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Stem cell membrane engineering for cell rolling using peptide conjugation and tuning of cell-selectin interaction kinetics

Hao Cheng[a,b], **Marta Byrska-Bishop**[b], **Cathy T. Zhang**[a], **Christian J. Kastrup**[c], **Nathaniel S. Hwang**[d], **Albert K. Tai**[e], **Won Woo Lee**[f], **Xiaoyang Xu**[b], **Matthias Nahrendorf**[g], **Robert Langer**[a,b,h], and **Daniel G. Anderson**[a,b,h,*]

[a]Department of Chemical Engineering, Massachusetts Institute of Technology, 77 Massachusetts Ave., Cambridge, MA 02139, USA

[b]David H. Koch Institute for Integrative Cancer Research, Massachusetts Institute of Technology, 77 Massachusetts Ave., Cambridge, MA 02139, USA

[c]Michael Smith Laboratories and Department of Biochemistry & Molecular Biology, University of British Columbia, Vancouver, BC V6T 1Z4, Canada

[d]School of Chemical and Biological Engineering, Seoul National University, 1 Gwanak-ro Gwanak-gu, Seoul 151-744, Korea

[e]Department of Pathology, Tufts University School of Medicine, Boston, MA 02110, USA

[f]Department of Nuclear Medicine, Seoul National University Bundang Hospital, Seoul National University College of Medicine, Seongnam, Gyeonggi-do 463-707, Korea

[g]Center for Systems Biology, Massachusetts General Hospital and Harvard Medical School, Boston, MA 02114 USA

[h]Division of Health Science and Technology, Massachusetts Institute of Technology, 77 Massachusetts Ave., Cambridge, MA 02139, USA

Abstract

Dynamic cell-microenvironment interactions regulate many biological events and play a critical role in tissue regeneration. Cell homing to targeted tissues requires well balanced interactions between cells and adhesion molecules on blood vessel walls. However, many stem cells lack affinity with adhesion molecules. It is challenging and clinically important to engineer these stem cells to modulate their dynamic interactions with blood vessels. In this study, a new chemical strategy was developed to engineer cell-microenvironment interactions. This method allowed the conjugation of peptides onto stem cell membranes without affecting cell viability, proliferation or multipotency. Mesenchymal stem cells (MSCs) engineered in this manner showed controlled firm adhesion and rolling on E-selectin under physiological shear stresses. For the first time, these biomechanical responses were achieved by tuning the binding kinetics of the peptide-selectin interaction. Rolling of engineered MSCs on E-selectin is mediated by a Ca^{2+} independent interaction, a mechanism that differs from the Ca^{2+} dependent physiological process. This further illustrates the ability of this approach to manipulate cell-microenvironment interactions, in

*Corresponding author. Tel.: +1 617 258 6843; fax: +1 617 258 8827. dgander@mit.edu (D.G. Anderson).

particular for the application of delivering cells to targeted tissues. It also provides a new platform to engineer cells with multiple functionalities.

1. Introduction

Cell membrane engineering (CME) is an emerging research area [1–12]. It refers to the modification of cell membranes with biological, chemical or physical methods to alter cell functions [1–3, 6–17]. Engineering cells to present natural or synthetic ligands on their membranes has become a promising modality to control cell-microenvironment interactions that are crucial in tissue regeneration. One potential application of CME is to navigate cells, such as stem cells, to targeted tissues [2, 10]. Stem cells hold great promise for regenerative medicine. However, one of the major obstacles of stem cell-based therapy is the homing and retention of cells in tissues requiring regeneration [18, 19]. One goal of CME is to modify human bone marrow-derived mesenchymal stem cells (hMSCs) to target receptors expressed on inflamed vessels of injured tissues. A natural response to injury is inflammation, during which certain circulating cells are recruited. Inflammatory cytokine-activated endothelial cells express adhesion molecules, E- and P- selectins to induce the rolling of leukocytes in postcapillary venules in the presence of Ca^{2+}, as well as vascular cell adhesion molecule 1 to mediate the firm adhesion of leukocytes [20].

For leukocytes and other cells to tether and roll on inflamed vessels, specific receptors on their membranes must be glycosylated with sialyl Lewis X (sLex), a tetrasaccharide [21–23]. hMSCs do not express receptors binding to E or P-selectin after they have been expanded *ex vivo*, due to the lack of sLex. In a previous report, sLex was generated on the membranes of hMSCs by α-1,3-fucosyltransferase, which allowed hMSCs to target the bone marrow of mice after intravenous administration [2]. However, this method requires a specific enzyme to generate one particular function, limiting its potential applications. In another report, sLex was anchored on the membranes of hMSCs via streptavidin-biotin bridges, which also resulted in cell rolling [10]. However, methods with less steps and that avoid the use of streptavidin, which may be immunogenic, would be favored for clinical applications [24]. Ligands with lipid or alkyl chains can be anchored on cell surfaces by inserting hydrophobic tails into lipid bilayer of cells [5]. This approach, though convenient, is less suitable for applications that exert strong forces on the ligands. This is due to the weak interaction between hydrophobic tails and membrane lipids, particularly in serum [25]. This problem can be reduced by using ligands containing multiple hydrophobic tails [15, 16].

Herein, we present a new facile chemical method to conjugate synthetic ligands on cell membranes to control cell-microenvironment interactions, in particular the binding of stem cells on adhesion molecules. This strategy overcomes the limitations of previous approaches. It is general and suitable for manipulating cell-cell and cell-matrix surface interactions with controlled binding dynamics and kinetics. Cell rolling is a mechanically delicate process with well balanced dynamics of bond formation and breakage, which requires proper binding kinetics between adhesion molecules and ligands on cell membranes. We engineered hMSCs to firmly adhere or roll under physiologically relevant shear stresses by tuning the kinetics of the interaction between peptides and selectins. This interaction is artificial and is Ca^{2+} independent. In this study, mouse MSCs (mMSCs) were also engineered to roll. The comparison of engineered hMSCs and mMSCs revealed that the size of cells is an important parameter to consider when engineering cells in this manner.

2. Materials and Methods

2.1 Materials

Succinimidyl-[(N-maleimidopropionamido)-diethyleneglycol] ester (NHS-PEG_2-Maleimide) and Immobilized TCEP disulfide reducing gel were purchased from Pierce Protein Research Products. Human mesenchymal stem cells (hMSCs), mesenchymal stem cell growth medium, hMSC adipogenic differentiation medium and hMSC osteogenic differentiation medium were purchased from Lonza. Formalin free fixative, Fast Blue RR Salt and Naphthol AS-MX phosphate were purchased from Sigma-Aldrich. Oil red O was purchased from Electron Microscopy Science. SYLGARD® 184 SILICONE ELASTOMER KIT (PDMS) for microfluidic device was purchased from Dow Corning. E-selectin/CD62E/Fc chimera and Recombinant human IgG_1 FC were purchased from R&D systems. AlarmaBlue, fetal bovine serum (FBS), EDTA, MEM alpha medium and DMEM medium were purchased from Invitrogen. hPGK-eGFP lentivirus and hPGK-mCherry lentivirus were obtained from Burnham Institute for Medical Research. All the peptides were custom synthesized by Biomatick Corp with purity >95%. CM5 Sensorchip, anti-human IgG Fc monoclonal antibody, amine coupling kit and HBS-EP+ buffer were purchased from GE Healthcare. HL-60s and Iscove's Modified Dulbecco's Medium were obtained from American Type Culture Collection (ATCC). Mouse mesenchymal stem cells (mMSCs) were a generous gift from the Lab of Phillip A. Sharp. StepOnePlusTM Rreal-Time PCR System and Taqman Gene Expression Assays were purchased from Applied Biosystems.

2.2 Coating microfluidic devices

Microfluidic devices were fabricated by rapid prototyping in polydimethylsiloxane (PDMS) using SU-8 photoresist masters [26]. Autoclave-sterilized PDMS microfluidic devices were washed 3 times with phosphate buffered saline (PBS). The interior of the microfluidic channels was coated with recombinant human E-selectin/CD62E/Fc chimera by filling the channels with indicated concentrations of E-selectin-PBS solution and incubating for 4 h at 37 °C, followed by incubation in 1% BSA-PBS blocking solution for 30 min. Recombinant human IgG_1 FC at 5 μg/mL instead of E-selectin was used to coat microfluidic channels in one control experiment. Devices were washed 3 times with PBS before use.

2.3 Cell culture and chemical modification of cell membranes

hMSCs were cultured at 37 °C/5% CO_2 in mesenchymal stem cell growth medium. hMSCs expressing GFP and hMSCs expressing mCherry were generated by infecting cells with hPGK-eGFP lentivirus and hPGK-mCherry lentivirus respectively. The titer of virus was 5×10^7 TU/mL. To infect cells, 5 μL of viral vector solution was used per 20,000 hMSCs in one well of a 6-well plate. The medium was replaced with fresh medium after 24 h.

To chemically modify the cell membranes, MSCs were cultured in a flask until the confluency reached ~70%. Cells were rinsed 3 times with PBS and then incubated with 100 μM NHS-PEG_2-maleimide for 15 min at room temperature. After the first step of membrane modification, cells were rinsed 3 times with PBS to wash away the unreacted reagent. Cells were further treated with peptide ligands by incubating them in a solution of 6 μM peptides for 15 min at room temperature. Prior to use, peptides were reduced with immobilized TCEP disulfide reducing resin for 2 h. After conjugating the peptide ligands onto cell membranes, cells were trypsinized for further testing.

To measure the density of peptides on cell membranes, 5-FAM-peptide (N-terminal modification) was used for cell membrane modification. The concentration-fluorescence intensity working line was generated by using the peptide at concentrations ranging from 0.049 μM to 1.25 μM. The fluorescence intensity of 10^5 engineered hMSCs was measured

at the same time on a plate reader (PerkinElmer 1420 Multilabel Counter). The intensity of unmodified cells as the control was found negligible. Cells were also imaged with a laser scanning confocal microscope (Zeiss LSM 510) to determine peptide distribution on cell membranes. Z stack images were reconstructed by Zen 2009 light edition software.

HL-60s were cultured at 37 °C/5% CO_2 in Iscove's Modified Dulbecco's Medium supplemented with 20% FBS. mMSCs were cultured at 37 °C/5% CO_2 in MEM alpha medium supplemented with 10% FBS.

2.4 Stem cell proliferation and differentiation

The viability of cells was tested by trypan blue. To test cell proliferation, the unmodified hMSCs and hMSCs conjugated with peptides on membranes were placed in a 96-well plate at 4000 cells/well. At 1, 4 and 7 d following seeding, the mesenchymal stem cell growth medium was replaced with phenol red free DMEM medium supplemented with 10% FBS, and the AlamarBlue assay was performed. The AlamarBlue reagent was added to each well according to the manufacturer's instructions and incubated for 90 min. The supernatant from each well was transferred to a black 96-well plate and the fluorescence intensity was measured on a plate reader.

To test hMSCs differentiation, 5×10^4 cells/ well and 1.5×10^4 cells/well were seeded in 12-well plates for adipogenesis and osteogenesis respectively. After 24 h, growth medium was replaced with osteogenic differentiation medium for osteogenesis, while cells for adipogenesis were allowed to grow in growth medium for 3 d to reach >95% confluency before replacing the medium with adipogenic differentiation medium. Fresh differentiation medium was added to cells every 3 d. At the 24th day after cell seeding, all cells were fixed with Formalin free fixative at room temperature for 20 min. Oil red O was used to probe cells under adipogenesis and control cells growing in regular growth medium. 6 mL Oil red O in isopropanol was mixed with 4 mL ddH_2O and filtered by 0.2 μm pore size membrane after staying at room temperature for 10 min. Oil red O in working solution was added to each well at 0.6 mL. The cells were washed with PBS after 20 min and imaged using phase contrast model of a microscope (Zeiss Axiovert). To probe alkaline phosphatase activity of osteogenic cells, Fast Blue RR Salt capsule was dissolved in 48 mL ddH_2O with 2 mL Naphthol AS-MX phosphate alkaline solution. Fixed cells were carefully washed with ddH_2O before and after applying probing solutions. Cells were imaged at a brightfield model.

To quantify the stem cell differentiation, mRNA marker expression was analyzed by real time PCR. Total RNA was isolated from cells using the TRIzol method. Total mRMA (0.5 μg) was reverse-transcribed using SuperScript reverse transcriptase with random hexamer. Quantitative PCR was performed using Taqman Fast Universal PCR Master Mix on a StepOnePlusTM Rreal-Time PCR System. Osteogenic differentiation was examined using Taqman Gene Expression Assays including OCN (Hs00754870_s1) and Runx2 (Hs00231692_m1). Adipogenic differentiation was examined using PPARG (Hs01115512_m1) and GTF3A (Hs01047486_g1). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Hs99999905_m1) was used as a reference control. The level of expression of each target gene was then calculated as $2^{-\Delta\Delta Ct}$.

2.5 Flow channel adhesion assay

The binding capacities of chemically engineered cells and unmodified cells to E-selectin were evaluated in microfluidic channels mimicking some aspects of the flow conditions in inflammatory blood vessels. To facilitate comparison, GFP-hMSCs modified with peptide K were mixed with unmodified mCherry-hMSCs at the same cell density. The mixed cells

were injected into microfluidic channels coated with E-selectin or with human IgG_1 FC as a control. The cells were allowed to attach to the substrates for 2 min before initializing the flow of pre-warmed medium through the channels. The flow speed was controlled by a syringe pump (Harvard Apparatus PHD 2000), and the shear stress was calculated based on the channel size according to a reference [27]. Cells in the channels were monitored and recorded under flow conditions by time-lapse imaging (total time = 2 min; interval = 1 s) with a fluorescent microscope (Zeiss Axiovert). Experiments were repeated 3 times under each fluid shear stress. For cell rolling experiment, the membranes of cells were conjugated with peptide M. The cells were allowed to attach to the substrates for 2 min before initializing the flow. For shear stress >1.25 dyn/cm^2, cells were first exposed under flow with shear stress 1.25 dyn/cm^2 for 30 s before applying the targeted shear stresses. Cells in the channels were monitored and recorded under flow conditions by time-lapse imaging. The rolling distances were measured by ImagJ, and the rolling velocities were calculated with the information of time. At each shear stress, 15 cells with rolling time longer than 30 s were randomly selected for calculations.

2.6 Measurement of binding kinetics of peptides and E-selectin

The binding kinetics of the two peptides to E-selectin was determined by surface plasmon resonance (SPR) using a Biacore T100 instrument via capture approach. Briefly, anti-human IgG Fc monoclonal antibody was immobilized to the flow cell (Fc) 1 and 2 of a CM5 Sensorchip via amine coupling per the manufacturer instructions. For each cycle, the recombinant human E-selectin protein (20 μg/mL) was captured onto the Fc2 to approximately 1300 response units (RU), followed by the injection of peptides (Peptide K and Peptide M) over both flow cells at 100 μL/min for 120 seconds and a dissociation phase of 300 seconds. Regeneration of the surface was achieved via injection of 3M magnesium chloride at 10 μL/min for 30 seconds. A concentration series (10, 3.33, 1.11, 0.37, 0.12, 0.04, 0.013 and 0 μM, in HBS-EP+ with 0.2% DMSO) of each peptides were used. The peptides were not modified at the N-terminus with CGS to prevent the formation of peptide dimer and the potential interaction between thiol group and the chip surface. The 1X HBS-EP+ was used as the running and sample dilution buffer. The resulted sensorgram were analyzed with Biacore T100 Evaluation software v1.1.1. The results were double referenced, the association, dissociation and steady state dissociation constant were determined with 1:1 binding curve fitting as well as steady state affinity analysis.

2.7 Measurement of cell tethering

Microfluidic channels were coated with 5 μg/mL E-selectin for cell tethering experiment. HL-60s and hMSCs and mMSCs engineered with peptide K were flushed into the microfluidic devices individually at a concentration 500,000 cells/mL. Cells were monitored and recorded under flow conditions by time-lapse imaging (total time = 2 min; interval = 2 s) with a fluorescent microscope (Zeiss Axiovert) at a phase-contrast model. The experiments were repeated a few times to find the maximum flow velocity, that cells could still attach to substrates.

3. Results and Discussion

To engineer the membranes of hMSCs to present synthetic peptides, a linker molecule, NHS-PEG_2-maleimide, was first reacted with amine groups on membrane proteins (Fig. 1). Then peptides containing a thiol on the N-terminus were anchored on the membranes by reacting with maleimide on the linker. For this study, a known E-selectin strong binding peptide [28] was modified with glycine-serine as a flexible linker and a cysteine providing the thiol group, H_2N-CGSDITWDQLWDLMK-COOH (peptide K). The number of synthetic ligands on engineered cells was determined by using a fluorescently labelled 5-

FAM-peptide K, and measuring the fluorescence intensity of engineered cells. There was an average of 1.5×10^7 peptides per cell when hMSCs were modified using 100 μM NHS-PEG_2-maleimide and 6 μM peptide. Cells were modified at this condition in all experiments unless otherwise specified. When the concentration of NHS-PEG_2-maleimide was increased to 800 μM, the average number of peptides per cell increased to 9×10^7. No peptide conjugation was detected in a control experiment when cells were treated with 6 μM 5-FAM-peptide K peptide alone, without the linker. This result indicates that the peptides were anchored on cells by reacting with the linker molecules during the CME process. Cells were attached on a tissue culture-treated substrate during modification. This is a rapid procedure with membrane engineering taking only 30 min to complete. After the cells were detached, the ligands were distributed on the cells relatively uniformly (Fig. 2a, Supplementary Mov. 1), even though it is possible that the chemical reactions occurred only on a portion of the membranes exposed to solution. This distribution is different from our reported asymmetrical distribution of nanoparticles on cell membranes [11]. In contrast to nanoparticles that can bind to several receptors on a membrane, each peptide only binds to one receptor, allowing diffusion on semi-fluid cell membranes. When engineered cells were suspended in medium at 37 °C after surface modification, most peptides stayed on cell membranes without being internalized for at least 6 h (Supplementary Fig. 1).

The viability of hMSCs after modification was > 95%, similar to untreated cells. There was no difference in cell proliferation rates between engineered cells and untreated cells at day 4 or day 7 after treatment (Supplementary Fig. 2). However when peptides were conjugated on hMSCs at a high concentration of 9×10^7/cell, the cells could not reattach to the tissue culture-treated substrates, even though the cells were viable. It is possible that the chemically conjugated peptides block the interactions between cell adhesion molecules and proteins adsorbed on substrates at this high peptide density. The multipotency of stem cells is critical for their regenerative ability. To evaluate the multipotency, the ability of engineered hMSCs to differentiate into adipocytes and osteogenic cells was analyzed and compared to those of unmodified cells (Fig. 2bc). Cells were allowed to grow in adipogenic differentiation medium, osteogenic differentiation medium, or standard hMSC growth medium for three weeks. Differentiated adipocytes from engineered and unmodified hMSCs showed a bright red color when stained with oil red O, which stains fat globules (Fig. 2b). The alkaline phosphatase activity of cells increased after osteogenesis, indicated by the purple-blue color of histochemically stained cells. Cells that grew in growth medium did not show staining for either adipogenesis or osteogenesis. The differentiations of hMSCs were further quantified by RT-PCR analysis of RNA markers (Fig. 2c). The mRNAs of PPARg and GTF3A were quantified for adipogenesis, while those of Runx2 and OSCN were quantified for osteogenesis. Engineered hMSCs retained their multipotency under the appropriate conditions.

To determine if the membrane-engineered hMSCs have the potential to target and remain in regions presenting E-selectin, we used polydimethylsiloxane (PDMS) microfluidic devices as an *in vitro* model of inflamed blood vessels (Fig. 3). The cross-section of the microfluidic channel was 1250×450 μm. The internal surface of the channels was coated with recombinant human E-selectin. The ability of cells to bind to the E-selectin surface was tested simultaneously for hMSCs presenting peptide K and for unmodified hMSCs. To facilitate visualization and distinction between these two groups of cells in the devices, the cells were transduced to internally express fluorescent proteins. Peptide K-modified hMSCs expressed green fluorescent protein, GFP (GFP-hMSCs), while unmodified hMSCs expressed red fluorescent protein, mCherry (mCherry-hMSCs). The cells were mixed together, and injected into the devices. Fluid shear stresses in channels were controlled by changing the flow rate of the growth media through the devices. Cells under shear stresses were visualized and recorded (Representative movies at each shear stress are available in

Supplementary Mov. 2). Only membrane-engineered hMSCs were bound under flow (Fig. 3). As the shear stress was increased, more engineered cells were flushed away. Physiologically, leukocytes tether and roll in postcapillary venules, where the shear stress is 1–4 dyn/cm^2 [29]. More than 80% of the membrane-engineered hMSCs were retained under shear stresses up to 5 dyn/cm^2. The highest shear stress tested, 10 dyn/cm^2, is close to the upper end of shear stress in humans [30]. The intracellular expression of GFP or mCherry did not influence the relative binding affinity of cells. When the labelling was switched, such that engineered cells were mCherry-hMSCs and unmodified cells were GFP-hMSCs, the engineered cells still remained bound while the unmodified cells were flushed away (Supplementary Mov. 3). Recombinant human E-selectin is composed of human IgG_1 FC and the extra-cellular domain of E-selectin. To test whether the binding of cells was due to the interaction between peptide K and E-selectin, engineered GFP-hMSCs and unmodified mCherry-hMSCs were placed into a microfluidic channel coated with human IgG_1 FC only. Without the extracellular domain of E-selectin, both types of cells were quickly flushed away at a low shear stress of 0.25 dyn/cm^2 (Supplementary Mov. 3).

Retention on inflamed vessels is an important step for cells to home to targeted tissues, and it requires firm adhesion. However, cell rolling is a dynamic process of the formation of new bonds and dissociation of existing bonds between cells and vascular adhesion molecules. The rate constants of peptide-selectin bond formation (k_{on}) and dissociation (k_{off}) are kinetic parameters governing the interaction of engineered cells and selectins. According to the Bell model, $k_{off}=k_{off}^{o}\exp(\gamma F/\mathrm{n}k_{B}\mathrm{T})$, where k_{off}^{o} is the k_{off} at zero pulling force, γ is the bond interaction range, F is the flow force on a cell, n is the number of bonds under force and k_BT is the thermal energy [31]. In order for cells to roll, fast k_{off} and k_{on} are needed so that the existing bonds break at the trailing edge, allowing cells to move, while at the same time, new bonds can create at the leading edge [32–34]. Additionally, although Ca^{2+} independent cell rolling on other biomolecules has been studied [32, 35], previously described mechanisms for cell rolling on E-selectin are Ca^{2+} dependent [21–23], but peptide K is known to bind to E-selectin through a Ca^{2+} independent mechanism [28]. We asked if the artificial interaction could be tuned to assist engineered cells to roll under physiologically relevant shear stresses. To this end, we removed Lysine from the C-terminus of the peptide K to give the sequence CGSDITWDQLWDLM (peptide M), which was reported to have a moderate binding IC50 with E-selectin [28]. We employed surface plasmon resonance to detect binding kinetics and dissociation constants of peptides. The SPR sensorgrams of binding curves of peptides on immobilized E-selectin were shown in Fig. 4. The k_{off} of peptide M is 10 times faster than that of peptide K at a compensation of 54% decrease of k_{on} (Table 1).

When hMSCs displaying peptide M were placed in the microfluidic channels coated with E-selectin, the cells rolled under physiological shear stresses (Fig. 5a). The average rolling velocity was substantially lower than the fluid velocity. For example, the average medium flow speed at shear stress 10 dyn/cm^2 was 11.9 cm/s, while the average cell rolling velocity was 0.8 μm/s and 1.8 μm/s for surfaces coated with 5 μg/mL and 2 μg/mL E-selectin (Fig. 5b). The potential for cells to roll on E-selectin substrates depend on surface density of E-selectin and the size of cells. A leukemia cell line, HL-60 is known to express selectin natural ligand, PSGL-1 [36], and was used as a control in our experiment. HL-60s showed stable rolling on 0.1 μg/mL and 0.5 μg/mL E-selectin coated surfaces (Fig. 5c), while engineered hMSCs dissociated from these surfaces and did not roll under high shear stresses. One reason is the difference in the sizes of cells. hMSCs have a mean diameter (d) around 22 μm, whereas HL-60s are 13 μm. Larger cells experienced higher flow force and torque than smaller ones, as the fluid force and torque are proportional to d^2 and d^3 respectively [33, 37]. Higher fluid forces increase k_{off} exponentially. Mouse MSCs with a mean diameter 15 μm, after engineered with peptide M, were able to roll on 0.5 μg/mL E-

selectin coated surfaces, as well as on 0.1 μg/mL E-selectin coated surfaces but at low shear stresses (Fig. 5c). Unmodified mMSCs did not show detectable interaction with E-selectins. There was an average of 4×10^6 peptides per engineered mMSC. Considering the size of cells, peptide density on mMSCs was about half of that on engineered hMSCs, indicating the size of cells instead of ligand density may be the reason for the different biomechanical response of hMSCs and mMSCs under shear stress. Many engineered MSCs detached after rolling less than 100 μm, not as stable as HL-60s. The fast k_{on} and k_{off} of PSGL-1 binding to selectin is believed one of the major contributors of the stable rolling of HL-60s on P or E-selectins [32]. The reported values are 4.4×10^6 M^{-1} s^{-1} and 1.4 s^{-1} [38]. The relative slow k_{on} of peptide M can also explain why engineered MSCs rolled at lower velocities than HL-60s. At higher rolling velocity, cells had less time to generate new bonds, leading to unbalanced dynamics of bond formation and breakage. The *in vivo* E-selectin-mediated leukocyte rolling speed was measured in one experiment to be around 4 μm/s in inflamed vessels.[39] Although high rolling velocities may not be necessary for cell homing, it is expected that higher rolling velocities of engineered cells can be obtained in the future using peptides with fast k_{on} and k_{off}. We observed that HL-60 rolling was Ca^{2+} dependent as 5 mM EDTA in medium eliminated the interaction between HL-60s and selectins. However, there was no detectable difference in the interaction between engineered MSCs and selectins with or without 5 mM EDTA.

Cell tethering (initial attachment) *in vivo* occurs in postcapillary venules. The small size of the venules and the collision between leukocytes and blood cells promote cell tethering [40]. Our model does not mimic these conditions, and cells have less chance to contact the substrate to initiate binding. By flushing cells directly into microfluidic channels without stopping flow, we have found cells effectively tethered on substrates coated with 5 μg/mL E-selectin at shear stress 0.5 dyn/cm^2, 0.25 dyn/cm^2 and 0.2 dyn/cm^2 for HL-60s, peptide K-mMSCs and peptide K-hMSCs respectively (Fig. 6). HL-60s were more efficient than stem cells that were engineered with peptide K in tethering.

A major advance of the CME strategy presented here is that the binding kinetics of cells can be controlled for the desired application. The rolling of engineered stem cells *in vivo* will be investigated in the future and optimized for targeted tissues by tailoring the binding of peptides to selectins. The reaction of NHS-PEG_2-maleimide with cell membranes has less specificity than enzymatic coupling. This provides versatility, but may not be beneficial for all applications. The enzymatic approach also introduces less immunogenic epitopes and may have lower barrier for clinical trials. In the future, bioorthogonal and site specific chemistry on cell surfaces [41] may be necessary to apply membrane-engineered stem cells to clinical applications. Future work aimed at testing if engineered stem cells are capable of extravasation into injured tissues will also be highly valuable. Another interesting aspect of this approach is that the rolling of engineered cells on E-selectin is Ca^{2+} independent. This further illustrates the ability of this approach to manipulate cell-microenvironment interactions in the context of regenerative medicine.

4. Conclusions

We have developed a chemical method to conjugate synthetic ligands on stem cell membranes to control cell-microenvironment interactions. This approach does not affect cell viability, proliferation or multipotency. It is convenient, robust, inexpensive and complementary to other CME strategies, providing broad application potentials. We showed that, at proper binding kinetics and dynamics, rolling of engineered cells under physiologically relevant shear stresses could be realized via an artificial interaction between membrane conjugated peptides and E-selectins. Theoretical models of cell rolling focused on the molecular interactions and the fluid mechanics. But knowledge in this aspect has only

been used to explain phenomena of natural cell rolling and rolling of beads, and had not been used to design engineered cells. Our work validated these theoretical studies and will allow refined theoretical models to be developed to guide the design of better engineered cells. Our cell engineering strategy has the potential to render cells with the ability to target any receptors with controlled binding affinity and kinetics. This technique may be applicable for navigating dendritic cells to lymph nodes and in the therapy of inflammation-associated diseases, such as myocardial infarction, atherosclerosis and cancer. In addition to cell targeting, our chemical approach may also be useful to engineer cells with multiple functionalities, such as cell labelling and sensing.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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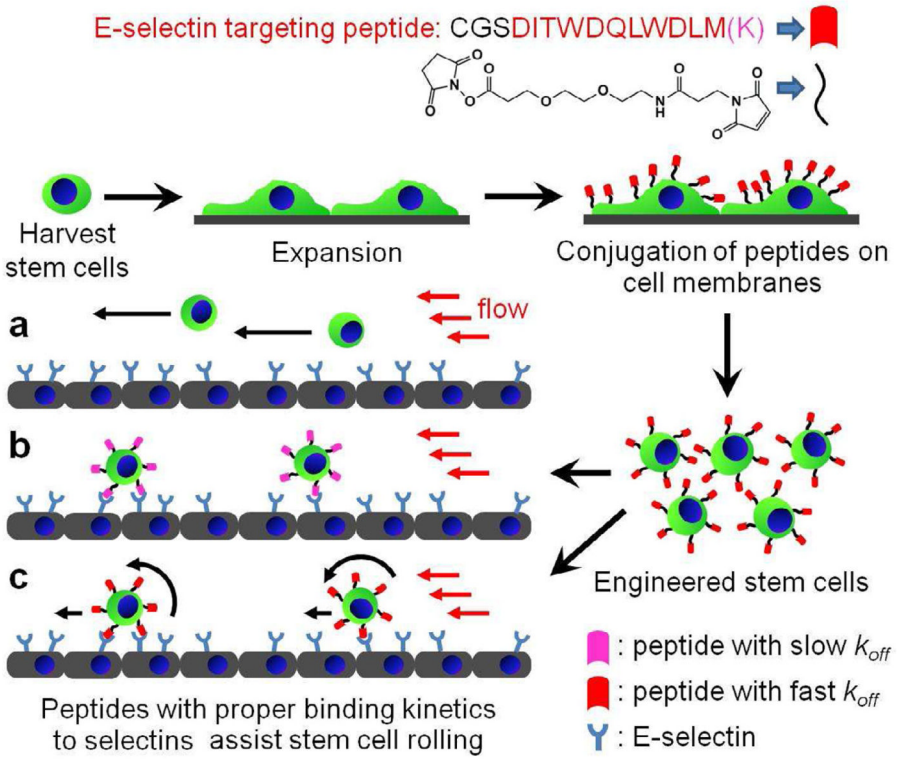

Fig. 1.

Schematic illustration of conjugating peptides to the surfaces of stem cells to assist stem cell rolling under flow. E-selectin targeting peptides are conjugated on cell membranes by using NHS-PEG_2-maleimide as a linker molecule and the thiol-maleimide reaction for subsequent peptide conjugation, creating engineered stem cells. a) Unmodified stem cells do not bind to selectins and are quickly flushed away under flow. b) Stem cells modified with peptides that strongly bind to selectins remain bound under physiological flow and do not roll. c) Stem cells modified with peptides that bind to selectins with fast dissociation rate constant (k_{off}) roll in the direction of flow.

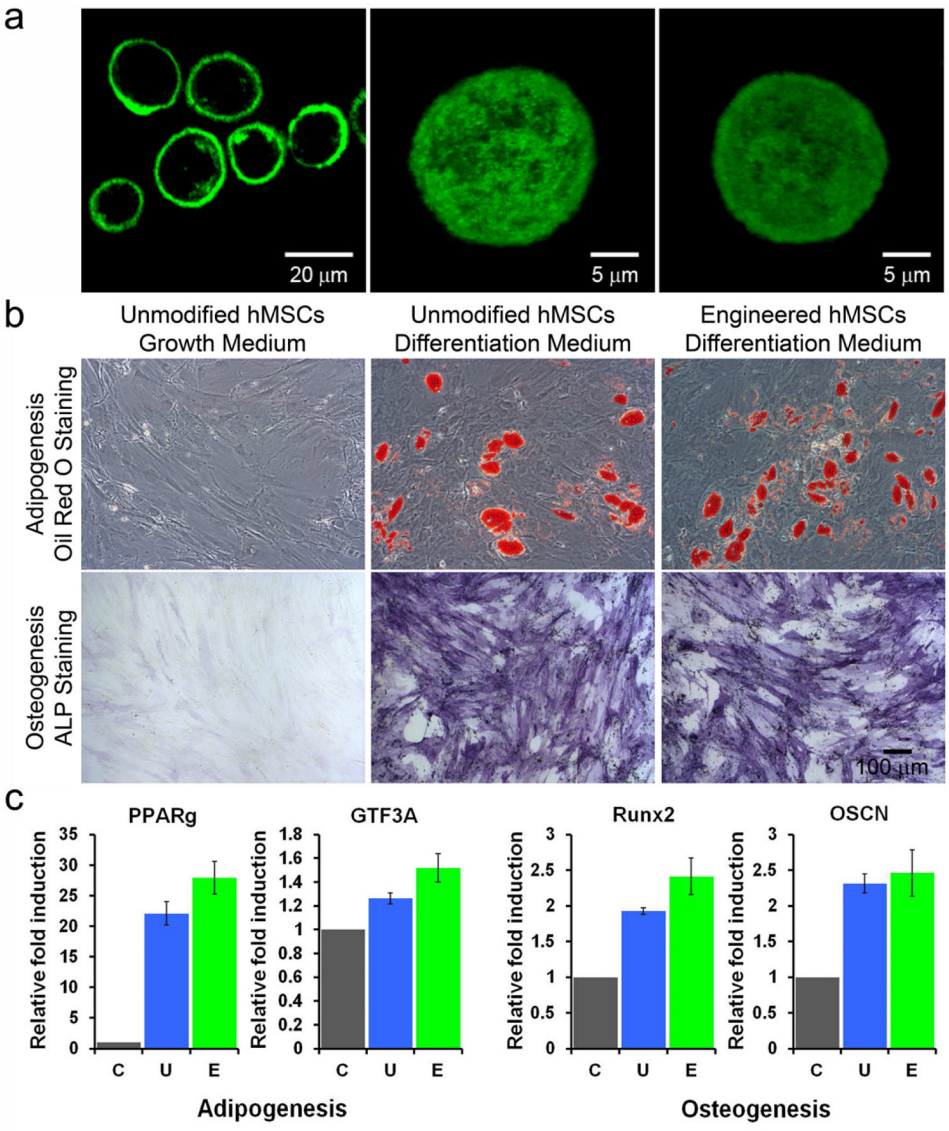

Fig. 2.

Stem cells modified with peptides on their membranes retain their multipotency. a) Representative confocal images of 5-FAM-peptide K-modified hMSCs. The cells were detached and resuspended in medium after modification. Left: the confocal image was obtained by focusing in the middle of the cells. Middle and right: three-dimensional reconstructed confocal microscopy images of one engineered hMSC. The middle image is the top view, and the right image is the bottom view. b) Staining of differentiated adipocytes (red) and osteogenic cells (purple blue) from unmodified hMSCs and membrane-engineered hMSCs. c) RT-PCR analysis of RNA markers of adipogenesis and osteogenesis. Values are mean ± s.d. C: unmodified hMSCs in growth medium for three weeks; U: unmodified hMSCs in differentiation medium; E: engineered hMSCs in differentiation medium.

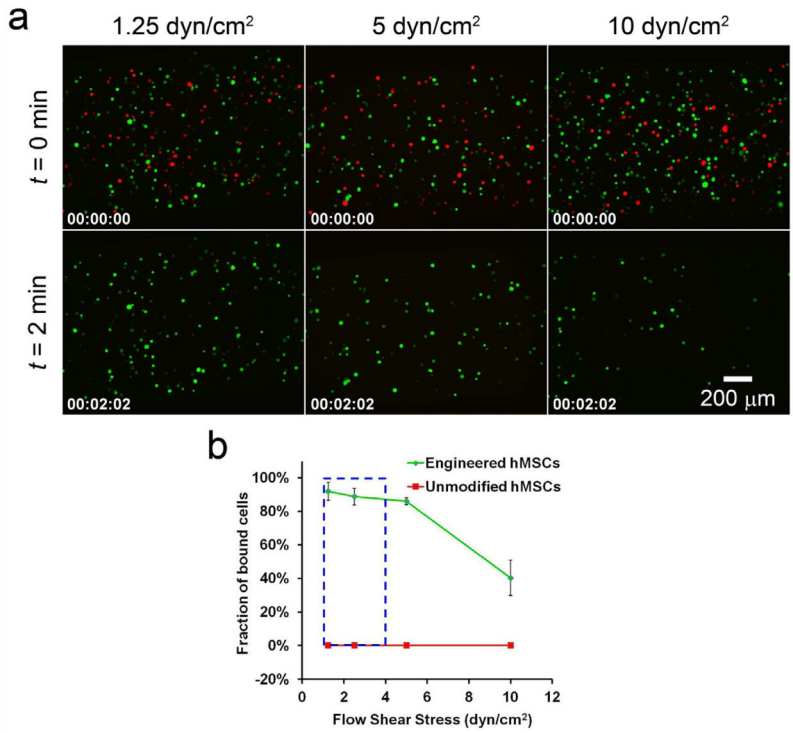

Fig. 3.

hMSCs modified with peptide K strongly bound to E-selectin coated microfluidic channels under flow. a) Representative images of membrane-engineered hMSCs (GFP, green) and unmodified hMSCs (mCherry, red) in microfluidic channels before applying flow and 2 min after flow. Each column of images corresponds to a different shear stress that the cells were exposed to (left to right: 1.25 dyn/cm^2, 5 dyn/cm^2 and 10 dyn/cm^2). b) Fraction of bound cells under different shear stresses. Values indicate mean ± s.e.m. (n=3 for each group). Blue-dashed box indicates the physiological range of shear stresses in human postcapillary venules.

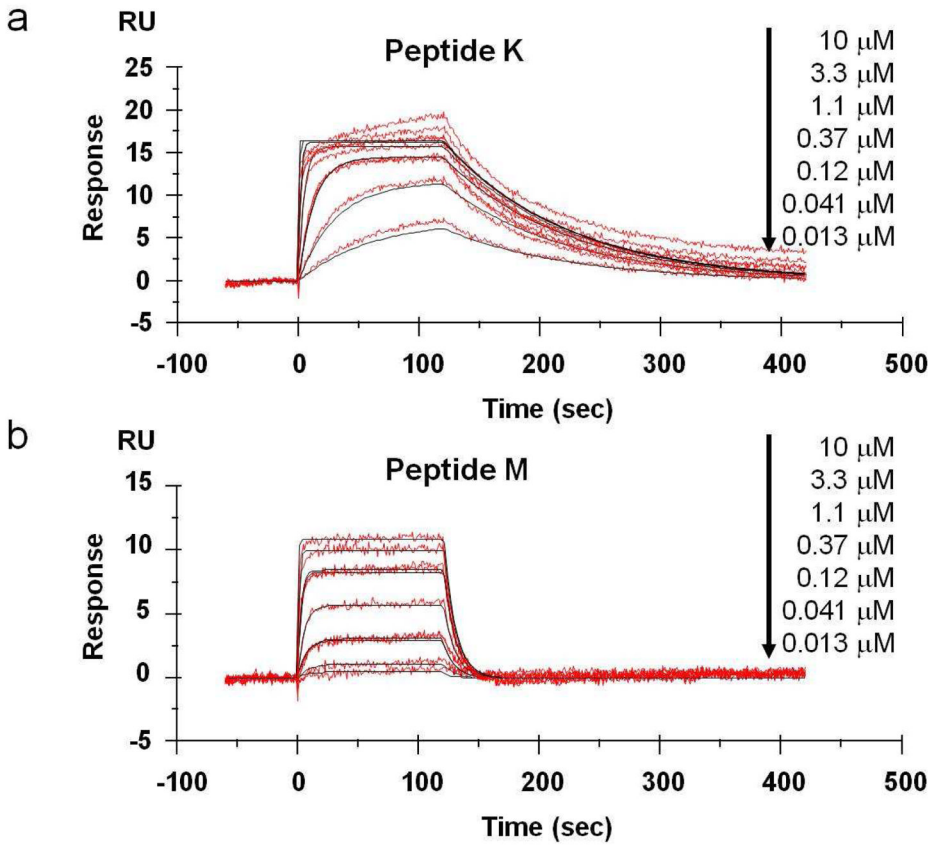

Fig. 4.

SPR sensorgrams of peptide binding on immobilized E-selectins. The red-colored lines are the adjusted data curves, and the black lines are the fitting curves using 1:1 binding model. The peptide concentrations of individual curves were from 10 μM to 0.013 μM as marked by the black arrow. a) Sensorgrams of peptide K binding on E-selectin. b) Sensorgrams of peptide M binding on E-selectin.

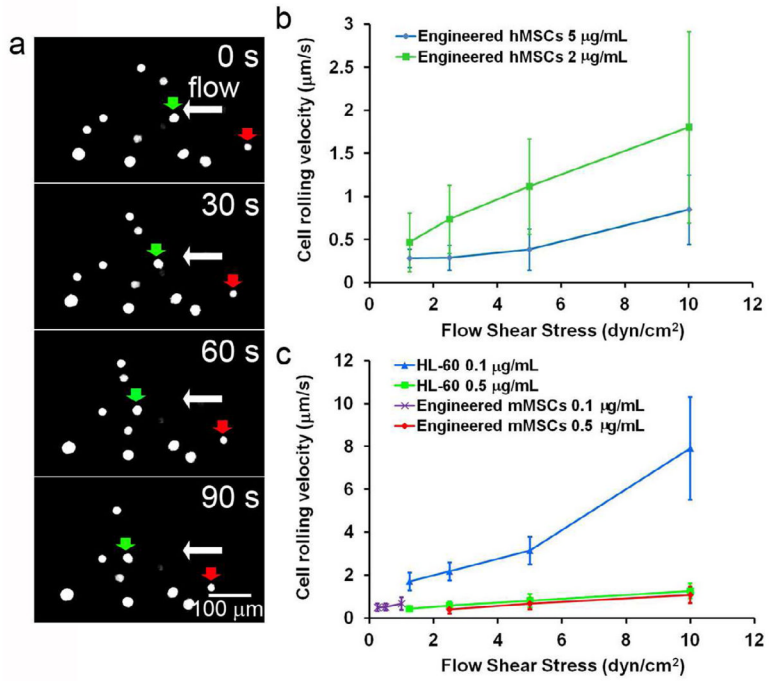

Fig. 5.

MSCs modified with peptide M roll on E-selectin under physiologically relevant shear stresses. a) Time-lapse images of engineered hMSCs rolling on a 5 μg/mL E-selectin-coated surface at shear stress 10 dyn/cm^2. White arrows mark the flow direction. Green and red arrows mark two rolling cells respectively. Quantification of rolling velocity of engineered cells on surfaces coated with different concentrations of E-selectin, b) engineered hMSCs; c) engineered mMSCs and HL-60s. Values indicate mean ± s.d.(n=15 for each group).

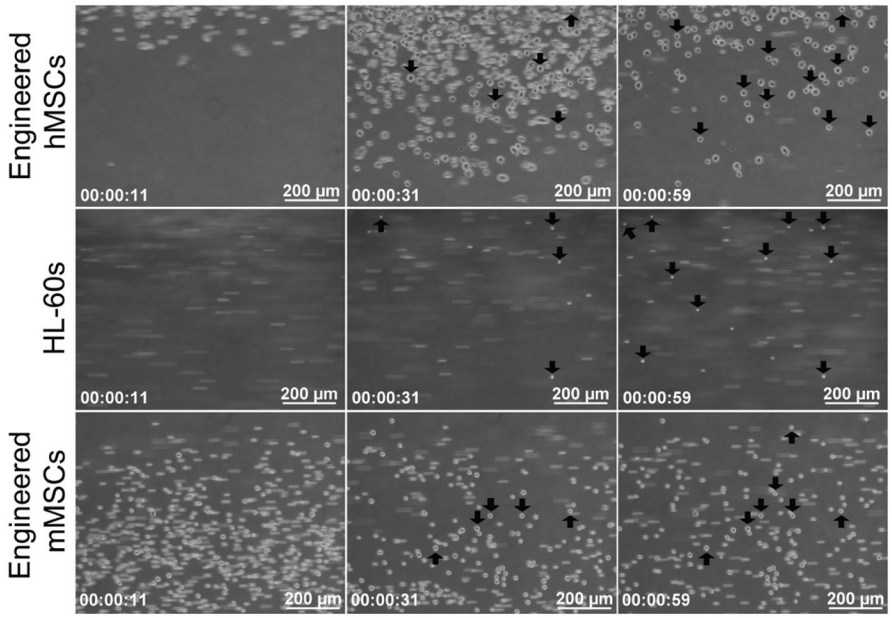

Fig. 6.

Tethering of membrane engineered stem cells and HL-60s on E-selectin coated substrate at different time points. hMSCs and mMSCs were modified at 100 μM NHS-PEG_2-maleimide and 6 μM peptide K. After trypsinization and resuspension in medium, cells at 500,000 cells/mL were flushed into microfluidic devices that were coated with 5 μg/mL E-selectin before use. Images were taken at shear stress 0.2 dyn/cm^2, 0.5 dyn/cm^2 and 0.25 dyn/cm^2 for hMSCs, HL-60s and mMSCs respectively. Black arrows mark some of the tethered cells.

Table 1

Dissociation constant and binding kinetics of peptides and E-selectin. Parameters were detected by surface plasmon resonance.

peptide[a]	K_D [nM]	k_{on} [$\times 10^5$ M^{-1} s^{-1}]	k_{off} [s^{-1}]
Peptide K	17.4	5.58±0.02	0.0097±0.0000
Peptide M	412.2	2.56±0.02	0.1055±0.0006

[a]peptides were not modified with CGS at the N terminus.