A Novel Strategy to Enhance Secretion of ECM Components by Stem Cells: Relevance to Tissue Engineering

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Short title: Peptide for promoting specific ECM components in hADSCs

Abstract

The ability of cells to secrete extracellular matrix (ECM) proteins is an important property in the repair, replacement and regeneration of living tissue. Cells that populate tissue engineered constructs need to be able to emulate these functions. The motifs KTTKS or palmitoyl-KTTKS (peptide amphiphile) have been shown to stimulate the production of collagen and fibronectin in differentiated cells. Molecular modelling was used to design different forms of the active peptide motifs to enhance the efficacy of the peptides to increase collagen and fibronectin production using terminals KTTKS/SKTTK/SKTTKS connected by various hydrophobic linkers, V₄A₃/V₄A₂/A₄G₃. Molecular dynamic simulations showed SKTTKS-V₄A₃-SKTTKS (P3), with palindromic (SKTTKS) motifs and SKTTK-V₄A₂-KTTKS (P5) maintained structural integrity and favourable surface electrostatic distributions that are required for functionality. *In vitro* studies showed that peptides P3 and P5 showed low toxicity to human adipose-derived stem cells (hADSCs) and significantly increased the production of collagen and fibronectin in a concentration-dependent manner, compared to the original active peptide motif. The 4 days treatment showed the stem cell markers of hADSCs remained stable with the P3. The molecular design of novel peptides is a promising strategy for the development of intelligent biomaterials to guide stem cell function for tissue engineering applications.

Key words: Molecular modelling; KTTKS; collagen; fibronectin; Human adipose-derived stem cells; Peptides; tissue engineering; *in-vitro* validation

Introduction

Tissues are living structures that rely on interactions between the cells within the tissues and the extracellular matrix (ECM) to maintain structural integrity, durability and optimal function. Replication of these properties is a pre-requisite for the success of strategies to tissue engineer living tissues. Communication between cells and their ECM relies upon physical attachment of cells to specific proteins in the ECM via integrin molecules on the surface of the cells. Proteins of particular importance for cell-ECM communication are collagen and fibronectin.¹⁻³ Collagen provides structural integrity to the ECM and acts as a docking site for integrins to regulate essential functions of cells including differentiation and motility.⁴⁻⁶ It has been extensively used in several wound healing and tissue engineering applications.^{7,8} Fibronectin, a master organizer in matrix assembly, plays a vital role by providing multiple binding sites for cells and regulating various signals within those cells that attach to the protein.^{3,9} However, the use of native collagen and fibronectin for tissue engineering has several disadvantages, including the risk of infection and immune cross-reactivity.^{10,11} These limitation can be circumvented by developing strategies to stimulate cells to secrete these vital ECM components.

Recently, human adipose-derived stem cells (hADSCs) have become an attractive source of cells for regenerative medicine and for tissue engineering applications such as heart valve tissue engineering due to their abundance, availability, pluripotency and similarity to valvular interstitial cells.^{12,13} With bioactive small molecules such as peptides, one can target specific functions for tissue repair.¹⁴⁻¹⁸ A well-known pentapeptide motif from pro-collagen with the amino acid sequence KTTKS has been shown to stimulate production of collagen and fibronectin in human dermal fibroblasts and corneal fibroblasts, but never on stem cells.¹⁹⁻²¹ The molecular design of the peptide could be optimised further to be used for this specific purpose.

Molecular modelling provides efficient tools to design the molecular basis of biomimetic peptides.^{22,23} We here describe a novel peptide modification capable of enhancing the capacity of hADSCs to produce collagen and fibronectin that are involved in crosstalk between cells and ECM which is relevant to tissue engineering.²⁴

Materials & Methods

Computational modelling

Molecular modelling of a novel design of peptides with dual motifs

The 3D molecular models of peptides were generated using discovery studio v3.5 [Accelrys, San Diego, USA]²⁵ (Table 1). Seven peptides (named peptide (P1) to peptide (P7)) were designed. Three types of terminals (derived from the functional motif KTTKS) were used in the modelling to assess their structural contributions: i) KTTKS, ii) SKTTK, an inverse form of KTTKS and iii) SKTTKS, a palindromic motif. To identify suitable linkers for connecting these hydrophilic motifs, three types of hydrophobic linkers composed of small amino acids were examined: i) V₄A₃, ii) V₄A₂²⁶ and iii) A₄G₃. For each system, four peptide chains were produced and aligned at regular spatial intervals to provide possibilities to increase stability by inter-molecular hydrogen bonds and hydrophobic interactions.

Molecular dynamics simulations

The peptides were prepared to perform molecular dynamics (MD) simulations using GROMACS simulation package V4.5.4.^{27,28} The GROMOS96 force field was applied to the structures.²⁹ The peptides were solvated with SPC3³⁰ water model in a cubic box with a size of 1.5 nm. Periodic boundary conditions were applied in all directions and the systems were neutralized by adding counter ions. The resulting systems contain ~50000 atoms. A twin range cut-off was used for long-range interactions: 0.8 nm for van der Waals interactions and 1.4 nm for electrostatic interactions. All bond lengths were constrained with the LINCS³¹ algorithm. The SETTLE³² algorithm was applied to constrain the geometry of the water molecules. The steepest descent algorithm was applied to energy minimize the systems with a tolerance of 2000 kJ/mol/nm. The energy minimized systems were subjected to 100 ps pre-equilibration. They were subsequently subjected to 30 ns of production MD simulations with a time-step of 2 fs at constant temperature (300 K), pressure (1 atm) and number of particles, without any position restraints.³³ Snapshots were collected at every 10 ps. The tools available within GROMACS were used to analyse the collected trajectories. The initial phase of the MD simulation (5 ns) was an equilibration period for the systems, thus it was not considered for the analyses.

Cluster analysis

The representative structures were selected using cluster analysis. In which, the collected trajectories with 3000 structures were grouped into clusters based on their structural deviation.

The top ranked cluster that represented a frequently occurring conformation was chosen from each system for representation. The collected structures were analysed using discovery studio and PyMOL (www.pymol.org).

Surface electrostatic potential calculation

The representative structure from each system was used to calculate electrostatic distribution by solving a linear Poisson-Boltzmann equation. The PARSE force field was applied to the structures using PDB2PQR v1.8.³⁴ The Adaptive Poisson-Boltzmann Solver tools were utilized to calculate the electrostatic surface potential with the dielectric constant of 80 and 2 for solvent and solute, respectively.³⁵

In-vitro experiments

Materials

All chemicals were purchased from Sigma Chem. corp. (St. Louis, United States of America) unless otherwise specified.

Peptides

Four peptides (Table 2) were purchased from Activotech, UK with peptide purity greater than 95% provided by the company through analytical high-performance liquid chromatography and mass spectrometry.

Methods

Cell culture

hADSCs were purchased from Lonza (PT-3273, Lonza, US) and cultured in culture medium composed of adipose-derived stem cell basal medium, 10% Fetal Bovine Serum (FCS), 1% L-glutamine and 0.1% gentamicin-amphotericin B (PT-4503, Lonza, US). The cells were fed every 3 days and sub-cultured at 90% confluency.

Peptide preparation

Peptides P0, PA, P3 and P5 were prepared at 1.25 μ M, 12.5 μ M and 125 μ M. The samples in the form of powder were weighed out in a biological class II safety cabinet (Thermo-Scientific, MSC-Advantage, US), dissolved in minimal dH₂O with sonication (Ultrawave, US) for 10 min and reconstituted with 2% FCS culture medium.

Cell morphology and metabolic activity assay for hADSCs

Triplicates of hADSCs between passages 2-4 were seeded into 24-well plates with 10,000 cells/well in 500 μ l of 10% FCS for 24 h to allow cell adhesion. Medium was changed to 2% for 24 h to allow cell synchronization. This was followed by exchanging the medium with 500 μ l of 2% FCS as negative control, 2% FCS with 250 μ M ascorbic acid additive as positive control and 2% FCS with 125 μ M of peptides P0, PA, P3 and P5. All samples were cultured for 4 days followed by observation under a light microscope (Nikon) and cell metabolic activity was determined using the MTS assays (CellTiter 96 Aqueous One Solution Cell Proliferation Assay, Promega, Madison, WI).. After 4 h of incubation at 37 °C, 100 μ l aliquots were taken and the absorbance at 450 nm was determined with a 96-well plate reader (TECAN, Infinite F200 Pro).

Immunostaining of fibronectin and collagen

hADSCs between passages 2-4 were seeded in 8-well chamber slides with 3000 cells/well in 200 μ l of 10% FCS for 24 h to allow cell adhesion. Medium was changed to 2% for 24 h to allow cell synchronization. This was followed by exchanging the medium with 200 μ l of 2% FCS as a negative control, 2% FCS 1mM L-ascorbic acid 2-phosphate, 0.025 ng/ml TGF β 1 and 0.01 mg/ml insulin as a positive control and 2% FCS with peptides P0, PA, P3 and P5 (at 1.25 μ M, 12.5 μ M and 125 μ M). All samples were cultured for 4 days before analysis. At day 4, media was removed and washed with 1X phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 10 min, washed in PBS and treated with 0.25% hyaluronidase in PBS for 15 min. The slides were then blocked with 3% bovine serum albumin (w/v) (BSA) in PBS for 30 min, followed by incubation with the primary antibodies (fibronectin and collagen) at the appropriate dilution (1:100 for collagen (Biologo), 1:500 for fibronectin (Sigma) in 3% BSA in PBS for 1 h. Appropriate secondary antibody (Alexa goat anti-mouse 488; or Alexa goat anti-rabbit 594, Invitrogen) was diluted in 2% normal goat serum in PBS at 1:1000 and were applied for 30 min at room temperature. Cells were washed twice with PBS and stained with DAPI (1:2000) for 3-5 min each for nuclear staining. Slides were viewed under a Zeiss LSM 880 confocal microscope.

Collagen quantification

Triplicates of hADSCs between passages 2-4 were seeded in 8-well chamber slides with 3000 cells/well in 200 μ l of 10% FCS for 24 h to allow cell adhesion. Medium was changed to 2% for 24 h to allow cell synchronization. This was followed by exchanging the medium with 200 μ l of 2% FCS as a negative control, 2% FCS with 250 μ M ascorbic acid as a positive control and 2% FCS with (1.25 μ M, 12.5 μ M and 125 μ M) peptides P0, PA, P3 and P5. Ascorbic acid was used as a positive control

due its ability to activate pro-collagen gene transcription and stabilisation of its mRNA and to stimulate crosslinking between the collagen triple-helix by stimulating the enzyme prolyl hydroxylase.^{19, 36-38} All samples were cultured for 4 days before analysis. The level of collagen expression was measured using the protocol modified from Jones et al., 2013.²¹ At day 4, media was removed and the collagen deposited on the surface was analyzed. 70% ice cold ethanol was used to fix deposited materials on the surface of the culture dishes. To ensure complete fixation of the collagen deposited on the surface, samples were transferred to -80°C for 30 min. The ethanol was removed and samples were treated with 0.1% Sirius red/ picric acid solution overnight at 4°C with gentle agitation. The unbound dye was removed on the following day with 6 gentle washes using distilled water. Samples were then treated with 150 µl of 1 M NaOH to dissolve the collagen-dye complex with gentle agitation for 30 min at room temperature. Then, 100 µl of the aliquots were transferred into a 96-well plate and absorbance measurements were taken at 540 nm using a plate reader (TECAN, Infinite F200 Pro). Type I rat tail collagen (Corning®, US) was used to construct a standard curve by drying known concentrations of the collagen on tissue culture plastic and treating with the protocol described above. All data are represented as mean ± standard deviation.

Fibronectin ELISA

Triplicates of hADSCs between passages 2-4 were seeded in 8-well chamber slides with 3000 cells/well in 200 µl of 10% FCS for 24 h to allow cell adhesion. Medium was changed to 2% for 24 h to allow cell synchronization. This was followed by exchanging the medium with 200 µl of 2% FCS as a negative control, 2% FCS containing 1mM L-ascorbic acid 2-phosphate, 0.025ng/ml TGFβ1 and 0.01mg/ml insulin as a positive control and 2% FCS with (1.25 µM, 12.5 µM and 125 µM) peptides P0, PA, P3 and P5. All samples were cultured for 4 days before analysis. At day 4, media was removed and washed with 1X phosphate-buffered saline (PBS) for twice, followed by addition of 200µl of RIPA buffer for 20 min with gentle shaking. The RIPA solubilized samples were diluted at 1:40 dilution with ELISA sample diluent, followed by fibronectin ELISA analysis (Human Fibronectin ELISA Kit, Bosterbio) according to the manufacturer's protocol. All data are represented as mean ± standard error of mean

Flow cytometry analysis

Flow Cytometry Analysis (FACS) analysis was used to investigate the incidence of stem cell surface marker expression for hADSCs treated with P3. Triplicates of 1×10^6 hADSCs were plated in T25 cell culture flasks and allowed to adhere overnight. Medium was changed to 2% FCS for 24 h to allow cell synchronization. hADSCs were treated with 125 µM P3 for 4 days at 2% FCS before being

harvested using trypsin (Sigma). hADSC (5×10^5 /ml) were incubated with the primary antibody (all primary antibodies (CD44, CD90, CD105, CD73, CD45, CD61, CD31) obtained from BD Biosciences except negative control which is protein G purified antibody (OX8 hybridoma obtained from ECACC) in binding buffer (1X PBS (Sigma) containing 2% FCS (Sigma) for 30 min on ice. These were washed in binding buffer twice then incubated with the secondary antibody (rabbit anti-mouse Ig FITC (Dako)) for 30 min on ice and kept in a dark room. Prior to FACS analysis, the cells were washed twice in the binding buffer and data was acquired on Beckman Coulter FC500 flow cytometer

Statistical analysis

All the *in-vitro* experiments were repeated a minimum of three times. All statistical analyses were performed with one way ANNOVA post hoc Tukey's multiple comparison test using PRISM6 software (GraphPad Software, Inc., San Diego, CA) and $p < 0.05$ was considered significant. Data are presented as mean $\pm$ standard deviation unless stated otherwise.

Results

Structural analyses of the peptides

The peptides with V_4A_3 linker: To analyse the structural stability of the peptides to maintain their functional motifs, the representative structure for each peptide was selected from the simulations using the cluster analysis. P1 did not maintain its stability during MD simulations (Fig. 1A) especially chains of the peptide were highly deviated. The number of hydrogen bonds (that maintain the structural integrity) in P1 and P2 were comparatively less than the P3 during the MD simulations (Fig. 1A-B and Fig. 2A). In P1 and P2, most of the hydrogen bonds were formed in their disordered states. On the other hand, the stability of P3 illustrates high possibility for ordered alignment of chains using the linker, V_4A_3 , through inter-molecular interactions (Fig. 1C and 2A).

The peptides with V_4A_2 linker: P4 and P6 displayed a disordered state that appeared like a spherical structure with a minimal possibility for projection of the motifs on the N and C terminals (Fig. 1D and 1F). Although they maintained their total number of hydrogen bonds in the course of the MD simulations (Fig. 2B), most of them were randomly formed in their disordered state. Interestingly, P5 was stable and exhibited possibility for ordered alignment with significant number of inter-chain hydrogen bonds, which were maintained in the course of the MD simulation (Fig. 1E and 2B).

The peptide with A₄G₃ linker: In order to examine the positional contribution of the residues in the linker region, P3 and P7 were modelled with the same length but with a modified hydrophobic linker. The latter has A₄G₃ instead of V₄A₃. However, this modification did not appear to be sufficient for the stability of the chains (Fig. 1G). It probably leads the chains of P7 to form random self-folding or a disordered state. Although we observed an increase in the total number of hydrogen bonds during the simulation of P7 (Fig. 2C), the inter-chain hydrogen bonds were minimal and inconsistent. This demonstrates the importance of positioning four valine residues at the hydrophobic core followed by three alanine residues for maintaining the stability. This is consistent with the experimental study on the linker V₄A₂ of a peptide amphiphile, where the position of V₄ is essential for the mechanical stability.²⁶ These results show that the linkers of P3 and P5 appear to be stable to present the motifs at the terminals for their function.

Map of surface electrostatic distribution

The distribution of surface electrostatics demonstrated that the peptides are positive in nature due to the four lysine residues in each chain except the negatively charged carboxylic group of C-terminals and a few hydrophobic patches in the middle (Fig. 3). The packing of hydrophobic residues using hydrogen bonds can be essential for the association of the chains of the peptides and presentation of charged motifs. P1 showed that its' hydrophobic patches were not packed consistently (Fig. 3A). In peptides 2, 3 and 5, the hydrophobic patches were packed in an ordered fashion in the middle (Fig. 3B, 3C and 3E). However, peptides 3 and 5 showed a proper hydrophobic segmentation to act as a spacer between the charged terminals. The surface potential of peptides 4, 6 and 7 were irregularly packed and the motifs appeared not to be presentable (Fig. 3D, 3F and 3G). Noticeably, in P7, both the terminals found each other and formed a ring-like structure. The results of P3 and P5 illustrate the importance of the molecular design with proper segmentation of electrostatic properties for presentation of motifs.

Radius of gyration

We have analysed the conservation of association of chains during MD simulations by measuring the distribution of the atoms around the central axis for each system (Fig. 4). P1 showed maximal fluctuation along the time scale and was unable to maintain the compactness (Fig. 4A). Whereas, peptides 2 and 3 were able to preserve the association of their chains and this is explained by the plateaus in the graph after the initial period of equilibration. In particular, P3 maintained the plateau at the same level throughout the simulation. Peptides 4, 6 and 7 exhibited a considerable decrease in association (Fig. 4B). In contrast, P5 preserved the integrity all over the simulation. The

radius of gyration indicates that the association of the chains of the peptides 3 and 5 maintained their overall structural integrity.

Selection of peptides for *in-vitro* experiments

Peptides 3 and 5 showed potential characteristics to form stable structures at the inter-chain level by regular hydrogen bonds. The possibilities were high for the presentation of their charged motifs due to the positioning of suitable segmentation of electrostatic properties. The structural packing of the linkers of these two peptides consistently maintained a similar pattern throughout their simulations. Therefore, these two peptides were prioritized from the modelling and examined along with the known peptide KTTKS (P0) and palmitoyl-KTTKS (PA) (Table 2) for their toxicity and their ability to stimulate hADSCs to secrete collagen and fibronectin *in-vitro*.

Cell morphology and metabolic activity

The representative bright field images of hADSCs (Fig. 5) showed (at the 125 μ M peptide concentration) a typical fibroblast like morphology after 4 days in culture with P0, P3, P5, except PA that adopted a spherical morphology as shown in Fig. 5B.

Figure 6 shows the assessment of cell metabolic activity after 4 days of culture with the peptide via MTS assay. The negative control, ascorbic acid, P0, P3 and P5 showed optical density reading of 0.188 (± 0.027), 0.159 (± 0.029), 0.146 (± 0.037), 0.169 (± 0.020), 0.149 (± 0.028), respectively. Positive control, P0, P3 and P5 showed no statistical significant difference between negative controls at $P < 0.05$. Except the PA that showed significant reduction of MTS with optical density reading of 0.016 (± 0.003).

Immunofluorescence of fibronectin and collagen

Figure 7 shows the immunofluorescence images of the collagen (red) and fibronectin (green) with increase dosages of the peptide concentration from 1.25 μ M, 12.5 μ M to 125 μ M at 20X magnification. Qualitatively, P0, P3 and P5 showed increased staining of collagen and fibronectin in a dosage dependence manner. However PA showed no increase in staining of collagen and fibronectin with increasing peptide concentration. Furthermore, at 125 μ M, PA had a negative influence on the cell proliferation, therefore a lack of staining was observed most likely due to cell detachment during the staining process. At 125 μ M, the P0, P3, P5 all showed

significant increases in the staining of collagen and fibronectin compared to the negative control. P3 showed the most prominent fibronectin and collagen staining, followed by P5 and P0.

Collagen stimulation

Figure 8 shows the quantitated collagen deposition via Sirius red analysis. The negative control showed deposition of 22.44 (± 1.20) $\mu\text{g/ml}$ and the positive control showed 29.46 (± 0.91) $\mu\text{g/ml}$. Increasing peptide concentrations of P0 from 1.25 μM , 12.5 μM and 125 μM showed increasing collagen deposition from 21.25 (± 0.75) $\mu\text{g/ml}$, 22.95 (± 1.45) $\mu\text{g/ml}$ and 29.03(± 3.91) $\mu\text{g/ml}$, respectively. Increasing peptide concentration of PA from 1.25 μM , 12.5 μM and 125 μM showed increasing collagen deposition from 24.98(± 2.73) $\mu\text{g/ml}$, 24.78 (± 5.99) $\mu\text{g/ml}$ and 39.39(± 3.39) $\mu\text{g/ml}$, respectively. Increasing peptide concentration of P3 from 1.25 μM , 12.5 μM and 125 μM showed increasing collagen deposition from 21.86(± 1.41) $\mu\text{g/ml}$, 23.07 (± 1.61) $\mu\text{g/ml}$ and 56.00(± 3.12) $\mu\text{g/ml}$, respectively. Increasing peptide concentration of P5 from 1.25 μM , 12.5 μM and 125 μM showed increasing collagen deposition from 23.53(± 1.34) $\mu\text{g/ml}$, 24.50 (± 1.29) $\mu\text{g/ml}$ and 42.86(± 1.53) $\mu\text{g/ml}$, respectively.

There were no statistical significant differences between the negative control and P0, PA, P3 and P5 at 1.25 μM and 12.5 μM . However, at 125 μM , all these peptides showed statistically significant increase of collagen production compared to the negative control. Statistical comparison at $P < 0.05$ shows that the P3 peptide at 125 μM had the most significant increase (56.00(± 3.12) $\mu\text{g/ml}$) in collagen production compared to P5 (42.86(± 1.53) $\mu\text{g/ml}$), PA (39.39(± 3.39) $\mu\text{g/ml}$) and P0 29.03(± 3.91) $\mu\text{g/ml}$.

Fibronectin stimulation

Figure 9 shows the quantitated fibronectin deposition via ELISA. The negative control showed 29.97(± 2.61) ng/ml and the positive control showed 88.03 (± 1.18) ng/ml. Increasing peptide concentration of P0 from 1.25 μM , 12.5 μM and 125 μM showed increasing fibronectin deposition from 33.37 (± 2.40) ng/ml, 36.10(± 0.85) ng/ml and 43.42(± 1.68) ng/ml, respectively. There were no statistical significant differences between the negative control and P0 at 1.25 μM and 12.5 μM . However, 125 μM of P0 showed increased fibronectin production compared to the negative control. 1.25 μM

and 12.5 μ M of PA showed no significant change in fibronectin deposition compared to the negative control at 37.12($\pm$ 0.60) ng/ml, 34.06 ($\pm$ 1.80) ng/ml, respectively. However, 125 μ M of PA showed a statistically significant decrease of fibronectin production compared to the negative control at 17.62 ($\pm$ 1.15) ng/ml

Increasing peptide concentration of P3 from 1.25 μ M, 12.5 μ M and 125 μ M showed increased fibronectin deposition from 41.04 ($\pm$ 2.59) ng/ml, 49.81($\pm$ 4.64) ng/ml and 61.90($\pm$ 0.31) ng/ml, respectively. Increasing peptide concentration of P5 from 1.25 μ M, 12.5 μ M and 125 μ M showed increased fibronectin deposition from 36.10 ($\pm$ 4.093) ng/ml, 43.59($\pm$ 1.45) ng/ml and 47.17($\pm$ 2.82) ng/ml, respectively. There were no significant differences between the negative control and P3 and P5 at 1.25 μ M. However, at 12.5 μ M and 125 μ M, P3 and P5 showed significant increased fibronectin production compared to the negative control. Statistical comparison showed that the P3 peptide at 125 μ M had the most significant increase (61.90($\pm$ 0.31) ng/ml) in fibronectin production compared to P5 (47.17($\pm$ 2.82) ng/ml) and P0 (43.42($\pm$ 1.68) ng/ml), whereas the PA at 125 μ M showed a significant decrease in fibronectin production.

Maintaining stem cell phenotype

P3 showed the most significant stimulation in the synthesis of collagen and fibronectin by the hADSCs. Therefore, the typical expression profile of hADSCs (highly positive for CD44, CD90, CD105, CD73, CD61 and negative for CD31 and CD45) was analysed for P3 treated and untreated hADSCs by flow cytometry. As shown in Table 3 and figure 10, the P3 had no effect on the profile of the typical hADSCs surface markers during its 4 days' of treatment. This suggests that the hADSCs phenotype was maintained with P3 treatment.

Discussion

Designing bioactive molecules to stimulate production of ECM has attracted great interest in developing biomaterials for biomedical applications. Here, we describe the use of molecular modeling to design a peptide that is capable of enhancing secretion of both collagen and fibronectin by hADSCs.

The previous studies on KTTKS and/or palmitoyl-KTTKS in non-pluripotent cell types showed stimulation of collagen and/or fibronectin for various applications including wound healing^{19-21,39}. This study demonstrated that hADSCs are also capable of responding to these peptides to produce specific ECM proteins and the cell type is relevant to a wide range of tissue engineering applications¹³, including heart valves, wound healing and vascular structure. Enhancement of the effects of the peptide KTTKS on collagen and fibronectin synthesis by hADSCs was achieved by design of a new peptide, P3 (SKTTKS-V₄A₃-SKTTKS), while conserving the phenotypic characteristics of the pluripotent cell type. The new architecture of P3 with palindromic motifs, SKTTKS, could be the reason for enhancing the stimulation.

Katayama *et al.*¹⁹ examined several effective possibilities from native pro-collagen and reported that the KTTKS is the minimum requirement to have maximum activity. Our P2 and P5 support the same, and these have a disordered or reverse motif (SKTTK) and show less stability in the modeling and decreased functionality of P5 compared to P3 under experimental conditions. The mechanism of the original peptide KTTKS is still unknown and there is a detailed review explaining the complications which prevent a clear understanding of the mechanism.³⁹

The design of P3 (SKTTKS-V₄A₃-SKTTKS) was developed with the palindromic motifs thus it can present the functional groups of the peptide at both terminals, which makes this as a unique peptide compared to the previously reported functional peptides with single motif KTTKS¹⁹ and palmitoyl-KTTKS.²¹ In the palindromic motif, the additional serine residue plays a crucial role to form a bioactive motif S/KTTK/S on both sides. The modeling suggests that regular hydrogen bonds between the linkers among the chains of the P3 are responsible for intra- and inter-molecular stability (Fig. 2A). In addition, the specific arrangement of surface electrostatic properties (hydrophilic-hydrophobic-hydrophilic) in P3 appears to be essential for the structural packing in the middle and for the presentation of motifs (Fig.3C). The hydrophobic segmentation seems to be sufficient to keep the functional groups away at both ends. This segmentation pattern mimics the property of bolamphiphiles^{40,41} which has two polar heads on both ends of a hydrophobic segment also the bolamphiphiles have potential implications in various biomedical applications.

In the P5 (SKTTKV₄A₂KTTKS), the linker V₄A₂ forms regular hydrogen bonds for the stability and alignment of chains for structural packing. This correlates with a study on a peptide amphiphile that has the same linker (V₄A₂), which contributes to mechanical stiffness by forming hydrogen bonds.²⁶ Though P5 fulfills the specifications of the modelling, the production of collagen and fibronectin is comparatively lower than P3. This might be due to the minimal functionality of the reverse motif (SKTTK) at the N-terminal. Nevertheless, this reverse motif along with KTTKS at the C-terminal appears to be stimulating hADSCs to produce increased amount of collagen compared to peptide KTTKS (P0) and palmitoyl-KTTKS (PA). This is consistent with a previous study, where the presence of serine at the C-terminal of the KTTKS plays an active role in collagen stimulation.¹⁹

P3 has a palindrome design with structural stability to induce possible structural packing for the presentation of the motifs, which seems to be the most efficient design to stimulate collagen production compared to P5. The magnitude of the effects on collagen synthesis needs to be assessed against other known stimulants of collagen production (which may be greater than that observed with ascorbic acid). In addition, these two peptides can promote fibronectin production while peptide 3 can maintain typical hADSCs phenotype as demonstrated by FACS analysis. In contrast, palmitoyl-KTTKS (PA) showed reduced cell viability, despite this effect, it still showed significant collagen stimulation in hADSCs. This is consistent with a previous finding²¹ where palmitoyl-KTTKS (PA) reduced viability of human corneal and dermal fibroblasts.

Conclusions

We have experimentally demonstrated that the proposed P3 can stimulate hADSCs to promote the production of collagen and fibronectin while maintaining the phenotype of the hADSCs. Further assessments of the peptide is underway to prove the efficacy of the peptide *in vivo*. The design of dual palindromic motifs might present multiple functional groups for the stimulation. The ability to instruct stem cells to produce essential components of ECM has profound implications in tissue repair, for wound healing and biomedical applications. In particular, this bioactive peptide can be extremely useful to generate an intelligent biomaterial to orchestrate ECM production *in-situ* for regenerative medicine and tissue engineering.

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Table 1. Designed peptides with different forms of motifs

Sequence	Peptide (P)	Length
NH2-KTTKSV ₄ A ₃ KTTKS-COOH	1	17
NH2-SKTTK V ₄ A ₃ KTTKS-COOH	2	17
NH2-SKTTKSV ₄ A ₃ SKTTKS-COOH	3	19
NH2-KTTKSV ₄ A ₂ KTTKS-COOH	4	16
NH2-SKTTKV ₄ A ₂ KTTKS-COOH	5	16
NH2-SKTTKSV ₄ A ₂ SKTTKS-COOH	6	18
NH2-SKTTKSA ₄ G ₃ SKTTKS-COOH	7	19

Table 2. Peptides for *in-vitro* experiments

Name	Sequence	Molecular Weight	Purity
P0	NH2-KTTKS-COOH	563.66	>96%
PA	Palmitoyl-KTTKS-COOH	802.41	>95%
P3	NH2-SKTTKSV ₄ A ₃ SKTTKS-COOH	1893.23	>95%
P5	NH2-SKTTKV ₄ A ₂ KTTKS-COOH	1647.99	>99%

Table 3: Expression profile of hADSCs

Surface protein	Antigen/ marker	Untreated hADSCs (% expression)	Peptide 3 treated hADSCs (% expression)
Hyaluronate	CD44	99.9(±0.2)	99.8(±0.0)
Thy-1	CD90	99.7(±0.1)	99.3(±0.8)
Endoglin	CD105	88.5(±5.3)	97.0(±2.4)
5'-Nucleotidase	CD73	99.6(±0.2)	99.7(±0.2)
Leukocyte common antigen	CD45	1.5(±0.3)	1.3(±0.2)
Integrin beta-3 (β3)	CD61	5.1(±3.7)	4.8(±2.58)
PECAM-1	CD31	1.6(±0.5)	1.4(±0.2)
Negative control	protein G	2.0(±0.1)	2.0(±0.1)

Table 3: The typical expression profile of hADSCs (CD44, CD90, CD105, CD 73, CD45, CD 61 and CD31) as analysed by flow cytometry. There were no significant difference in marker expression between untreated hADSCs and P3 treated hADSCs ($P < 0.05$, $N=3$, Mean $\pm$ SD).

Figure Legends

Figure 1: Molecular interactions of the linkers. The representative structures of the seven peptides are shown as ribbons. Here, A) peptide 1, B) peptide 2, C) peptide 3, D) peptide 4, E) peptide 5, F) peptide 6 and G) peptide 7. The residues in the linkers are shown in ball and stick model and the hydrogen bonds are shown as dotted lines. The residues valine, alanine and glycine are colored in blue, gold and violet, respectively.

Figure 2: Dynamic interactions of the peptides. Total number of inter- molecular hydrogen bonds of the peptides during MD simulations. A) Peptides with V4A3 linkers (P1- P3), B) peptides with V4A2 linkers (P4-P6) and C) peptide 7 with A4G3 linker is shown with peptide 3 for comparative purpose. Here, peptides 1 to 7 are labelled as P1 to P7, respectively.

Figure 3: Electrostatic potential surface. Surface map of the representative structures are shown. Here, A) peptide 1, B) peptide 2, C) peptide 3, D) peptide 4, E) peptide 5, F) peptide 6 and G) peptide 7. Where, sky blue, red and pale blue/white indicates positive, negative and hydrophobic surfaces, respectively.

Figure 4: Radius of gyration. Dynamic molecular association around the central axis of each system A) Peptides with V4A3 linkers and B) Peptides with V4A2 and A4G3 linkers shown together for comparative purpose. A plateau after the equilibration period indicates the integrity. Here, peptides 1 to 7 are labelled as P1 to P7, respectively.

Figure 5: Bright field images of hADSCs. The cells treated with 125 μ M of peptides at 4 days. (A) KTTKS, B) Palmitoyl-KTTKS-COOH C) peptide 3, D) peptide 5, E) negative control with 2% FBS and F) positive control with 250 μ M ascorbic acid.

Figure 6: Metabolic activity of hADSCs. After 4 days of treatment with negative control (2% FCS), Positive control (250 μ M ascorbic acid) and 125 μ M of peptides KTTKS (P0), Palmitoyl-KTTKS-COOH (PA), peptide 3 (P3) and peptide 5 (P5). (Data expressed as Mean $\pm$ S.D. * = $P < 0.05$ compared to the negative control, $n=3$).

Figure 7: Immunostaining of Fibronectin and Collagen. Immunofluorescence staining of the fibronectin (green) and collagen (red) of KTTKS (P0), Palmitoyl-KTTKS-COOH (PA), peptide 3 (P3) and peptide 5 (P5) with increase concentration from 1.25 μM,12.5 μM to 125 μM.). Negative control is 2% FCS and Positive control is 2% FCS 1mM L-ascorbic acid 2-phosphate, 0.025ng/ml TGF Beta 1 and 0.01mg/ml insulin

Figure 8: Concentration dependent stimulation of collagen by hADSCs. A) KTTKS (P0), B) Palmitoyl-KTTKS-COOH (PA), C) peptide 3 (P3) and D) peptide 5 (P5). Negative control is 2% FCS and positive control is 2% FCS with 250 μM ascorbic acid. All data expressed as Mean $\pm$ S.D. * = $P < 0.05$ compared to the negative control, $N=3$. Observed on 8-well chamber slide with increased concentration of peptide from 1.25, 12.5 to 125 μM over 4 days.

Figure 9: Concentration dependent stimulation of fibronectin by hADSCs. A) KTTKS (P0), B) Palmitoyl-KTTKS-COOH (PA), C) peptide 3 (P3) and D) peptide 5 (P5). Negative control is 2% FCS and Positive control is 2% FCS 1mM L-ascorbic acid 2-phosphate, 0.025ng/ml TGF Beta 1 and 0.01mg/ml insulin. All data expressed as Mean $\pm$ S.D. * = $P < 0.05$ compared to the negative control, N=3. Observed on 8-well chamber slide with increased concentration of peptide from 1.25, 125 to 125μM over 4 days.

Figure 10: FACS histogram of hADSCs. The typical expression profile of hADSCs (CD44, CD90, CD105, CD 73, CD45, CD 61 and CD31) as analysed by flow cytometry. There were no significant difference in marker expression between untreated hADSCs and P3 treated hADSCs ($P < 0.05$, $N=3$, Mean $\pm$ SD).