of addition of a paclitaxel to dose-escalated VIP protocol[19]. Addition of paclitaxel to high dose-VIP (HD-VIP) protocol resulted in higher response rate of 79%, and five-year PFS and OS of 64.1% and 75.2% respectively.

The only completed randomized phase Ⅲ study is the one of Motzer *et al*[20]. This study included 219 untreated patients with metastatic TGCC intermediate and poor prognosis. One group of patients was treated with standard therapy, four cycles of BEP, while the experimental group received two cycles of BEP and afterwards two cycles high dose CEC (HD-CEC) protocol. Proportion of one year complete remission was not different in the two groups of patients (52% PEB + HD-CEC *vs* 48% BEP, P = 0.53). Benefit of a high dose chemotherapy, in this clinical trial, was observed only in those patients with unsatisfactory tumor markers decline. The study concludes that there is no benefit of adding a HDCT in this group of patients. Two other studies have started the third phase, but due to poor recruiting of patients they are not fully completed[21-23]. The analysis of the included patients from high dose chemotherapy did not show the expected benefit in first-line treatment of metastatic TGCC with a poor prognosis. A review of studies of the first line is given in Table 2.

HDCT IN SECOND-LINE THERAPY

Therapeutic options of SDCT in patients with relapsed/refractory testicular cancer can achieve long-term remission of 25% of the cases with vinblastine, etoposide, ifosfamide (VeIP) protocol[24], to about 65% of patients treated with paclitaxel, ifosfamide, cisplatin (TIP)[25]. Considering the chemosensitivity of TGCC and relative modest results of conventional chemotherapy protocols, a large number of researchers have designed a variety of studies which applied HDCT with the support of haematopoietic autologous stem cell transplantation (Table 3).

Rodenhuis *et al*[26] have demonstrated a 54% PFS after a median follow-up of 37 mo in a phase Ⅱ study on 35 patients. In this study they used two cycles of a HDCT after the induction with conventional chemotherapy. Similar design study was published in 2000[27]. Two cycles of intensification were used and the results were almost the same as in the previous study. The same year, Motzer *et al*[28] demonstrated overall survival of 54% after a median of nearly three years of follow-up. They used the induction regimen with paclitaxel and ifosfamide, and three cycles of high dose protocol carboplatin/etoposide (TI-CE). A slightly worse result was achieved in the study by Rick *et al*[29] which included only one cycle of high dose protocol after the induction with three cycles of TIP. Three-year survival in this study was 30%. The explanation for the slightly

Table 2 Studies of first line high dose chemotherapy for poor prognosis patients

Ref.	Type of study	Number of patients	Protocol	OS (%)	PFS (%)	Median follow-up (mo)
Motzer *et al*[15]	Phase II, prospective	28	VAB-6 × 2 + HD-CE × 2	57	46	31
Motzer *et al*[16]	Phase II, prospective	30	VIP × 2 + HD-CEC × 2	48 (5 yr)	48 (5 yr)	60
Bokemeyer *et al*[17]	Comparative, retrospective	147 (HDCT) *vs* 309 (SDCT)	VIP × 2 + HD-VIP × 2 *vs* BEP/VIP × 4	82 *vs* 72 (2 yr) $P = 0.0184$	75 *vs* 59 (2 yr) $P = 0.0056$	21
Schmoll *et al*[18]	Phase I/II, prospective	221	VIP + HD-VIP × 3-4	73 (5 yr)	68 (5 yr)	48
Hartmann *et al*[19]	Phase I/II, prospective	52	VIP + T-HD-VIP	75 (5 yr)	64 (5 yr)	41
Motzer *et al*[20]	Phase III, prospective	108 (HDCT) *vs* 111 (SDCT)	BEP × 2 + HD-CEC × 2 *vs* BEP × 4	71 *vs* 72 (2 yr)	60 *vs* 57 (2 yr)	33
Daugaard *et al*[23]	Phase III, prospective	65 (HDCT) *vs* 66 (SDCT)	VIP + HD-VIP × 3 *vs* BEP × 4	86.1 *vs* 83 (2 yr)	66.1 *vs* 48 (1 yr)	NR
Necchi *et al*[22]	Phase II, prospective	43 (HDCT) *vs* 42 (SDCT)	BEP × 2 + HD-CpE + HD-Carbo *vs* BEP × 4	54.8 *vs* 55.8 (5 yr)	59.3 *vs* 62.8 (5 yr)	114

BEP: Bleomycin, etoposide, cisplatin; HD: High dose; HD-CE: High dose carboplatin, etoposide; HD-CEC: High dose carboplatin, etoposide, cyclophosphamide; HDCT: High dose chemotherapy; HD-VIP: High dose, etoposide, ifosfamide, cisplatin; NR: Not reported; OS: Overall survival; PFS: Progression free survival; SDCT: Standard-dose chemotherapy; VAB: Actinomycin D, vinblastine, cyclophosphamide, bleomycin, cisplatin.

Table 3 High dose chemotherapy as second line treatment

Ref.	Type of study	Number of patients	Protocol	OS (%)	PFS (%)	Median follow-up (mo)
Rodenhuis *et al*[26]	Phase II, prospective	35	Conventional chemotherapy + HD-CTC × 2	NR	54	37
Bhatia *et al*[27]	Phase II, prospective	65	VeIP × 1-2 + HD-CE × 2	NR	57	39
Motzer *et al*[28]	Phase II, prospective	37	TI × 2 + HD-CE × 3	54	49	31
Rick *et al*[29]	Phase II, prospective	62	TIP × 3 + HD-CET × 1	30 (3 yr)	25 (2 yr)	36
Pico *et al*[30]	Phase III, prospective, randomized	135 (HDCT) *vs* 128 (SDCT)	VIP/VeIP × 3 + HD-CE × 1 *vs* VIP/VeIP × 4	53 *vs* 53 (3 yr)	42 *vs* 35 (3 yr)	45
Einhorn *et al*[31]	Retrospective	135	HD-CE × 2	NR	70	48
Lorch *et al*[32]	Phase II, prospective, randomized	111 (sequentional HDCT) *vs* 105 (single HDCT)	VIPx 1 + HD-CE × 3 *vs* VIP × 3 + HD-CE × 1	47 *vs* 45 (5 yr)	49 *vs* 39 (5 yr) $P = 0.057$	90
Feldman *et al*[33]	Phase I/II, prospective	107	TI × 2 + HD-CE × 3	52 (5 yr)	48 (5 yr)	61
Lorch *et al*[34]	Comparative, retrospective	821 (HDCT) *vs* 773 (SDCT)		53.2 *vs* 40.8 (5 yr) $P < 0.001$	49.6 *vs* 27.8 (2 yr) $P < 0.001$	NR
Selle *et al*[36]	Phase II, prospective	45	Epi-Tax × 2 + HD Thio-Tax + HD-ICE × 2	66% (2 yr)	50% (2 yr)	26
Berger *et al*[37]	Comparative, retrospective	95 (HDCT) *vs* 48 (SDCT)	HDCT *vs* SDCT	$P = 0.931$	Median 8 *vs* 42 mo $P < 0.001$	NR
Nieto *et al*[64]	Phase II, prospective	42	BEC-GDMC + BEV + HD-ICE	65% (2 yr)	63% (2 yr)	NR

BEV: Bevacizumab; Epi-Tax: Epirubicine, paclitaxel; GDMC: Gemcitabine, docetaxel, melphalan, carboplatin; HD: High dose; HD-CE: High dose carboplatin, etoposide; HD-CET: High dose carboplatin, etoposide, thiotepa; HDCT: High dose chemotherapy; HD-CTC: High dose carboplatin, thiotepa, cyclophosphamide; HD-ICE: High dose ifosfamide, carboplatin, etoposide; NR: Not reported; OS: Overall survival: PFS: Progression free survival; SDCT: Standard-dose chemotherapy; Thio-Tax: Thiotepa, paclitaxel; TI: Paclitexel, ifosfamide; TIP: Paclitexel, ifosfamide, cisplatin; VeIP: Vinorelbine, ifosfamid, cisplatin; VIP: Etoposide, ifosfamide, cisplatin.

worse result in this study could be the application of only one cycle of high dose protocol.

The only prospective, randomized phase III study by Pico *et al*[30] compared four cycles of conventional chemotherapy VeIP or VIP with three cycles of of SDCT with the addition of one cycle of high dose carboplatin/etoposide (HD-CE) protocol. This study included a total of 263 patients. It did not demonstrate the superiority of the addition of one cycle of HDCT. Based on that study and study by Rick *et al*[29], it was concluded that one cycle of HDCT was not sufficient to achieve better results in treatment compared to conventional chemotherapy, so further studies had two or even three cycles of HDCT.

The study which probably had the greatest impact on the practice of treating relapsed TGCC and utilization of HDCT was that by Einhorn *et al*[31].

One hundred and eighty-four patients were retrospectively analysed, and 135 of 184 patients received two cycles of HD-CE protocol in the first relapse, while the other 49 were treated in second and subsequent relapses with the same protocol. After a median follow-up of four years, progression free survival in patients treated in the first relapse was 70%. Lorch *et al*[32] compared one cycle of high dose therapy with three cycles of to HD-CE. After long-term follow-up PFS was 49% *vs* 39% in favor of the sequential approach while overall survival did not differ between these two groups.

Table 4 High dose chemotherapy for third or subsequent lines, refractory/absolute refractory

Ref.	Type of study	Number of patients	Setting	Protocol	OS (%)	PFS (%)	Median follow-up (mo)
Vaena *et al*[38]	Retrospective	80	Second and subsequent lines, refractory	HD-CE × 2	40 (2 yr)	32 (2 yr)	24
Lotz *et al*[39]	Prospective	45	Second and subsequent lines, refractory/absolute refractory	Epi-Tax × 2 + HD Thio-Tax × 1 + HD-ICE × 2	23.5 (3 yr)	23.5 (3 yr)	36
Kondagunta *et al*[40]	Prospective	47	Second and third line, refractory/absolute refractory	TI × 2 + HD-CE × 3	NR	51	40
Einhorn *et al*[31]	Retrospective	49	Third or subsequent	HD-CE × 2	55	45	48
Lorch *et al*[41]	Retrospective	49	Third or subsequent, refractory	Various	17 (5 yr)	26 (5 yr)	48
Popovic *et al*[42]	Prospective	8	Forth or fifht line, refractory	Epi-Tax × 2-3 + HD-CE × 1-2	Median 11 mo	NR	NR

Epi-Tax: Epirubicine, paclitaxel; HD: High dose; HD-CE: High dose carboplatin, etoposide; HDCT: High dose chemotherapy; HD-ICE: High dose ifosfamide, carboplatin, etoposide; NR: Not reported; OS: Overall survival; PFS: Progression free survival; SDCT: Standard-dose chemotherapy; Thio-Tax: Thiotepa, paclitaxel; TI: Paclitexel, ifosfamide.

Feldman *et al*[33] demonstrated in a prospective study of 107 patients a five-year PFS of 48% using the TI-CE protocol with three cycles of high dose chemotherapy.

A multicenter retrospective analysis of 1984 patients by Lorch *et al*[34] compared the standard and high dose chemotherapy in patients with metastatic TGCC after progression on first-line chemotherapy. Patients were divided into five prognostic groups according to previously established criteria: Very low risk, low risk, intermediate risk, high and very high risk[35]. Total of 1594 patients had all the data necessary for analysis, 773 of which received conventional chemotherapy, while 821 patients received HDCT. Two-year PFS and five-year OS was longer in the group with HDCT: 49.6% *vs* 27.8% (HR = 0.44; $P < 0.001$), 53.2% *vs* 40.8% (HR = 0.65; $P < 0.001$). This difference was seen in all prognostic groups except in low-risk group[34].

Selle *et al*[36] in the study TAXIF II demonstrated efficiency of a complex protocol which included several high dose cycles of paclitaxel, thiotepa, ifosfamide, carboplatin and etoposide, after induction with a combination of paclitaxel/epirubicin. The median PFS was 22 mo and OS was 32 mo. Two-year PFS was 50%, with Kaplan-Meier curve that showed a plateau at that value, and two-year OS of 66%[36]. German Testicular Cancer Study Group retrospectively analyzed 143 patients and compared the HDCT ($n = 95$) with CDCT ($n = 48$). They showed a significantly longer median PFS 8 mo *vs* 42 mo ($P < 0.001$) with HDCT, but this difference was not seen when they analyzed overall survival[37].

HDCT FOR REFRACTORY AND HEAVILY PRETREATED PATIENTS

Patients who progressed during standard cisplatin based chemotherapy have the worst prognosis. This group of patients also includes those who have not been cured after two lines cisplatin protocol. For this group of patients there have been several studies that, despite the very poor prognostic characteristics, showed some benefit from HDCT (Table 4).

Vaena *et al*[38] retrospectively analyzed the results of HDCT in platinum-refractory patients. Two-year PFS was 32%, while two-year OS was 40%. Lotz *et al*[39] applied a different concept in refractory patients. In TAXIF study they prospectively treated 45 patients with absolutely refractory metastatic TGCC. After mobilization therapy with paclitaxel/epirubicin, they gave two cycles of high dose ifosfamide, carboplatin, etoposide (ICE) protocol. Three-year PFS and OS were 23.5%[39]. Kondagunta *et al*[40] prospectively treated 47 refractory patients with high dose chemotherapy. After a median of 40 mo of observation the PFS was 51%. The Study by Einhorn *et al*[31] treated 49 patients with HDCT in third and subsequent lines of chemotherapy. Time to disease progression and OS in these patients was 45% and 55% respectively. In patients with third and subsequent lines of therapy, Lorch *et al*[41] reached five-year OS of 17% at five-year PFS 27%. In a pilot study, we have treated 8 heavily pretreated patients with HDCT. We used a modified TAXIF protocol[39]. All patients had previously received four lines of different therapies. The median OS was 11 mo, with no long-term survival[42,43].

HDCT FOR EXTRAGONADAL GCC

Extragonadal GCC tumors occur most often in retroperitoneum and mediastinum and have worse prognosis compared to TGCC[2]. Several studies with high dose chemotherapy administration has addressed this subgroup of patients (Table 5). Bokemeyer *et al*[44] have treated patients with primary mediastinal germ cell tumors (PMNSGCT) initially with high dose chemotherapy. They included 28 patients and achieved 56% and 64% PFS and OS, respectively. Banna *et al*[45] also used HDCT in the first line of treatment PMNSGCT and reached a three-year OS 41%. Rosti *et al*[46] retrospectively analyzed 22 patients who had primary extragonadal non-seminomatous germ cell tumor (EGNSGCT) and received HDCT. Five-year survival in this group of patients was 75%. Hartmann *et al*[47] and De Giorgi *et al*[48] in two studies published in 1999 showed retrospective results of a treatment of

Table 5 High dose chemotherapy for extragodadal germ cell cancer

Ref.	Type of study	Number of patients	Setting	Protocol	OS (%)	PFS (%)	Median follow-up (mo)
Bokemeyer *et al*[44]	Phase I/II, prospective	28	PMNSGCT, first line	VIPx 1 + HD-VIP × 3	64 (5 yr)	56 (5 yr)	43
Banna *et al*[45]	Prospective	21	PMNSGCT, first line	BEP or VIP × 4 + HD-CEC × 1	41 (3 yr)	43 (5 yr)	52
Rosti *et al*[46]	Retrospective	22	EGCT, poor prognosi, first line	Various	75 (5 yr)	67 (5 yr)	50
Hartmann *et al*[47]	Retrospective	142	EGNSGCT, salvage	Various	12 (3 yr) (PMNSGCT only)	11 (3 yr) (PMNSGCT only)	45
De Giorgi *et al*[48]	Retrospective	59	EGNSGCT, salvage	Various	14 (PMNSGCT only)	14 (PMNSGCT only)	58

EGCT: Extragonadal germ cell tumor; EGNSGCT: Extragonadal non-seminomatous germ cell tumor; HD: High dose; HD-CEC: High dose carboplatin, etoposide, cyclophosphamide; HDCT: High dose chemotherapy; HD-ICE: High dose ifosfamide, carboplatin, etoposide; NR: Not reported; OS: Overall survival; PFS: Progression-free survival; PMNSGCT: Primary mediastinal non-seminomatous germ cell tumor.

Table 6 International Germ Cell Cancer Collaborative Group-2 prognostic criteria for relapsed germ cell cancer patients

Parameter	Score points			
	0	1	2	3
Primary site	Gonadal	Extragonadal	-	Mediastinal non-seminoma
Prior response	CR/PRm-	PRm+/SD	PD	-
PFI, mo	> 3	⩾ 3	-	-
AFP salvage	Normal	⩽ 1000	> 1000	
HCG salvage	⩽ 1000	> 1000	-	-
LBB	No	Yes	-	-
Score sum (0-10)				
Regroup into categories: (0) = 0; (1 or 2) = 1; (3 or 4) = 2; (5 or more) = 3				
Add histology points: Seminoma = -1; Non-seminoma or mixed = 1				
Final prognostic score: -1 = Very low risk; 0 = Low risk; 1 = Intermediate risk; 2 = High risk; 3 = Very high risk				

AFP: Alpha-fetoprotein; CR: Complete remission; HCG: Human chorionic gonadotrophin; LBB: Liver, brain, bone; PD: Progressive disease; PFI: Progression free interval; PRm-: Partial remission, markers negative; PRm+: Partial remission, markers positive; SD: Stable disease.

EGNSGCT after progression on first-line therapy. Results were rather modest with 12%-14% long term survival.

RISK ADAPTED APPROACH FOR USING HDCT

The first prognostic score related to the outcome of a HDCT was developed Beyer *et al*[49]. They have analyzed a series of 310 patients treated with HDCT in four centers in Europe and the United States and by multivariate analysis of prognostic factors determined the data which had influenced the outcome. Progressive disease before HDCT, primary mediastinal localization, refractory or absolute refractory disease to cisplatin therapy and the value of human chorionic gonadotropin (HCG) over 1000 were independent factors for failure-free survival (FFS) after HDCT. These parameters separated patients into groups with good, intermediate and poor prognosis. Patients with good, intermediate and poor prognosis had 51% FFS after HDCT, 27%, and 5% ($P < 0.001$) respectively. The International Prognostic Factors Study Group (Table 6) analyzed data of 1984 patients with TGCC, who have progressed after at least three cycles of cisplatin based chemotherapy. Patients' data were collected from 38 centers worldwide and 1594 patients had sufficient data for analysis. Patients were treated with SDCT or HDCT based on carboplatin. Factors that influenced the outcome were: Site of primary tumor, previous response to therapy, progression free survival on previously applied therapy, alpha-fetoprotein and HCG above 1000 and the presence of metastases in the liver, bone and/or bone[35].

Based on these factors, patients were divided into five categories: Very low risk with a two-year PFS of 72%, low risk with PFS 51%, medium risk with 40%, high risk with 26% and very high risk with 6%. This is followed by the already mentioned retrospective analysis by Lorch *et al*[34] which showed benefits in all prognostic categories, except in the low risk group. Given that the benefit was demonstrated even in the category of very low risk, there is a question in which prognostic groups, in patients with relapsed GCC, HDCT should be applied and which groups should receive conventional chemotherapy. Opinions differ greatly, and certain groups of authors believe that high dose chemotherapy should be applied in all patients with relapsed GCC, while some groups believe that patients with a low risk should be treated with the conventional chemotherapy in the second line, and HDCT should be applied in patients with medium and higher risk as well as in those with a low risk who relapse after second-line of conventional chemotherapy[50]. Our position is closer to the second opinion.

STEM CELLS MOBILISATION AND OPTIMAL PROTOCOL OF CONDITIONING

Collection of sufficient numbers of hematopoietic stem cells is a key step in the further implementation of

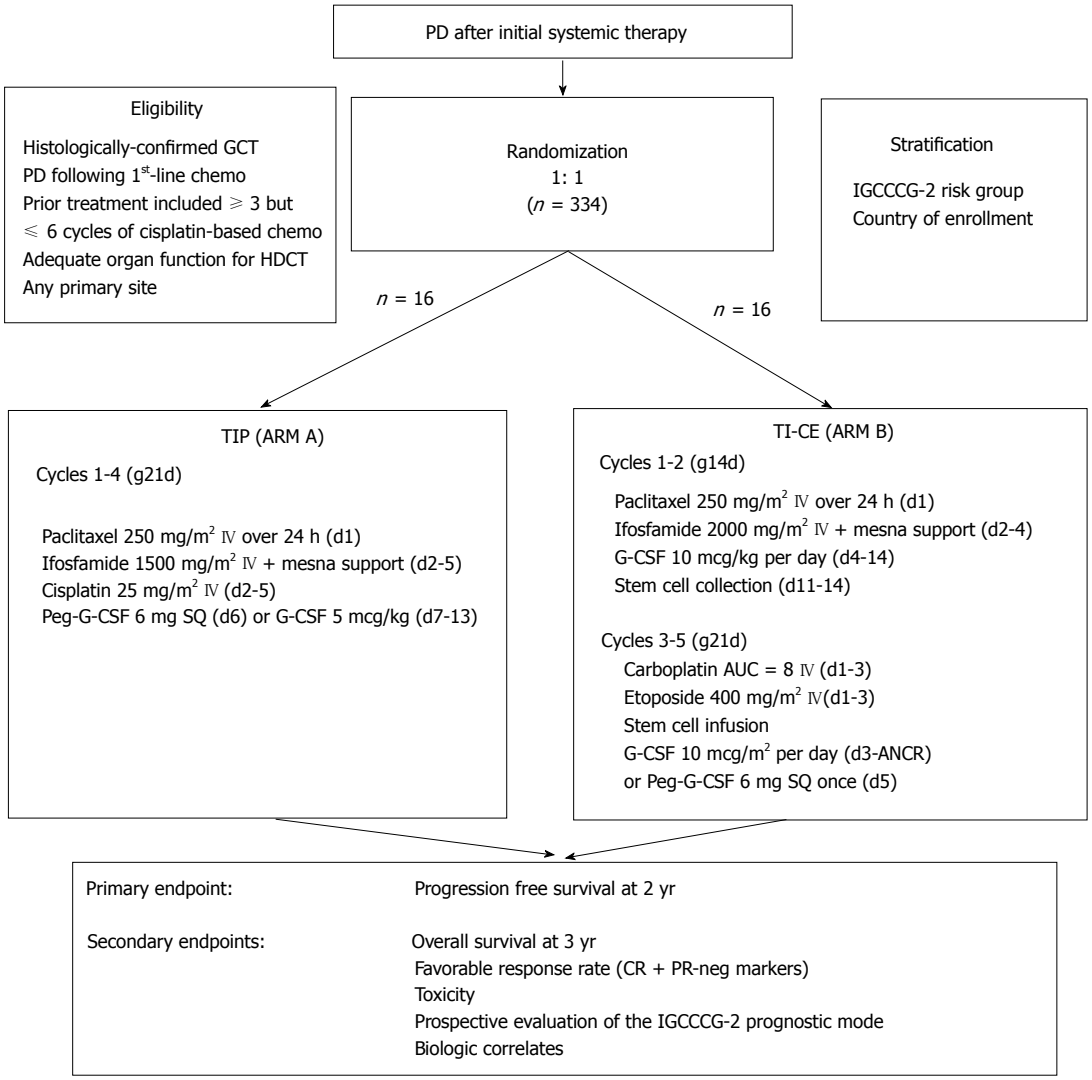

Figure 1 Training intervention and genetics of exercise response study design[62]. PD: Progressive disease; GCT: Germ cell tumors; HDCT: High dose chemotherapy; G-CSF: Granulocyte colony-stimulating factor.

a HDCT, and the possibility of treating patient with multiple cycles of HDCT. Combination of chemotherapy with granulocyte growth factor (G-CSF) is a standard for the mobilization of hematopoietic stem cells. However, in heavily pretreated patients, this method of mobilization is not enough to collect a sufficient number of stem cells. In our cohort of heavily pretreated patients median collected hematopoietic stem cells was 3.6×10^6 cells/kg of BW. Consequently, it was not possible for us to apply tandem transplantation in some patients[43]. Some other authors as well conclude that the mobilization with chemotherapy + G-CSF was inadequate for obtaining a sufficient number of stem cells, especially in cases of highly pretreated patients[4,51-53].

Plerixafor is the CXCR4 receptor antagonists which separate hematopoietic stem cells from bone marrow stroma and can lead to better mobilization of these cells into peripheral blood[54]. After the positive outcome in poor-mobilisers with lymphoma and multiple myeloma increased enthusiasm for using plerixafor to mobilize hematopoietic stem cells in patients with TGCC. The four smaller cohorts and three case studies[52-60] showed the efficiency of plerixafor in heavily pretreated patients with TGCC. Worel *et al*[55] showed the efficiency of plerixafor in 33 patients with non-hematologic diseases, of which 11 were metastatic GCC. A total of 28 (85%) patients gathered a sufficient number of stem cells. Kobold *et al*[57] showed a series of 6 patients who had previously received chemotherapy for 3.5 lines metastatic GCC and were not able to mobilize a sufficient number of stem cells for transplantation. After the use of plerixafor, five of these six patients mobilized an adequate number of cells for a minimum one transplant. Kosmas *et al*[52], in a pilot study, showed stem cells mobilization in pretreated patients with GCC, in which 7 out of 10 patients could yield an adequate number of hematopoietic stem cells for transplantation. The remaining three, poor-mobilisers, have amassed an adequate number of stem cells after applying plerixafor. In all these publications, engraftment of stem cells obtained after the mobilization with plerixafor was adequate.

Despite attempts with different drugs that would

supplement carboplatin, such as thiotepa and ifosfamide, panelists of the third European consensus conference on the treatment of GCC, agreed that a combination of carboplatin and etoposide is a standard high dose protocol[50].

FUTURE DIRECTIONS

Although HDCT is considered standard treatment option for relapsed GCC in most major cancer centers, there are still no level IA evidence for applying HDCT in the current recommendations for the treatment of GCC[2,3,61]. The reason for this is the series of negative results of the randomized phase Ⅲ studies by Motzer *et al*[20] in the first line, and Pico *et al*[30] in metastatic relapsed GCC, therefore TIGER (randomized phase Ⅲ trial of initial salvage chemotherapy for patients with germ cell tumors) has been initiated[62]. The study design is shown in Figure 1. The plan is to include 390 patients, a group of which will receive four cycles of TIP protocol, while the second group will receive TI-CE protocol with three cycles of a HDCT. The hypothesis is that the overall survival of patients should be 13% higher after the treatment with the HDCT.

The second concept is target therapy in addition to the treatment of relapsed GCC, for conventional, as well as for high dose chemotherapy. Vascular endothelial growth factor over-expression is an independent factor of poor prognosis for non-seminomatous germ cell tumor (NSGCT), especially for teratoma NSGCT which is the most refractory to chemotherapy[4]. Voigt *et al*[63] showed a case of successful treatment of patients absolutely refractory to cisplatin with bevacizumab and HD-ICE protocol. At the last ASCO meeting, Nieto *et al*[64] presented a phase Ⅱ study for the first and the second relapse of intermediate and high risk metastatic GCC. They combined bevacizumab with tandem HDCT. In the first cycle of HDCT they combined bevacizumab with gemcitabine, docetaxel, melphalan and carboplatin, while in the second cycle they combined bevacizumab with HD-ICE protocol. One-year and 2 year-OS were 72% and 65% respectively[64]. In addition to these studies, there is an ongoing study TAXIF Ⅲ, of the French group, with the addition of bevacizumab to HD-ICE protocol[65]. Approximately 70% of embryonic GCT express CD30 receptor on cell surface[66]. Brentuximab-vedotin is an anti-CD30 conjugated to a monoclonal antibody that has shown significant results in the treatment of Hodgkin's and peripheral T-cell lymphomas[67]. The Italian group has started a phase Ⅱ clinical study of efficiency brentuximab vedotin in refractory CD30-positive metastatic testicular cancer[68].

CONCLUSION

Although there are no randomized phase Ⅲ trials that support HDCT as an effective treatment option for patients with metastatic GCT, the majority of centres use this type of therapy in patients with intermediate and high risk according International Germ Cell Cancer Collaborative Group-2 score[35]. TIGER study might give a definitive answer whether HDCT should be a standard treatment for these patients, and a better understanding of tumor biology, detection of markers of resistance to cisplatin, as well as if adding target therapy such as bevacizumab should improve the treatment of GCT, especially in the group of patients with a poor prognosis.

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REVIEW

Towards *in vivo* amplification: Overcoming hurdles in the use of hematopoietic stem cells in transplantation and gene therapy

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Abstract

With the advent of safer and more efficient gene transfer methods, gene therapy has become a viable solution for many inherited and acquired disorders. Hematopoietic stem cells (HSCs) are a prime cell compartment for gene therapy aimed at correcting blood-based disorders, as well as those amenable to metabolic outcomes that can effect cross-correction. While some resounding clinical successes have recently been demonstrated, ample room remains to increase the therapeutic output from HSC-directed gene therapy. *In vivo* amplification of therapeutic cells is one avenue to achieve enhanced gene product delivery. To date, attempts have been made to provide HSCs with resistance to cytotoxic drugs, to include drug-inducible growth modules specific to HSCs, and to increase the engraftment potential of transduced HSCs. This review aims to summarize amplification strategies that have been developed and tested and to discuss their advantages along with barriers faced towards their clinical adaptation. In addition, next-generation strategies to circumvent current limitations of specific amplification schemas are discussed.

Key words: Gene therapy; Hematopoietic stem cells; *In vivo* selection; Chemical Inducer of Dimerization; Chemo-selection; Lentivirus

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Core tip: Though hematopoietic stem cell (HSC)-directed gene therapy is becoming a viable therapy for many disorders, optimization of clinical output needs improvement. One approach to circumvent lower efficiencies of gene transfer and/or engraftment is to apply *in vivo* amplification strategies. Here we review

various modules that have been developed and tested to mediate amplification of HSCs after gene transfer.

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INTRODUCTION

Hematopoietic stem cells (HSCs) are long-term, multipotent, self-renewing cells that reside in specialized bone marrow (BM) niches and are capable of generating and repopulating the entire spectrum of blood and lymphoid cells[1,2]. Due to these unique properties, HSCs are targets for therapy for a number of hematological malignancies and many inherited blood disorders including β-thalassemia, sickle cell anemia, chronic granulomatous disease, and severe combined immunodeficiencies (SCID-X1 and ADA-SCID) among others[3-8]. Additionally, HSC transplants have been used in attempt to correct other monogenic deficiencies, such as the mucopolysaccharidoses and Gaucher disease[9-11].

There are still numerous drawbacks of allogeneic transplantation despite its clinical utility. Often, HSCs are collected from the patient's sibling, parents, or a matched donor. HLA-identical donors can be difficult to find and there are risks involved with the use of HLA-haploidentical or non-identical donors including rejection or poor engraftment of HSCs along with the occurrence of graft-versus-host disease (GVHD). Conditioning is also necessary for engraftment of HSCs, which can increase the risk of infections[12-14]. As a consequence, HSC allo-transplantation is still considered a fairly risky intervention and is applied with caution in the clinic.

Gene therapy targeting patient-derived HSCs is a viable solution for some monogenic diseases[15] (Figure 1A). Autologous transplantation has been well studied and detailed clinical protocols are available for this procedure[3]. Additionally, autologous transplantation does not have a risk of GVHD associated with it and immune reconstitution after ablation occurs in a shorter period of time[16,17]. Gene transfer into HSCs has been traditionally achieved by stable transduction of target cells using replication-incompetent retroviruses[15]. There the expression of transgenes can be driven by constitutive or tissue-specific promoters, giving a range of control over the intended therapeutic intervention. Next-generation strategies are also being developed to correct original nucleotide mutations with the use of gene-editing technologies, such as TALENs and CRISPR-Cas9, though these remain to be optimized for clinical application[18-20].

Over 2000 clinical gene therapy trials have been conducted to date[4,15,21,22]. Most earlier trials employed onco-retroviral vectors, which have shown to be clinically disadvantageous because of their tendency to integrate close to genes that are important for cell growth and proliferation, enhancing their expression and increasing the likelihood of developing leukemias[4,15,23-25]. So far it appears that this genotoxicity and tendency towards insertional mutagenesis has been diminished with the introduction of HIV-1-derived, replication-incompetent, and self-inactivating lentiviral vectors (LVs), which do not show preferential integration near genes involved in cell growth and/or proliferation[4,26-30].

There are other caveats to using HSCs as target cells for gene therapy that are a result of their unique biology. HSCs can be more difficult to transduce than some other cell types, partially owing to the difficulty of culturing them *ex vivo*. Longer-term culturing ensures that the cells will differentiate. Transduction also requires transient activation of the cell cycle, especially with onco-retroviral-based vectors since their downstream integration requires a breakdown of the nuclear membrane. As a consequence of *ex vivo* manipulation and cell-cycle activation, transduced HSCs often have lower engraftment potential and reduced longevity once engrafted. These additional limitations have also been partially addressed with the use of LVs, which need shorter transduction times and do not require target cells to be fully cycling[31-35].

In spite of the progress made in HSC gene therapy with the implementation of recombinant LVs, there is ample room for additional improvements to increase therapeutic efficacy. Many active fields of research are geared towards optimizing gene therapy for HSCs. Efforts are under way to hone GMP-grade LV production to subsequently allow modulation of multiplicities of infection at a clinical level, whilst reducing the cost of gene therapy[36,37]. Improvements are also being made to protocols for *ex vivo* handling and culture of HSCs with studies demonstrating enhanced transduction with shorter culture times and less activation, which have resulted from better understanding of the biology of HSCs and their BM microenvironments[4,38,39]. In addition, in-depth studies of HSC biology have identified molecular targets for drugs that allow more efficient and safer mobilization of patient stem cells[40-42]. The gene therapy field has also sought out methods to provide extrinsic selective pressure for transduced cells, though clear clinical utility of any system has yet to be demonstrated, especially in the context of reconstitutive HSC-directed gene therapy.

Reconstitution of deficient gene products in some inherited blood diseases leads to innate positive selective pressure *in vivo* for mature cells derived from transduced progenitor cells, especially when the gene product is necessary for the development or function of those cells. For example, reconstitution of the common gamma chain (γc/CD132) in SCID-X1 allows immune cells to develop normally, thus progeny cells are derived from successfully transduced HSCs almost exclusively[17,43-45]. Selectivity for donor-derived late-stage

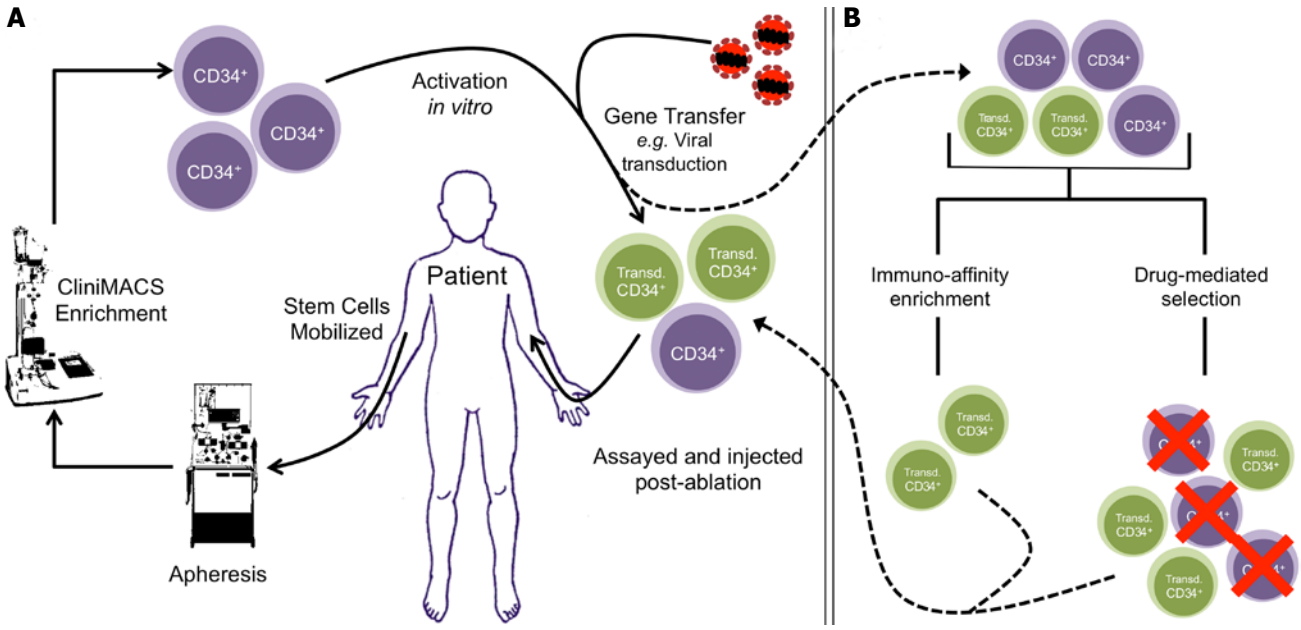

Figure 1 General outline of *ex vivo* hematopoietic stem cell gene therapy and pre-selection methods. A: $CD34^+$ cells are enriched by CliniMACS after apheresis of peripheral blood of patients following mobilization. These cells are then briefly activated *ex vivo* and can be modified, commonly by viral transduction, to express a desired therapeutic protein. Cells are then assessed for quality control metrics and engrafted into patients following ablation; B: Pre-selection of transduced cells. Cells can be engineered to express an inert surface marker that can be used to immuno-enrich for the transduced population prior to engraftment. This strategy can increase the chances of hematopoietic reconstitution from the transduced population. Alternatively, cells can be given resistance to cytotoxic drugs. Pre-treatment of the cells *ex vivo* with drugs can kill off the non-transduced population. *Ex vivo* treatment allows the use of drugs that would normally not be efficacious in the bone marrow environment at a tolerable dose.

erythrocytes has also been observed in β-thalassemia patients that have received allo-transplantations; and a similar trend has been observed with gene therapy in mouse models of the disorder[7,46,47]. HSC gene therapy may therefore become routine clinical practice some day for patients suffering from such hematological disorders that do not have matched donors, considering the innate advantage of such reconstituted cells.

There is also great potential of HSC-directed gene therapy for the treatment of non-hematological, monogenic enzyme deficiencies, such as those that lead to lysosomal storage disorders (LSDs) and other metabolic indications. In most cases, functional enzymes expressed after gene transfer into LSD patient cells have the potential to be secreted and subsequently taken up by other cells that do not have the transgene, a process termed metabolic cooperativity or "cross-correction". This occurrence has been demonstrated for a number of LSDs including Gaucher, Farber, and Fabry diseases[48-51]. The current standard of care for many LSDs, enzyme replacement therapy (ERT), is actually a corollary of this phenomenon. HSC-directed gene therapy presents numerous putative advantages over conventional ERT, including sustained and continuous secretion of therapeutic enzyme by ubiquitously circulating cells, improvements in patient lifestyle by reducing the need for biweekly enzyme infusions, and overall cost savings. It is necessary to tailor HSC gene therapy for individual patients, however, which is incongruous with many current industrial business models, highlighting the necessity of shifting industrial focus from general to personalized therapeutics.

Our laboratory is currently pursuing first-in-man HSC-based gene therapy for Fabry disease and is concomitantly demonstrating the utility of gene therapy for amelioration of Farber disease. However, in these cases, as with many such target disorders, expression of the functional gene product imparts no innate growth advantages to transduced cells. Vector-encoded transgenes alone or in tandem that allow extrinsic selective pressure to be applied *in vivo*, leading to an increased percentage of vector-transduced cells over background could therefore be highly beneficial in the context of HSC gene therapy for LSDs and many other monogenic deficiencies. Additionally, application of positive selective pressure could result in cell populations that have higher transgene expression, resulting in an increased therapeutic benefit.

In this review, we aim to summarize the various strategies that have been employed to date in attempts to increase vector-transduced HSC numbers, thereby increasing the efficacy of HSC-targeted gene therapy. In addition, we will discuss putative next-generation strategies aimed at addressing current shortcomings of applying selective positive pressure on transduced HSCs.

EX VIVO PRE-SELECTION STRATEGIES

Resistance to cytotoxic drugs

Selection of genetically modified cells is a compilation of laboratory techniques commonly applied to acquire

polyclonal cell lines after gene transfer. To achieve this, target cells are engineered to express proteins that confer resistance to drugs or proteins that allow selection by immune-affinity methods such as fluorescence- and magnetic-activated cell sorting (FACS and MACS, respectively)[52,53] (Figure 1B). Ideally, proteins expressed for enrichment should have low or no endogenous expression in target cells, and should have no effect on the biology of the transduced cells or their progeny. Traditionally, xenogenic enzymes have been used to confer cells with resistance to pan-toxic drugs in this context. For example, neomycin and hygromycin phosphotransferases (NeoR and HygR) derived from bacteria are commonly used to provide protection against neomycin and hygromycin B, respectively[54,55]. As such, first attempts at conferring resistance to cells for engraftment were made with these enzymes. However, the use of xenogenic enzymes in clinical protocols has been limited by their tendency to be highly immunogenic once such modified cells are engrafted[56-58]. To address this, mutants of various endogenous enzymes have been used to confer resistance to other cytotoxic drugs. These enzymes are discussed in the section below in the context of *in vivo* selection. However, most drugs require prolonged *ex vivo* culture to effectively enrich for the gene-modified population. Prolonged *ex vivo* handling of HSCs reduces their usefulness post-selection due to a loss of "stemness" and engraftment potential[31]. Thus, drug-mediated *ex vivo* pre-selection may not be ideal in current iterations for clinical purposes.

Cell-surface marking for immuno-enrichment

Transduced HSCs can also be enriched *ex vivo* with the use of cell-surface markers. Selectable cell-surface markers that have been studied for HSC marking and pre-selection include truncated forms of the human low-affinity nerve growth factor receptor (ΔLNGFR)[59-62], the heat stable antigen (HSA/CD24)[48,50,63,64], the human lymphocyte antigen T1 (CD5)[65,66], and the human interleukin-2 receptor alpha chain (IL-2Rα/huCD25)[67]. In mouse allograft experiments, long-term engraftment of transduced and FACS-enriched BM cells along with hematopoietic cell marking has been demonstrated using CD24[64] and CD5[66] as selectable markers. However, it must be noted that those experiments did not include a control in which no pre-selection was applied prior to engraftment. This makes it difficult to unequivocally assess the contribution of pre-selection to the engraftment and repopulating ability of transduced cells.

Despite positive results in pre-clinical settings with the use of FACS for enrichment, it has more detrimental effects on cell survival, viability, and function than MACS, even though FACS can lead to higher purity[68]. It is also difficult to physically and/or temporally achieve enrichment of large numbers of clinically-applicable cells by FACS. As such, MACS and analogous schemas are preferred for enrichment prior to engraftment in patients. Over 90% purity has been achieved with MACS enrichment of ΔLNGFR-marked HSCs *in vitro*[60] and similarly marked lymphocytes in clinical trials[69-71]. In an allograft experiment with a mouse model of Fabry disease, BM mononuclear cells were transduced with a therapeutic vector capable of co-expressing α-galactosidase A and huCD25[67]. Pre-selection of transduced cells by MACS led to a long-term increase of huCD25-marked peripheral blood mononuclear cells when compared to controls. Therapeutic benefit of pre-selection was demonstrated by a higher α-galactosidase A activity in plasma and most organs. Additionally, the utility of pre-selection in long-term HSC marking has been demonstrated in some cases by secondary transplant experiments[64,67].

Since pre-selection strategies reduce the size of the transduced cell population[60], however, their application to HSC gene therapy can be critically limited if there are difficulties in collecting large numbers of patient HSCs. *Ex vivo* expansion of HSCs is currently not a viable solution in order to compensate for reduced cell numbers since over-activation can have detrimental effects on their "stemness" and engraftment potential[31]. In addition, pre-selection increases time of *ex vivo* manipulation, which increases costs and risks of contamination. It is therefore difficult to obtain a post-selection yield high enough to exert a therapeutic effect. Nevertheless, these studies demonstrate the benefit of enriching transduced cells *ex vivo* and that clinical translation may be augmented by higher yields of HSCs during their acquisition.

IN VIVO CHEMO-SELECTION STRATEGIES

Various proteins have been shown to grant variable degrees of chemoprotection in the context of cancer therapy, such as ATP-binding cassette, sub-family B, member 1 (ABCB1), dihydrofolate reductase (DHFR), and O^6-alkylguanine DNA alkyltransferase (MGMT). Overexpression of these proteins in HSCs has been pursued with the aim of protecting the hematopoietic compartment from the severe toxicity of many cytotoxic drugs used in cancer chemotherapy[72].

Pan-resistance to chemotherapeutic agents using ABCB1

ABCB1 [also known as multidrug resistance protein 1 (MDR1); or P-glycoprotein 1 (P-gp1)] is a cell membrane transporter with broad specificity that pumps foreign compounds out of the cell and is also involved in lipid translocation[73,74]. ABCB1 mediates chemoresistance in cancer cells in which its expression is upregulated[75]. Overexpression of ABCB1 in murine BM was shown to confer protection to many chemotherapeutic agents such as vinblastin, doxorubicin, daunomycin, taxol, vincristine, etoposide, actinomycin D, colchicine, and paclitaxel[76,77]. Early studies with mouse allografts showed *in vivo* selection of hematopoietic cells derived

from *ABCB1*-overexpressing HSCs, but it is unclear whether selection occurred at the stem cell level[78,79]. Later studies demonstrated successful selection of human HSC-derived cells in the BM of murine xenograft models[80,81]. In contrast to these outcomes, early autograft experiments in large animals and clinical trials demonstrated rather disappointing results. In a canine model, high toxicity was documented despite long-term ABCB1$^+$-peripheral blood cell enrichment in the only surviving animal[82]. In a study involving non-human primates, there was low initial ABCB1 cell marking, drug-induced neutropenia, and no significant increase of neutrophil counts after drug treatment[83]. In clinical trials, selection after drug treatment has been low and predominantly transient, albeit with little or no toxicity[84-87]. The inefficacy of ABCB1-mediated selection may have been due to insufficient expression of the transgene in hematopoietic cells[88]. Onco-retrovirally-mediated expression of *ABCB1* was found to be unstable due to cryptic splice sites within the cDNA[89]. This issue was resolved by introducing a silent mutation that inactivates that splice site, which subsequently increased expression of onco-retrovirally-delivered *ABCB1*[90,91]. Nevertheless, the robustness of this system must be reliably demonstrated in large animal models before it can be considered a feasible strategy to enrich HSCs after transplant for clinical gene therapy in patients.

Antifolate resistance using mutant DHFR

DHFR catalyzes the reduction of dihydrofolate to tetrahydrofolate, a precursor required for the *de novo* synthesis of purines and some amino acids. Antifolate drugs such as methotrexate (MTX) and trimetrexate (TMTX) inhibit DHFR activity, thus blocking cell proliferation and promoting apoptosis in dividing cells. HSCs and myeloid progenitor cells, however, can employ nucleotide salvage mechanisms to escape antifolate toxicity[92]. In order to overcome this, the nucleoside transport inhibitor nitrobenzylthioinosine 5'-monophosphate (NBTI/NBMPR-P) has been used in combination with MTX or TMTX[92]. Transplanted HSCs have been engineered to overexpress mutant forms of DHFR, such as DHFR^{L22Y}, that are resistant to antifolate agents[93]. *In vivo* enrichment of transduced HSCs in murine allogeneic transplants has been demonstrated[94,95]. However, translation of this method into large animal models has been rather discouraging. In a study in rhesus macaques that used a recombinant onco-retrovirus to deliver DHFR^{L22Y}, enrichment of cells derived from the transduced graft was only transient, indicating poor selection at the HSC level[96]. To address this problem, enrichment of CD34$^+$ progenitor cells in a xenograft transplant of human embryonic stem cells (hESCs) into mice has been demonstrated, though this enrichment was only modest and no clinically established methods to transplant hESCs exist[97]. High toxicity and lethality has been documented in antifolate-mediated selection studies in dogs and rhesus macaques[96,98]. Additionally, antifolate toxicities are well documented when such compounds are indicated for treatment of cancer patients[99-102]. These toxicities and the lack of positive evidence suggest that DHFR-mediated *in vivo* selection may not be useful for HSC gene therapy targeting monogenic diseases. Instead, it may be better suited to prevent graft rejection after HSC transplants, because antifolates would spare highly-proliferating T lymphocytes arising from transduced donor HSCs while eliminating alloreactive recipient T cells as shown recently *in vitro*[103] and in a canine model[104].

Selectivity using O^{6}BG-resistant MGMT

MGMT repairs DNA damage by removing adducts from the O^6 position of guanine, and thus confers resistance to the cytotoxic effects of alkylating agents such as dacarbazine, temozolomide (TMZ), procarbazine, and nitrosoureas such as 1,3-bis-(2-chloroethyl)-1-nitrosourea (BCNU)[105,106]. MGMT is expressed at very low levels in the BM[107,108]. MGMT overexpression was attempted in murine[105,109,110] and human[105] HSCs to confer BCNU resistance. A modest increase in resistance to BCNU was achieved in murine progenitors, both *in vitro* and *in vivo*[105,109,110]. Human HSCs, however, had poor resistance *in vitro*[111]. In order to sensitize the HSC compartment to alkylating drugs to achieve better *in vivo* selection, BCNU or TMZ have been co-administered with O^6-benzylguanine (O^6BG), a pseudosubstrate that irreversibly inactivates endogenous MGMT[112]. O^6BG-resistant mutants MGMT^{P140K} and MGMT^{G156A} have also been studied[113]. The former is more commonly used in selection strategies, despite having a modest reduction in its DNA-repair activity compared to the wild-type enzyme[113]. Indeed, MGMT^{P140K} has been shown to mediate selection of transduced HSCs in murine and canine allograft and autograft models[114-117], and in murine xenograft models with human HSCs[30,118,119]. However, high dose administration of BCNU and/or TMZ has been shown to cause toxicity[120-122]. For example, selection experiments have shown up to 75% mortality in mice treated with TMZ[120] or BCNU[121], and 88% mortality in rhesus macaques treated with TMZ[122].

Optimization of drug dosing by co-administering high doses of O^6BG with low doses of BCNU or TMZ has partially ameliorated the cytotoxic effects of the alkylating drugs and allowed better engraftment of HSCs transduced at low MOIs[121,123,124]. The improvements in survival are thought to be a result of lowering the threshold of MGMT expression required for resistance, which allows partial fulfillment of conditions expected in clinical trials. This dose-adjusted protocol has conferred successful chemoprotection of the hematopoietic compartment in a canine[125] and a nonhuman primate model[126], with no significant toxicity reported in the former. Despite survival of the macaques in the latter study, administration of chemoselective agents led to substantial peripheral blood cell depletion and enrichment of different blood lineages was highly variable[126]. In the same study, use of a multi-

cistronic vector to co-express C46, which is an anti-HIV transgene, MGMT^{P140K}, and enhanced green fluorescent protein (eGFP) resulted in lower selective potential[126]. From a translational point of view, the risk-to-benefit ratio is currently not in favor of implementation of such chemoselective strategies though further adjustments to drug regimens can be done.

In the context of gene therapy for murine models of β-thalassemia[123], hemophilia A[124], and hemophilia B[127], amelioration of the disease phenotype has been enhanced with the use of bicistronic LVs encoding the therapeutic gene and an MGMT^{P140K}-based selection module. Additionally, increased expression of the therapeutic gene after drug selection in secondary[123,124] and tertiary[127] recipients of serial BMT demonstrated enrichment at the HSC level. MGMT-mediated enrichment of eGFP^{+} BM and peripheral blood cells has also been demonstrated in a murine model[128]. Despite these promising results, there are still no reports of successful MGMT-mediated selection following gene therapy in large animals that we are aware of, wherein HSC selection may be less efficient because of their lower replication rates[129]. However, autologous MGMT^{P140K}-transduced HSC transplants have been attempted in MGMT^{hi}, TMZ-resistant glioblastoma patients[130]. Drug selection resulted in no significant extra-medullary toxicity and all three participants surpassed the median survival (12 mo) for glioblastoma patients with unmethylated MGMT-promoter status. Despite these promising results in the context of chemoprotection of the hematopoietic compartment during glioblastoma treatment[130], it must be noted that gene-modified circulating blood cells were depleted from the patients with termination of treatment. As such, repeat administration of chemotherapy may be required for the use of this system for amplification of transduced HSCs for gene therapy.

Hypoxanthine-guanine phosphoribosyltransferase inactivation for 6-thioguanine resistance

While the strategies described above rely on the overexpression of a protein that confers chemoprotection to transduced cells, down-regulating endogenous enzymes necessary to activate cytotoxic drugs can achieve analogous outcomes. Hypoxanthine-guanine phosphoribosyltransferase (HPRT) is an enzyme involved in the purine nucleotide salvage pathway. HPRT can catalyze the addition of ribose 5-phosphate to the purine analog 6-thioguanine (6TG) to generate thioguanosine monophosphate (thio-GMP)[131]. Thio-GMP is then converted into thiodeoxyguanosine triphosphate (thio-dGTP), which can be incorporated into DNA inducing futile mismatch repair and consequent apoptosis. It has been shown that BM cells of HPRT-deficient mice are resistant to 6TG treatment[132]. Transplantation of HPRT-deficient BM into wild-type HPRT mice under 6TG selection resulted in good engraftment and long-term hematopoietic reconstitution in primary and secondary recipients[133]. Furthermore, transduction of murine hematopoietic progenitor cells with a LV encoding a short-hairpin RNA (shRNA) that targets HPRT can confer resistance to 6TG *in vitro*[134]. The same knockdown strategy has shown effective enrichment in murine allograft models[135] and human HSC xenograft models[136]. This approach has some advantages over other drug selection methods described above. 6TG can be used for both pre-conditioning and chemoselection, and the shRNA sequence is very short, which makes it easier to include in a dual-gene vector. Also, ample information about 6TG dosage and toxicity is available because it has been used in the clinic for decades[137]. A recent study suggests, however, that this method may be limited to enrichment of committed progenitor cells, which would decrease long-term efficacy with single-dosing regimens[136]. Also, hereditary HPRT deficiency is the cause of Lesch-Nyhan syndrome, which has been associated with megaloblastic anemia[138]. Therefore, it is necessary to carefully assess long-term consequences of HPRT deficiency in the hematopoietic lineage, especially in large animal models. Nevertheless, selective induction of an enzyme deficiency, as demonstrated by virally-induced HPRT knockdowns, may be a powerful method of introducing selective pressure following gene therapy.

ENGINEERED INDUCIBLE GROWTH AND SELECTION MODULES

Cytotoxic chemical inducers of dimerization

Discoveries relating to functional consequences of forcing proteins such as receptor tyrosine kinases into proximity with each other have allowed the use of protein engineering to confer specific biological characteristics to a subset of modified cells with exposure to various stimuli. For example, chemical inducers of dimerization (CIDs) are synthetic compounds that can be used to induce dimerization of proteins that are expressed as fusions to CID-binding domains (CBDs). With the use of CBDs, cell-fates can be made dependent upon the addition of CIDs (Figure 2A). One of the first examples of such a system utilized FK1012, a synthetic dimer of the immunosuppressant FK506 (Tacrolimus)[139]. Proteins that can modulate cell biology, including proliferation[140] and apoptosis[141], have been engineered from growth factor receptors and the FK506 binding domain from FK506-binding protein (FKBP12). However, FK1012 retains the ability to bind endogenous FKBP12[139]. This is undesirable from a clinical point of view because endogenous FKBP12 could sequester the drug, preventing its intended effect. In addition, FK1012 administration could affect the normal physiological role of FKBP12[142-145]. As a result, the use of these systems has been limited, though thorough clinical evaluation of the drug has yet to be completed. However, amplification protocols can be envisioned wherein the underlying toxicities of FK1012, which are expected to be similar to FK506, are exploited. Future systems can be developed using other cytotoxic agents with their respective binding targets as CBDs fused to survival or growth signaling

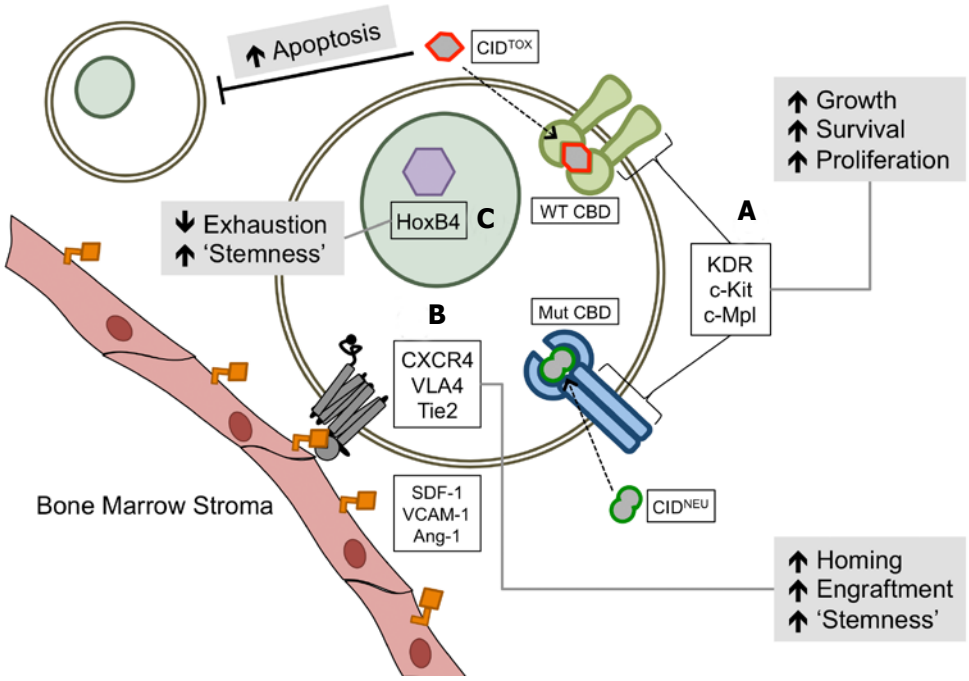

Figure 2 Summary of next-generation amplification modules. A: Fusion proteins comprised of chemical inducer of dimerizations (CBDs) such as FKBP12 (WT CBD) or F36V (Mut CBD) and receptors involved in hematopoietic stem cells (HSC) growth, proliferation, and survival. Activation of signaling by CIDs allows expansion of the transduced population. The use of cytotoxic CIDs (CID^{TOX}) can allow simultaneous depletion of the non-transduced population. FK1012 is a putative cytotoxic CID-binding domain. Examples of inert or neutral CIDs (CID^{NEU}) include AP20187 and AP1903; B: Controlled overexpression of HSC homing and adhesion molecules can increase the potential for therapeutic cells to survive and can promote long-term engraftment. Examples of such molecules include but are not limited to CXCR4, VLA4, and Tie2. Their corresponding ligands (SDF-1, VCAM-1, and Ang-1, respectively) are usually expressed on osteoblasts, osteoclasts, MSCs, and other cells that make up the bone marrow stroma; C: Downstream effectors of key signaling pathways involved in maintaining HSC phenotypes that are down-regulated during *ex vivo* handling of CD34$^+$ cells can be reconstituted to prevent stem cell exhaustion and to increase long-term engraftment of transduced cells. HoxB4 is an example of a transcription factor that is activated in response to Wnt signaling and is key to maintenance of the stem phenotype of HSCs.

factors[146]. Careful dosing can allow for simultaneous depletion of non-transduced cells and expansion of the transduced population (Figure 2A). Such studies have yet to be performed in clinically-relevant settings. Conversely, the application of such current systems remains risky as the continuous use of cytotoxic agents in general can have detrimental effects on patient quality of life. A potential solution is to aim for selective pressure to be applied to more mature cells, where dose reduction can be envisioned whilst clinical benefit is still achieved.

Neutral CIDs

Reverse-engineering of proteins and drugs that bind to them have led to numerous other CIDs and their respective CBDs. Progress in understanding the modularity of protein signaling has yielded numerous opportunities to generate CBD-signaling domain fusions. From a gene therapy perspective, ideal CIDs are those that would have little to no effect on any cells other than the transduced population. Ideal CBDs should also have no effect on the biology of transduced cells without the presence of a CID. As well, engineered polypeptides with CBDs should be derived from endogenous proteins where possible to minimize the potential immunogenicity of the fusions. In addition, unexpected effects of CID-induced dimerization of CBD fusion proteins should not occur in cells derived from transduced HSCs. "Bump and hole" engineering of FK1012 and FKBP12 has yielded derivative CIDs such as AP20187 (B/B homodimerizer; Takara) and AP1903 (Rimiducid; Bellicum)[147]. Such CIDs are chemically modified to prevent them from binding the original CBD. The derivative CBDs, such as FKBP12^{F36V} (F36V) for AP20187 and AP1903, have mutations that confer ability to bind the engineered CIDs[147] (Figure 2A). These modifications allow such CID-CBD systems to be acceptable for use in gene therapy, as outlined above.

Amplification of HSCs using neutral CIDs

Multiple studies have been performed in cell, mouse, and canine models that utilize AP20187 and protein receptors fused to F36V[148-154]. Our lab has previously shown the utility of a kinase insert domain receptor (KDR/CD309)-F36V fusion to control cell-fate in an AP20187 dependent manner, and we characterized the molecular mechanisms that are induced as a consequence of KDR dimerization in TF1 cells[150]. The utility of this system has yet to be demonstrated in an animal model of HSC engraftment. The characterization of HSC cells and their signaling components have yielded other targets for F36V fusion and CID-mediated control of cell fate, such as c-Kit (SCF receptor)[155], and c-Mpl (thrombopoietin receptor)[149,152,156] (Figure 2A). It must be noted that these signaling components are not fully

unique to the HSC compartment and subsequent risk of unwanted proliferation can exist. That said, restricted expression of CBD-receptor fusions using HSC-specific promoters in gene transfer vehicles could reduce effects on non-target cells.

A long-term study in canines using an engineered thrombopoietin receptor, Mpl-F36V, has demonstrated the utility of intermittent use of AP20187 and AP1903 administration over the course of a number of years with no effect on the normal physiology of the dogs and no effect on the HSC compartment without drug administration[152]. However, this study only involved long-term monitoring of two dogs and did not test the utility of such a system in a reconstitutive gene therapy context. Studies in healthy human volunteers (see below) may need to be conducted to unravel the effects of long-term administration of AP20187 in order to inform the FDA prior to administration in patients.

Unlike AP20187, AP1903 has gained more momentum with respect to its translation into the clinic. It induces dimerization through the same CBD as AP20187, extending the utility of studies that use F36V fusions[150-156]. Most importantly, a phase I clinical trial in which a single infusion of AP1903 was administered to 28 healthy volunteers at a range of doses has already been conducted[157]. No relevant adverse effects were observed at any of the doses. Furthermore, donor T cells that are genetically modified with a CID-inducible caspase-9 (iCasp9) suicide system have been administered to leukemia patients to enhance immune reconstitution in recipients of allogeneic HSC transplants[158,159]. A single dose of AP1903 was sufficient to ameliorate the GVHD. T cell counts were reduced in as little as 30 min after drug administration followed by resolution of GVHD symptoms in 24 h. Further administration of AP1903 is being tested in patients receiving cell products that have been gene modified with iCasp9 (www.bellicum.com). The dosing for these patients has been informed by the pharmacokinetics observed in healthy volunteers[157]. Long-term follow-up of patients from one study has been published with up to 4-year occurrence-free survival of patients receiving iCasp9-modified T cells and AP1903[160]. However, it must be noted that repeated administration of the drug and associated safety profiles have not been published to the best of our knowledge. This is mainly because single-administration of the drug resolved symptoms in the studies conducted thus far and eliminated the need for further intervention[158,159]. Additionally, no information about biodistribution and pharmacogenomics of AP1903 in humans is available - that we are aware of. As such, the drug is still considered experimental and there is no prior knowledge about its efficacy in the bone marrow niche other than that which has been observed in animal models[152,161]. Further studies conducted in healthy volunteers to assess a full set of biological parameters can boost the clinical development of enrichment modules relevant to HSC-directed gene therapy.

Engineering alternative amplification modules

While FDA approval and consequential routine administration of existing CIDs in patients may be achievable in the future, there is value in stepping away from sole confinement to FKBP12-derived systems. Multiple other technologies exist that utilize either compounds known to be benign along with reverse engineered signaling domains or compounds and their respective binding domains derived from other species. For example, a recently demonstrated degron-system that uses an auxin-inducible domain derived from plants[162] could be engineered to enhance Wnt signaling in HSCs by targeting axin degradation in an auxin analog-dependent manner[163]. Mouse studies have shown no effect of this analog on normal physiology[164]. In addition, reverse engineering of G-protein-coupled receptors (GPCRs) have led to the development of a variety of Designer Receptors Activated by Designer Drugs (DREADDs)[165]. One DREADD has been designed to mediate chemotaxis of monocytes and neutrophils in a drug-dependent manner and highly promising results have been demonstrated *in vivo*[166]. This DREADD is activated in response to Clozapine-N-oxide (CNO), a benign metabolite of the clinically-approved anti-psychotic agent, Clozapine[167]. With our growing understanding of protein modularity, it is not difficult to envision the development of a similar DREADD that potently activates HSC-specific survival and proliferative signals. Development of systems that utilize compounds with known pharmacokinetics and biodistribution that can be fast-tracked for FDA approval can provide a significant boost to *in vivo* enrichment strategies for HSC-directed gene therapy.

MODULES TO ENHANCE ENGRAFTMENT OF THE TRANSDUCED HSC SUB-POPULATION

HSCs are housed in specialized compartments in the BM. They use cell surface receptors and ligands to anchor themselves in their niche and to modulate signals for long-term survival and self-renewal[4,168]. Roles of multiple signaling pathways have been elucidated; as well, cell surface proteins that can demarcate HSCs as single cells that can stably engraft and that are capable of reconstituting the entire hematopoietic system have been uncovered[169]. It is therefore not difficult to envision novel clinical roles for these cell surface proteins. For example, plerixafor (Mozobil, Sanofi) is a drug that was developed to disrupt the interaction of C-X-C chemokine receptor type 4 (CXCR4, fusin) and C-X-C motif chemokine 12 (CXCL12, or SDF-1)[170]. Greater numbers of $CD34^+$ cells can be acquired with administration of plerixafor over the traditional mobilization strategy that uses granulocyte-colony stimulating factor[171,172]. Recently, experiments have shown the potential of another drug, Bortezomib (Velcade, PS-341), in HSC mobilization *via* disruption of very late antigen 4 and

vascular cell adhesion molecules (VCAM-1) in mice[173].

Enhancement of engraftment can also be envisioned using information resulting from such aforementioned studies. Specifically, interactions that can be disrupted for HSC mobilization can also be used to increase engraftment potential. To this effect, studies have shown stage-specific roles for CXCR4 in BM homing and survival of engrafted HSCs[174,175] (Figure 2B). In addition, the benefit of virally-mediated CXCR4 transgene expression for HSC engraftment into a humanized mouse model has been shown with *ex vivo*-cultured human $CD34^+$ cells[176,177]. Concerns of long-term side effects of CXCR4 expression, such as diminished repopulating potential of the transduced cells and off-target expression in non-HSCs, still remain. For example, high CXCR4 expression is associated with worse prognosis in acute myeloid leukemia, amongst other cancers[178,179]. Developments in transient cDNA introduction, conditional control of transcription, and/or robust, tissue-specific control of expression may minimize these concerns, though they remain to be tested. Should such technologies be developed in a clinically-relevant manner, multiple other cell surface targets for enhancing HSC homing, survival, and "stemness" could be utilized, such as Tie2, VLA4, and c-Kit[168,180-182] (Figure 2B). Other methods relying on expression of downstream components of survival and self-renewal signaling such as homeobox protein Hox-B4 (HoxB4) can also be envisioned to be employed in a controlled manner[183] (Figure 2C). Though introduction of transgenes may provide the ultimate solution to these challenges, other groups are focusing on development of small molecules and co-injection protocols as alternative methods; such as co-injection of BM-derived mesenchymal stem cells[184-186].

LINEAGE-SPECIFIC ENRICHMENT

As discussed previously, it can be conceptually difficult to target therapeutic HSCs for *in vivo* amplification due to the unique microenvironment in the BM niche in which they reside and because of their inherently quiescent nature. Whilst it is generally agreed that the most clinical benefit can be obtained by targeting long-term precursor cells, it is not conclusively proven to be so. Examples of gene therapy that result in enrichment of cells derived from transduced HSCs, such as in SCID-X1 and β-thalassemia, provide proof-of-concept for the clinical utility of targeting a subset of the mature hematopoietic compartment for selection[7,17,43-47]. Application of enrichment strategies in such a manner can overcome many of the hurdles of attempting to enrich the original HSC engraftment, such as bioavailability of the compounds used for enrichment and toxicities towards the core center of hematopoiesis. In addition, targeting mature cells, and more importantly, excluding the HSC compartment, reduces the chances of long-term complications in patients that have already gone through a risky intervention and reduces the likelihood of sporadic malignant disease arising from alterations in "stem-like" progenitor cells. One conceptual disadvantage of such a targeted system is the lack of persistence of selection, since mature hematopoietic cells will be replaced over time. Enrichment protocols can be developed, however, that utilize either drug dosing that has little or no toxicity in normal cells, or compounds that specifically act on cells that arise from transduced HSCs. As such, continuous administration of the respective agent can provide for prolonged enrichment with minimal or no side effects.

IMPDH2 mutants for MMF resistance in T/B cell progeny

Inosine monophosphate dehydrogenase 2 (IMPDH2) is the rate-limiting enzyme involved in the *de novo* biosynthesis of guanosine monophosphate (GMP)[187]. While most cells in the body have a salvage pathway, T cell activation and proliferation as well as B cell maturation are highly dependent upon this biosynthetic pathway[188,189]. Mycophenolic acid (MPA) is a potent, non-competitive, reversible inhibitor of IMPDH2[190-192]. Its prodrug, Mycophenolate Mofetil (MMF, Roche), is routinely used in the clinic as an immunosuppressant to control GVHD amongst other indications[193]. Mutants of IMPDH2 that have diminished binding affinity for MPA have been described[194,195]. The most potent amongst these is the combination of T333I and S351Y ($IMPDH2^{IY}$). The utility of this double mutant has been demonstrated in the context of donor T cell selection, both *in vitro* and in mouse models using primary human T cells[196,197]. It should be noted that the total lymphocyte count in these experiments was dramatically lowered with MMF treatment, reducing the benefit of such a system with respect to T cell gene therapy[197]. The dosage of drug used in that study, however, was considerably higher than that used to treat patients for GVHD. The effect of low-dose MMF treatment on engrafted cells expressing $IMPDH2^{IY}$ has yet to be shown. In addition, it has been demonstrated that there is no biological effect of constitutive expression of this mutant enzyme on HSC differentiation[194]. Therefore, use of such an enrichment strategy could exclude HSCs and all hematopoietic progeny other than T and B cells from being affected. That said, previous work has only described the use of $IMPDH2^{IY}$ for application in T cell-related disorders, such as HIV treatment and prevention of GVHD[194,197]. To our knowledge, *in vivo* use of this enrichment module in *HSC* gene therapy has not yet been demonstrated.

CID-dependent enrichment of gene modified progeny

Numerous examples exist of receptor-CBD fusions that can provide a proliferative advantage to subsets of mature cells. Most recently, an erythropoietin receptor (EpoR)-F36V fusion has been developed for use in facilitating AP20187-dependent erythropoiesis[198]. The fusion is engineered with the minimal components of EpoR required for dimerization-induced signaling along with a myristoylation signal. The fusion is expressed under the control of an erythrocyte-specific promoter. The goal of that study was to design a system to

replace the necessity of recombinant Epo administration in anemic patients. The authors have successfully demonstrated CID-dependent erythropoiesis *in vivo* in a mouse model[198]. Though this group has not shown the utility of their system in the context of HSC gene therapy, it can be postulated that it would be applicable for enhancement of treatment for disorders that affect erythropoiesis. It must be noted that such systems are hindered to date in their clinical translation due to their use of clinically-unavailable CIDs. Future studies utilizing clinically-available compounds and a variety of lineage specific growth signals are anticipated.

DISCUSSION

Safety of LV-mediated gene therapy

With the initial implementation of recombinant onco-retroviruses in gene therapy strategies, an emergent obstacle to be considered when genetically modifying long-term, stem-like cells is the potential for the development of malignancy[4,15,23-25]. As discussed above, LVs greatly diminish the likelihood of integration near known oncogenes or tumor suppressors[4,26-30]. However, multiple other mechanisms that can lead to gross cellular aberrations or changes in function of cells derived from transduced HSCs are still of concern. Though improbable, integrative modification of gene loci can lead to alternative splicing of putative oncogenes, or to the insertional inactivation of tumor suppressors[199,200]. Additionally, a comprehensive understanding of oncogenesis has yet to be achieved in all its different forms, especially within the complex network of cells in the hematopoietic system. As such, continuation of long-term studies investigating the effects of transplanting patients with transduced HSCs is a necessity, especially with new knowledge being acquired regarding the functional importance of intergenic "junk" DNA.

One strategy to circumvent putative side effects of HSC gene therapy is to include suicide modules (or "cell-fate control" systems) in the transfer vector. Suicide modules refer to elements of therapeutic vectors that are capable of inducing specific cell-death of transgenically modified cells. This is especially important when considering the inclusion of amplification modules, which have not been thoroughly tested in patients. These systems can be designed to induce cell death by providing a surface target for antibodies, by inclusion of an inducible component that activates the apoptotic pathway, or by being able to activate a normally non-toxic prodrug. For example, there are multiple CID-based systems that bring together components of the apoptotic-signaling pathway[158,159,201-203]. One of these, iCasp9, is currently being tested in patients receiving haploidentical donor T cell infusions amongst other indications, as discussed previously, though the CIDs being used have yet to acquire FDA approval[158,159]. Additionally, such systems have been tested for their ability to eliminate autologous HSC engraftments in rhesus macaques[204].

The ability to conditionally activate prodrugs has been a useful tool in molecular biology to induce killing of subsets of cells, though many of the enzymes used are derived from other species. For example, thymidine kinase (tk), derived from the herpes simplex virus (HSV-tk), can be used to render cells sensitive to the drug ganciclovir, a commonly employed laboratory technique[205]. HSV-tk has been used to ameliorate GVHD in patients receiving allogeneic transplants and in anti-tumor suicide gene therapy[71,206-209]. However, use of this system is limited by concerns of immunogenicity of non-human proteins that can cause elimination of otherwise useful cells[56,57]. Additionally, ganciclovir and acyclovir are commonly prescribed for viral infections following engraftment, and use of HSV-tk can lead to unintended elimination of transduced cells[210]. Our lab has developed a fusion protein comprised of the extracellular and transmembrane components of LNGFR (CD271) along with an engineered variant of human thymidylate kinase[211]. This module combines the advantages of being able to overexpress a cell surface marker for tracking transduced HSCs and their progeny, since CD271 expression is absent in circulating blood cells, and the ability to activate azidothymidine to a toxic form.

Stem cell exhaustion and clonal selection

One of the principal advantages of *in vivo* selection is the potential for an increase in therapeutic benefit from an initially lower number of transduced repopulating cells. Yet, proliferative stress on few selected HSCs can occur, resulting in a gross negative, long-term impact on HSC proliferation and lineage differentiation. This is termed "stem cell exhaustion" and can eventually lead to BM failure in recipients. Such concerns have been studied in mice[212] and dogs[213] that underwent serial $MGMT^{P140K}$-expressing HSC transplantation under prolonged O^6BG/BCNU treatment. Importantly, these studies revealed no apparent impairment in HSC repopulation, proliferation, or differentiation, suggesting that stem cell exhaustion may not be an issue, at least in the context of that mode of chemoselection. Further studies in long-lived, clinically-relevant models need to be conducted, however, especially in the context of HSC gene therapy, to demonstrate lack of long-term exhaustion within primary autologous recipients. *Ex vivo* selection may also be achievable without stem cell exhaustion with the co-expression of factors that maintain HSC "stemness", such as HoxB4[183].

In vivo drug selection can also exacerbate clonal dominance, a phenomenon readily observed with the use of recombinant onco-retroviruses. Amplification strategies could augment the proliferative advantage of cells with proviral integration sites in or near proto-oncogenes, risking the development of hematopoietic malignancies. For example, analysis of tertiary MGMT-transduced BM recipients showed only 17 unique retroviral integration sites (RIS) following chemo-selection[212]. Most RIS in that study were in or near

genes involved in important cell regulatory processes, such as cell growth, cell development, and/or cell differentiation. However, this observation may be an artifact of the use of derivatives of murine stem cell retrovirus. In contrast, studies in canines and humanized mice that utilized recombinant LVs, and a mouse allograft study utilizing a foamy viral vector, showed no observable evidence of clonal dominance following chemoselection[30,213,214]. Taken together, current data suggests that stem cell exhaustion and clonal dominance are unlikely to occur with amplification strategies, especially with the use of recombinant LVs. Nevertheless, a persistent concern remains when considering HSC gene therapy in patients. As such, an excellent strategy to address issues surrounding *in vivo* amplification of transduced HSCs is to apply enrichment to mature hematopoietic cells, as discussed previously. However, more studies need to be conducted to demonstrate clinical efficacy and safety of amplification of mature cells subsequent to HSC gene therapy.

Future considerations

Many of the enrichment strategies that have been suggested or tested for use in gene therapy applications are designed to be expressed in a constitutively "on" manner. While this may increase the potency of the given strategy, unexpected secondary effects on normal physiology may occur. Therefore, conditional expression cassettes for clinical use should be developed in order to minimize unwanted expression of amplification modules. This is paramount when considering the use of highly engineered or trans-species proteins, which can, over time, elicit immune responses against target cells. In addition, to avoid similar issues at a genomic level, use of innate promoters, such as that derived from the elongation factor 1-alpha locus (EF1α), in contrast to virally-derived promoters, such as that derived from cytomegalovirus (CMV), should be considered for clinical application[215].

Greater focus in the gene therapy field is placed upon the improvement of gene delivery methods and, in the context of HSC gene therapy, efforts are being made to increase the efficacy of various aspects of HSC acquisition, engraftment, and patient care. However, there is limited research to develop next-generation strategies for other aspects of HSC gene therapy that can improve clinical efficacy of this treatment modality. Clinically-feasible strategies need to be developed that allow for selection or enrichment of transduced, therapeutic HSCs after engraftment. In addition, strategies that use other target cell types, such as MSCs, should also be considered for tandem gene therapeutics, to increase the efficiency of correction mediated by HSC gene therapy. More specifically, enrichment strategies that utilize clinically-approved compounds with known pharmacokinetics and pharmacodynamics, such as MMF, need to be developed. Such systems have the potential to be employed in the clinic more quickly and can allow for repeated administrations or continuous low dosing for long-term benefit. Dose adjustments can also be safely made to compensate for variability in patient pharmacogenomics. The development of modules that allow resistance to drugs used for the treatment of benign hematopoietic hyperplasias can encompass many of the aforementioned advantages.

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REVIEW

Multipotent pancreas progenitors: Inconclusive but pivotal topic

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Abstract

The establishment of multipotent pancreas progenitors (MPP) should have a significant impact not only on the ontology of the pancreas, but also for the translational research of glucose-responding endocrine β-cells. Deficiency of the latter may lead to the pandemic type 1 or type 2 diabetes mellitus, a metabolic disorder. An ideal treatment of which would potentially be the replacement of destroyed or failed β-cells, by restoring function of endogenous pancreatic endocrine cells or by transplantation of donor islets or *in vitro* generated insulin-secreting cells. Thus, considerable research efforts have been devoted to identify MPP candidates in the pre- and post-natal pancreas for the endogenous neogenesis or regeneration of endocrine insulin-secreting cells. In order to advance this inconclusive but critical field, we here review the emerging concepts, recent literature and newest developments of potential MPP and propose measures that would assist its forward progression.

Key words: Multipotent pancreas progenitors; Regeneration; Self-renewal; Clonogenesis; Differentiation

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Core tip: Diabetes mellitus is a pandemic health problem that currently affects approximately 400 million people worldwide and its incidence is increasing by 2%-3% per year. At present, insulin deficiency in diabetes is treated by exogenous insulin given as either multiple daily injections or continuous subcutaneous infusion (pump), which is associated with acute, potentially life-threatening metabolic disturbances as well as chronic, vascular complications with significant morbidity and mortality. The ultimate solution would therefore be regenerative therapies by which lost β-cells in disease processes could be restored/replaced by surrogate insulin-secreting cells including those derived from multipotent pancreas progenitors.

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INTRODUCTION

In contrast to what we would expect, modern humans encounter unprecedented health challenges, including the pandemic diabetes mellitus (DM), which is a major metabolic disorder worldwide with a progressively climbing incidence. This disease currently affects 387 million individuals, with one dying in every 7 s due to severe complications (http://www.idf.org/diabetesatlas). Among them, approximately 10% suffer from type 1 DM (T1DM), due to the absolute lack of glucose-responding β-cells destroyed by the patient's own immune system. Provided autodestruction of β-cells is under control, a permanent replacement approach may therefore be an ideal solution for T1DM, through regeneration *in situ* of endogenous β-cells, or by the replacement with donated glucose-responding islets, or of in *vitro* produced insulin-secreting β-cells from stem cell sources such as pluripotent stem cells (PSCs). The other 90% diabetic subjects are currently affected by type 2 DM (T2DM), resulting from the inability to react to insulin regulation by key metabolic tissues, the inability to regulate the generation of glucose from the liver and the dysfunction of endocrine β-cells[1]. The latter is typically believed to be cause by the increase of β-cell death[2]. Recently, accumulating evidence has suggested that β-cell dysfunction in T2DM is also caused by the dedifferentiation of glucose-regulating β-cells[3,4]. Thus, the ability to restore function of failed endocrine cells would provide a novel redifferentiative treatment for T2DM. In order to develop regenerative medicine therapies to T1DM and T2DM, the interest to both stem cells and progenitors in the pancreas has recently been progressively increased.

In general, the concept of multipotent progenitors is exchangeable with that of stem cells. The latter are usually used to define undifferentiated primitive cells that have the capacity for self-renewal themselves as well as of differentiation into terminal functional cells. For example embryonic stem cells (ESC) and induced pluripotent stem cells (iPSC, both together known as PSC) are able to produce all types of 210 cells that build up the body. Debatably, progenitors with multipotency are stem cells. Progenitors could be multipotent, oligopotent or unipotent based on their developmental potentials. Multipotent progenitors/stem cells are of fundamental importance to normal physiology and to regeneration during disease/injury repair. Multipotent pancreas progenitors (MPP) would be a rare subset present in developing and adult pancreas, and have a capacity for regeneration when required, even though their location and origin have not yet been completely established and are still controversial. In this article, we will summarize knowledge on the candidate MPP along the natural route of endocrine pancreas development and in three functional components of the pancreas. We will also propose future research perspectives on the potential MPP.

CLASSICAL EMBRYOLOGY

The pancreas is an important digestive and endocrine organ originating from the endoderm of posterior foregut. The latter along the other two germ layers is derived from the inner cell mass and would develop into the pancreas primordia when a localized area of thickened columnar epithelia along the dorsal and ventral posterior foregut appears. The columnar endodermal layer evaginates into the neighboring mesenchymal tissues of mesoderm origin and gives rise to the dorsal and ventral buds of the pancreas, respectively. Whilst continuously proliferating and branching, two buds gradually fuse together due to the gut rotation movement. Subsequently, the primordial pancreas continue to expand, transform and, finally, differentiate into the mature organ. The mature pancreas is composed of the acinar compartment that secretes digestive enzymes and fluid, that are drained into the intestinal lumen by the pancreatic ducts and, the endocrine pancreas that secrete hormones (glucagon, insulin, somatostatin, ghrelin or pancreatic polypeptide) responsible for maintaining normal glucose metabolism.

The key developmental events in the human pancreas are different from what we know about from developing pancreas in mice[5]. For instance, human dorsal bud is detectable at 26 d post conception (dpc), which is an equivalent age of embryonic day (E)9.5 in mouse embryos. The cellular developmental sequences in humans also differ from that in mice. Although they are not visible until 52 dpc, approximately 2 wk later than the equivalent stage at which they can be detected in mice, insulin-positive β-cells in humans appear precedent, unlike that in mice, to glucagon-positive α-cells at 8-10 wk of gestation[6]. All islet cells are detectable at the end of the first trimester in humans[6], but at later stages (E17.5) in mice[7]. Finally, the dynamics of gene expression during embryonic development and in pathophysiological conditions also differ between the two species[8]. Readers are referred to more reviews of human pancreas development elsewhere[9-12].

POTENTIAL MPP ALONG THE DEVELOPMENT OF ENDOCRINE PANCREAS

Remarkably, PSC can be guided to differentiate into definitive endoderm (DE)-like progenitors *ex vivo* by applying knowledge of *in vivo* developmental mechanisms (Figure 1). For example, this has been achieved

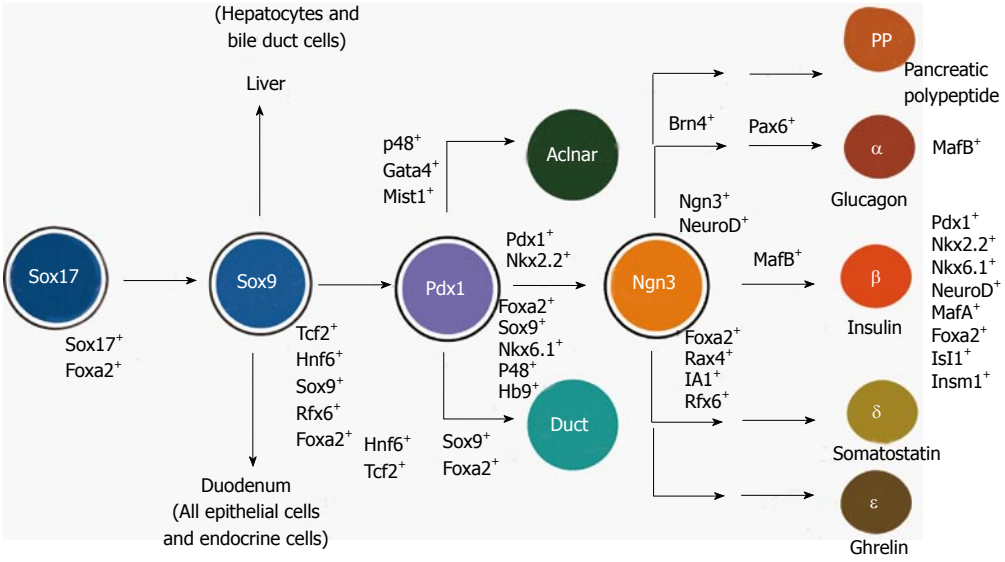

Figure 1 Multiple fate selections allow the development of the pancreas islet lineages. Committed from one of three germ layers (the ectoderm, mesoderm and endoderm) during gastrulation, the definitive endodermal cells (DE) are marked by the expression of Sox17 (the Sry-related HMG box transcription factor 17) and foxhead homeobox 2a (Foxa2). Along the anterior-posterior axis the DE is divided into foregut (giving rise to the lung, thyroid and esophagus), posterior foregut (PF), marked by the expression of the transcription factor hepatocyte nuclear factor 4a (Hnf4a) and hindgut (committing the intestine and colon). *In vitro*, retinoid acid would direct the DE cells to PF cells. Largely to the liver and duodenum, a fraction of the PF cells give rise to pancreatic progenitors (PP, marked by the expression of the transcription factor Pdx1). Mostly to the exocrine and ductal tissues, the PP commits to progenitors of the endocrine islet lineages [IP, marked by the expression of Neurog3, as well as neural differentiation 1 (NeuroD), insulinoma associated 1 (IA1), Islet 1 (Isl1), paired box factor 6 (Pax6) and Rfx6]. The IP then differentiates into at least five types of islet cells [α, β, δ (somatostatin), pancreatic polypeptide (PP) and ε (ghrelin)].

in culture by supplementing with a pharmacological level of activin A, a growth factor (GF) of the superfamily of the transforming GF β. Furthermore, human PSC-derived self-renewable DE-like cells have been reported to expand under the stimuli of four GFs[13]. These DE-like endodermal progenitors have proliferated over 24 passages with an astonishing increase in cell numbers. To establish their reliability and utility in developmental biology, drug screening and regenerative medicine, PSC-derived DE-like cells should be biologically and transcriptomically compared to embryo-derived DE cells. Although further research is required, these endodermal progenitors may indeed function as pre-MPP in addition to possessing an incredible capacity for expansion.

Sox9-expressing MPP

Cystic epithelial colonies expressing E-cadherin first were generated by our group (Figure 2). After dissociation, fetal pancreatic cells produced cystic colonies containing β-cells in the presence of the basement membrane glycoprotein laminin 1, 1, 1 and a bone morphogenetic protein[14,15]. Interestingly, spherical but not cystic colonies are generated in the presence of epidermal GF[16], suggesting these GFs play different roles during development. We have not investigated whether all or only some fractions of cystoid epithelial cells stochastically commits to different lineages, though it seems possible that these cystic colonies originated from multipotent progenitors that are Sox9$^+$ (see below).

Sox genes encode versatile regulators of stem/progenitor cell fate[17], belonging to members of transcription factor family that contain the Sry (sex determining region Y) box-related high-mobility group. Sox9 for example critically regulates the development of multiple embryonic organs including the pancreas. This pivotal transcription factor is first detectable at E10.5 in the dorsal and ventral pancreases[18] and at E13.5, Sox9-expressing progenitors (Figure 3) develop into the exocrine and endocrine lineages in the pancreas[19]. However, by E16.5 the expression of Sox9 is progressively restricted to pancreatic ductal cells[19]. Genetic tracing studies reveal that Sox9 is also expressed in organs of other posterior foregut origin such as the liver, the bile duct and the duodenum. For example, it is present in bile ductal cells adjacent to the portal vein from E16.5. Sox9 also is broadly detectable in the intestinal epithelia at E13.5 but confined to the crypt region from E18.5[19]. Thus All Sox9-expressing cells in the posterior foregut region could be MPP. Supportive to these analyses, Sox9-expressing (Sox9$^+$) multipotent progenitors purified from E11.5 Sox9-eGFP embryos generate expandable cystoid colonies that contain hormone-expressing cells in a laminin 1, 1, 1-enriched Matrigel-coated culture condition[20].

Sox9$^+$CD133$^+$ ductal cells generates duct-like "ring/dense" colonies (1/5) in the culture of the Matrigel-containing methylcellulose-based semisolid medium. With the addition of the roof plate-specific spondin 1, a Wnt agonist, these ring/dense colony-forming cells can be serially dissociated and replated in the presence of Matrigel with an expansion of more than 100000-fold[21]. In a laminin-containing hydrogel, the Sox9$^+$CD133$^+$ (cluster differentiation 133) cells are able to give rise to acinar/endocrine colonies[21]. Further investigations

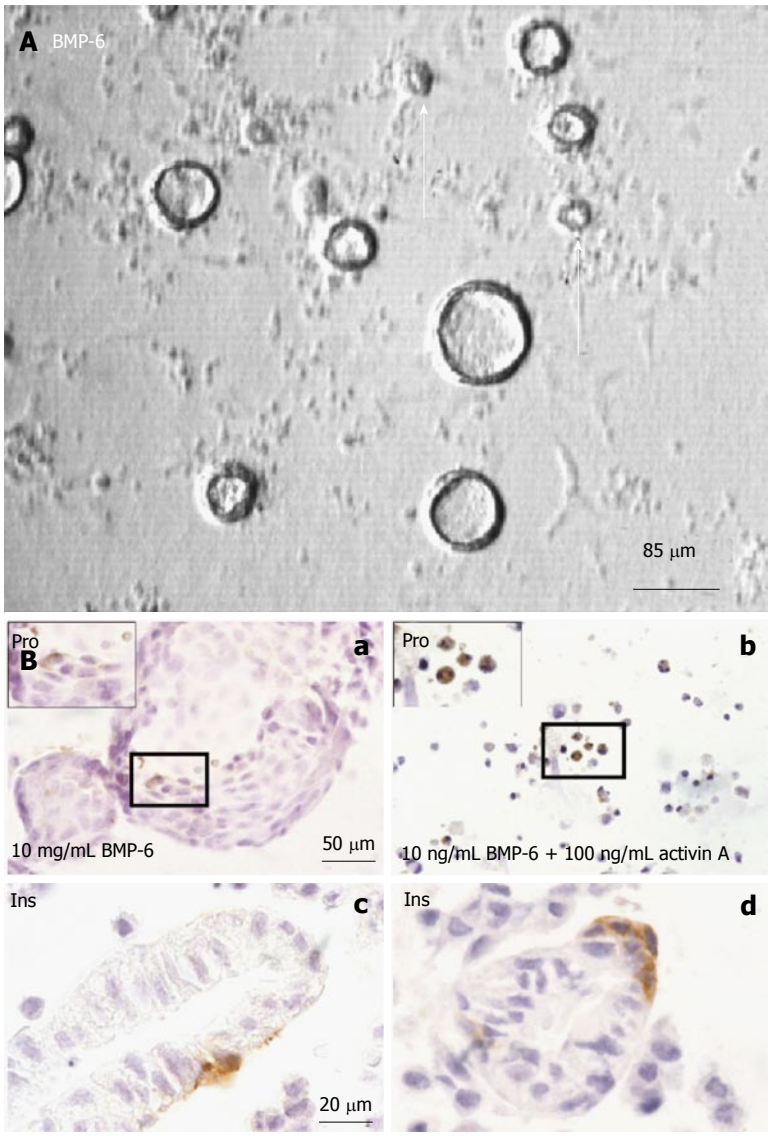

Figure 2 Cystic colony formation from dissociated fetal mouse pancreas cells. A: Phase contrast image showing that BMP-6 promotes colony formation. Open arrows indicate colonies ⩽ 30 μm; B: Immunocytochemical analyses: a: Proinsulin staining. Fixed colonies were stained with proinsulin antibody (brown); b: Activin A antagonizes colony formation; c, d: Insulin staining. Histological sections of harvested colonies were stained with anti-insulin antibody (brown). Adapted and modified from ref.[14].

are needed to ascertain whether $Sox9^+CD133^+$ cells are identical to, or distinct from, the colony-forming Lgr5 [leucine-rich repeat-containing G-protein coupled receptor 5, also known as GPR49 or GPR67 (G-protein coupled receptor 49 or 67)]-expressing cells described below. However, Sox9-positive ductal and centroacinar cells are unable to produce β-cells in a few experiments for regeneration or trans differentiation, such as with cerulean-induced acute pancreatitis, an *in vitro* culture experiment, pancreatic duct ligation (PDL), partial pancreatectomy and a streptozotocin-induced diabetic model[19,22]. Originated from the posterior foregut endoderm, a human bile duct progenitor population known as "biliary tree-derived cells"[23] may have a MPP potential and give rise to islet lineages. However, the purified population, molecular profile and detailed developmental potential of these "tree-derived cells" require further investigations.

Pdx1-expressing MPP

A cluster of unique cells in the thickened DE epithelium along the dorsal and ventral surfaces of the posterior foregut in E9.0-9.5 mouse embryos expresses the homeobox gene named Pdx1 (pancreas and duodenum transcription factor 1). The latter encodes a parahox homeobox-containing factor, critical for the establishment of primitive pancreas[24] as well as the maintenance of functional β-cells[25,26].

Studies have demonstrated that Pdx1-expressing ($Pdx1^+$) progenitors are MPP because they give rise to acinar, ductal and islet tissues of the pancreas[27]. Genetic lineage tracing studies revealed that these MPP reside in the termini of the tree-like branching ducts in the developing pancreas that also express the acinar transcription factor known as Ptf1a and the exocrine cell marker known as Cpa1[28]. The $Pdx1^+$ cells are capable of taking up the thymidine analogue bromodeoxyuridine (BrdU) and incorporating the latter into their genome during mitosis[29], revealing that these cells are proliferative.

Using developmental biological knowledge, PSC have been manipulated to generate $Pdx1^+$ cells that have been expanded for 16-fold co-cultured with pancreas-derived mesenchymal cells[30]. To verify the proliferation

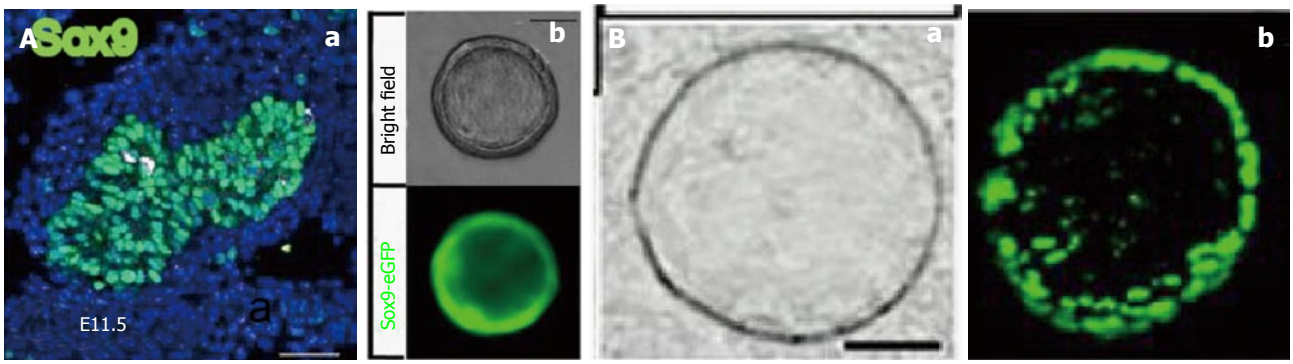

Figure 3 Cystic colonies generated from Sox9-tagged cells. A: Embryonic Sox9+ progenitors in the pancreas capable of generating cystic colonies; a: Sox9 is expressed in most ductal progenitors in E11.5 mouse pancreas; b: Cystic colonies are formed from purified Sox9-eGFP$^+$ progenitors in E11.5 mouse pancreas; B: Purified Sox9$^+$ cells in adult mouse pancreas capable of generating cystic colonies under a phase contrast (a) or a fluorescence microscope (b). Adapted and modified from[20,21].

of Pdx1$^+$ cells, independent confirmation of this report will be required. We also need to ascertain whether these PSC-derived Pdx1$^+$ cells share all or partial characteristics of embryonic pancreatic progenitors, because *Pdx1* is also expressed in extra-pancreatic tissues including other endoderm-derived non-pancreas organs[31].

In developing human pancreas, numerous PDX1$^+$ progenitors are present between 8 and 21 wk of gestation[32,33]. These PDX1$^+$ progenitors are frequently expressed SOX9 and highly proliferative[34], supporting the notion that pancreatic progenitors are committed from SOX9$^+$ multipotent progenitors. The progressive increase of PDX1$^+$ cells that co-express insulin or somatostatin is observed in this period of gestation[32]. Further research is required to investigate whether the PDX1$^+$ progenitor pool is established by its self-renewal or by direct differentiation of the posterior foregut progenitors or both.

Strategies for proliferation, self-renewal and differentiation of pancreatic progenitors will be of importance in developmental biology and regenerative medicine. Interestingly, reserpine and tetrabenazine that inhibit the vesicular monoamine transporter-2 are shown to direct PSC-derived Pdx1$^+$ cells to produce cells that express neurogenin 3 (Neurog3, also known as Ngn3)[35], a DNA-binding protein, belonging to the transcription factor family of the basic helix-loop-helix category. However, as a positive control, Pdx1$^+$ progenitors purified from embryonic pancreas would have been tested with these two molecules to examine their capacity and efficiency to give rise to islet progenitors. Furthermore, caution needs to be exercised for the use of genetic lineage tracing in PSC differentiation *in vitro* because temporospatial cues are essential for the success of *in vivo* lineage tracing studies (see review by[36]). To enrich PSC-derived genuine pancreatic progenitors for further expansion and differentiation, identification of their specific markers would be highly valuable.

Neurog3-expressing pancreatic endocrine progenitors

Approximately in E9.5 mouse embryos, within the thickened posterior foregut DE epithelium there is a small cluster of cells that express a high level of the Neurog3, an endocrine determinant[27,37,38]. The cells that express Neurog3 highly are the progenitors of endocrine pancreas as they develop into all islet lineage cells *in vivo*. Several studies verify the critical role of Neurog3 in the ontogenesis of endocrine pancreas: Islet cells are not observed in *Neurog3* targeted mouse pancreas[37]; genetic tracing demonstrates that Neurog3$^+$ progenitors differentiate into all five types of islet cells[27]; isolated adult Neurog3$^+$ cells reappeared after PDL can, after inoculation into an embryonic pancreas *ex vivo*, give rise to five types of endocrine cells[38]. Nevertheless, a few Neurog3$^+$ cells are observed to coexpress insulin in the dual fluorescence-tagged developing mouse pancreas[39]. Another laboratory reported that PDL allows the activation of Neurog3 expression but the Neurog3-expressing cells are not able to complete the entire β-cell regeneration program[22]. Furthermore a recent study found that β-cell mass and insulin content are totally unchanged after PDL-induced injury[40], unsupportive to the conclusion of an active β-cell regeneration. The reason for these inconsistencies is unknown and future investigation is warranted to confirm or refute the conclusion.

Whereas the expression of mouse *Neurog3* mRNA in the developing pancreas plateaus approximately E15.5[41] (approximately week 9 of gestation in humans), that of human *NEUROG3* is low prior to 9 wk, but increases sharply onward and remains very high until 17 wk[33]. Furthermore some cells coexpress both NEUROG3 and insulin in the embryonic pancreas from 10 to 21 wk[32]. Although earlier studies showed that Neurog3$^+$ cells could proliferate[42,43], clonal assays by "mosaic analysis with double marker" (MADM) have confirmed that Neurog3$^+$ cells are quiescent and commit to only one cell type of the endocrine pancreas[44]. A recent study consistently demonstrated that the activation of Neurog3 itself inhibits cell division by the activation of cyclin-dependent kinase inhibitor 1a[45]. The inconsistencies between the previous and recent reports require future research to reconcile. Again, it is formally

possible that PSC-derived Neurog3-expressing cells are not completely bona fide islet progenitors as Neurog3 is expressed in endoderm-derived non-pancreas organs.

Insulin$^+$ multipotent progenitors

Embryonic insulin-expressing cells may be endocrine progenitors in the pancreas as they can give rise to other islet cell types in addition to β-cells at least during development[46]. By employing fluorescence-tagged tracing strategies, multipotent progenitors that are insulin$^+$ (arguably to be MPP) in the pancreatic tissues are believed to be originated from the Pdx1$^+$ progenitors, instead from the ectoderm-derived neural crest. These MPP-like cells express several islet progenitor markers and are able to differentiate into all endocrine cells *in vivo*. The MPP express a low concentration of insulin and low copies or complete lack of glucose transporter-2, clearly different from functional mature β-cells[47]. Nevertheless, studies have demonstrated that the expression of insulin gene is activated in Pdx1$^+$ MPP, *via* Neurog3$^+$ precursors to adult β-cells[48,49], thus future investigation is needed to establish what stages at which insulin$^+$ MPP are present: The embryonic, fetal and/or adult endocrine pancreas.

Human insulin$^+$ multipotent progenitors have also been described with a similar developmental potential to the mouse ones. These cells, isolated from either mice or humans, could, after transplantation into diabetic mice, develop into functional cells to ameliorate hyperglycemia. Surprisingly, however, these insulin$^+$ multipotent progenitors also gave rise to neural lineage cells *in vivo*[47].

Ghrelin (ε)-expressing progenitors?

Ghrelin-expressing ε-cells are the fifth cell type in the endocrine pancreas and are first discovered in the stomach. Ghrelin is a polypeptide hormone composed of 28-amino acid residuals and known to negatively regulate insulin secretion from mouse, rat and human islets[50]. Now it is well established that pancreaticε-cells are detectable in mid-gestation in mice and humans and their number plateaus during the perinatal period[51]. However, there are a substantial number of ε-cells in only human but not mouse and rat adult islets[51], raising the possibility thatε-cells could participate in the regulation of glucose homeostasis.

Deletion of the *Arx* gene, encoding an α-cell transcription factor, drastically reduces the number of ε-cells[52]. In contrast, knockout of *Nkx2.2, Pax4* or *Pax6* significantly increases the number of these cells at the expense of reducing other pancreatic endocrine cell types[53,54]. Intriguingly, genetic studies of lineage tracing demonstrated that ε-cells give rise to α, PP and, to a lesser extent, β-cells in adult mouse pancreas[55], suggesting that these cells have a unusual plasticity for trans differentiation towards, and may even be progenitors of, other islet cells. However, whether ε-cells would act as MPP of functional islets remains to be established.

Can islet β-cells behave like unipotent "progenitors"?

Functional duplication of insulin-secreting β-cells is first described by Dor *et al*[56] by using RIP (rat insulin promoter)-driving genetic lineage tracing. They revealed that mouse β-cells in the endocrine pancreas could be reproduced by RIP-expressing cells under the physiological condition or after partial pancreatectomy. This report however did not preclude the presence of and the role of MPP by assuming that all RIP-expressing cells are functional β-cells. Similarly, in a transgenic model using the RIP to govern diphtheria toxin expression resulted in 70%-80% β-cell apoptosis, disrupted architecture of endocrine pancreas and eventually in the development of diabetes. Withdrawing the expression of diphtheria resulted in a significant recovery of β-cell mass, islet architecture and of normoglycemia[57]. Further analysis revealed that a subset of 20%-30% surviving "β"-cells played a major role in the β-cell regeneration and recovery of euglycemia[57]. These studies suggest that islet β-cells are indeed facultative unipotent progenitor cells.

In the sophisticated double transgenic MADM (designated RIP-CreER; Rosa26^{GR}/Rosa26^{RG}) mouse pancreas, each RIP-expressing clone is made up of only 5 cells after one month of chase, slightly increasing to 8 cells by two months[58]. The clusters that express RIP have been viewed as supportive evidence of regeneration of functional cells, but this should be treated with caution as discussed hereafter. A further study with a reductionist approach on the gene *Hnf4*a (hepatocyte nuclear factor 4a) suggested that the β-cell regeneration may be associated with the signaling cascade of the Ras/Erk pathway[59] and even be modulated by cell cycle regulators such as cyclin D2[60]. Collectively, it is critical to establish and identify the well-known RIP-expressing cells either in self-duplication or in dedifferentiation both *in situ* and *ex vivo*, as the latter may be a key MPP candidate for a T1DM replacement solution.

Furthermore, lineage tracing labeled with a thymidine analogue showed that β-cells are produced within an islet by rare self-renewable cells that have a long duplication-refractory time. Under stress conditions such as during pregnancy or after partial pancreatectomy, the number of self-renewable cells is dramatically elevated[61]. However the molecular nature of these rare cells and the replication-refractory length have not been established. Future studies should characterize their molecular identity and reveal whether these self-renewable replicating cells are fully functional. Due to the ethical barrier and technical difficulties, similar studies are not possible to be undertaken to label human endocrine pancreas *in vivo*, but a similar research should be repeated with larger mammals.

The above described investigations of β-cell self-renewal as a regenerative mechanism have drawn considerable interest in last several years, due to its promise as a way to increase β-cell mass for the treatment of diabetes. However, these studies have not excluded

whether the insulin transcript (as controlled, for instance, by the RIP transgene) is only expressed in functional β-cells. There is increasing evidence suggesting that is not the case. First, the expression of insulin gene is detectable in the Pdx1$^+$ progenitors, dramatically increasing in Neurog3$^+$ precursors and peaking in mature islet β-cells[48,49]. Second, the demonstration of insulin$^+$ multipotent progenitors[47] precludes insulin as an exclusive marker of functional β-cells. Third, insulin protein has been detectable in some mouse and human islet precursors as described above.

Taken together, the expression of insulin gene is clearly not exclusive for functional β-cells. It is formally possible β-cell populations in adults are maintained not only by the self-replication of functional glucose-regulating cells but also by the self-duplication and development of MPP. In order to demonstrate that MPP are indeed present in the adult endocrine pancreas, their clonogenesis must be established with isolated single cell MPP candidates *ex vivo*, with data generated from the intermediary stages of the clonogenesis, and with demonstrable ability to give rise to at least non-β endocrine cells *in vivo*.

MPP PRESENT IN THREE TYPES OF PANCREAS TISSUES

Accumulating evidence generated in *ex vivo* studies has suggested that MPP are present in three major tissues of the pancreas: The pancreatic ductal[62-64], acinar and islet compartments[65,66]. For instance, the application of flow cytometry has identified a potential MPP population in the mouse pancreas both in development and in adults[67]. These candidates are characterized by the exhibition of the receptor c-met for hepatocyte GF, without the presence of hematopoietic lineage markers including CD45, c-Kit (stem cell factor receptor), Flk-1 and TER119. The purified population is able to give rise to several types of pancreatic cells *ex vivo* and generate pancreatic endocrine and exocrine cells *in situ* after transplantation[67]. Nevertheless, the spatial localization and the molecular natures of these c-met positive cells are completely unknown and the clonogenesis has not been established at the single cell level.

MPP likely present in the ductal epithelium

Many studies suggest that MPP are present in the ductal epithelium. Bonner-Weir *et al*[68] first reported that human ductal epithelial cells in adult pancreas are able to differentiate into islet-like clusters containing insulin-secreting β-cells. Ramiya *et al*[63] showed that insulin-secreting islet-like clusters generated *in vitro* from mouse MPP are capable of ameliorating diabetes after being grafted under the kidney capsule. In cultures of human "pancreatic ductal cell aggregates" after isolation of the islets for transplantation, cells with fibroblast-like morphology appear known as pancreatic "mesenchymal stem cells (MSC)". These cells were passaged over 12 times and expressed an array of markers of bone marrow MSC such as CD13, CD29, CD44, CD49f (also known as α6 integrin subunit), CD54, CD90 (also known as Thy1) and CD105. The pancreatic MSC are shown to be able to give rise to cross-germ layer cells such as endoderm-originated non-pancreas lineages[69]. Utilizing culture protocols suitable for producing the neurospheres of ectoderm origin, ductal cells from mouse pancreas are shown to give rise to neurosphere-like clusters that subsequently give rise to a few types of endocrine cells, such as insulin-secreting β cells[66]. Nevertheless, the exact nature of the special cells has not been established. A major limitation of the forementioned reports is the use of unenriched/unpurified cell preparations in addition to not demonstrating single-cell self-renewability.

Furthermore, numerous CK19$^+$ ductal cells and islet cells are detected after PDL[38]. The lineage relationship between the ductal cells and islet cells has further been addressed using the genetic lineage tracing of the Cre-loxP system. Using the system, the Cre governs the promoter activity of carbonic anhydrase Ⅱ gene, encoding a marker of adult ductal epithelia and controls the removal of the stop sequence (Rosa-loxP-stop-loxP-lacZ) in the reporter Rosa26 (R26R) mice. This resulted in Cre-driving β-galactosidase expression in ductal epithelia. After 28 d in normal or PDL pancreas, the activity of β-galactosidase is detectable in numerous ductal epithelia, localized acinar tissue and in up to 40% islet cells[70,71]. These data have been viewed as further evidence that the ductal cells expressing carbonic anhydrase Ⅱ are able to regenerate mouse endocrine cells. It remains unclear, nevertheless, how many cells that express carbonic anhydrase Ⅱ have this regenerative capacity, how many potential MPP are present or whether a trans differentiation process also takes place.

Nevertheless, once an exon of the ductal epithelial marker gene *Tcf2* (T cell factor 2, also known as Hnf1b, hepatocyte nuclear factor 1b) is exchanged with the transgene containing Cre, the lineage tracing has demonstrated that the Tcf2$^+$ cells in the postnatal duct cannot develop into endocrine cells in both normal and PDL pancreas[72]. However, a complicating factor in this report is that one allele of *Tcf2* was non-functional, leading to reducing by half the transcription of *Tcf2*. As heterozygous *Tcf2* mutant does not support pancreas development in mice and humans[73,74], inactivation of one allele of *Tcf2* in the lineage tracing studies may have led to haploinsufficiency and affected the differentiation of potential MPP into functional islet cells.

Fbw7 (F-box and WD-40 domain protein 7), an ubiquitin ligase, is expressed in embryonic and adult ductal epithelial cells. Deletion of this gene stabilizes the heavily ubiquitinated Neurog3, and reprograms the ductal cells to α, β and somatostatin-producing δ-cells[75]. This study suggests that pancreatic ductal cells are a latent MPP and Fbw7 is a critical cell-fate

regulator. Nevertheless, this report did not describe whether all or a small fraction of the ductal cells express Fbw7 and what the frequency of the reprogramming event is; both are critical to assess whether this might become a viable strategy to regenerate islet cells by suppressing the Fbw7 signaling. A fundamental assay on reprogrammed cells to ameliorate diabetes has also not been reported.

Interestingly, PDL robustly activates the Wnt signaling pathway and allows the regenerating ducts to express Lgr5[76], a Wnt target which marks actively dividing stem cells such as those present in the intestine[77]. Purified ductal Lgr5-expressing cells are also responsive to spondin 1 and form clonal 3D pancreatic organoids within the gel-forming Matrigel that generate ductal as well as endocrine lineages upon transplantation[76].

MPP likely present in acinar tissue of the pancreas

In the clinic with experimental transplantation of donated islets from the cadaver's pancreas, the acinar cells are normally un-used. The discovery of a scientific and practical value for the acinar cells has hence drawn considerable attention. Cotransplantation with pancreatic cells from fetal mice under the kidney capsule in the immunocompromised mice, the acinar cells are reported to give rise to islet cells with undetectable β-cell division or cell fusion[78]. The results imply that MPP or progenitor cells might be present in the acinar tissue of human adult pancreas. Moreover, the Cre-loxP lineage tracing analysis showed that acinar cells expressing amylase and elastase gave rise to insulin-producing cells in a rotating culture system[79]. Nevertheless, a self-renewable assay at the single-cell level and its intermediate steps need to be demonstrated. Additionally, these studies have not excluded the possibility that some pancreatic acinar cells might have trans differentiated into surrogate insulin-expressing cells[80,81]. This likelihood was supported by a previous study that showed that mouse acinar cells could be directly re-programmed *in vivo* to β-like cells by forced expression of three DNA-binding genes, namely, *Pdx1*, *Neurog3* as well as *MafA*[82]. In addition, as the acinar cells from cadavers[78] were not isolated by FACS, residual ductal or even islet cells present in the system may have compromised their conclusion. Supporting this view, a lineage tracing study *in vivo* did not provide evidence that the mouse acinar cells would give rise to insulin-secreting β-cells after 70%-80% pancreatectomy[83]. Thus these inconsistencies remain to be addressed more carefully.

MPP likely present in the endocrine pancreas

A large body of evidence suggests that the islets of Langerhans harbor the MPP. The potential MPP are nestin$^+$ and hormone$^-$ present in both rat and human islets. These nestin$^+$ cells are reported to expand in culture for approximately 8 mo and differentiate into cells that exhibit markers of liver (α-fetoprotein) and pancreas lineages (including amylase, CK19, PDX1, glucagon, insulin and neural-specific cell adhesion molecule). These nestin$^+$ MPP candidates may thus have been involved in the generation of new pancreatic islet cells[65], potentially modulated by the incretin hormone GLP1, a processed product from the polypeptide pro-glucagon[84]. It, however, remains unclear whether these nestin$^+$ cells are either islet or duct-originated multipotent progenitors that transform into neurosphere-like structures as well as differentiated into β-like cells of the endocrine pancreas[66], or the outgrown fibroblast-like cells that expand readily and differentiate *in vitro* into aggregates of non-typical hormone-expressing endocrine cells[85]. Human endocrine cells transduced with a RIP-controlling transgene dedifferentiated into fibroblast-like cells that proliferate up to 16 population doublings without detectable insulin expression[86]. Nevertheless, *in vivo* Nestin$^+$ cells are mostly present in non-endodermal-derived compartments[87,88]. Governed by their promoters, Pdx1- and RIP-expressing cells have not committed significantly *ex vivo* to the observed cells with fibroblast-like morphology[89]. These discrepancies require clarifications in the future studies.

CONCLUSION

The presence of MPP remains to be conclusively established. Future development of the field needs to: establish essential criteria for MPP; screen and select cell surface antigens that can be used to generate antibodies for purification of candidate MPP and establish a simple, effective and robust *in vitro* assays and *in vivo* experimental protocols for the examination of the multipotency and lineage commitments of isolated MPP candidates.

The following minimal criteria have to be satisfied: (1) enriched or purified cell population by FACS or other technologies should form colonies at the single cell level; (2) single cells after purification should be studied *ex vivo* with their self-renewability; (3) colony cells would be able to differentiate *in vitro* into multiple functional cellular lineages; and (4) the clonogenic cells post transplanted into a recipient should be able to give rise *in vivo* to different terminally differentiated lineages. Thus, embryonic mouse Sox9$^+$ multipotent progenitors and probably adult Sox9$^+$ ductal cells satisfy some but not all criteria of MPP. Research into MPP would therefore be highly valuable for two reasons. First, MPP differentiation and trans differentiation of non-β-cell types in the pancreas may provide an important source for surrogate β-cells. Second, as there is a significant difference in regeneration capability of islets from mice, rats to humans, we should not extrapolate directly from rodent regenerative data to humans.

Future MPP research should apply integrated approaches, different from many previous *in situ* or *ex vivo* studies that did not target defined cell populations. Application of flow cytometry and cell surface markers would allow the separation of interested subsets for demonstrating *in vitro* the capacity of self-duplication, clonogenesis and differentiation. The latest technologies

such as single-cell RNA-seq, single-cell genomics and single-cell epigenomics[90-92] should be applied to examine their developmental dynamics, differentiation pathways, gene interactions and genetic heterogeneity, and along with genetic studies to characterize their growth pattern, biological potential and lineage commitment *in vivo*. Lastly cross-sector, cross-institutional and global collaborations as well as the involvement of the biotechnological and drug companies will eventually deepen our understanding of MPP that assists the establishment of a platform towards a regenerative therapy for both T1DM and T2DM.

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ORIGINAL ARTICLE

Basic Study

Induction of CXC chemokines in human mesenchymal stem cells by stimulation with secreted frizzled-related proteins through non-canonical Wnt signaling

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Author contributions: Bischoff DS performed many of the experiments, designed some of the experiments, analyzed the data and contributed to writing the manuscript; Zhu JH performed the majority of the experiments; Makhijani NS maintained cell lines; Yamaguchi DT coordinated the research, designed some of the experiments, interpreted the data and contributed to writing the manuscript.

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Abstract

AIM: To investigate the effect of secreted frizzled-related proteins (sFRPs) on CXC chemokine expression in human mesenchymal stem cells (hMSCs).

METHODS: CXC chemokines such as CXCL5 and CXCL8 are induced in hMSCs during differentiation with osteogenic differentiation medium (OGM) and may be involved in angiogenic stimulation during bone repair. hMSCs were treated with conditioned medium (CM) from L-cells expressing non-canonical Wnt5a protein, or with control CM from wild type L-cells, or directly with sFRPs for up to 10 d in culture. mRNA expression levels of both CXCL5 and CXCL8 were quantitated by real-time reverse transcriptase-polymerase chain reaction and secreted protein levels of these proteins determined by ELISA. Dose- (0-500 ng/mL) and time-response curves were generated for treatment with sFRP1. Signal transduction pathways were explored by western blot analysis with pan- or phosphorylation-specific antibodies, through use of specific pathway inhibitors, and through use of siRNAs targeting specific frizzled receptors (Fzd)-2 and 5 or the

receptor tyrosine kinase-like orphan receptor-2 (RoR2) prior to treatment with sFRPs.

RESULTS: CM from L-cells expressing Wnt5a, a non-canonical Wnt, stimulated an increase in CXCL5 mRNA expression and protein secretion in comparison to control L-cell CM. sFRP1, which should inhibit both canonical and non-canonical Wnt signaling, surprisingly enhanced the expression of CXCL5 at 7 and 10 d. Dickkopf1, an inhibitor of canonical Wnt signaling prevented the sFRP-stimulated induction of CXCL5 and actually inhibited basal levels of CXCL5 expression at 7 but not at 10 d post treatment. In addition, all four sFRPs isoforms induced CXCL8 expression in a dose- and time-dependent manner with maximum expression at 7 d with treatment at 150 ng/mL. The largest increases in CXCL5 expression were seen from stimulation with sFRP1 or sFRP2. Analysis of mitogen-activated protein kinase signaling pathways in the presence of OGM showed sFRP1-induced phosphorylation of extracellular signal-regulated kinase (ERK) (p44/42) maximally at 5 min after sFRP1 addition, earlier than that found in OGM alone. Addition of a phospholipase C (PLC) inhibitor also prevented sFRP-stimulated increases in CXCL8 mRNA. siRNA technology targeting the Fzd-2 and 5 and the non-canonical Fzd co-receptor RoR2 also significantly decreased sFRP1/2-stimulated CXCL8 mRNA levels.

CONCLUSION: CXC chemokine expression in hMSCs is controlled in part by sFRPs signaling through non-canonical Wnt involving Fzd2/5 and the ERK and PLC pathways.

Key words: CXC chemokines; Mesenchymal stem cell; Osteogenesis; Differentiation; Wnt signaling pathway; Frizzled-related protein; Frizzled receptors

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Core tip: Chemokines have multiple functions during bone formation and fracture repair. The ELR$^+$ chemokines classically have a role in blood vessel formation and were found to be stimulated by the non-canonical Wnt5a protein and also by soluble frizzled-related proteins (sFRPs) that are known inhibitors of both canonical and non-canonical Wnt signaling. This stimulation was mediated *via* the p44/42 extracellular signal-regulated kinase and phospholipase C pathways signaling through the non-canonical frizzled receptors 2 and 5. This is a newly identified role for the sFRPs in stimulation of ELR$^+$ chemokines which may be involved in blood vessel formation during wound repair.

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INTRODUCTION

Bone fracture repair proceeds through a series of sequential steps including an inflammatory phase resulting in recruitment and differentiation of mesenchymal stem cells (MSCs) into osteoblasts, restoration of blood supply, subsequent soft (cartilaginous, in the case of endochondral repair) and hard (bone, in both endochondral and intramembranous) callus formation, and ultimately remodeling of the new woven bone into lamellar bone. During the initial inflammatory stage, neutrophils, macrophages, and lymphocytes migrate to the wound, fight infectious organisms, scavenge tissue debris, and begin the process of granulation tissue formation[1]. Cytokines, chemokines, and growth factors released from these cells are necessary to initiate bone repair in the adult. The pro-inflammatory cytokine, tumor necrosis factor-α (TNF-α) is critical in both long bone fracture as well as intramembranous bone repair[2,3]. TNF-α can highly induce members of the CXC chemokine family *via* NF-κB signaling in osteoblasts[4]. CXC chemokines can be grouped as to whether or not they contain a Glu-Leu-Arg (ELR) motif. ELR$^+$ CXC chemokines, such as CXCL8 (IL-8), are present during the inflammatory phase to serve as chemoattractants for neutrophils[5,6] and exhibit angiogenic activity[7-9]. Chemokines without the ELR sequence are anti-angiogenic[9].

Human MSCs (hMSCs) express CXCL8 mRNA[10-12] and it has been reported that TNF-α can prime hMSCs to upregulate production of several CXC chemokines (highest upregulation with CXCL5 and CXCL8) and induce hMSC migration[13]. In humans, CXCL8 is a ligand for both CXC receptor 1 (CXCR1) and CXCR2 whereas CXCL5 interacts solely with CXCR2. Angiogenesis in response to CXCL8 has only been associated with CXCR2 signaling[14-16]. We previously demonstrated that CXCL8 expression can be stimulated with dexamethasone treatment during osteoblastic differentiation[17] and by low extracellular pH[18] in hMSCs. We also demonstrated that secreted CXC chemokines induced angiogenic tube formation of a human microvascular endothelial cell line (HMEC-1)[17] consistent with *in vitro* angiogenesis.

The mouse CXC receptor (mCXCR) is functionally related to hCXCR2[19]. Mice lacking the mCXCR (mCXCR2$^{-/-}$) have been described[20] and some healing[21] and bone[22-24] defects have been reported. A second murine CXCR (mCXCR1) has also been identified; although, it has no discernable defect phenotype when inactivated (Jackson Laboratory Stock #005820). We have shown by DEXA and micro computerized tomography analysis that the mCXCR2$^{-/-}$ mice (Jackson Laboratory Stock #002724) have an osteopenic phenotype with decreased trabecular bone volume, number, and thickness without any changes in bone formation and resorption indices[25]. However, bone quality was affected as femurs had reduced stiffness and a lower ultimate load breaking point[25]. There was

also a reduction in the blood vessel density in the newly repaired bone in a cranial defect model[25]. During bone regeneration, ingrowth of blood vessels is required for endochondral bone formation[1]. These results suggest a potential coupling of mMSC differentiation, bone formation, and angiogenesis in response to mCXCR signaling.

The Wnt family of secreted glycoproteins is involved in differentiation of an assortment of tissues[26]. Wnts signal through specific seven transmembrane spanning G-protein coupled frizzled (Fzd) receptors *via* both canonical β-catenin signaling, and non-canonical Wnt/calcium and Wnt/planar cell polarity pathways[27,28]. The highly conserved and redundant nature of the Wnt/Fzd system (19 Wnts and 10 Fzd in humans) only adds to the complexity of this system and confusion as to its role in osteogenesis.

The canonical pathway is characterized by Wnt binding to both Fzd and LRP5/LRP6 co-receptors resulting in activation of Disheveled (Dsh) which inhibits glycogen synthase kinase 3β (GSK3β) phosphorylation. In the absence of Wnt binding, GSK3β phosphorylation ultimately results in β-catenin degradation, preventing its nuclear translocation for activation of target genes. In murine models, evidence suggests that canonical Wnt/β-catenin signaling is necessary for lineage commitment of pluripotent MSCs to osteochondroprogenitor cells, then to osteoprogenitor cells, and for differentiation to mature osteoblasts while suppressing both chondrogenesis and adipogenesis[29,30]. However, in hMSC models, β-catenin and canonical Wnt3a can negatively regulate the differentiation of MSCs into the skeletal precursor cells that precede the appearance of the osteochondroprogenitor cells[31-35]. Additionally, de Boer *et al*[36] reported a dose-response relationship in which lower levels of β-catenin stimulated hMSC proliferation while blocking adipogenesis; whereas, higher levels induced expression of alkaline phosphatase. The authors thus concluded that canonical Wnt/β-catenin signaling could initiate osteogenic differentiation in the human system[36].

Signal transduction through the non-canonical or β-catenin-independent Wnt pathways has also been shown to inhibit adipogenesis and chondrogenesis in MSC models and to stimulate osteogenesis[33,37-40] mediated through activation of phospholipase C (PLC), and then through activation of the calcium-calmodulin kinase, nuclear factor of activated T-cells (NFAT), and protein kinase C (PKC) pathways or through the mitogen-activated protein kinase (MAPK) and RhoA pathways. Non-canonical Wnt signaling through traditional canonical Wnt ligands, Wnt3a and Wnt7b, and the non-canonical Wnt ligand, Wnt4, can also lead to osteogenic differentiation in both murine and human MSCs models through the activation of PKC and/or MAPK pathways[33,41,42]. Levels of non-canonical Wnt5a are increased in the inflammatory environment during early fracture healing[43] and non-canonical signaling (Wnt4 and Wnt5a) can affect the transition from proliferative osteoprogenitors to mature osteogenic cells[44,45]. However, as with canonical signaling, there are conflicting reports as to whether non-canonical Wnt5a can induce osteogenesis[30,33].

Wnt antagonists which include secreted frizzled related proteins (sFRPs), that can inhibit both canonical and non-canonical Wnt signaling[46], or the canonical Wnt/β-catenin Dickkopf (Dkk) inhibitors may also contribute to osteoblast differentiation and mineralization[30,38,47]. sFRP1 knock-out mice exhibit increased trabecular bone mass due to reduced osteoblast and osteocyte apoptosis[48] suggesting that Wnt signaling is involved in bone formation. Additionally, long bone fracture healing is enhanced in sFRP knockout mice through canonical Wnt signaling as a consequence of MSCs directed to differentiate into osteoblasts rather than towards cartilage[49]. However, high sFRP1 levels expressed early in fracture repair in this model would suggest that both canonical and non-canonical Wnt signaling are inhibited in early callus formation. Wnt5a/5b expression was decreased in the sFRP1 knockouts; although, contrary to conventional thought, canonical Wnt7a and Wnt1 were elevated[49]. There have been other reports of sFRPs enhancing rather than inhibiting Wnt activity[50] through mechanisms which may involve: (1) sFRP-Wnt binding to each other and facilitating transport and binding of Wnts to Fzd receptors on distant cells; or (2) binding to both Wnt molecules and Fzd receptors simultaneously to activate downstream Fzd signaling[51]. Many repair processes are stimulated by sFRP2-Wnt interactions including the enhancement of vascular density during granulation tissue formation[52]; inhibition of cardiomyocyte apoptosis during cardiac repair[53]; establishment of MSC-endothelial and smooth muscle contacts to stabilize new blood vessel formation[54]; and stimulation of angiogenesis by sFRP1 (FzlA) independent of VEGF, bFGF2, or angiopoietin1[55].

A key observation made by several laboratories is that canonical Wnt/β-catenin signaling may be important in osteoblastogenesis through the cooperation of Wnt signaling with other known osteogenic factors such as BMP-2 and BMP-4[47,56]. Thus taken together, canonical Wnt/β-catenin signaling appears to be involved in determining a specific tissue fate of MSCs. However, further effects of Wnt signaling (both canonical and non-canonical) on osteogenic differentiation is dependent on several factors including: The species from which the MSCs are derived, the specific Wnt (and Fzd receptors) expressed, the stage of osteogenic differentiation, the amount of β-catenin available to translocate to the cell nucleus, and other biologically active molecules (*e.g.*, growth factors) present in the MSC's microenvironment[30,57]. These other factors could include the ELR^+ CXC chemokines which are also elevated in the inflammatory phase of healing and which have been shown to be stimulated by non-canonical Wnt5a[58,59]. In this article, we report the observation that sFRP treatment of hMSCs leads to an increased expression of ELR^+ CXCL5 and CXCL8 which may serve

Table 1 siRNA oligonucleotide sequences

Gene	Qiagen product name	Qiagen catalog No.	Human target sequence
siFZD2	Hs_FZD2_5	SI02757433	CACGGTCTACATGATCAAATA
siFZD5	Hs_FZD5_5	SI02757650	TAAGGTTGGCGTTGTAATGAA
siROR2	Hs_ROR2_6	SI00287525	CTGGTGCTTTACGCAGAATAA
siScambled	Ctrl_Control_1	SI03650325	AATTCTCCGAACGTGTCACGT

Table 2 Reverse transcription-polymerase chain reaction primer sequences

Gene	Human primer sequence
hCXCL5	5′GCTGGTCCTGCCGCTGCTGTG3′
	5′GTTTTCCTTGTTTCCACCGTC3′
hCXCL8	5′GCCTTCCTGATTTCTGCAGC3′
	5′TCCAGACAGAGCTCTCTTCC3′
18S Ribosomal	5′CGGGTCATAAGCTTGCGTT3′
RNA	5′CCGCAGGTTCACCTACGG3′
FZD2	5′CCTCAAGGTGCCATCCTATCTCAG3′
	5′GTGTAGCAGCCCGACAGAAAAATG3′
FZD5	5′CCTACCACAAGCAGGTGTCC3′
	5′GGACAGGTTCTTCCTCGAAA3′
ROR2	5′TCCTTCTGCCACTTCGTGTTTCC3′
	5′TGCTTGCCGTTCCTCTGTAATCC3′

to attract MSCs to the wound or to couple angiogenesis to osteogenesis in the early phase of bone repair.

MATERIALS AND METHODS

Cell culture

hMSCs, growth supplements, and basal medium were purchased from LonzaWalkersville, Inc. (Walkersville, MD). hMSCs from several donors were used: 19 years old male (Lonza Lot #6F4393; race unknown); 20 years old Caucasian male (Lot #0000351482); 27 years old Black male (Lot #0000318006). Cells were grown in complete medium (HMSCGM) at 37 ℃ under 95% air/5% CO_2 atmosphere and subcultured once a week at 60%-70% confluence.

For osteoblastic differentiation, hMSCs were treated every 3-4 d with osteogenic medium (OGM) consisting of complete growth medium with 50 mmol/L ascorbic acid-2-phosphate, 10 mmol/L β-glycerophosphate, and 10^{-7} mol/L dexamethasone (Sigma-Aldrich, St. Louis, MO).

Cells (passage 2-7) were plated at 5000-10000 cell/cm^2 in HMSCGM and allowed to adhere for 4 h prior to exposure to OGM ($n \geqslant 3$ for all experiments). Differentiation toward the osteoblastic lineage was monitored by detection of mRNA levels of the reporter gene alkaline phosphatase or by Alizarin Red staining for calcium at 28 d as previously described[17]. Qiagen RNeasy Miniprep columns (Qiagen, Inc., Valencia, CA) were used to isolate RNA at the specified time-points. In some experiments exogenous sFRPs (varying concentration from 0-500 ng/mL; PeproTech Inc., Rocky Hill, NJ), Dkk1 (50 ng/mL, PeproTech), and L-cell or Wnt5a-conditioned medium (CM) (1:1 mixture with HMSCGM) were added as needed. The PLC signal transduction inhibitor (U73122) and control (U73343) were used at 10 μmol/L (Calbiochem, San Diego, CA). Effects of siRNA inhibition of receptors were determined by transfection of hMSCs with siRNA (150 ng/mL) using the HiPerFect transfection reagent (Qiagen) followed by treatment of the cells with sFRPs for 48 h before gene expression analysis. siRNA were purchased from Qiagen and the nucleotide sequences indicated in Table 1. A scrambled oligonucleotide siRNA was used as a negative control.

Quantitative reverse transcriptase-polymerase chain reaction

Relative mRNA levels of various genes were determined by real-time RT-PCR using the Opticon Continuous Fluorescence System (Bio-Rad Laboratories, Inc., Hercules, CA) and the SYBR Green RT-PCR kit (Qiagen). Primers used for RT-PCR are indicated in Table 2.

PCR reactions were performed in triplicate. Reactions consisted of reverse transcription at 50 ℃ (30 min), inactivation at 95 ℃ (15 min); followed by 50 cycles of denaturing at 94 ℃ (15 s), annealing at 60 ℃ (30 s), and extension at 72 ℃ (30 s). Gene expression changes were calculated and normalized to 18S ribosomal levels and the reference time point using the $2^{-\Delta\Delta C(T)}$ method[60].

ELISA analysis

Secreted CXCL5 protein levels were determined with the human CXCL5/ENA-78 DuoSet (R and D Systems, Minneapolis, MN) after concentration of supernatants with microcon centrifugal filters (EMD Millipore Inc, Billerica, MA). Culture supernatant samples were compared to CXCL5 standard curves and were run in duplicate.

Western blot analysis

Cells were plated in 35 mm dishes and treated with OGM medium (7 d). sFRP1 (150 ng/mL) was added and cell lysates isolated at indicated time points in PhosphoSafe Extraction Reagent (EMD Chemicals, Gibbstown, NJ). Proteins were separated (SDS–PAGE), transferred to polyvinylidenedifluoride membrane, and probed with ERK-specific pan or phospho-antibodies (Cell Signaling Technology, Danvers, MA). Immunoreactive proteins were detected using the ECL kit (GE Healthcare Bio-sciences, Piscataway, NJ) and levels quantitated using AlphaView Software (ProteinSimple, San Jose,

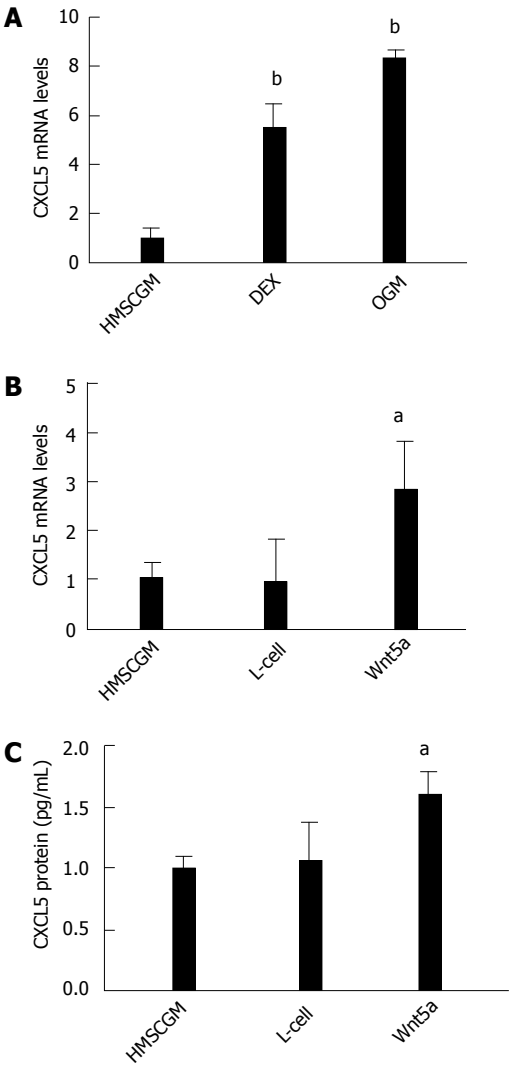

Figure 1 CXCL5 chemokine induction in human mesenchymal stem cells treated with osteogenic medium or non-canonical Wnt5a. A: CXCL5 mRNA levels are induced at 7 d in complete osteogenic medium or medium containing 0.1 μmol/L dexamethasone. Conditioned medium containing non-canonical Wnt5a induces (B) mRNA expression and (C) protein secretion. All values are mean ± SD. $^{a}P < 0.05$; $^{b}P < 0.01$ *vs* HMSCGM-treated group. HMSCGM: Human mesenchymal stem cell growth medium; DEX: Dexamethasone; OGM: Osteogenic medium; L-cell: Conditioned medium from L-cells; Wnt5a: Conditioned medium from L-cells expressing Wnt5a.

CA).

Statistical analysis

Data values are reported as mean ± SD. Statistical analysis (1-way ANOVA with the Bonferroni method for multiple comparisons between pairs or non-parametric Mann-Whitney *t* test) was performed using GraphPad Prism software. Differences from negative controls were considered to be statistically significant at the $P < 0.05$ level.

RESULTS

We had previously demonstrated that mRNA and protein for CXCL8 (IL-8) and CXCL1 (GROα)[17] were induced in hMSCs exposed to osteogenic differentiation medium (OGM) containing ascorbate-2-phosphate, β-glycerophosphate, and dexamethasone. To see if another angiogenic CXC chemokine, CXCL5 (ENA-78), was also induced by osteogenic differentiation, RNA from hMSCs treated with OGM was analyzed for CXCL5 expression levels. OGM treatment for 7 d stimulated CXCL5 mRNA levels approximately 8-fold ($P < 0.01$; Figure 1A). Dexamethasone alone (0.1 μmol/L) in the presence of proliferating medium (HMSCGM) increased CXCL5 mRNA by 5.5-fold ($P < 0.01$).

Non-canonical Wnt signaling has also been associated with osteogenic differentiation of hMSCs[32,61]. Since osteogenic differentiation using OGM or dexamethasone alone resulted in CXCL5 mRNA expression, we then explored if treatment of hMSC cells with non-canonical Wnt5a protein was able to stimulate the expression of CXCL5. hMSC cells were treated for 7 d with CM from L-cells overexpressing Wnt5a protein or control L-cell CM. CM containing Wnt5a induced the expression of CXCL5 mRNA 3-fold ($P < 0.05$) compared to the lack of stimulation of CXCL5 in both non-osteogenic medium (HMSCGM medium) or control L-cell medium (Figure 1B). CXCL5 protein secretion was also increased 1.5-fold ($P < 0.05$) above controls (Figure 1C).

We next sought to inhibit all Wnt signaling, both canonical and non-canonical Wnt signaling, using sFRPs. Surprisingly and unexpectedly, sFRP1 increased CXCL5 mRNA levels 3-fold ($P < 0.01$) at 7 d and approximately 8-fold ($P < 0.01$) at 10 d of culture in HMSCGM medium (Figure 2A). To see if canonical Wnt signaling inhibition was responsible for the unexpected stimulation of CXCL5, hMSCs were treated with Dkk1 which binds to the low density lipoprotein receptor related protein 6 (LRP6) to inhibit canonical signaling. Unlike sFRP-1, Dkk1 addition did not induce an increase in CXCL5 levels at 7 or 10 d and in fact significantly inhibited basal mRNA expression levels more than 50% ($P < 0.01$) at 7 d (Figure 2A). To see if the effect of sFRP1 on CXCL5 was unique amongst the other sFRP family members, sFRPs 2, 3, or 4 was each added separately to the medium (150 ng/mL) for 7 d and levels of CXCL5 protein secreted into the medium determined. All four sFRPs added independently significantly stimulated CXCL5 protein secretion 3-4-fold ($P < 0.01$) over unstimulated vehicle control (Figure 2B).

We next tested if CXCL8 mRNA levels are also stimulated by sFRP1 treatment. sFRP1 treatment increased CXCL8 mRNA levels in a dose-dependent manner (Figure 2C). Maximum stimulation of CXCL8 mRNA expression (approximately 5-fold) was observed at a concentration of 150 ng/mL sFRP1 ($P < 0.05$). A time-course study of CXCL8 mRNA expression stimulated by sFRP1 (150 ng/mL) showed maximal expression levels ($P < 0.05$) between 5 and 7 d of culture (Figure 2D).

In an effort to explore the mechanism of the sFRP1-stimulated increase in both CXCL5 and CXCL8 expre-

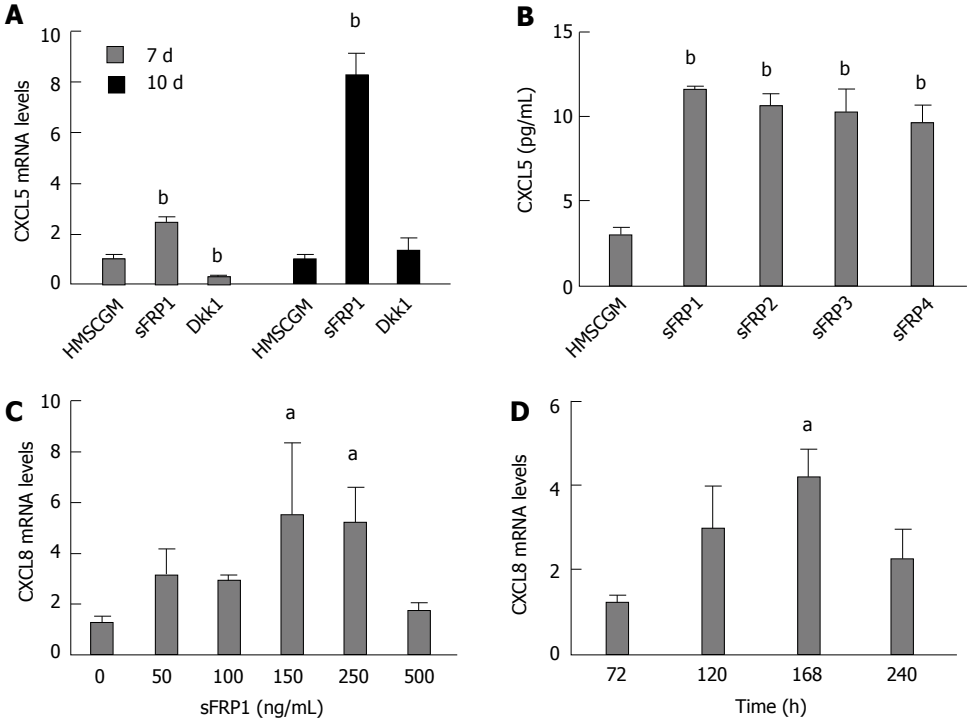

Figure 2 Secreted frizzled-related protein-stimulated expression of CXCL5 and CXCL8 in human mesenchymal stem cells. A: sFRP1, an inhibitor of canonical and non-canonical Wnt signaling, induces expression of CXCL5 mRNA. Dkk1, an inhibitor of canonical Wnt signaling does not induce CXCL5; B: All four of the sFRPs stimulated CXCL5 protein secretion as determined by ELISA analysis of cell supernatants. sFRP1 induces expression of CXCL8 mRNA in a dose- (C) and time- (D) dependent manner with maximum expression at 150 ng/mL and 7 d post-treatment. All values are mean ± SD. $^{a}P < 0.05$; $^{b}P < 0.01$ *vs* HMSCGM-treated group (A, B), *vs* untreated (C), or 3-d CXCL8 mRNA levels (D). HMSCGM: Human mesenchymal stem cell growth medium; sFRP: Secreted frizzled-related protein; Dkk1: Dickkopf-related protein 1.

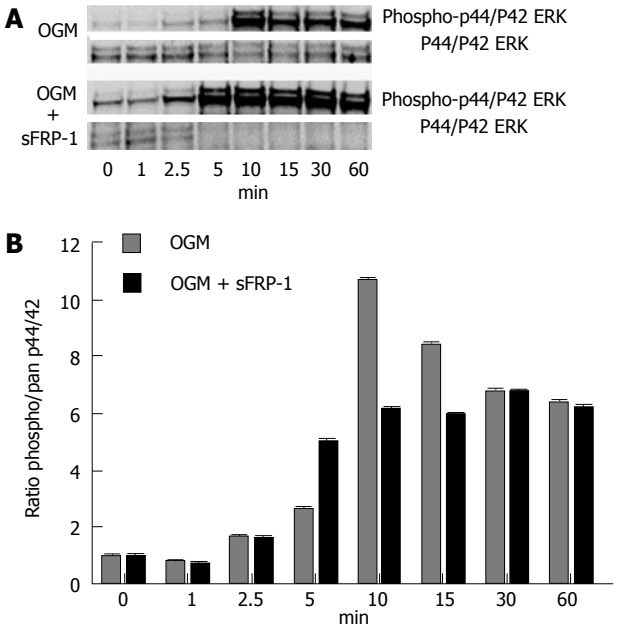

Figure 3 Phosphorylated state of mitogen-activated protein kinase p44/42 extracellular-signal-regulated kinases in response to secreted frizzled-related protein 1 stimulation. Western Blot time-course analysis of pan and phosphorylated states of the MAPK p44/42 ERK proteins (A); The ratio of phospho/pan p44/42 ERK reaches a maximum at 5 min in the presence of OGM and sFRP1 (B); whereas, in OGM alone the maximal ratio level is not reached until 10 min post stimulation (representative experiment). sFRP1: Secreted frizzled-related protein 1; MAPK: Mitogen-activated protein kinase; ERK: Extracellular-signal-regulated kinases; OGM: Osteogenic medium.

ssion, we characterized the phosphorylated state of the extracellular signal-regulated kinase (ERK) p44/42, a member of the MAPK pathway, since it has been previously reported that CXC ligand expression can be increased *via* MAPK activation[62-66]. p44/42 was shown to be phosphorylated maximally at 5 min in the presence of OGM and sFRP1, whereas maximal p44/42 phosphorylation occurred at 10 min in OGM alone (Figure 3).

To see if other G-protein coupled signaling mechanisms could be involved in sFRP stimulation of CXCL8 mRNA expression, the PLC inhibitor, U73122, was added (10 μmol/L) to the HMSCGM medium and CXCL8 mRNA levels determined after 3 d of sFRP treatment. Both sFRP1 and sFRP2 in the presence of the inactive isomer, U73343, enhanced CXCL8 mRNA by approximately 3 to 5 fold ($P < 0.05$). However, U73122 prevented the increase in both sFRP1- and sFRP2-stimulated CXCL8 mRNA levels returning them back to HMSCGM control levels (Figure 4).

Since Fzd receptors are G-protein coupled seven transmembrane receptors, it was further investigated if the sFRP stimulation of CXCL8 could be through interactions with specific frizzled receptors. Fzd2 and Fzd5 have been associated with non-canonical Wnt signaling[67,68]; whereas, Fzd7 is associated with canonical signaling[69]. sFRP1, 2 and 3 all stimulated CXCL8 mRNA levels in the presence of a scrambled

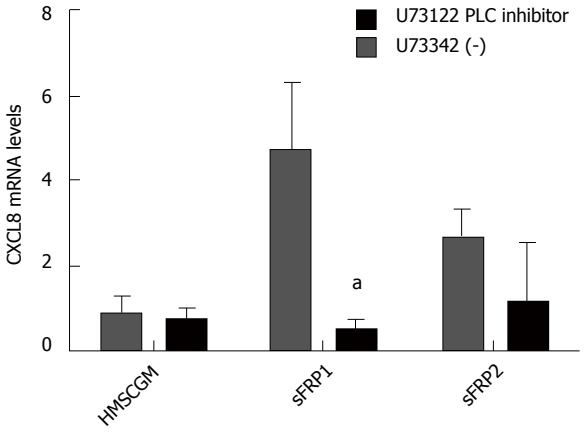

Figure 4 Modulation of secreted frizzled-related protein-stimulated induction of CXCL8 mRNA. sFRP1/2-stimulated CXCL8 mRNA expression is suppressed in the presence of the PLC inhibitor U73122 suggesting that signaling is *via* the Wnt/calcium non-canonical pathway (values are mean ± SD). $^{a}P < 0.05$ *vs* HMSCGM-treated levels. sFRP1/2: Secreted frizzled-related protein 1/2; PLC: Phospholipase C.

siControl siRNA (7 to 10-fold for sFRP1 and sFRP2; 3-fold for sFRP3). In the presence of siRNA to Fzd2 and 5, the sFRP-stimulation was almost entirely inhibited back to baseline levels ($P < 0.05$; Figure 5A and B). siRNA to Fzl7 did not have any effect on sFRP1 or sFRP2 induction of CXCL8 mRNA (data not shown). The receptor tyrosine kinase-like orphan receptor-2 (RoR2) has been shown to be either a stand-alone receptor or co-receptor with Fzd in non-canonical Wnt signaling[70,71]. siRNA directed against RoR2 inhibited sFRP1-stimulated CXCL8 mRNA expression by approximately 65% but did not have an effect on sFRP2-stimulated CXCL8 mRNA expression (Figure 5C).

DISCUSSION

sFRPs have been traditionally thought to act as Wnt signaling antagonists by binding to Wnt molecules and preventing them from binding to Fzd receptors thus inhibiting signal initiation[72]. Interaction of Wnts with Fzd receptors has been hypothesized to occur *via* interaction with the extracellular cysteine-rich domains (CRDs) found in Fzd receptors. sFRPs also contain N-terminal CRDs, but without the transmembrane domain characteristic of Fzd receptors[73], and it has been demonstrated that sFRP interaction with Wnts occurs through binding within this CRD[74,75]. Additionally, Bafico *et al*[75] found that at least one sFRP could also bind to a selected Fzd (*e.g.*, Fzd6) and hypothesized that heteromeric complex of Fzd and sFRPs would render Fzd receptors nonfunctional.

However, there has been emerging evidence that sFRPs may not only function as Wnt antagonists or anti-morphogens but also serve as molecules that promote differentiation of specific tissues. It has been suggested that sFRPs may aid in Wnt protein distribution and signaling within tissues. Wnt proteins interact with heparin sulfate proteoglycans thereby limiting diffusion

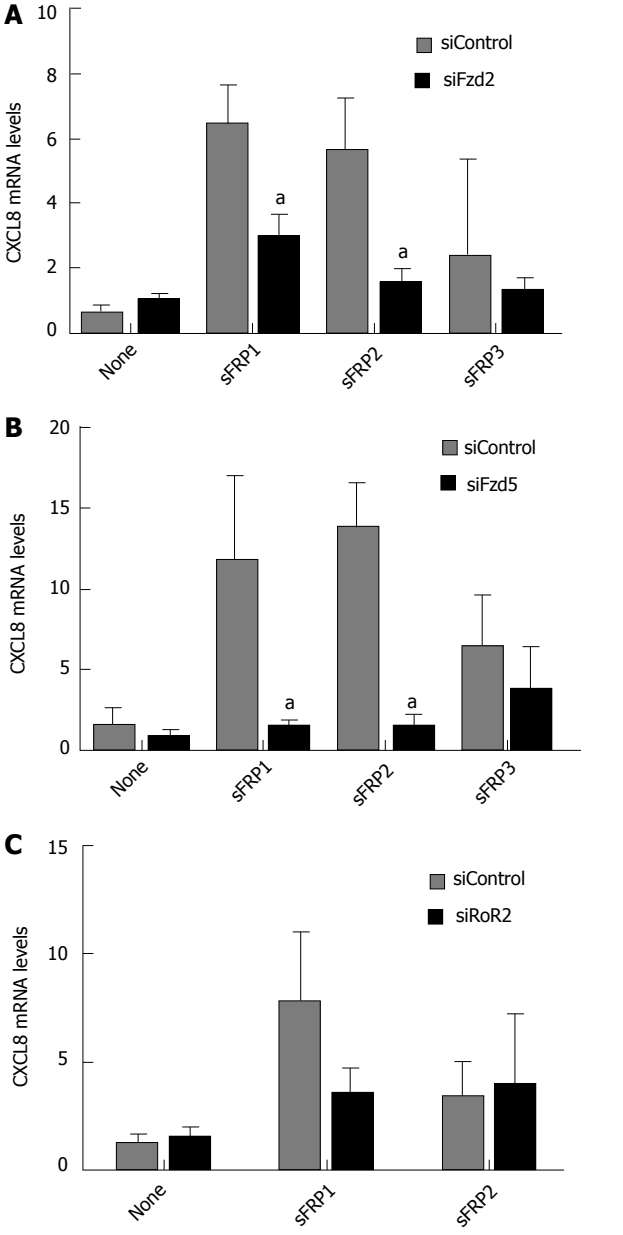

Figure 5 Effect of siRNA directed to frizzled receptors or receptor tyrosine kinase-like orphan receptor-2 on secreted frizzled-related protein-stimulated CXCL8 mRNA levels. sFRP-stimulated CXCL8 mRNA expression is inhibited in the presence of siRNAs directed toward non-canonical (A) Fzd2 and (B) Fzd5 or (C) the non-canonical Frizzled co-receptor RoR2. All values are mean ± SD. $^{a}P < 0.05$ *vs* untreated siControl levels for each graph. sFRP1-3: Secreted frizzled-related protein 1-3; siFzd2/5: siRNA to the non-canonical frizzled receptor 2/5; RoR2: Receptor tyrosine kinase-like orphan receptor-2; siRoR2: siRNA to RoR2; siControl: Scrambled control siRNA.

of the Wnt proteins within tissues. sFRPs may compete with this binding, enabling the Wnt-sFRP "transport" complexes that are formed to diffuse along a more extended gradient allowing for longer range Wnt signaling within the tissue[76] thereby aiding in tissue generation or differentiation. In one mouse model system, MSCs engineered to overexpress protein kinase B (also known as Akt) produce high levels of sFRP2 which can effectively limit cardiac muscle infarct size through the inhibition of cardiomyocyte apoptosis[53].

The increased sFRP2 led to increased levels of nuclear β-catenin thus enhancing canonical Wnt signaling and increased transcription of anti-apoptotic genes such as Birc1b and to a lesser extent Bcl2.

Others have reported links between non-canonical Wnt signaling and sFRP-mediated differentiation processes. Chung *et al*[77] reported that sFRP3 could increase osteoblast differentiation in the mouse pre-osteoblastic cell line, MC3T3-E1, by increasing alkaline phosphatase, osteocalcin, and promoting mineralization of MC3T3-E1 cultures. Endostatin, which promotes degradation of β-catenin independent of GSK3β, did not abrogate sFRP3-stimulated osteogenic differentiation suggesting that non-canonical Wnt signaling may be involved in the sFRP3 effect. Esteve *et al*[78] also reported that chick sFRP1 enhanced retinal differentiation by increasing the generation of retinal ganglion and photoreceptor cells independent of cell proliferation. This group noted that canonical Wnt-β-catenin signaling was not involved in this process; although, they did find that phosphorylation of GSK3β down-regulated its activity while promoting retinal cell differentiation. The authors were unclear if non-canonical Wnt signaling was involved in the sFRP1 findings at that time. In a subsequent communication, this group reported that chick sFRP1 binds to Fzd2 to stimulate axonal outgrowth from retinal neurons[79]. Furthermore, the action of sFRP1 on the retinal ganglion cells was dependent on cAMP and cGMP in a pertussis toxin-sensitive manner suggesting that sFRP1 acted as an agonist for Fzd2 non-canonical Wnt signaling.

Several reports have shown that sFRPs were involved in stimulating angiogenesis through canonical Wnt signaling but independent of VEGF signaling[54,55,80]. In studies utilizing MRL/MpJ mice, which have enhanced regenerative capacity, it was found that MRL/MpJ bone marrow MSCs showed decreased expression of cyclin D1, Sox2, and Axin2, which are target genes of canonical Wnt signaling. Concomitantly, sFRP2 and sFRP4 expression was found to be significantly up-regulated[52] in these cells. It was also reported that sFRP2 overexpression in mouse MSCs that were then injected into the cardiac peri-infarct area reduced infarct size and improved cardiac function similar to that seen when MRL/MpJ MSCs were injected. Of note, vascularization of granulation tissue was also enhanced by sFRP2 overexpression. This was also reported in another MRL/MpJMSC engraftment wound healing model[52] whereby sFRP2 overexpression in mouse MSCs increased levels of several angiogenic factors including FGF2 receptor, PDGF receptor beta, VEGF, and angiopoietins among others. While the authors concluded that the increased sFRP2 inhibited canonical Wnt signaling which may be related to the increased angiogenesis, non-canonical Wnt signaling was not examined. Furthermore, the expression of ELR^+ CXC chemokines that are also angiogenic was not assessed.

There are a number of steps that occur in new blood vessel formation, several of which have been linked to sFRP1 signaling, including endothelial cell (EC) spreading, proliferation and migration, vascular channel formation, and blood vessel stabilization. sFRP1 has been shown to enhance angiogenesis in a chick chorioallantoic membrane model of angiogenesis and to increase blood vessel density in a tumor implantation model[55]. EC spreading was hypothesized to be a result of an interaction of sFRP1 with Fzd4 and Fzd7 thereby blocking Fzd activity and has also been shown to be independent of canonical Wnt-β-catenin signaling; although, this process still involving GSK3β upstream of Rac 1 signaling[80]. sFRP1 has also been shown to stimulate EC migration and chemotaxis *in vitro*, increase EC branching in capillary structures when cultured on Matrigel, and inhibit EC apoptosis[55]. EC and vascular smooth muscle cell proliferation are also inhibited as evidenced by slower entry into S-phase as well as decreased expression of the cell cycle components cyclin D1 and cdk4[81]. This latter inhibition of vascular cell proliferation appeared to be dependent on inhibition of canonical Wnt-β-catenin signaling but not MAPK signaling through ERK1/2. Vessel maturation and stabilization of EC channels by pericytes or MSCs were also enhanced by sFRP1 stimulated cell-cell interactions between MSCs and ECs or smooth muscle cells in a GSK3β-dependent manner. Interestingly, sFRP1 increased α-smooth muscle actin expression in MSCs suggesting differentiation of MSCs to pericytes which are involved with blood vessel stabilization. Furthermore, the localization of β-catenin at cell-cell junctions rather than intranuclear locations could further support a non-canonical Wnt signaling mechanism. Others have also reported that sFRP2 can also stimulate EC migration and tube formation as well as inhibit hypoxia-induced EC apoptosis through a non-canonical Wnt-calcium pathway involving an increase in NFATc3 nuclear translocation[82,83].

The mechanism of how sFRPs stimulate angiogenesis is currently unknown. sFRP1, which did not induce expression of the known angiogenic factors VEGF or FGF2, did increase expression of PDGF-BB which is involved in postnatal blood vessel maturation[84]. Expression of other angiogenic factors such as the ELR^+ CXC chemokines could potentially be the result of sFRP1 actions. Indeed we are the first to report here that sFRPs are able to induce the ELR^+ CXC chemokines CXCL5 and CXCL8 in hMSCs. Rauner *et al*[85] has shown that human bone marrow MSCs stimulated with pro-inflammatory factors (lipopolysaccharide or TNF-α) resulted in Wnt5a and RoR2 increases in mRNA and protein. The expression of the ELR^+ CXC chemokines, CXCL1, CXCL2, and CXCL5, was also increased with Wnt5a treatment of these hMSCs. Additionally the CC chemokines, CCL2, CCL5, CCL7, and CCL19 were also upregulated, although, to a lesser extent than the CXC chemokines CXCL1 and CXCL5. Albers *et al*[59] also reported that Fzd9 knockout mice demonstrated an osteopenic phenotype caused by decreased bone formation which was unrelated to canonical Wnt signaling[59]. The presumed non-canonical Wnt regulation

of bone mass in Fzd9-deficient mice was also shown to have significantly decreased CXCL5 expression. In these studies, treatment of wild type osteoblasts with Wnt5a showed a 12-fold increase in CXCL5 mRNA. In a fracture repair model in Fzd9-deficient mice, protein expression of CXCL5 and CCL2 in the healing callus was diminished in comparison to wild-type, and overall new bone in the Fzd9 knockout mice was reduced[86]. Most recently, Zhao *et al*[87] reported that in human dental pulp cells, non-canonical Wnt5a significantly induced CXCL8, CCL2, and CCL5 mRNA and protein expression, as well as increasing CXCL1 mRNA expression. CXCL5 expression was not tested in response to Wnt5a stimulation in this model.

The signaling mechanism(s) responsible for sFRPs induction of ELR$^+$ CXC chemokine expression are unknown. Our results suggest that ELR$^+$ CXC chemokine stimulation by sFRPs in human bone marrow-derived MSCs is *via* non-canonical Wnt signaling. MAPK, specifically though ERK1/2, and PLC pathways appear to play a role in sFRP stimulation of the ELR$^+$ CXC chemokines. PLC signaling can be upstream of MAPK/ERK[88] and perhaps the non-canonical Wnt-calcium pathway is involved. Our findings that inhibition of sFRP-induced ELR$^+$ CXC chemokine expression by PLC as well as demonstration of ERK phosphorylation upon sFRP stimulation of MSCs are consistent with potential sFRP signaling thru serpentine G protein-coupled receptors such as the Fzd receptors. Furthermore, our data demonstrating prevention of sFRP1-stimulated CXCL8 mRNA induction with siRNA-directed inhibition of the non-canonical Fzd2 and Fzd5 are also consistent with a role of sFRP-Fzd receptor interaction in ELR$^+$ CXC chemokine genesis. How RoR2 either acting as a co-receptor with non-canonical Fzd receptors or independently fits in to the regulation of ELR$^+$ chemokine expression is currently unknown. Since these angiogenic chemokines are expressed during the inflammatory phase of wound healing, these chemokines could contribute to several aspects of bone repair including attraction of additional MSCs to the site for differentiation or attraction of endothelial cells for generation of vascularized granulation tissue and stimulation of angiogenesis as we had previously demonstrated[17]. Thus, in addition to regulation of Wnt signaling as inhibitory substances, our study adds to a growing body of knowledge on the stimulatory functions of sFRPs. Specifically, a novel function of sFRPs in stimulating angiogenic chemokines can be envisioned that may aid in wound and bone repair.

COMMENTS

Background

Mesenchymal stem cells (MSCs) have the capability to differentiate into several cell types including adipocytes, chondrocytes, and osteoblasts and thus have high potential as treatment for repairing bone defects. This process requires the interaction of various growth factors, chemokines, and signaling pathways resulting in the necessary inflammatory, angiogenic, and osteogenic stages of bone repair.

Research frontiers

Although much attention has been placed on the role of the major angiogenesis proteins (vascular endothelial growth factor and fibroblast growth factors) and the Wnt system in bone repair, not much research has been conducted on the role of the ELR$^+$ chemokines in this process. These chemokines also have important functions in inflammation and blood vessel formation and have been shown to be stimulated by non-canonical Wnt signaling and during osteogenic differentiation of MSCs.

Innovations and breakthroughs

In this report, the authors demonstrate that treatment of human MSC (hMSC) with the soluble frizzled-related proteins (sFRPs), which should inhibit both canonical and non-canonical Wnt signaling, actually stimulates the expression of the angiogenic CXC ELR$^+$ chemokines CXCL5 and CXCL8. CXC ELR$^+$ chemokine stimulation was mediated through the non-canonical frizzled receptors 2 (Fzd2) and Fzd5 Wnt receptors and the RoR2 co-receptor. This adds to the data suggesting non-canonical Wnt control of several bone formation processes through expression of the ELR$^+$ chemokines and identifies a potential new role for the sFRPs in coupling ELR$^+$ chemokine angiogenesis to bone repair.

Applications

Many recent reports have focused on the use of native or genetically engineered MSCs as a treatment to speed up or enhance the quality and mineralization of bone in wound and bone defect models. The ability of the sFRPs to stimulate ELR$^+$ CXC chemokines detailed in this study may suggest another avenue for manipulation of bone formation pathways and may eventually lead to a therapeutic treatment to hasten bone healing and return bone strength back to pre-injury levels.

Terminology

MSCs: Multipotent stromal cells that can be differentiated into several cells types including cartilage (chondrocytes), bone (osteoblasts), fat (adipocytes) and muscle (myocytes); ELR$^+$ CXC chemokines: Family of small cytokines secreted by cells and containing a Cys-X-Cys domain. CXC chemokines can be further divided into those with or without a Glu-Leu-Arg (ELR$^+$) motif. ELR$^+$ CXC chemokines are angiogenic. ELR$^-$ CXC chemokines are angiostatic. Wnt signaling: family of signaling molecules (Wnts) and Fzds that are important in many developmental pathways including cell fate, proliferation, and differentiation; sFRPs: Family of proteins that inhibit Wnt signaling by acting as soluble, decoy receptors preventing Wnt binding to Fzds.

Peer-review

The paper found that CXC chemokine expression in hMSC is controlled in part by sFRPs signalling through non-canonical Wnt involving Fzd2/5 and the ERK and PLC pathways. The results are interesting.

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