



A potential bioactive peptide candidate for biomaterial and tissue engineering applications

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ABSTRACT

The surface modification of biomaterials with matrikines for tissue engineering application is one of the recent approaches to improve their biocompatibility. In an earlier study, a peptide containing 21 amino acid isolated from bovine tendon collagen was shown to promote good cell adhesion in HeLa cell, and a smaller region in the peptide was identified using bioinformatics tool to mediate cell-peptide interaction. Hence, the present study was undertaken to validate the cell adhesion property of the smaller region of the peptide and elucidate probable peptide-cell interaction pathway. Cell adhesion and proliferation properties of the peptide were studied on cells cultured on surfaces coated with varying concentrations of peptide. Expression of focal adhesion related proteins like paxillin and pFAK Tyr397 was confirmed by immunoblotting and immunofluorescence microscopy respectively. The anti-pFAK Tyr 397 stained confocal micrographs and mRNA transcription levels of Cdc42 and Rho further confirmed peptide mediated cell spreading. The change in the expression levels of integrin $\alpha 1$ and $\beta 1$ indicates an integrin mediated cell-peptide interaction for cell survival and proliferation. Integrin mediated adhesion was further confirmed by anti-integrin blocking assay. The modulation of ECM components by the peptide was assessed by expression of COL1A1, TIMP mRNA levels and gelatin zymography for MMPs. The results of the study confirm the role of the small region of the larger collagen peptide in cell adhesion and proliferation and hint at the possible use of such small peptides as biocompatible surface modifiers for tissue scaffolds.

1. Introduction

Collagen, the main protein component of extracellular matrix (ECM) accounts for about 25–35% of the whole body proteins in humans, and about 28 types of collagen have been identified till date. Type I collagen is the most prevalent form, and other types are present in lower proportions in various tissues and organs, which play specific functions in the particular organ or tissue [1,2]. Recently, there has been an increasing research focus on peptide sequences within the parent collagen molecule for identifying cryptic peptides with diverse physiological and functional roles [3]. Novel peptides identified in ECM proteins are being investigated for use in biomaterial, therapeutic and tissue engineering applications. Cell adhesion is an important process that plays a crucial role in the development of multicellular organisms. Cell adhesion occurs due to interaction of the ECM binding sites with the cell adhesion receptors, which then triggers the downstream proteins leading to cell attachment followed by remodeling of the cytoskeletal filaments supporting the cell shape and spreading of the cell on the substratum [4]. Among the cell adhesion receptors, integrins are the

largest family discovered till date [5]. Integrins facilitate the interaction between cell and collagen, and they are involved in the anchorage and bi-directional signal transfer. The whole of the signal cascade is operated through a series of protein signals, which initiates the formation of protein aggregates termed focal adhesions. The focal adhesions link the integrins to the cytoskeletal proteins leading to a cascade of other cellular events involved in cell growth and development [6,7]. The cell movement and differentiation are fundamental processes involved in events such as wound healing, tumor growth, tissue regeneration, immune response and metastasis [8,9].

In an earlier study, a peptide isolated from collagen hydrolysate with the sequence, GPOGPOGKNGDDGEAGKPGRPG, was found to possess anti-oxidant, ACE inhibitory and cell adhesive properties [10,11]. Using bioinformatics tool, a small region, GKNDDGEA in the peptide was predicted to be responsible for interaction with integrin receptor thereby promoting cell adhesion [12]. The present study was initiated with the objective of validating the cell adhesion property of the smaller peptide and also to understand the pathways through which the cell adhesion is brought about by the peptide. The polystyrene

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surface, commonly used for cell adhesion experiments, being is hydrophobic in nature is usually coated with hydrophilic materials to render the surface suitable for cell attachment. In this study, the polystyrene surface was pre-coated with a layer of purified collagen hydrolysate over which the peptide was coated. The coating of collagen hydrolysate was done to facilitate uniform spreading of peptide.

2. Methods

2.1. Reagents and chemicals

The custom-made peptide was procured from Neo Scientific, USA and HeLa (human epithelial) cell line was sourced from National Centre for Cell Science, Pune, India. The T-flasks (Nunc surface) were obtained from Nunc, Roskilde, Denmark and disposable culture dishes (35 × 10 mm) were from Fischer Scientific, Hanover Park, IL, USA. The culture media DMEM and fetal bovine serum were from GIBCO, and antibiotic solution was obtained from HiMedia, India. Total RNA was isolated using RNA iso Plus (Takara Bio Inc., USA). 10 × trypsin-EDTA solution, anti-paxillin polyclonal antibody (SAB4502553) β -actin antibody and phalloidin-tetramethylrhodamine B isothiocyanate were procured from Sigma-Aldrich, USA. pFAK Try 397-R (SC-11765) and anti-integrin α 1 A-9 antibody (SC-271034) were from Santa Cruz Biotechnology, USA. Secondary antibody m-IgGK BP-HRP (SC-516102) and alexa fluor 568 (A11011) goat anti-rabbit IgG (H + L) were purchased from Thermo Fisher Scientific, USA.

2.2. Peptide coating

The peptide diluted to desired concentrations was coated on to the sterile surface pre-coated with collagen hydrolysate purified by ion-exchange chromatography (Fast flow liquid chromatography – Sephadex G100). In order to study the effect of peptide on cell adhesion, a dish coated with only collagen hydrolysate was used as control. Experiments were conducted with varying peptide coating concentrations based on the earlier study performed with larger peptide [12]. The concentrations of peptide in terms of coating density ranged from 0.178 to 0.893 ng/cm². A commercially available cell culture dish was used as a positive control for comparison.

2.3. Cell viability assay

To 96 well cell culture plate coated with peptide as mentioned in Peptide coating section, 1 × 10⁴ cells per well was seeded in DMEM medium. After 24 h incubation, MTT solution (20 μ l, 5 mg/ml in PBS) was added to each well and incubated for 4 h in a humidified atmosphere of 5% CO₂ at 37 °C. 100 μ l of DMSO was added to each well after removal of the medium to dissolve the tetrazolium salt. The plates were gently agitated on a shaker for 10 min to allow homogenous dissolution of the precipitate. The plates were immediately placed on a microplate reader, and the absorbance was measured at 570 nm.

2.4. Cell adhesion and proliferation assay

3 × 10⁶ and 1 × 10⁵ cells were seeded in each dish for cell adhesion and proliferation assay respectively and incubated in a 5% CO₂ chamber. The cells were harvested after 6 h, and the cell count was assessed by trypan blue exclusion assay for cell adhesion, while the cell proliferation was assessed after 48 h using inverted light microscope.

2.5. Cell adhesion inhibition assay

1 × 10⁴ cells were taken in two tubes, cells in one tube was incubated for 30 min in 1: 500 dilution of mouse α 1 integrin antibody and another tube was left untreated (Control). The cells were seeded in 96 well plates and incubated for 45 min. The non-adhered cells were

washed with PBS and adhered cells were fixed with 2% formaldehyde for 10 min. The fixed cells were stained with 0.5% crystal violet in 20% methanol and washed with distilled water. Plates were air-dried, dissolved in 10% acetic acid and absorbance was measured at 600 nm which is directly proportional to adherent cells.

2.6. Immunofluorescence microscopy

The adhesion complex proteins, pFAK and actin filaments were immuno-stained with fluorescent dyes. Peptide was coated in the same concentration levels as described in Section 2.2 on a 12 mm circular cover slip, and cells were seeded at a concentration of 1 × 10³ and cultured for 48 h. The cover slips were washed with 1 × PBS twice and fixation was done with 4% formaldehyde for 10 min at 30 °C. Blocking was done with 1% BSA in PBS containing 0.05% Triton X-100 for 1 h at 30 °C and washed with PBS. Anti-rabbit pFAK Try397 primary antibody was added to the coverslips at a dilution of 1:500, incubated for 1 h at 30 °C and washed with 1 × PBS followed by treatment with secondary antibody Alexa Flour 568 at a dilution of 1:1000 in PBS for 1 h at 30 °C. For actin staining, phalloidin was used at a dilution of 200 units/ml in blocking buffer (BSA in PBS) and stained for 30 min at 30 °C. Nuclear staining was done with Hoechst 33258 in Hanks buffer at a dilution of 1:100 for 30 min at 30 °C. All the staining procedures were carried out in the dark. The cover slips were mounted onto clean glass slides and stored at 4 °C until analysis. Imaging was done using Olympus FV1000 laser scanning confocal microscope.

2.7. Western blot analysis

Cells were washed twice with PBS and treated with lysis buffer (RIPA) containing protease inhibitor cocktail. Lysate was passed through a 26-gauge needle to shear the DNA and kept for 30 min with the entire process being carried out at 4 °C. The cell lysate was centrifuged at 10,000g for 10 min at 4 °C and the supernatant was transferred to a micro centrifuge tube. The total protein was estimated by BCA method. 50 μ g/ml protein sample were loaded in 8% polyacrylamide gels under reducing condition. The gel was incubated in transfer buffer for 30 min at 30 °C and PVDF membrane was pre-treated with methanol for 20 min. Blotting was done overnight at 30 V, 100 mA at 4 °C. The membrane was blocked with 5% skimmed milk in tris buffer saline, treated with Tween 20 for 1 h followed by primary anti-paxillin incubation for 6 h at 4 °C. Membrane was washed and probed with HRP conjugated secondary antibody for 1 h. Protein bands were detected by enhanced chemiluminescence method. The blot was stripped and re-probed with anti- β actin antibody as loading control.

2.8. Gelatin zymography

Cells were grown up to 80% confluence on peptide coated dishes, the cultured cells were placed in serum-free media for 12 h and used for the assay. Zymography was performed in 7% native and SDS-PAGE gels co-polymerized with gelatin. SDS-PAGE gel was incubated with 1 × renaturation buffer (2.5% Triton X-100 in distilled water) for 45 min to remove SDS. The gels were then washed with distilled water and equilibrated with 1 × developing buffer (50 mM Tris-base, 50 mM Tris-HCl, 0.2 mM NaCl and 5 mM CaCl₂, pH adjusted to 7.8–8) for 45 min at 30 °C. The gels were then placed in freshly prepared 1 × developing buffer for 18 h at 37 °C. The gel was stained using Coomassie blue and destained until clear sharp bands were visible in a blue background.

2.9. Real-time PCR

The total RNA quantification and purity was determined using Nano drop 2000 (Thermo Fisher Scientific). 1 μ g of total RNA was transcribed to complementary DNA with the high capacity cDNA kit according to the manufacturer instructions. Gene-specific primers were purchased

Table 1
Primer sequences used for quantitative real-time PCR analysis.

Gene	Forward	Reverse
GAPDH	5'TCACCAGGGCTGCTTTAAC3'	5'GACAAGCTTCCCCTTCTCAG3'
COL1A1	5'CCTCAAGGGCTCCAACGAG3'	5'TCAATCACTGTCTTGCCCCA3'
TIMP1	5'CTTCTGGCATCCTGTTGTTG3'	5'AGAAGGCCGTCTGTGGGT3'
Rho	5'ACCTGCCTCCTCATCGTCTTC3'	5'CACCTGCTTGCCGTCCAC3'
cdc42	5'GGCTGTCAAGTATGTGGAGTGTTC3'	5'GCTCCAGGGCAGCCAAT3'
Integrin α 1	5'CAGCCCCACATTTCAAGTCGT3'	5'ACCTGTGTCTGTTTAGGACCA3'
Integrin β 1	5'CAAAGGAACAGCAGAGAAGC3'	5'ATTGAGTAAGACAGTCCATAAGG3'

from indigenous DNA Pvt. Ltd. India. The gene expression of TIMP-1, COL1A1, integrin α 1, integrin β 1, Rho and Cdc42 was analyzed. The reaction mixture was prepared with 1 μ l of diluted cDNA template, 5 μ l SYBR green PCR master mix and 1 μ l of each forward and reverse primers (0.2 μ M). Real-time PCR was performed in triplicates using CFX 96 touch (Bio-Rad, USA) with an initial denaturation temperature of 95 °C for 1 min followed by 40 cycles of 15 s at 95 °C, 20 s at 57 °C and then 30 s at 72 °C. Subsequently, a melt curve analysis was performed to ensure specific amplification. For each target gene, relative levels of expression were normalized using the GAPDH signal. Relative quantification was performed using the $2^{-\Delta\Delta Ct}$ value, where $\Delta Ct = Ct$ (target) – Ct (endogenous control) and $\Delta\Delta Ct = \Delta Ct$ (sample) – ΔCt (calibrator) (Table 1).

2.10. Statistical analysis

The data is presented as mean \pm SD, and the statistical analysis between the experimental groups was carried out using one-way analysis of variance (ANOVA) by Tukey post-test using graph pad 5.0 for windows. * $P < 0.05$ is considered statistically significant.

3. Results

HeLa cell was cultured onto the peptide-coated and uncoated sterile surfaces as per the coating density mentioned in Methods section.

3.1. Effect of peptide on cell viability

An increased percentage of viable cells were found on peptide treated surface compared to the uncoated control (40.4%) and hydrolysate coated surface (44.3%). The cell viability was found to be maximal at a peptide coating density of 0.563 ng/cm² (denoted as P3) with 87% viability (Fig. 1). The cells grown on commercial coated plates were taken to be 100% viable. There was significant decrease in cell viability with higher coating density (P4-70.3% and P5-60.4%).

3.2. Effect of peptide coating on cell adhesion

Cell adhesion was analyzed on peptide coated surface in comparison with the uncoated control. The peptide coating improved the adhesion of HeLa cells when compared to the uncoated control dish. A maximal cell count of $12.07 \pm 0.29 \times 10^5$ was observed with a peptide coating of P3 (* P value < 0.05). The effect of various peptide coating on adhesion is depicted in (Fig. 2).

3.3. Peptide enhanced cell proliferation efficiency

The confluence of the cells was monitored microscopically to study cell proliferation. The cells grown at P3 concentration were nearly confluent at 48 h. At highest concentration (P5), many cells appeared rounded and not proliferated (Fig. 3). The uncoated control surface showed complete absence of proliferating cells.

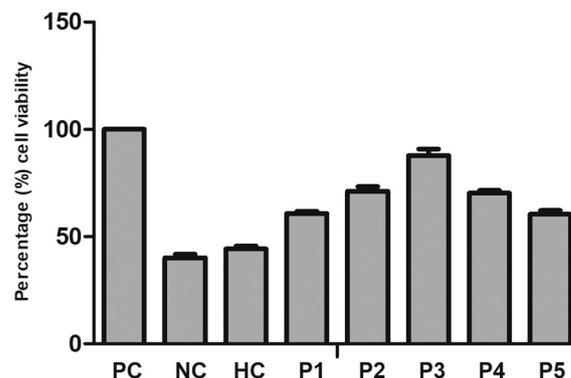


Fig. 1. Effect of the peptide on HeLa cell viability. NC (Negative control) represents the uncoated surface, HC (hydrolysate control) coated with hydrolysate alone and P1 - P5 peptide coated onto hydrolysate, PC (positive control) commercially available coated plate. The amount of peptide coated was as follows, P1 – 0.178, P2 – 0.357, P3 – 0.536, P4 – 0.714 and P5 – 0.893 ng/cm² in 96 well plate. The cell viability is represented as percentage of viable cells in comparison to positive control. Data presented are as mean of triplicates \pm SD.

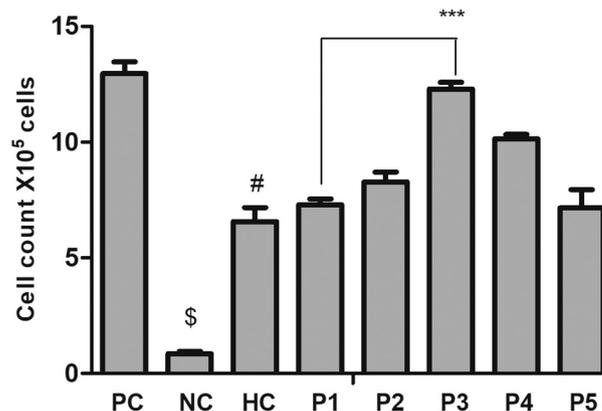


Fig. 2. Effect of HeLa cell adhesion on peptide coated surface. \$ - comparison made with control versus HC, P1, P2, P3, P4 and P5 (P value < 0.05 ***), # - comparison of HC with P3 and P4 (P value < 0.05 ***). The cell count was enumerated as ($\times 10^5$) mean of three individual experiments \pm SD. * P value < 0.05 was considered statistically significant.

3.4. Peptide guides cell adhesion through integrin

The number of cells that adhere after anti-integrin treatment was monitored by the amount of crystal violet dye taken up by the adhered cells. The assay results are mentioned as absorbance of the resultant solution at 600 nm. The dye uptake was lower in anti-integrin treated cells, indicating decrease in cell adherence (Fig. 4) shows the cell adherence inhibition of anti-integrin on peptide coated surface. A significant reduction in adhesion in P3 was observed in comparison with the antibody untreated cells (P value < 0.05).

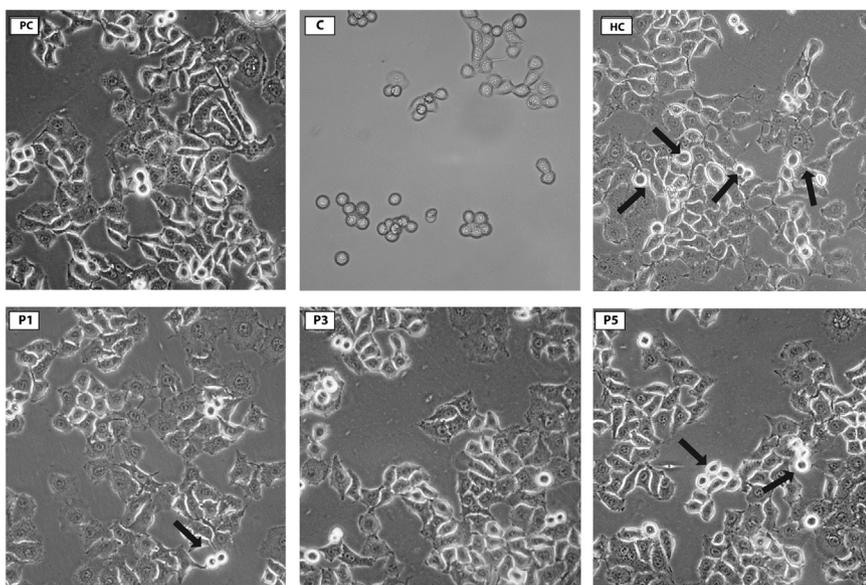


Fig. 3. Role of the peptide on cell proliferation. PC (Positive control) commercially available coated dishes, NC (Negative control) untreated control surface, HC – hydrolysate coated, P1 – 1.72 ng peptide coated, P3 – 5.16 ng and P5 – 8.6 ng. Images were captured with an inverted phase contrast microscope at 20× magnification. The black arrows points on rounded cells.

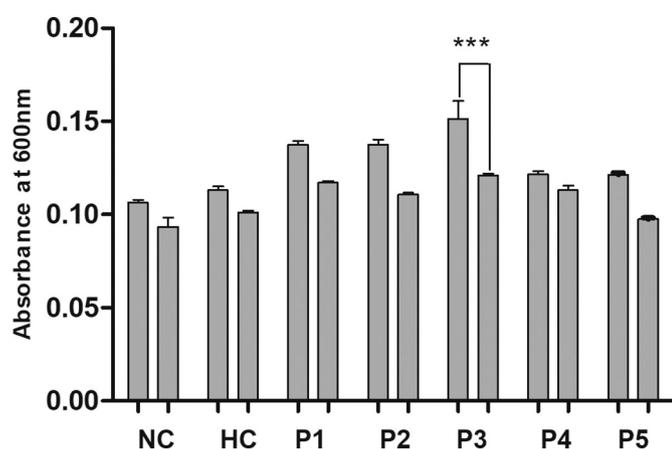


Fig. 4. $\alpha 1$ integrin blocking assay. Mouse anti $\alpha 1$ integrin antibody was used to treat the cells before seeding onto the peptide coated and uncoated surface. The control group of cells were seeded to peptide coated and uncoated surface without antibody treatment. Comparison was made between the antibody treated and untreated cells within the groups. Statistical significance was observed within the group i.e. integrin antibody treated cell and untreated cells seeded on to peptide coated group. *P value < 0.05 was considered statistically significant. The peptide coating amounts were same as mentioned in MTT assay (Fig. 3). The data presented are as means of triplicates \pm SD.

3.5. Pathway mediated by peptide in cell adhesion and proliferation

Western blotting and immunofluorescence were performed to analyze paxillin expression and the activation of focal adhesion kinase (pFAK) respectively. The immunofluorescence imaging of pFAK protein on cells cultured on peptides (Fig. 5A & B), showed significant increase in the expression of pFAK along the leading edges of the proliferating cells in peptide coated surface (P3) in comparison with other dishes such as control and hydrolysate alone treated. Paxillin protein expression was observed to be significantly higher in P3 (P value < 0.05) in comparison with uncoated surface (Fig. 6).

3.6. Involvement of actin filaments in cellular protrusion and proliferation

The cytoskeletal actin filaments were stained with phalloidin (green), nuclei with Hoechst 33258 (blue), and pFAK was stained with alexa fluor 568 (orange). The cytoskeletal protrusions were

significantly well spread in cells grown on peptide coated surface (P3) in comparison with the uncoated control and hydrolysate coated surfaces (Fig. 5C). The control surface was observed to have rounded cells with lesser cellular protrusions.

3.7. The peptide modulates the ECM microstructure to enhance cell adhesion and proliferation

Integrin $\alpha 1$ showed no significant difference between the varying peptide coating density (P3) but a significant decrease (2 fold) was found in control surface (P value < 0.05) (Fig. 8a). In case of integrin $\beta 1$, the expression was observed to be increasing significantly with increasing peptide coating (P value < 0.05) (Fig. 8b). COL1A1 expression was found to be increased by 40% in P3 in comparison with control dish (P value < 0.05) but there was no difference observed with increase in coating density (P3 and P5 in Fig. 8e). Increase in TIMP1 expression was found to be statistically insignificant compared to uncoated control surface, but there was a one-fold increase in P5 in comparison to other peptide coating concentrations (Fig. 8f). The genes responsible for cell spreading were analyzed with Cdc42 and Rho expression, which was not significantly different at the optimal coating density (P3) in comparison with control, PC and P1. There was a significant 0.7 fold increase in expression at P5 compared to uncoated control.

Gelatin zymography was performed with cell culture supernatant of all the control and peptide coated groups. The cells were cultured in serum-free culture medium to determine the MMPs secreted by the cells. Clear bands under blue background were observed in the molecular size range of around 50 to 90 kDa. There was no visible change in the band intensity with varying peptide coating (Fig. 7). The clear bands suggest the presence of MMPs but under native conditions bands were not visible. This may be possibly due to the bound TIMP (inhibitors of MMPs) keeping the MMPs inactive under native condition. When the samples were run under denaturing condition, the bound TIMP molecule was released and the activity of MMP visualised as clear bands.

4. Discussion

Screening ECM derived peptides for improving surface properties of tissue engineering and biomedical devices would be a robust, versatile and convenient approach. Cell adhesion is an important surface property of a biomaterial and HeLa cells were used as model cell line to

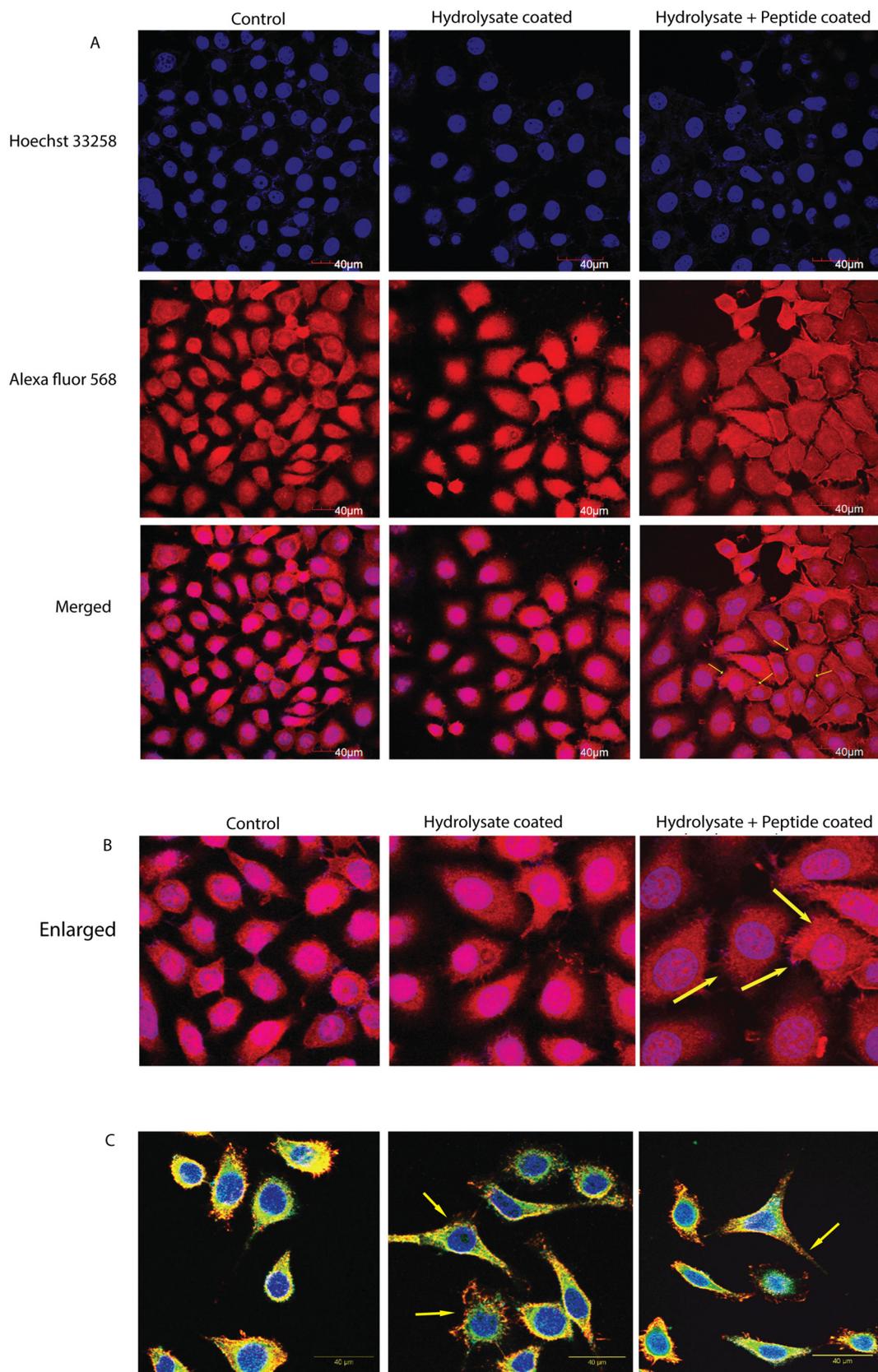


Fig. 5. Confocal imaging of HeLa cells cultured on peptide coated surface. Images were captured at λ_{ex}/em 352/461 nm for blue channel (Hoechst 33258) to stain the nucleus and λ_{ex}/em 578/603 nm for red channel (Alexa fluor 568) to stain pFAK. A - (Control) cells cultured on untreated surface, (Hydrolysate) coated and (Peptide) coated surfaces $5.36 \text{ ng}/\text{cm}^2$ (P3) coated surface. Images are represented as nuclear stain, pFAK Tyr 397, Alexa fluor 568 and merged image from top to bottom. B - enlarged image of peptide-coated and uncoated surface showing the focal adhesion sites at the edges of the cells (bright dots – marked with arrows). C - Effect of peptide on cell proliferation. Actin 544 filaments of the cytoskeleton is stained with Phalloidin (green), nucleus with Hoechst 33258 (blue) and pFAK stained with alexa fluor 568 (orange) the arrows indicate the expression of pFAK at the leading edges of the cells and nucleus at the centre. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

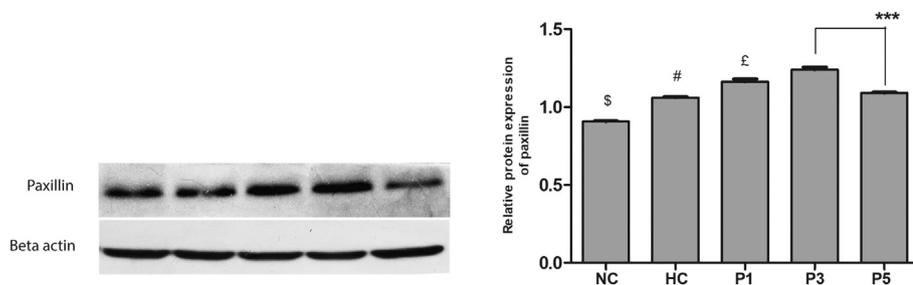


Fig. 6. Western blot analysis on paxillin protein expression. \$ - comparison made with control verses HC, P1, P3 and P5 (P value ***), # - comparison made with HC verses P1 and P3 (P value ***), £ - comparison made with P1 verses P3 and P5 (P value **), and comparison made with P3 and P5 (P value ***). The peptide coating was as mentioned in cell adhesion assay (Fig. 1). *P value < 0.05 was considered statistically significant. The blots were confirmed with three individually performed experiments.

study the adhesive behaviour of the peptide under study [13]. The cell adhesion and proliferative role of a larger peptide of 22 amino acid identified in bovine Achilles tendon type I collagen on HeLa and Vero cells was confirmed in an earlier study. Prediction made using docking studies suggested that a smaller sequence GDDGEA in the larger peptide to be responsible for interaction with surface receptors integrin $\alpha 1\beta 1$ and $\alpha 2\beta 1$ [12]. In the present study, the role of the peptide GKNGD-DGEA with a molecular weight of 861 Da in modulating the cell behaviour was investigated. Type I collagen hydrolysate purified with CM-Sepharose columns [11] containing the larger 22 amino acid peptide was used to uniformly coat all dishes before coating with peptide. The amount of hydrolysate coating was kept constant throughout the study in all the surfaces. The uncoated polystyrene surface was used as negative control and surface coated with hydrolysate alone was used as hydrolysate control (PC).

The peptide exhibited an enhanced cell adhesion and proliferation role in HeLa cell line. HeLa cells showed highest cell viability at a peptide coating density of 0.536 ng/cm^2 . Polystyrene surface is hydrophobic in nature, which renders it inappropriate for cell adhesion. Hence, altering the surface with application of a protein could reduce the hydrophobicity and provide multiple substrates for cell adhesion [14]. Cells in the uncoated dish were not adhered well, very fragile and rounded or not well proliferated (Figs. 1 & 2). Cell proliferation assay was carried out at similar concentrations of peptide as shown in (Fig. 2). The peptide was found to improve cell proliferation, which increased with the peptide coating significantly. This study confirmed that the optimum coating amount for cell adhesion and proliferation to be 0.536 ng/cm^2 . Cell adhesion depends on the number of binding sites for the cell receptors. The adhesion strength affects the migration of cells and hence there is a need for a balance in the density of ligands and its cell receptors [15]. This could be the probable reason for decreased adhesion of cell with higher coating concentrations of peptide.

Integrins are important mediators of cell-ECM interaction, and the results from the integrin $\alpha 1$ mediated cell adhesion inhibition (Fig. 4)

showed a partial involvement of the $\alpha 1$ integrin receptor in the peptide mediated cell adhesion. Integrins, a class of cell-matrix heterodimeric receptor recognized by a large family of integral membrane proteins, play central role in all aspects of cell fate determination] and alternative splicing further increases the integrins diversity [15,16]. Various combinations of α and β subunits of the heterodimeric receptor bring about bidirectional signalling by binding to ligands in the extracellular matrix molecules like laminin, fibronectin and collagens. At a time, a single cell can express many integrin type receptors for a single matrix molecule and at the same time, one integrin heterodimer can have multiple ligands. $\beta 1$ intracellular domain is the connecting bridge to the cytoskeleton, whereas the α chains are proposed to be responsible for the integrin signal transduction function. Cell phenotype, differentiation, gene expression and migration are regulated by integrin-mediated signal [17]. Integrins are also classified into ECM binding specific molecules, which include collagen binding like $\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 10\beta 1$ and $\alpha 11\beta 1$. The major integrin receptor for HeLa cells is $\alpha 1\beta 1$, and the antibody targeting $\alpha 1\beta 1$ heterodimer (SR-84) used in an earlier study could almost completely inhibit the adhesion of HeLa cells to type I and IV collagens [18–20]. To study the role of integrin in the pathway activated by the peptides for promoting cell adhesion, the adhesion of the cells were monitored after treating the cells with anti-integrin $\alpha 1$ antibody. The cell adhesion was significantly reduced in antibody treated cells than the untreated cells, and the optimum concentration for cell viability and adhesion (P3) showed a significant reduction in cell adhesion in antibody treated cells (P value < 0.05) (Fig. 4).

Another important aspect of cell binding to ECM substrate is integrin clustering and spacing between the molecules. The cytoplasmic proteins such as actin-binding, signalling and adaptor proteins linking integrin to cytoskeletons are recruited upon ligand binding [21,22]. The enrolled protein like kindlin and talin promote integrin clustering with additional recruitment of signalling proteins to focal adhesions [23,24] and integrin mediated adhesion site through the reinforcement of actin cytoskeleton linking [25,26]. Integrins signal to the interior of

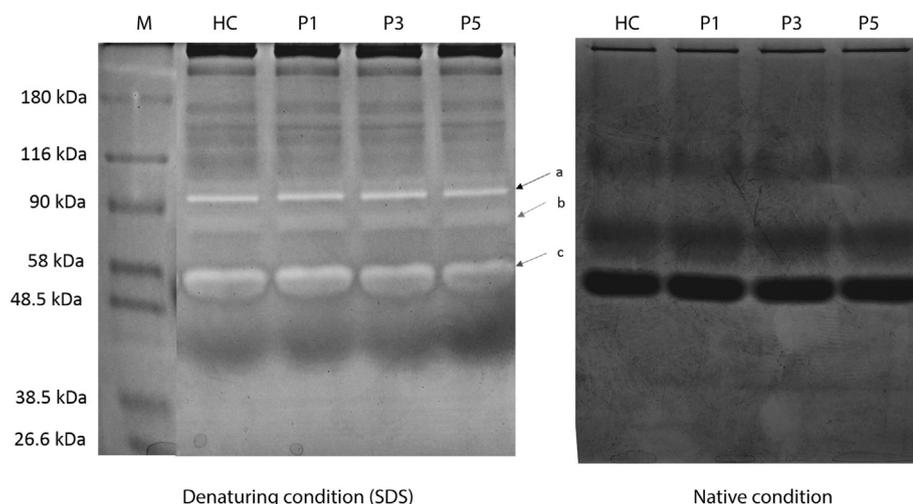


Fig. 7. ECM modulation by peptide. Cells were grown up to 80% confluence on peptide coated dishes, the cultured cells were placed in serum-free media for 12 h and used for the assay. Zymography was performed in 7% native and SDS-PAGE gels copolymerized with gelatin. Clear bands detected under blue background indicates the presence of MMPs under reducing condition but no clear visible bands were seen under non-reducing condition which hints on the presence of TIMP bound with MMPs under non-reducing condition. M – Protein marker, HC – hydrolysate alone coated, P – peptide coated with the respective concentration already mentioned in cell proliferation (Fig. 2). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

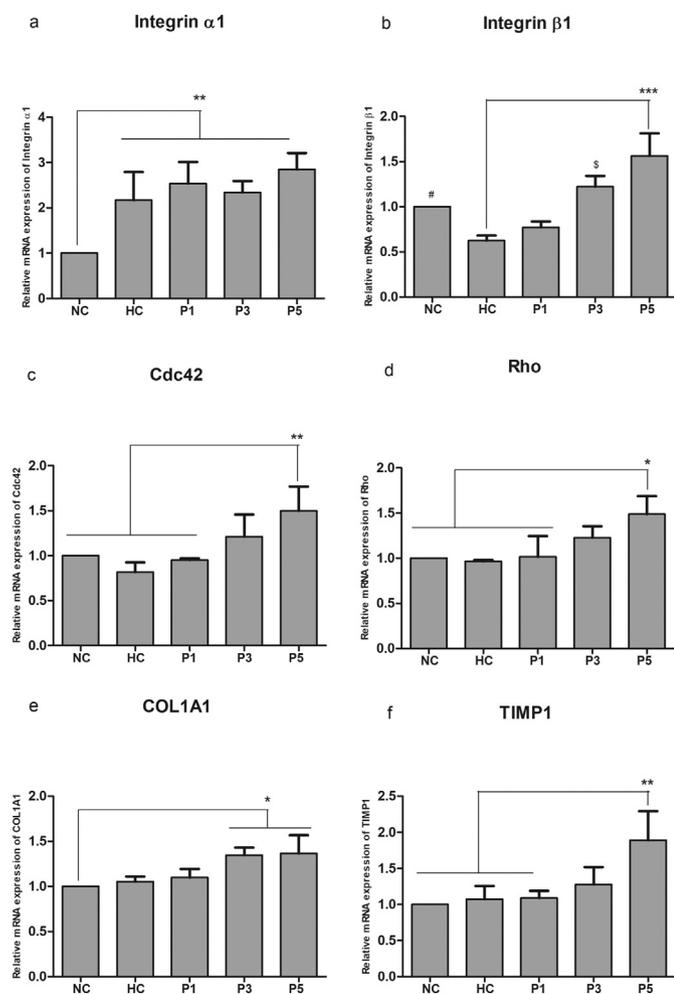


Fig. 8. mRNA expression levels of cell surface receptors integrin $\alpha 1$, $\beta 1$, Cdc42, Rho, COL1A1 and TIMP1. The expression of integrin $\alpha 1$ (a) the comparison was made with uncoated control and peptide coating. (b) Integrin $\beta 1$ # - comparison made with P5, \$ - comparison made with P1 and comparison with P1 and P5. (c) COL1A1 comparison made with control and P3, P5. (d) TIMP1 comparison made with P5 and Control, PC and P1. (e) Cdc42 comparison was made with P5 and control, PC and P1. *P value < 0.05 was considered statistically significant.

the cell through adhesion complexes, which are assembled around the cytoplasmic face of the plasma membrane. Focal adhesions, focal complexes, podosomes and fibrillar adhesions are specialized sites for integrin-associated cell interaction with ECM [27]. Integrin adhesions provide control over the internal architecture of the cell via sites of cytoskeletal assembly and cell-cell contact points. The adaptor proteins such as paxillin, enzymes like FAK, Src and Jnk, Rho family GTPases and lipid kinases are responsible for the distal signalling that control cell-fate events such as growth, survival, differentiation and migration [28,29]. To investigate cell migration and adhesion related signalling pathway, the phosphorylation level of signalling molecules was studied. FAK is a key factor in controlling FA dynamics, and phosphorylation of FAK (p-FAK) at Tyr397 plays an important role in FAK activation. Focal adhesion kinase (FAK) is a crucial protein of focal adhesions (FAs), and it regulates several cytoskeletal and focal adhesion proteins. FAK interacts with α -actinin [30], paxillin [31], integrin [32] and several other proteins that interact with FAs to the actin cytoskeleton [33]. It is not fully characterized in terms of detailed molecular interactions, but in general, it is believed that FAK enhances a high FA turnover through a rho-kinase dependent pathway (ROCK) [34,35]. The linking plaque between the ECM and actin cytoskeleton is formed by FAs consisting of

complex network of cytoplasmic proteins, but the real time interactions between the FA molecules during FA turnover with regard to their spatial and temporal changes are unclear. The 3D molecular conformation and 3D location within the FA complex may partly contribute to the different degradation properties displayed by FA [36].

The process of cell migration is a coordinated and dynamic change involved in the actin filaments with subsequent formation and disassembly of cell adhesion sites [37]. Membrane protrusions and traction forces are the result of interplay between actin cytoskeleton and cell adhesion sites [38]. Many external stimuli affect cell migration through the biochemical signals generated intracellularly by the interactions of transmembrane integrins, which bind to the ECM proteins or mechanical stimuli like shear stress, which induce deformation of the actin cytoskeleton [39]. The cells cultured on uncoated surfaces when stained with phalloidin and pFAK-alexa fluor 568 showed no distinct membrane protrusions during the early hours of attachment. There was significantly more number of non-rounded cells and cells with protrusions in peptide coated dishes at 6 h of incubation (Fig. 5C). This observation suggests that the peptide induced formation of cell protrusions and filopodia for cell spreading. Studies have shown that leading edge of a cell has a role in stabilizing the formation of focal adhesions, thus maintaining adhesion and motility [40]. Researchers have observed integrins to induce FAK phosphorylation at Try397 that served as a binding site for Src family kinases, which ultimately controls FA turnover [41]. pFAK expression was observed more at the leading edges of the cells in peptide coated dishes than the control and hydrolysate treated dishes. The expression profile clearly suggests the involvement of active FAK in cell proliferation and migration (Fig. 5C). Actin protein is highly conserved and found in most eukaryotic cells as essential element of cytoskeleton, and exists freely in monomeric state or linear filamentous state within the cytoplasm and nucleus [42]. Actin cytoskeletons are formed by F-actins to form large scale network which is formed by the regulation of actin-binding proteins and adaptor molecules. This process plays critical roles in maintaining the cell shape, enabling cell motility and providing mechanical support [43]. Paxillin protein showed an increase with increasing peptide coating concentration till P3. This result correlates well with results of the studies on cell adhesion, proliferation and pFAK expression and hence P3 was concluded as optimum concentration for cell growth. As paxillin recruitment occurs early at the site of adhesions, it serves as an important adaptor protein in recruiting other signalling components to the membrane [44]. A Schematic representation of role of peptide on cell adhesion and migration in association with focal adhesion proteins (Fig. 9).

The effect of peptide on ECM modulation by MMPs was analyzed by gelatin zymography, COL1A1, and TIMP1 expression by real time PCR. Zymography gel showed clear bands under blue background at the molecular size range of 50–90 kDa, but there is no visible change in the band intensity of the MMPs in the gel (Fig. 7). The clear bands marked with coloured arrows approximately correspond to the molecular weight of 72 kDa for pro MMP2 and 92 kDa for pro MMP9. More in-depth study on mRNA and protein expression levels of the MMPs needs to be carried out to further substantiate the role of varying coating amounts of peptide in influencing levels of MMPs.

There was significant increase in COL1A1 expression in peptide coated dishes (Fig. 8e). This result suggests that the expression of collagen $\alpha 1$ chain significantly increased in peptide coated dishes probably to support the cell growth in its micro-environment. The expression of MMPs with increased COL1A1 could be to support cell adhesion and migration. On the other hand, TIMP1 expression was significantly increased with increased peptide coating concentration with P5 showing one fold higher expression than the uncoated control dish (Fig. 8f). TIMP1 regulates MMP activity and induces the growth of various cells through MMP independent pathway [45]. TIMP1 plays dual role both as inhibitor of proteases and mitogenic stimulus [46,47]. The role of TIMP1 might be concentration dependent, with lower TIMP1 levels

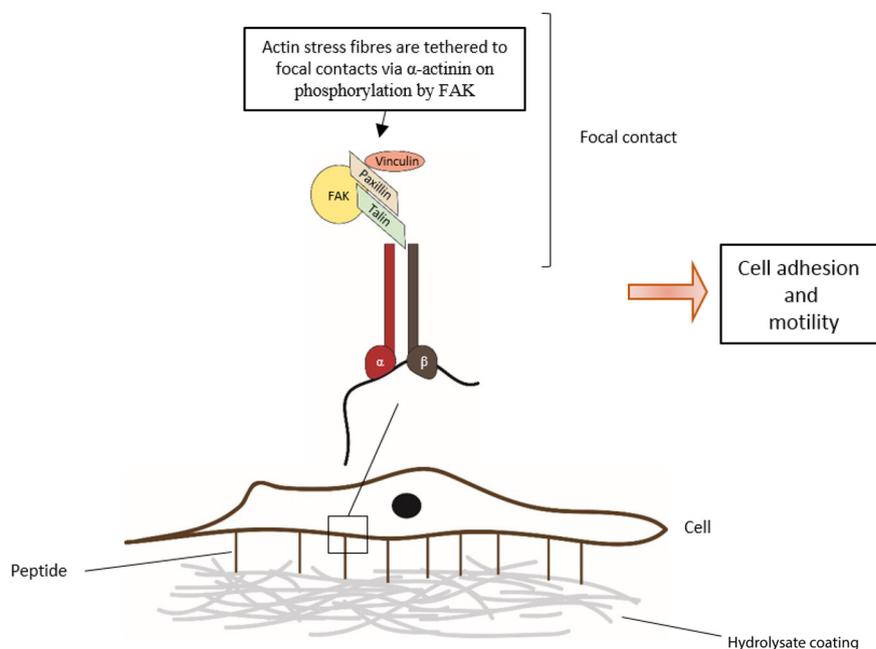


Fig. 9. Schematic representation of role of peptide on cell adhesion and migration in association with focal adhesion proteins.

favours cell growth and higher levels inhibiting proteolysis and slowing down cell growth and propagation within ECM. These results suggest the probable role of peptide in modulating ECM.

Gene expression analysis using real-time PCR on cell surface receptors integrin $\alpha 1$ showed no significant difference among different peptide concentrations coated. In case of integrin $\beta 1$, there was an increase in expression levels with increasing peptide concentration hinting at the possible involvement of integrin $\beta 1$ in cell growth. The role of Rho and Cdc42 activation on actin cytoskeleton organization has been observed in many cell types, including endothelial, epithelial and astrocytes, as well as macrophages [48,49]. The Rho family of GTPases are important regulators of protrusions, and they play crucial role in actin and adhesion organization to control the formation of filopodia and lamellipodia [50]. Rac, Cdc42 and RhoG are important for the formation of protrusions like lamellipodia and filopodia [51]. Cdc42 activation was found to stimulate actin-rich surface protrusions called filopodia. There was slight increase in expression of both Cdc42 and Rho with increasing concentration of peptide which correlated well with formation of filopodia and cell spreading. Cytoskeletal changes induced by Cdc42 are also linked to distinct integrin grounded adhesion complexes. Cdc42 is considered the master regulator of cell polarity in eukaryotes ranging from humans to yeast. They are present in the cell front in migrating cells [41]. Both activation and inhibition of Cdc42 disrupt directionality of cell migration [51]. Cdc42 determines cell polarity by controlling the formation of lamellipodia in the specific direction [52]. Cell migration and its directionality are guided by the regular turnover of the adhesion and signalling molecules, which necessitates further in-depth research.

5. Conclusion

The bioactive peptide from bovine Achilles tendon collagen type I was found to be effective in cell adhesion and proliferation involving the integrin mediated bidirectional signalling. Further studies on the effect of the peptides on different cell types will give more insight into the bidirectional signalling that would promote cell growth, spreading and ECM modulation. The use of such ECM derived peptides has found increasing application in various fields. The bioactive peptide from the present study could find application in improving the cell adhesive properties of scaffolds and hydrogels used for surface modification in

tissue engineering applications.

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Conflict of interest statement

The authors declare no conflict of interest.

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