Chapter 4

Functional Activation of Autologous Human Diabetic Stem Cells for Cell Therapy

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Additional information is available at the end of the chapter

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Abstract

Diabetic retinopathy (DR) is a common cause of vision loss and blindness. Healthy CD34+ stem cells are capable of homing to vascular lesions and facilitating vascular repair. However, many diabetic patients have dysfunctional CD34+ stem cells with no reparative potential. CD34+ dysfunction is corrected by transiently inhibiting endogenous transforming growth factor-β1 (TGF-β1) within the patient's own dysfunctional CD34+ stem cells using phosphorodiamidate morpholino oligomers (PMOs). Antisense TGF-β1-treated dysfunctional CD34+ stem cells are now functional, no longer require growth factor stimulation to evade apoptosis, and are stable at 37°C ex vivo for >5 days. We identified three markers of restored stem cell function: (1) upregulation of CXCR4 expression necessary for stem cell homing and adhesion, (2) SDF-1-mediated nitric oxide (NO) production required for cell mobility, and (3) restoration of the ability of CD34+ cells to migrate and repair vascular lesions. The antisense targets autocrine TGF-β expression, whereas neutralizing antibodies do not. The PMO antisense triggers a cascade of hematopoietic proliferation and differentiation that paracrine TGF-β cannot alter. We describe optimal PMO manipulation of CD34+ stem cells ex vivo for transplantation, screening multiple gene targets leading to the identification of TGF-β1, and a lead TGF-β1 inhibitor evaluated in clinical studies.

Keywords: diabetic retinopathy, stem cell therapy, transforming growth factor-β1 (TGF-β1), phosphorodiamidate morpholino oligomers (PMOs), transient antisense

1. Introduction

Hematopoietic stem cells (HSCs) are capable of self-replication and clonal expansion generating differentiated progenitors, which give rise to all blood cell lineages [1]. These cells

co-express transforming growth factor-β (TGF-β) type I and type II receptors and one or more of the three isoforms of the TGF-β ligand as a latent complex [2]. The three TGF-β ligands qualitatively and quantitatively differ in the responses they elicit with TGF-β1, a multifunctional regulator of hematopoietic progenitors in vivo and in vitro, depending on cell differentiation, growth factors, ligand concentration, and cell–cell contacts [3]. Autocrine signaling by TGF-β1 plays a critical role in lineage-specific reconstitution [4] and enables non-canonical signaling involving mTOR, Ras, MAPK, PI3K, AKT, RhoA, and JNK [5]. TGF-β is an important part of the stromal microenvironment that regulates several niche cells, which in turn regulate HSC. Selective manipulation of endogenous TGF-β1 in HSC represents a therapeutic approach to transplantation to maintain, enhance, and restore tissue viability and organ function.

Transient inhibition of TGF-β1 in HSC accelerates the engraftment of long-term repopulating HSC (LTR-HSC), permits successful transplantation with as few as 60 LTR-HSCs to rescue mice from lethal irradiation, and promotes the survival of LTR-HSC in the absence of growth factors [6]. This permits LTR-HSC transplant without cell expansion ex vivo prior to transplant. TGF-β1 regulates LTR-HSC entry into the cell cycle at G0 [7]. Conditional knock-out of the TGF-β1 type II receptor in adult mice has increased stem cell cycling and reduced transplantation ability [8]; likewise, the inhibition of TGF-β in normal HSC with neutralizing antibodies releases cells into the cell cycle [9]. Inhibiting SMAD 4 signaling, key to TGF-β signaling, decreased HSC self-renewal in vivo [10]. The rapid generation of donor neutrophils that are the last cells to regenerate in bone marrow transplantation (BMT) is observed in transplanted mice after LTR-HSCs were treated with an antisense TGF-β1 PMO [6].

Phosphorodiamidate morpholino oligomers (PMOs) resist degradation [11], enhance specificity through a no-pucker six-membered morpholine ring in place of the five-membered ribose or deoxyribose [12], and are net charge neutral with one non-bridging oxygen substituted with a dimethylamine residue [13]. PMOs binds to the target RNA, forming a PMO:RNA heteroduplex that can inhibit translation [14] or pre-mRNA splicing [15]. The cellular internalization of PMOs in different cell types is mixed and not robust unless entry is assisted by cell uptake technologies [16]. Given the impressive safety profile for unmodified PMO [17–19], conjugation with delivery enhancements adds risk. An unmodified PMO represents the most specific and least risk to the modulation of gene expression in HSC.

Antisense TGF-β will reverse HSC growth arrest induced by TGF-β ligand, informing the reversibility of the ligand [20]. The antisense approach gains access to autocrine RNA expression over neutralizing antibodies, which targets protein. Transient antisense inhibition of autocrine TGF-β1 in HSC triggers a cascade of hematopoietic proliferation and differentiation that paracrine TGF-β cannot alter [21]. We report the kinetics of HSC internalization and efflux of PMO essential to transient inhibition of TGF-β1.

2. Feasibility: PMO entry into CD34+ stem cells

Evidence for receptor-mediated internalization of DNA came from studies with leukocytes over 30 years ago [22]. Uptake involves endocytosis based on chloroquine, a lysosomotropism

agent, enhanced intracellular fluorescence, sodium azide inhibited internalization, and a punctate pattern observed in the cytoplasm [23]. Scavenger receptors on rat liver endothelial cells participate in uptake and play a prominent role in plasma clearance [24]. Many oligonucleotide uptake pathways have been described, but the adaptor protein AP2 M1 is involved in phosphorothioate oligonucleotide (PSO) uptake. siRNA targeting clathrin and caveolin had no effect on antisense activity but did decrease the uptake of fluorescently labeled oligonucleotides, highlighting multiple subcellular compartments that accumulate oligonucleotides but not all are associated with antisense activity. Abasic oligomers, backbone and sugar without nucleobase oligomers, were not transported into cells by the AP2 M1 pathway [25], pointing to the nucleobase as a recognition site for uptake. The neutral charge of PMO compounds sets them apart from ionic forms like PSO.

We explored techniques to deliver PMO into cells in culture to improve bioavailability and efficacy including scrape loading [26], syringe loading [27], microinjection [28], osmotic loading [29], and complexation with cationic lipids [30]. These techniques suffer from limited efficiency and poor reproducibility and often leave residual biologically active carrier molecules in the culture media. We then explored a variety of cationic peptides conjugated to the PMO for an enhanced delivery including HIV-TAT [31] and a broad spectrum of arginine-rich peptides [32–34]. At present, the optimal delivery peptide is still composed of multiple arginines [35]. A concern for loading arginine into a stem cell was the role arginine plays in generating NO, a complication in interpreting observations of CD34+ activation. Thus, we examined unassisted entry in stem cells.

Earlier studies evaluating unassisted PMO entry into cultured cells revealed that primary cell cultures are more efficient in uptake than established cell lines. Uptake is independent of PMO sequence or the position of FITC conjugation (5′ vs. 3′ ends are equivalent) (**Figure 1A**) but dependent on concentration (**Figure 1B**), time, and temperature. There is a direct relationship between fluorescence intensity of CD34+ cells and PMO concentration. Localization to both cytoplasmic and nuclear compartments is observed, so that both pre-mRNA and mRNA targets are feasible. Uptake into hematopoietic lineages reveals that monocytes and dendritic cell uptake are efficient, while entry into CD8+ T-cells, CD4+ T-cells, and B-cells is minimal [36]. Viral infection activates some T-cell populations, resulting in permissive PMO uptake [37]. Current understanding of mechanisms involved in activation associated with PMO uptake is limited.

The first evidence of unassisted PMO entry into CD34+ cells came from microscopic observation of cells in which the visible uptake of FITC-PMO came within 15 min. Stem cells are unique in permissive unassisted PMO uptake. The maximal saturation of PMO uptake into HSC occurs within 2 h (**Figure 2**). Optimal uptake in terms of activation of HSC occurs after 16 h of FITC-PMO incubation. Stem cell positivity after 16 h was observed at 37°C with 70 ± 12% FITC-positive CD34+ cells (n = 6), room temperature incubation led to 56 ± 8% positive cells (n = 5), and 4°C incubation led to 30 ± 19% positive cells (n = 6). The percent FITC-PMO-positive CD34+ cells were mirrored by cellular fluorescence defined by mean channel fluorescence with 112 ± 47 at 37°C, 56 ± 22 at room temperature, and 31 ± 24 at 4°C incubation. Negative controls included CD34+ cells incubated with no FITC-PMO at 4°C, RT, and 37°C (n = 6) for each group, and no FITC-PMO-positive cells were observed.

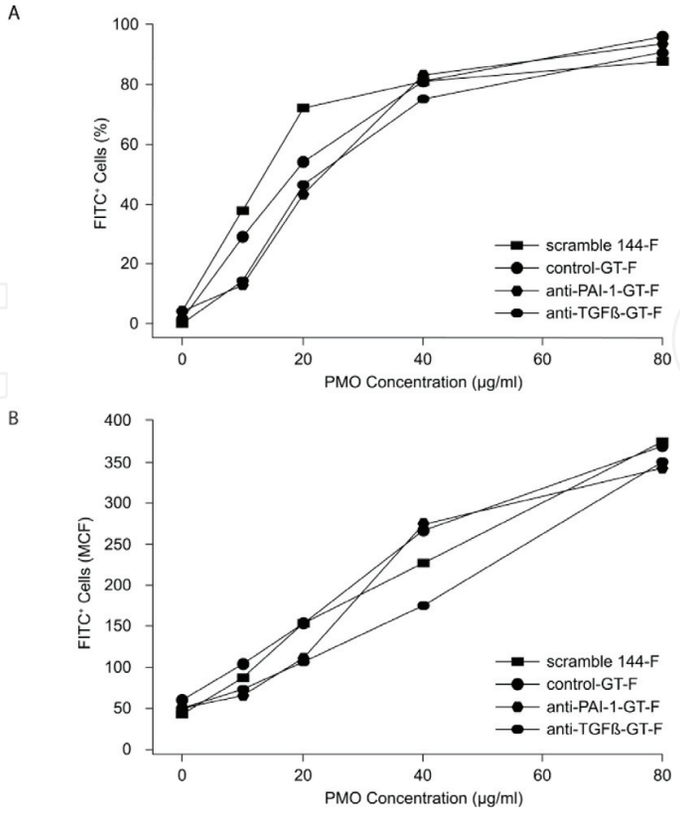

Figure 1. Concentration-dependent uptake of FITC-PMO into CD34+ human stem cells. A. Percent-positive CD34+ HSC on the ordinate and PMO concentration on the abscissa. B. Mean channel fluorescence of CD34+ HSC on the ordinate and PMO concentration on the abscissa. The uptake of PMOs is not sequence specific: 20-mer PMOs have similar entry kinetics, and percent-positive CD34+ cells are directly proportional to the PMO concentration in the medium. CD34+ cells were isolated from the blood of healthy subjects by pre-enriching the CD34+ by a lineage negative selection followed by FACS sorting of CD34+ CD45+ cells.

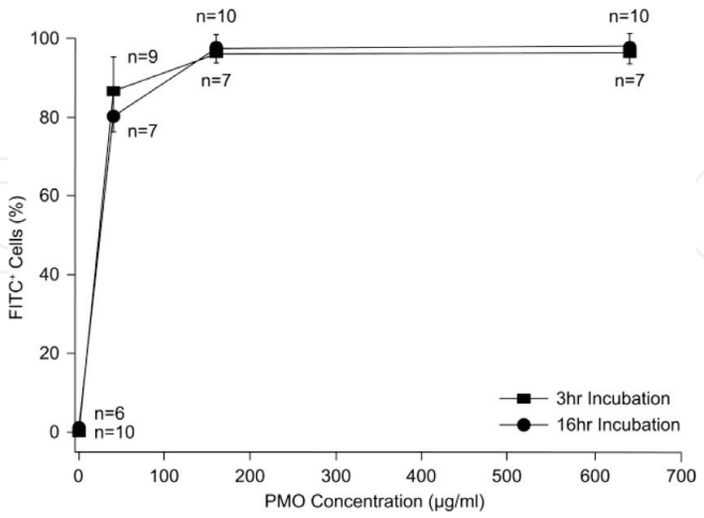

Figure 2. Time-dependent uptake of FITC-PMO into CD34+ human stem cells. After 3 h at 37°C at 150 µg/mL 144-F (a FITC-control PMO), >95% of CD34+ cells became FITC-labeled. At 16 h in culture, the degree of FITC-144-F PMO was the same as the 3-h point. CD34+ cells were isolated and FACS sorted from the blood of healthy subjects as described in **Figure 1**.

PMO uptake was found to be time and dose dependent. Uptake reached 100% FITC-PMO-positive CD34+ cells between 1 and 6 h of incubation at 37°C. Uptake determined as percent-positive CD34+ cells measured over time were linear (r2 0.94–0.94) (**Table 1**). Comparison of CD34+ cells recovered from diabetic individuals to non-diabetic individuals reveals that uptake is three to five times more rapid in non-diabetic CD34+ cells compared to those from diabetic individuals (**Table 1**, **Figure 3**). No loss in cell viability has been observed in protocols involving a 6-h incubation sufficient for 100% PMO-positive cells. Preliminary data suggest that treated stem cells will carry less than 5 μg PMO into the eye as a result of a combination of

Treatment group	Rate percent pos./H (r^2)[c]	Ratios (expected)	Time to 100% pos.	Saturation ratio
Norm[a] 40 μg (N = 2)	17.6 ± 1.1 (0.95)	3.3 (N/D 40)	6 h	3.0 (D/N 40)
Diab[b] 40 μg (N = 1)	5.3 ± 5.2 (0.95)	3.4 (D 160/40) [4]	18 h	3.6 (D 40/160) [4]
Norm 160 μg (N = 2)	98.0 ± 7.3 (0.94)	5.6 (N 160/40) [4]	1 h	6.0 (N 40/160) [4]
Diab 160 μg (N = 1)	18.1 ± 8.2 (0.94)	5.4 (N/D 160)	5 h	5.0 (D/N 160)

[a]CD34+ cells recovered from normal donors.
[b]CD34+ cells recovered from diabetic donors.
[c]Correlation coefficient from linear regression analysis.

Table 1. PMO uptake kinetics in CD34+ cells.

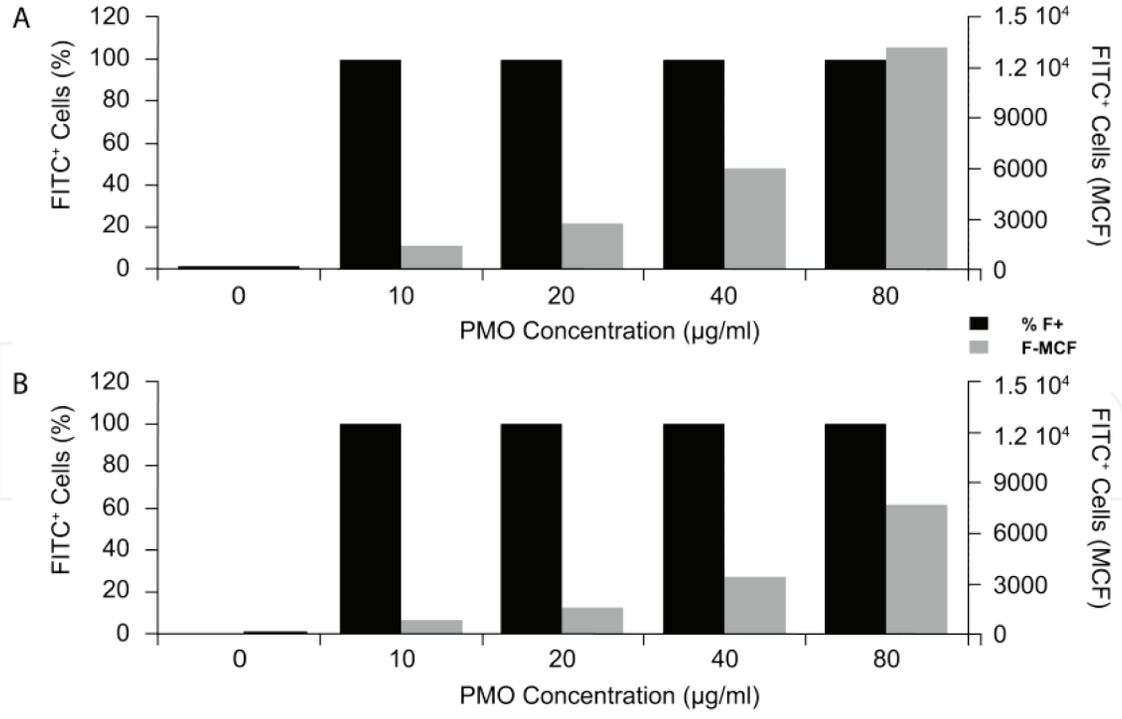

Figure 3. The percent of blood-derived CD34+ cell taking up FITC-PMO. A. Percent-positive (black bar) and mean channel fluorescence (gray bar) CD34+ cells from healthy donors. B. Percent-positive (black bar) and mean channel fluorescence (gray bar) CD34+ cells from diabetic subjects. Uptake is not different in healthy and diabetic subjects, but the rates of maximum saturation of FITC-PMO (measure by MEAN CHANNEL FLUORECENCE) are delayed in diabetic CD34+ cells relative to non-diabetic cells. CD34+ cells were pre-enriched and FACS sorted as described above.

efflux out of the cell, and tissue half-life will quickly lead to undetectable PMO and a transient inhibition of TGF-β in the stem cells. The overall exposure of PMO will be below 100,000 times the reported no observed adverse effect level (NOAEL) for a similar PMO in GLP toxicology studies [38, 39]. The use of PMO-treated CD34+ stem cells to treat patients with diabetic retinopathy is expected to be safe and feasible.

3. Optimal TGF-β PMO inhibitors in human Lin-CD34+ CD45+ HSC

TGF-β is a family of multifunctional peptide cytokines with the capacity to regulate proliferation, differentiation, adhesion, migration, and other functions in many cell types. TGF-β receptors are found on most cells, and their signal transduction positively and negatively regulates many other growth factors. Secreted TGF-β is cleaved into a latency-associated peptide (LAP) and a mature TGF-β1 protein. TGF-β is latent in the form of a TGF-β1 homodimer, a LAP homodimer, and a latent TGF-β1-binding protein (LTBP). However, TGF-β1 homodimer can be active, and the mature protein may also form heterodimers with other TGF-β family members.

The HSC is pluripotent immature cell that can generate daughter cells committed to all nine types of mature blood cells, including trillions of white blood cells, red blood cells, and platelets. HSCs are found in the bone marrow and also circulate in the peripheral blood. HSC possesses two key properties: (1) the ability to self-renew (generating HSC replicates) and (2) the ability to generate daughter cells that differentiate into fully functional blood cells (namely, asymmetrical HSC division in which one daughter cell remains a HSC and the other daughter cells are destined to mature).

When the most primitive HSCs self-replicate, they produce daughter cells with a long (possibly unlimited) clonal life span. When HSC replication leads to differentiation divisions, they lose their multi-lineage potential and the corresponding lineage commitment accompanied by a progressive reduction in clonal life span. Previous studies have shown that *ex vivo* proliferation of HSC favors differentiation divisions at the expense of self-replication, resulting in a complete loss of HSC.

4. TGF-β is an optimal stem cell target for CD34+ stem cells

An inhibitor of c-myc was identified based on antiproliferative effects in differentiated cells that block translation as well as create a dominant-negative variant of c-myc [40]. This inhibitor was effective in preventing coronary restenosis [41], preventing cyst growth in kidneys of polycystic kidney disease models [42], and reducing tumor growth [43]. We investigated the c-myc inhibitor in c-kit+/sca-1+ cells incubated with IL-3, IL-6, and SCF to drive cell proliferation of the stem cells. Incubation of these cells with saline was associated with a cell doubling half-life of 2.66 days, a scrambled sequence PMO (5′-GCTATTACCTTAACCCAG-3′) had a doubling half-life of 2.554 days, and the c-myc inhibitor (5′-ACGTTGAGGGGCATCGTCGC-3′)

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had a doubling half-life of 2.53 days. Unlike differentiated cells, the stem cells show no difference in cell proliferation when c-myc was inhibited. While inhibiting, *c-myc* did not influence proliferation rate; however, it did enhance stem cell differentiation as high proliferation potential (HPP) colony forming counts (CFC) rose from 3.8 HPP CFC in controls to 8.0 HPP-CFC in *c-myc*-inhibited cultures. This surprising observation suggested that *c-myc* inhibition stimulates stem cell differentiation and regulates self-renewal inspired studies to look at upstream signaling pathways in these stem cells. We studied the inhibition of ecotropic virus insertion-1 (EVI-1), which inserts in the DNA of murine stem cells and c-Kit, a stem cell marker along with c-myc and found that PMO-antisense treatment in vitro decreased LTR-HSC repopulating ability (**Figure 4**). Furthermore, the intra-peritoneal administration of PMO anti-c-myc reduces HSC-repopulating ability in vivo (**Figure 5**). These results represent an excellent functional control for PMO-TGF-β1 since these PMO antisense treatments do not promote HSC engraftment while PMO-TGF-β1 does.

ID11, a neutralizing monoclonal antibody to three isoforms of TGF- β (TGF- β1, 2, 3), added to stem cell cultures can replace growth factors and prevent apoptosis in mouse HSC [7]. Adding 100 c-kit+/sca-1+ cells to 96-well plates with no IL-3, IL-6, or SCF led to no cells observed after 5 days in culture. Adding ID11 to those cultures led to 37 ± 7 cells, confirming that the antibody could replace growth factors. Further, the addition of the PMO targeting *c-myc* led to 10 ± 3 cells at 5 days in culture, leading us to conclude that *c-myc* expression is required for the loss of TGF-β phenotype. It became apparent that ID11 effectively blocks extracellular TGF-β, but a PMO (5′-GCA CTG CCG AGA GCG CGA ACA-3′) inhibitor of TGF-β translation could have the advantage of blocking autocrine signaling. Inhibiting TGF-β with either antibody or antisense PMO enhances HPP-CFC from progenitor cells [7, 20, 21] and can enhance hematopoietic reconstitution following bone marrow transplantation [6, 44, 45]. Importantly,

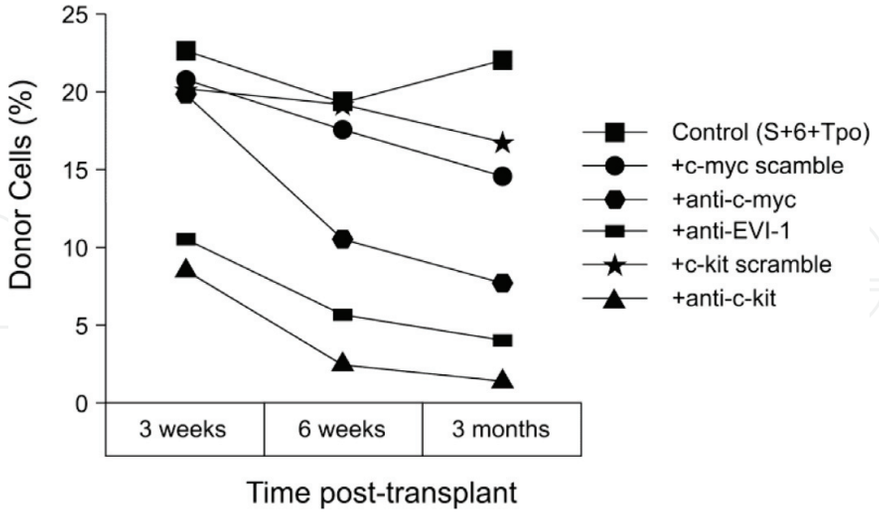

Figure 4. PMO targeting of *c-myc*, *c-kit*, and *EVI-1* in *ex vivo* cultures of highly purified murine LTR-HSC. LTR-HSCs were isolated as previously described, then 25 cells per well were incubated for 5 days with PMO and hematopoietic growth factors followed by intravenous transplant into lethally (950 rads) irradiated mice. CD45.2 congenic LTR-HSCs were transplanted into CD45.1 recipients, so that donor LTR-HSC could be detected by monoclonal antibodies. Significantly fewer ($p < 0.05$) LTR-HSCs were observed in cultures treated with *c-kit*, *EVI-1*, and *c-myc* PMO compared to control, c-myc scramble, and c-kit scramble PMO after 3 months post-transplant.

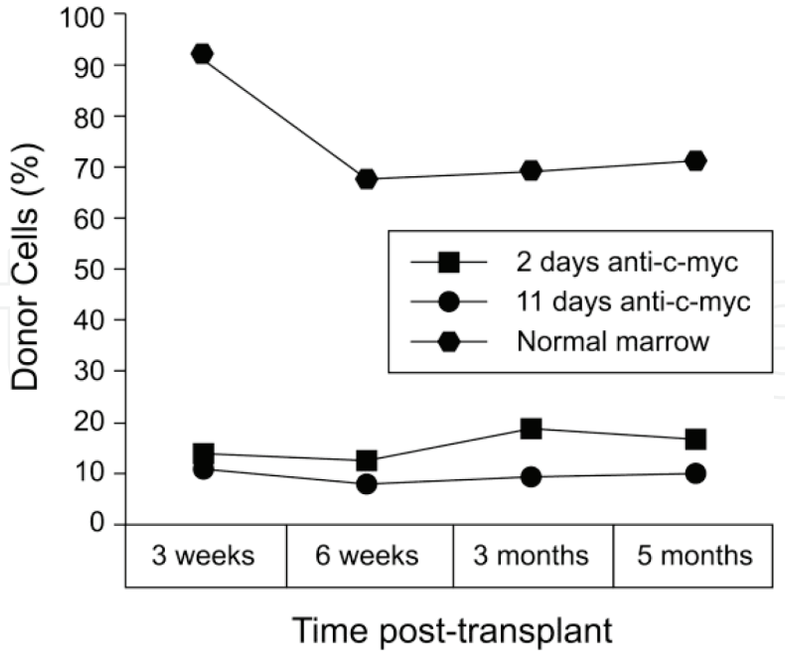

Figure 5. In vivo activity of PMO targeting *c-myc* in short-term and long-term hematopoietic stem cells. Mice were treated with *c-myc*-PMO in vivo (intraperitoneal injection) for 2 or 11 days. At each time point, mice were sacrificed, and the femoral marrow was assayed for HSC levels using a murine transplantation model. Significant reductions in repopulating HSC ($p < 0.05$) were observed in mice treated with c-myc PMO compared to normal bone marrow.

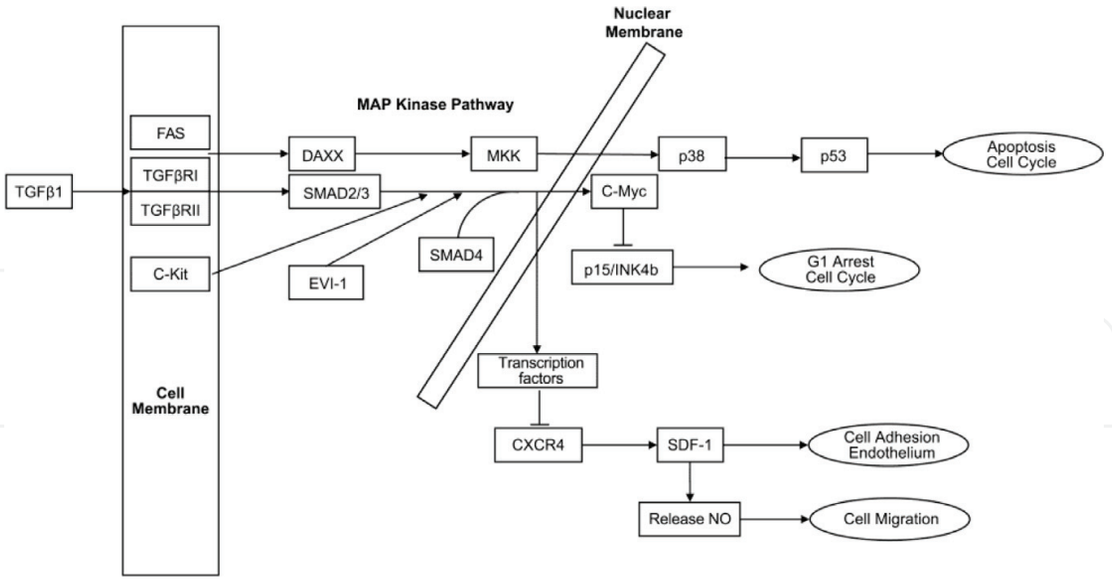

Figure 6. Targeting stem cell pathways. Studies were conducted targeting c-myc, SMAD4, EVI-1, c-kit, TGF-βRI, TGF-βRII, and TGF-β1 with PMOs designed to inhibit expression in HSC. Regulation of transcription factors by TGF-β1 is linked to stem cell homing through CXCR4 interaction with SDF-1 and release of nitric oxide and elevated migration. The MAP kinase pathway signaling reveals the potential mechanism for the prevention of apoptosis with TGF-β1 inhibition. The upstream regulation of c-myc and p53 by TGF-β1 inhibition allows stem cells to proliferate. Inhibition of TGF-β1 is the optimal target resulting in stem cell proliferation, homing, and migration of all favorable properties for autologous transplantation.

the transplantation of Tfgβ1$^{-/-}$ bone marrow into lethally radiated TGF-β1$^{+/+}$ recipients reconstitutes all hematopoietic lineages [46]. Taken together, these studies encouraged further examination of the TGF-β1-signaling pathway.

We investigated PMO inhibitors of TGF-β receptor I (5′CAT GGT CCC TGC AGA GAG GA-3′) and TGF-β receptor II (5′-GAC CCA TGG CAG CCC CCG TCG-3′) to reveal the same phenotype to the TGF-β1 ligand inhibitor. Subsequent studies targeting SMAD 4 (5′-AAT CAT ACT CAT CCT TCA CCA TCA T-3′) also led to the TGF-β-inhibited phenotype in CD34+ cells, confirming that the signal transduction pathway is responsible for the phenotype, while blocking other pathways did not (**Figure 6**). We focused on the TGF-β1 ligand due to the short half-life, enabling rapid onset and transient inhibition properties of the treatment.

5. The optimal TGF-β1 inhibitor

We investigated the use of an antisense PMO targeting the AUG translation start site for efficacy in inhibiting TGF-β1 expression by hybrid arrest of translation. One possible outcome of a PMO at AUG1 will be for translation slippage to a translation initiation start site at amino acid 38, AUG38 (**Figure 7**). The resulting protein will not have the signal peptide, leading to the loss of appropriate subcellular localization, altered autocrine regulation, and possibly a protein with a shorter half-life. The diminished protein product fails to provide a negative feedback to the promoter, so enhanced transcription is expected. To test this hypothesis, we evaluated six oligomers targeting translation and two scrambled control sequences (**Table 2**).

The compounds were evaluated in an in vitro translation assay using rabbit reticulocyte lysate and a luciferase fusion transcript with TGF-β1 mRNA. Each of the antisense PMOs effectively inhibited translation, and the scrambled control oligomers did not inhibit, confirming PMO sequence specificity. The TGF-β1 PMO included 13 guanines (G) in the 20-mer and presented water solubility limitations and reduced synthetic yield concerns. Replacing guanine with inosine improved both water solubility and synthetic yield. However, inosine pairing with cytosine involves two hydrogen bonds in contrast to the three hydrogen bonds between guanine and cytosine. The hypothesis is that the more inosine replacement of guanine in the oligomer will result in a lower binding energy between PMO and target RNA and subsequent

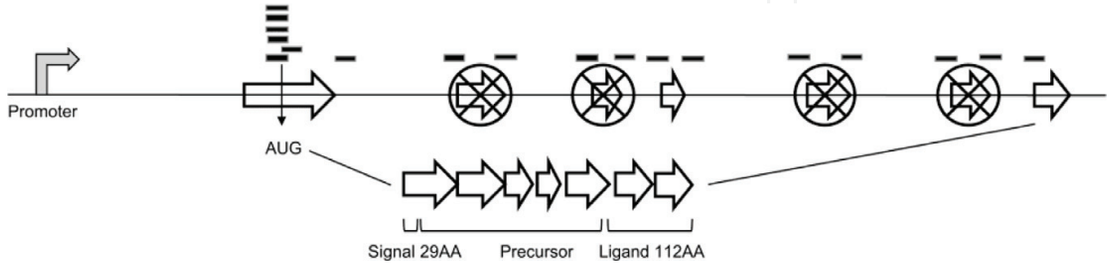

Figure 7. Optimal antisense strategy. Multiple PMOs were developed to inhibit translation initiation at the AUG site as well as targeting each exon at both splice donor and splice acceptor sites (black bars). Skipping exons 2, 3, 5, and 6 results in out-of-frame reading, and a nonsense-mediated decay (NMD) of the transcript is expected (X circles).

Name	Sequence 5′ → 3′	Mol Wt.
GT Control	CCTCCTACCTCAGTTACAATTTATA	—
144 Control	AGTCTCGACTTGCTACCTCA	7020
TGF-β1 GT	GCACTGCCGAGAGCGCGAACA	7642
TGF-β1	GAGGGCGG**CAT**GGGGGAGGC	7175
TGF-β1 1-I*	GAGGGCGG**CAT**GG I GGAGGC	7160
TGF-β1 3-I (1067)	GAGGGCGG**CAT**G III GAGGC	7130
TGF-β1 2-I	GAG I GCGG**CAT**GG I GGAGGC	7145
TGF-β1 4-I	GAG I GCGG**CAT**G III GAGGC	7115

*I refers to inosine, a strategy to limit "purine clash."

Table 2. Oligomer sequences employed to inhibit TGF-β1 translation.

diminished inhibition of translation. By contrast, the PMO with three inosines, TGF-β1 3-I (1067), inhibited translation more effectively than PMOs with all guanine or PMOs in which one or two guanines were replaced by inosine (data not shown).

TGF-β1 has seven exons transcribed into eight variant mRNAs, five alternately spliced variants and three unspliced forms (**Figure 7**). A small signal peptide (29 amino acids) is encoded in exon 1; the precursor LAP is encoded in exons 1 through 5; the active TGF-β1 is encoded in exons 6 and 7. The LTBP is encoded by a separate gene and binds directly to the LAP in the latent TGF-β complex prior to secretion. The amino terminus of LTBP binds to the extracellular matrix followed by proteolytic cleavage by a serine protease, plasmin, releasing the latent complex. A urokinase plasminogen activator (uPA) protease cuts the 391-amino acid TGF-β1 propeptide liberating the active 112 amino acid TGF-β1, which forms a homodimer ligand for the TGF-β receptors [47].

The TGF-β N terminal domain is present in a variety of proteins, which include TGF-β, decapentaplegic peptides, and bone morphogenetic proteins. The N-terminal domain expressed on the decapentaplegic protein acts as an extracellular morphogen guiding: (1) the proper development of the embryonic dorsal hypoderm, (2) viability of larvae, and (3) cell viability of the epithelial cells in the imaginal disks. When the N terminal domain is expressed on the bone morphogenetic protein (BMP), it induces cartilage and bone formation, possibly for epithelial osteogenesis. TGF-β1 is a protein composed of 112 amino acid residues liberated by proteolytic cleavage from the C-terminal of a precursor protein. A number of proteins are related to TGF-β1. The TGF-beta family is only active as homo- or heterodimers, the two chains being linked by a disulphide bond. X-ray studies of TGF-β2 reveal that all the other cysteines are involved in intrachain disulphide bonds. The four disulphide bonds in TGF-β and in the inhibin beta chains distinguish function from the other members of this family that lack the first bond. Concern has been noted as TGF-β not only exerts tumor-suppressive effects but also modulates cell invasion and immune regulation such that dysregulation of the TGF-β signaling pathway can result in tumor development.

In order to demonstrate PMO inhibition, THP-1 cells, which are human monocytes that express TGF-β, were studied. THP-1 grows equally well in RPMI supplemented with 10% fetal bovine serum and serum-free media. When grown in serum-free media, TGF-β is not secreted into the media (ELISA = 0 pg./mL). Media supplemented with 50 ng/mL PMA lead to TGF-β secretion (ELISA = 92 pg./mL). The addition of 10 μM atorvastatin (Lipitor) enhances TGF-β secretion by fivefold (ELISA >500 pg./mL) following 72 h of incubation.

The evaluation of mRNA from splice altering PMOs is shown (**Table 3**). The control fragment appears at the correct size. Cells treated with PMOs targeting SD Ex2, SD Ex4, and AUG show no variation in transcript size and thus no evidence of exon skipping. The AUG signal appears to be enhanced relative to the untreated control, possibly indicating a rebound induction of transcription. This may be anticipated as the translation start site inhibitor will lead to suppression of the propeptide including LAP, which may lead to loss of the negative feedback mechanism for TGF-β1 transcription. Cells treated with PMOs targeting SD Ex5 and SD Ex6 reveal smaller transcripts in addition to faint bands at the correct size. The SD Ex5 smaller transcript is consistent in size with the loss of exon 4 (74 bp), which would leave the mature mRNA in frame. Those transcripts skipping exon 5 would be smaller yet (148 bp) and are expected to be degraded by a nonsense-mediated decay (NMD), so that the product would not be observed. The SD Ex6 smaller fragment is approximately 800 bp in size, which is 300 bp smaller than the expected full-length transcript and consistent with loss of both exons 5 (148 bp) and 6 (156 bp), which would be 304 bp smaller than the full-length transcript.

Treatment (5 μM 96 h)	Hu TGF-β1 protein (pg/mL)	Mu TGF-β1 protein (pg/mL) 5 μM 65 h	Cell viability (% control)
No PMO	520 ± 2	—	100
Scr Ctr	502 ± 15	1700 ± 10	88
SD Ex1*	385 ± 7	—	—
SA Ex2	387 ± 5	—	—
SD Ex2	BLD	1320 ± 30	93
SA Ex3	594 ± 21	—	—
SD Ex3	222 ± 3	—	—
SA Ex4	465 ± 12	—	—
SD Ex4	23 ± 2	520 ± 20	66
SA Ex5		—	—
SD Ex5	BLD	120 ± 10	79
SA Ex6	404 ± 18	—	—
SD Ex6	BLD	330 ± 10	79
SA Ex7	101 ± 1	—	91

*SD refers to the splice donor site of the exon (Ex) and SA refers to the splice acceptor site.

Table 3. Exon skipping in THP-1 cells stimulated to secrete TGF-β1.

Skipping exon 6 alone or exon 5 alone would be degraded by NMD, and those transcripts would not be observed. Skipping exons 5 and 6 will also remain in frame.

The exercise to identify an optimal inhibitor of TGF-β1 involved screening multiple gene targets and dozens of PMO inhibitors. Qualitative differences between splice-altering strategies and translation inhibitors involve the preservation of feedback inhibition of the promoter. Translation inhibitors and splice-altering targets that induce a nonsense-mediated decay (NMD) prevent the synthesis of the negative feedback, resulting in compensatory transcription followed by rebound translation of TGF-β1. By contrast, skipping of exons 5 and 6 leads to translation products with altered function but includes the LAP portion of the translated product, resulting in a prolonged inhibition of TGF-β1. Transient inhibition of TGF-β1 is desired [48], so the optimal approach favors the AUG and NMD PMO over exon skipping and ligand-neutralizing antibodies. Translation inhibition is preferred over NMD because NMD responses may be less reliable.

6. Stem cell therapy for diabetic retinopathy

The Centers for Disease Control and Prevention report that 4.2 million (28.5%) of US diabetics aged ≥40 years have diabetic retinopathy (DR) or damage to the small blood vessels in the retina that may result in loss of vision [49]. The direct costs for DR in the US were over $4.5 billion, and the indirect economic impact was an additional $5 billion. Retinopathy occurs in almost all patients with type 1 diabetes and 75% of patients with type 2 diabetes within 15 years of the manifestation of diabetes [50]. Over 12,000 diabetic patients become blind each year due to ocular complications [51]. Current therapy addresses the end stages of DR including laser photocoagulation, intravitreal antivascular endothelial growth factor (VEGF) agents such as Bevacizumab and Aflibercept, intravitreal corticosteroids such as Triamcinolone, and vitreoretinal surgery. CD34+ stem cells from diabetic patients cannot generate endothelial cells to repair the vasculature, instead generating more inflammatory monocytes [52]. The CD34+ stem cell therapy described here exploits the ability of these cells to differentiate into a wide variety of cell types to stimulate both vascular and neural regeneration to treat early stages of DR.

CD34+ cells are capable of homing to vascular lesions in the eye, mediating vascular repair [53]. The use of autologous CD34+ cells eliminates the significant complication of transplant rejection. However, diabetic CD34+ cells are dysfunctional, contributing to the diabetic complication of DR [54]. While CD34+ cells from healthy subjects could repair retinal capillaries in streptozotocin-induced diabetic mice, spontaneously diabetic obese BBZDR/Wor rats and neonatal mouse oxygen-induced retinopathy animal models CD34+ cells from diabetic mice could not [55]. The approach described here restores function to dysfunctional diabetic CD34+ cells.

TGF-β1 is overexpressed and may cause dysfunction in diabetic CD34+ cells, and correction of this overexpression can restore the regenerative ability of those cells in diabetics. TGF-β1 is the major regulator of the balance between CD34+ proliferation, differentiation, and quiescence. Transient inhibition of TGF-β1 with an optimal PMO (1) activates human CD34+ proliferation, whereas ID11 antibody does not, (2) enhances CXCR4 cell surface expression and effective stem

cell homing to SDF-1 ligand, (3) increases nitric oxide (NO) release, stimulating stem cell migration, and (4) increases vascular repair by the activated diabetic CD34+ cells. The transient TGF-β1 inhibition approach holds potential to impact other diabetic microvascular complications and improve current bone marrow transplantation processes used in the treatment of blood cancers.

The transient inhibition of TGF-β1 in autologous diabetic CD34+ cells with an antisense PMO ex vivo represents a feasible approach that poses minimal potential for adverse events and has potential benefit to the patient with diabetic retinopathy. Challenges remain in development such as the selection of animal models that adequately predict the human response to treatment. Numerous features including the genetic basis of the retinal disease, anatomical differences in the eye, and the genesis of retinal damage limit the utility of animal models. Substantial differences in diabetic subpopulations, the presence of comorbidities, patient age, and diabetes severity will influence the success of our proposed therapy. A detailed understanding of the natural history of diabetic retinopathy deserves in-depth investigation, so that patient enrollment can be refined, and clinical trials will examine optimal patient populations and appropriate stage of disease. Current efforts are ongoing to address these limitations as our protocol advances to the clinic.

7. Conclusions

Damaged retinal vessels are repaired by HSC in individuals throughout their life. Diabetic HSC function is impaired, leading to the development of numerous clinically important conditions including diabetic retinopathy. Selective *ex vivo* manipulation of TGF-β1 in diabetic HSC represents a therapeutic approach to maintain, enhance, and restore vascular viability in the retina. The PMO offers transcript selective binding and transient interference with translation of TGF-β1. The PMO offers a feasible technology in which they enter HSC, can inhibit autocrine TGF-β1 signaling in HSC, and have an excellent safety profile. We presented the process of selecting TGF-β1 as an optimal transcript and the optimal PMO sequence targeting TGF-β1 mRNA. Our studies identified a transient interference with the translation of TGF-β1 in diabetic CD34+ HSC with an antisense PMO that will (1) upregulate the expression of CXCR4, enabling stem cell homing and adhesion to sites of vascular injury in the retina, (2) stimulation of nitric oxide production, enabling stem cell mobility, and (3) the release of cell cycle checkpoints, enabling stem cell proliferation and differentiation required for the repair of vascular lesions. The manipulated stem cell treatment strategy is making the transition from discovery to preparation for clinical evaluation.

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Conflict of interest

The authors of this chapter have financial interest in BetaStem Therapeutics, Sausalito CA.

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