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Cell behavior on extracellular matrix mimic materials based on mussel adhesive protein fused with functional peptides

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ABSTRACT

Adhesion of cells to surfaces is a basic and important requirement in cell culture and tissue engineering. Here, we designed artificial extracellular matrix (ECM) mimics for efficient cellular attachment, based on mussel adhesive protein (MAP) fusion with biofunctional peptides originating from ECM materials, including fibronectin, laminin, and collagen. Cellular behaviors, including attachment, proliferation, spreading, viability, and differentiation, were investigated with the artificial ECM material-coated surfaces, using three mammalian cell lines (pre-osteoblast, chondrocyte, and pre-adipocyte). All cell lines examined displayed superior attachment, proliferation, spreading, and survival properties on the MAP-based ECM mimics, compared to other commercially available cell adhesion materials, such as poly-L-lysine and the naturally extracted MAP mixture. Additionally, the degree of differentiation of pre-osteoblast cells on MAP-based ECM mimics was increased. These results collectively demonstrate that the artificial ECM mimics developed in the present work are effective cell adhesion materials. Moreover, we expect that the MAP peptide fusion approach can be extended to other functional tissue-specific motifs.

1. Introduction

Efficient cell adhesion onto extracellular scaffolds is a central issue in tissue engineering. Adherent anchorage-dependent cells interact with individual extracellular matrix (ECM) molecules during attachment, growth, migration, and even apoptosis, whereby biological responses determine morphogenesis and differentiation [1–4]. Thus, ECM environments appear to be important in controlling cellular processes for tissue regeneration, wound healing, and organ development. ECM is mainly composed of several proteoglycans as co-receptors and proteins such as collagen, laminin, and fibronectin. Integrin-mediated signaling provides the most significant contribution to cell adhesion, although proteoglycans display cooperative signaling activation in adhesion [5–7]. Collagens are the most abundant protein constituents of ECM. Among these, type IV collagen, a major component of the basal lamina, is associated with differentiation of stem cells [8] and osteoblast cells [9]. Laminin is the extensively characterized functional ECM component, and more than 40 active sites have been defined [10]. Laminin binds to type IV collagen and the cell membrane as a structural component of all basement membranes, and mediates cell–matrix interactions to promote adhesion and growth of neurite cells as well as bone cells [11,12]. Fibronectin, a large glycoprotein found in all vertebrates, is composed of a repeat arrangement of three types of modules with binding domains for fibrin, fibronectin, collagen, cells, and heparin. This protein is involved in cell adhesion, growth, migration, and differentiation [5,13].

ECM proteins have been directly utilized to prepare artificial ECM environments via covalent conjugation or physical adsorption [14–16]. However, several limitations, such as uneconomical production, random folding on surface, lack of defined characteristics, and immunogenicity, hamper their biomedical application [4,17]. The use of specific short peptides originating from the essential recognition sites of ECM proteins for cellular signaling can overcome these limitations, and thus be effectively applied to prepare ECM environments for tissue engineering [18,19]. The ECM-derived short peptides acting as receptor binding motifs are immobilized or incorporated onto various well-defined surfaces, including polymers, hydrogels, titanium, and nanofiber mesh, mainly via chemical modifications or biological linkers [20–25]. Immobilized ECM peptides on artificial surfaces efficiently promote

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cell adhesion, although free short peptides in solution can inhibit attachment by acting as an antagonist signal for integrins, and chemical linkers for surface immobilization are considerably toxic for attached cells [18,26,27].

Mussel adhesive proteins (MAPs) secreted from mussels strongly bind to various surfaces in a wet environment. In view of their superior adhesive properties, along with significant biocompatibility and biodegradability, MAPs are attractive candidate biomaterials with high potential in tissue and medical engineering [28–31]. Previously, we designed a hybrid MAP, fp-151, composed of six type 1 (fp-1) decapeptide repeats at both N- and C-termini of type 5 (fp-5), with the aim of overcoming poor yield and purification difficulties of natural MAPs [32]. The hybrid protein displayed strong adhesion ability [32] and was easily fused with short peptides, thus generating a potential efficient cell adhesion biomaterial for cell culture and tissue engineering. In the present study, we constructed artificial ECM materials based on a fusion strategy between fp-151 and specific ECM peptides. We propose that the fp-151-peptides can be effectively used to immobilize ECM-derived short peptides on surfaces for diverse cell cultures (Fig. 1A): the strong adhesion ability of the MAP facilitates efficient coating of ECM components on scaffold surfaces with no protein and/or surface modifications, and signaling-mediated ECM components efficiently promote cellular processes, including adhesion, spreading, proliferation, differentiation, and survival. We examined 4 short peptides, RGD (from fibronectin), YIGSR (from laminin), GEFYFDLRLKGDK (from type IV collagen), and CRPKPQQFFGLM (from substance P), as representative ECM components. The adhesive fusion proteins generated were applied to prepare artificial ECM environments, and cellular behaviors were investigated using three cell lines (pre-osteoblast, chondrocyte, and pre-adipocyte).

2. Materials and methods

2.1. Construction of expression vectors

We designed a forward primer based on the N-terminus of MAP fp-151 and reverse primers incorporating the ECM mimetic short peptide sequences (presented in Table 1). Genes encoding fp-151 with C-terminal short ECM peptides were amplified from pENG151 [32] using polymerase chain reaction (PCR), and introduced into pET-22b(+) vector (Novagen, Darmstadt, Germany) containing the T7 promoter for expression in *Escherichia coli* BL21 (DE3) (Novagen). *E. coli* TOP10 (Invitrogen, Carlsbad, CA, USA) was used as the host for gene cloning, and transformed cells were grown in Luria-Bertani (LB) with 50 μg/ml ampicillin. All cloned sequences were confirmed by direct sequencing.

2.2. Expression and purification of fp-151-peptides

The constructed plasmids were transferred into *E. coli* BL21 (DE3) for protein expression. The cells bearing the plasmids were cultured in 5 L LB medium with 50 μg/ml ampicillin at 37 °C and 300 rpm until optical density at 600 nm (OD_{600}) was reached to 0.4–0.6, induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and were harvested after 9 h by centrifugation at 4000 rpm and 4 °C for 10 min (Hanil Science Industrial, Incheon, Korea). Cell pellets were

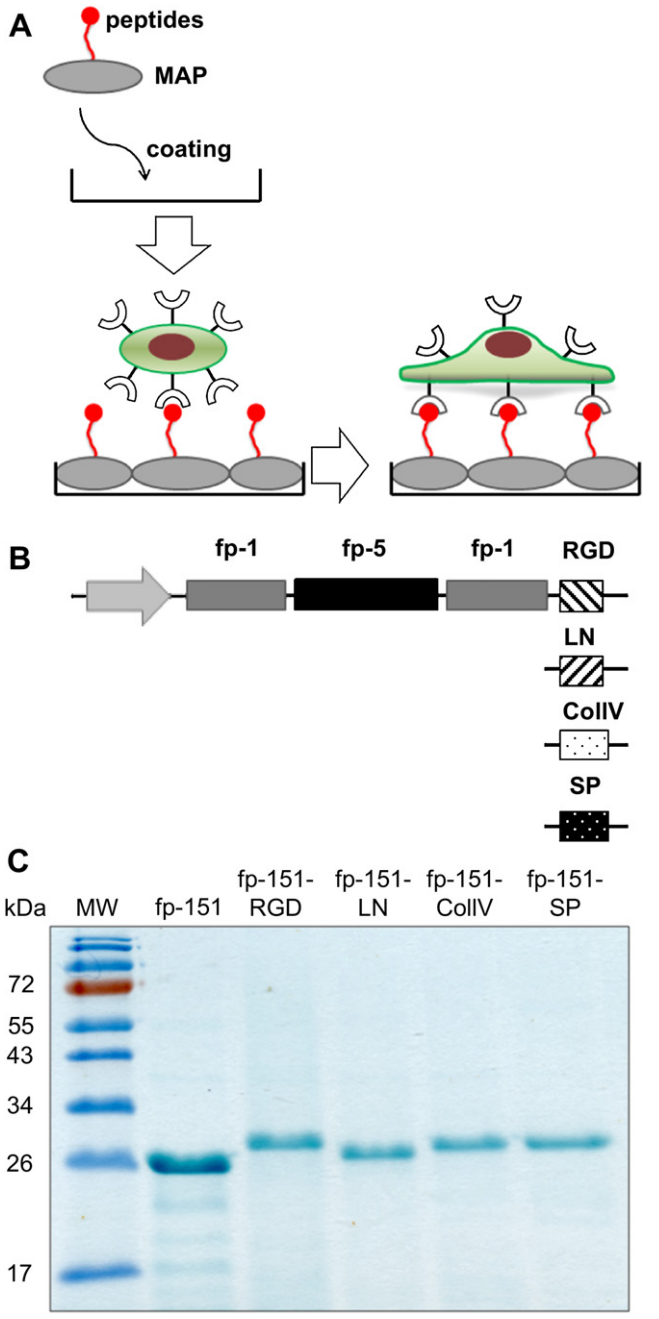

Fig. 1. (A) Schematic diagram of vector construction for fp-151-peptides. (B) SDS-PAGE analysis of purified fp-151-peptides. (C) Scheme for use of fp-151-peptides as ECM mimics. Abbreviations: MW, molecular weight marker; LN, laminin; ColIV, type IV collagen; SP, Substance P.

Table 1
Primers used in the study.

Primer	Nucleotide sequence (5′→3′)	Amino acid sequence
Forward for fp-151	GCCATATGGCTAGCGCTAAACCGTCTTAC	
Reverse for RGD	AAGCTTACGGGCTATCGCCACGGCCTTTGAAGTCGGGGGG	GRGDSP
Reverse for LN	GCAAGCTTTCAGCGGCTGCCAATATACTTGTAAGTCGGGGGGTAAC	YIGSR
Reverse for ColIV	GCAAGCTTTCATTTATCGCCTTTCAGGCGCAGATCAAAATAAAATTCGCCCTTGTAAGTCGGGGGGTAAC	GEFYFDLRLKGDK
Reverse for SP	GCAAGCTTTCACATCAGGCCAAAAAACTGCTGCGGTTTCGGGCGGCA CTTGTAAGTCGGGGGGTAAC	CRPKPQQFFGLM

resuspended in 5 ml lysis buffer (10 mM Tris–Cl, 100 mM sodium phosphate, pH 8.0) per gram wet weight and lysed by a cell disruption system (Constant Systems, Daventry, UK) at 20 KPSI. Cell debris was collected by centrifugation of the lysates at 18,000*g* and 4 °C for 20 min. The proteins were extracted using 25% (vol/vol) acetic acid, and purity of each sample was assessed by 12% (wt/vol) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Purified samples were freeze dried and stored at −80 °C for further analysis. Protein concentration was determined using the Bradford assay (Bio-Rad, Hercules, CA, USA) with bovine serum albumin (BSA) (Promega, Madison, WI, USA) as a protein standard.

2.3. Mammalian cell lines and cell culture conditions

The mouse pre-osteoblast cell line, MC3T3-E1, and mouse embryonal carcinoma-derived chondrogenic cell line, ATDC5, were obtained from RIKEN Cell Bank (Tsukuba Science City, Japan). The mouse pre-adipocyte cell line, 3T3-L1, was obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). MC3T3-E1 cells were maintained in Minimal Essential Medium-alpha (MEM-α; Hyclone, Logan, UT, USA) supplemented with 10% (vol/vol) fetal bovine serum (FBS; Hyclone) and penicillin/streptomycin (Hyclone) at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. ATDC5 cells were cultured in DME/F12 medium (Hyclone) supplemented with 5% (vol/vol) FBS, 10 μg/ml human transferrin, 30 μmol/ml sodium selenite, and penicillin/streptomycin under similar conditions. 3T3-L1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Hyclone) containing 10% (vol/vol) bovine calf serum (BCS; Hyclone) and penicillin/streptomycin under similar conditions. Subconfluent cells were collected from dishes using 25% trypsin–EDTA (Hyclone) and used for subsequent cell adhesion, spreading, and proliferation analyses.

2.4. Coating of culture surfaces

Untreated polystyrene 6- or 24-well culture plates (SPL Life Science, Pocheon, Korea) were coated with coating materials. Cell-Tak (BD Bioscience, San Jose, CA, USA) and poly-L-lysine (PLL; Sigma–Aldrich, St. Louis, MO, USA) was used as positive controls and uncoated wells were used as negative controls. The amount of coating material used was 50 μg per cm^2 of well area. Cell-Tak- and PLL-coated wells were prepared according to the manufacturer's instructions. For fp-151-peptides, coated wells were prepared based on the Cell-Tak manufacturer's instruction using sodium bicarbonate.

2.5. Cell attachment and proliferation analyses

All trypsinized cells were diluted to a concentration of approximately 2×10^5 cells per ml medium without serum. In total, 1×10^5 cells (>95% viable) in serum-free medium were pipetted onto each sample-coated plate to examine cell adhesion. Cells were allowed to adhere to the sample-coated culture plate in a humidified incubator (37 °C and 5% CO_2) for 1 h, and unattached cells removed from the coated surfaces by rinsing with phosphate buffered saline (PBS). The culture medium for each cell line was added to the wells, followed by 300 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; USB Corporation, Cleveland, Ohio, USA) to allow the formation of formazan crystals for 2 h. After dissolving MTT into dimethyl sulfoxide (DMSO), absorbance was measured at 570 nm using a microplate reader (Perkin Elmer, Waltham, Massachusetts, USA). MTT assays were performed in triplicate.

Cell proliferation was additionally evaluated using the MTT assay. 5×10^4 cells (>95% viable) in serum-free medium were pipetted onto each sample-coated plate to allow cell adhesion. Following attachment, serum-free medium was replaced with serum-containing medium and then, cells were incubated at 37 °C for 72 h. At 24 h intervals, 300 ml of MTT was added to wells to allow the formation of formazan crystals for 2 h. Absorbance was measured at 570 nm using a microplate reader.

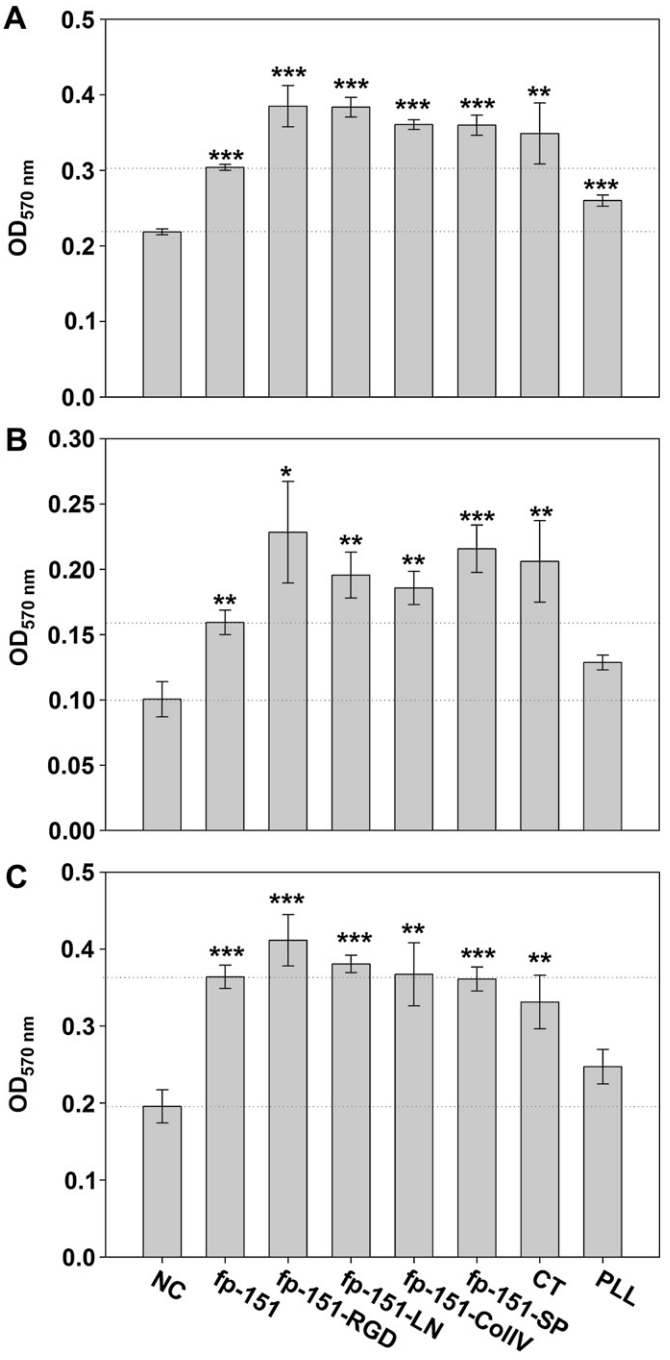

Fig. 2. Adhesion of (A) MC3T3-E1, (B) ATDC5, and (C) 3T3-L1 cells on fp-151-peptide-coated surfaces. The concentration of samples used in surface coating was 50 μg/cm^2. Cells (5×10^4) were incubated in fp-151-peptide-coated 24-well polystyrene culture plates for 1 h. Attached viable cells were measured with the MTT assay. Values and error bars represent the means of three independent experiments with triplicate samples and standard deviations with statistical significance ($^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$).

2.6. Cell spreading and cytoskeleton organization analyses

Prior to the cell spreading assay, all cells were incubated in serum-free medium to induce serum starvation. We employed 12 mM ϕ

coverglass (Superior Marienfeld, Lauda-Königshofen, Germany) to examine cell morphology. Cells in 500 μl of serum-free medium were placed on the sample-coated coverglass and incubated for up to 18 h. Actin filaments were labeled with fluorescein isothiocyanate (FITC)-conjugated phalloidin (Sigma–Aldrich), and the nuclei stained with 4′,6-diamidino-2-phenylindole (DAPI; Sigma–Aldrich). Specimens were analyzed using fluorescence microscopy (Olympus, Tokyo, Japan).

2.7. Apoptotic cell death analysis

Samples (50 μg/cm^2) were coated on polystyrene 6-well culture plates, and 5×10^5 cells in serum-containing medium seeded onto each plate to allow cell adhesion. Following aspiration of medium, unattached cells and serum remaining in the dish were removed by rinsing twice with PBS. Attached cells were cultured in serum-free medium for 72 h, and all detached cells collected by trypsinization. Collected cells were centrifuged at 3000 rpm for 5 min, and stained with FITC-conjugated Annexin V (BD Bioscience) and propidium iodide after resuspension of the cell pellet in Annexin V-binding buffer. Wild-type cells were used as the control to set the basal fluorescence level. All samples were evaluated using a fluorescence activated cell sorter FACSCalibur™ (BD Bioscience), and data analyzed with WinMDI 2.8 software (Joseph Trotter, La Jolla, CA, USA).

2.8. Cell differentiation analysis

MC3T3-E1 cells were seeded into 6-well plates and cultured in a similar manner as the proliferation experiment. Culture medium was replaced every 72 h. At 90% confluence, cells were induced with a mixture of 50 μg/ml ascorbic acid and 10 mM sodium phosphate monobasic in the culture medium for differentiation. Calcification of differentiated MC3T3-E1 cells was analyzed via alizarin red S staining at 20 days after induction of differentiation. The medium was aspirated from wells, and cells rinsed in PBS solution and fixed with 4% formalin. Next, cells were rinsed with ultra-pure water and stained with 1% alizarin red S solution (adjusted to pH 4.2 with ammonium hydroxide; Sigma–Aldrich) at room temperature for 30 min. After the removal of alizarin red S solution, cells were rinsed three times with ultra-pure water. Images of the alizarin red-stained area were obtained using an optical microscope (Olympus), the retained dye eluted into 20% acetic acid, and absorbance determined at 570 nm using a microplate plate reader. The intracellular calcium ion concentration in MC3T3-E1 cells at 15 days after differentiation was measured using the QuantiChrom™ Calcium Assay Kit (Bioassay systems, Hayward, CA, USA). Alkaline phosphatase (ALP) activity was additionally estimated in MC3T3-E1 cells at 15 days after differentiation using the SensoLyte® *p*NPP Alkaline Phosphatase Assay Kit (Anaspec, San Jose, CA, USA), following the manufacturer's protocol.

2.9. Statistical data analysis

Independent experiments were performed at least three times and triplicate samples were analyzed in each experiment. The significance of data obtained with the control and treated groups was statistically analyzed using the paired Student's *t*-test. A *p*-value <0.05 was considered statistically significant.

3. Results and discussion

3.1. Expression and purification of fp-151-peptides

Fibrous proteins and growth factors allow cells to anchor to artificial matrices, and consequently affect cellular behavior

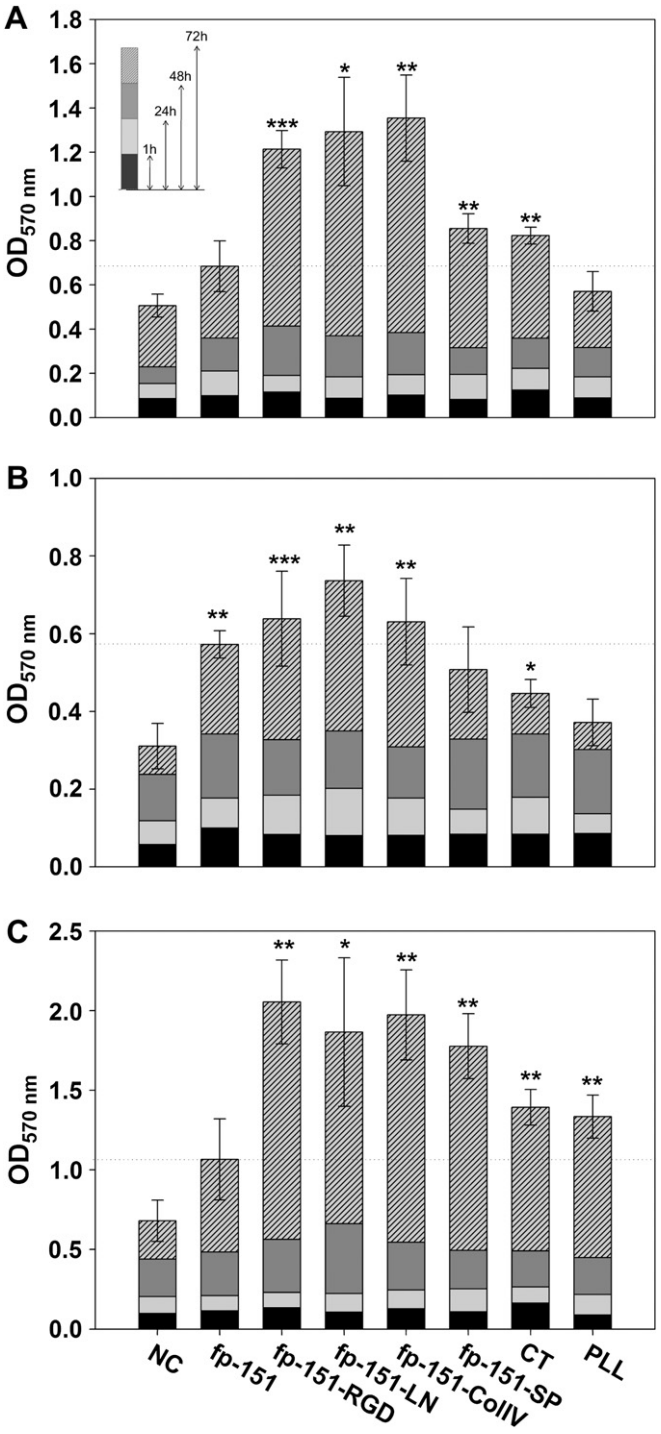

Fig. 3. Proliferation of (A) MC3T3-E1, (B) ATDC5, and (C) 3T3-L1 cells on fp-151-peptide-coated surfaces. All experimental conditions were similar to those of the cell adhesion assay, except that serum-containing medium was altered after cell adhesion for 1 h. Every 24 h, viable cells were measured with the MTT assay. The bar in the graph is based on data accumulated from each sample. Values and error bars represent the means of three independent experiments with triplicate samples and standard deviations with statistical significance ($^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$).

[34–39]. A number of biofunctional peptides originating from fibrous proteins and growth factors additionally display properties of ECM proteins [17,19]. Among these, we selected the RGD sequence from fibronectin, YIGSR from the β-chain sequence of laminin, GEFYFDLRLKGDK from the α1 chain of type IV collagen, and CRPKPQQFFGLM from substance P as representative ECM

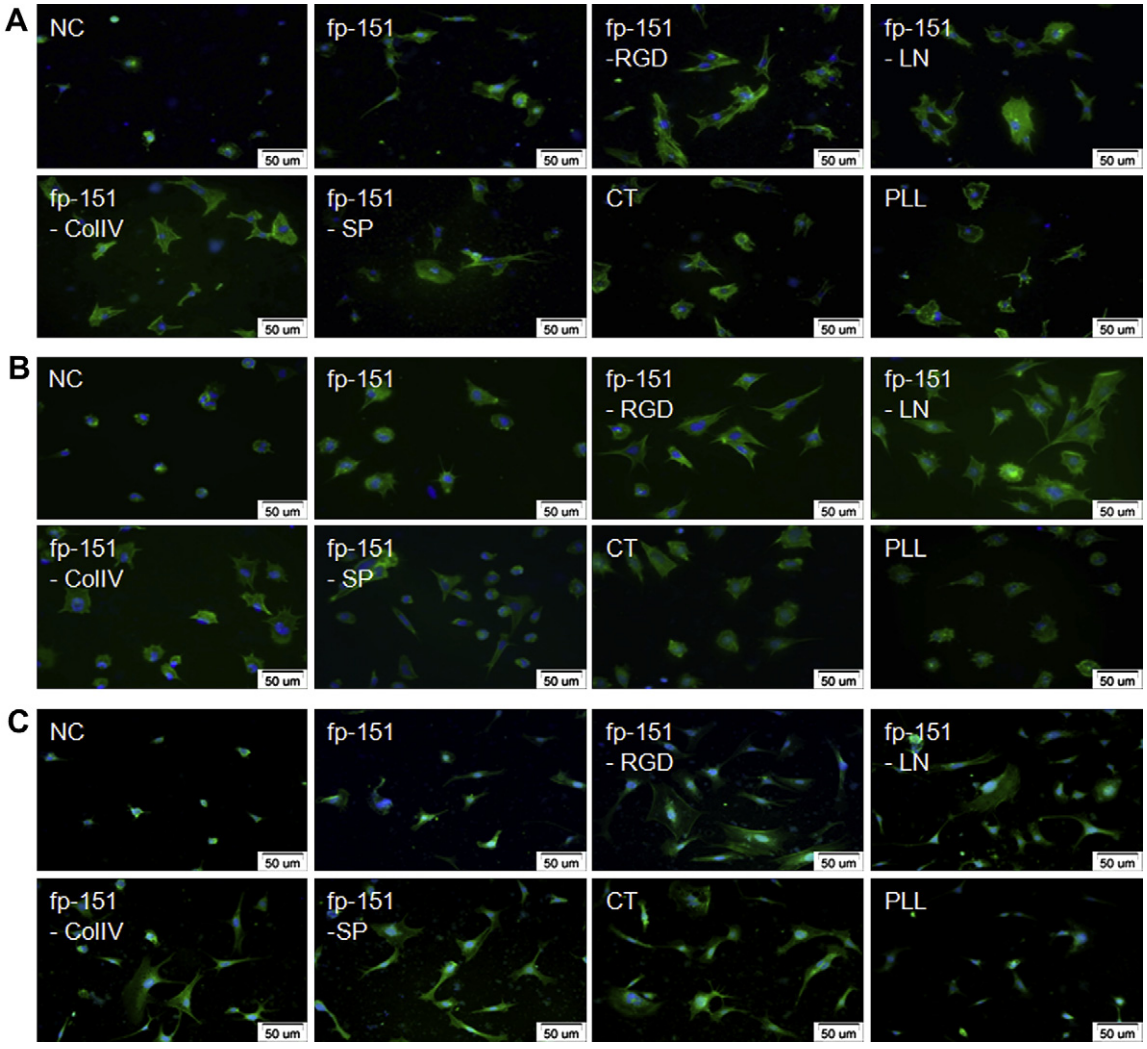

Fig. 4. Spreading of (A) MC3T3-E1, (B) ATDC5, and (C) 3T3-L1 cells on fp-151-peptide-coated surfaces. Cells (5×10^4) were added to a coverglass coated with 50 μg/cm^2 of sample without serum, and cultured for 18 h. Actin filaments stained with phalloidin-FITC are presented in green, and nuclei stained with DAPI are blue. The scale bar is 50 μm.

components. Substance P secreted from neuronal axons is a known neuromodulator that causes inflammation in several peripheral tissues [40]. Recent studies report positive effects of substance P in various cell lines as a potent growth factor [37–39]. Individual ECM peptides were fused to the C-terminus of MAP fp-151 (Fig. 1B), and the fusion proteins were overexpressed in *E. coli* and efficiently purified via acetic acid extraction from insoluble inclusion bodies (Fig. 1C). The introduction of short peptides at the C-terminus of the fp-151 did not significantly influence the expression level or purification yield. In terms of purity, all fusion proteins were >95% homogenous, as evident from Coomassie blue-stained SDS-PAGE (Fig. 1C). Purified fp-151-RGD, fp-151-YIGSR (fp-151-LN), fp-151-GEFYFDLRLKGDK (fp-151-ColIV) and fp-151-CRPKPQQFFGLM (fp-151-SP) fusion proteins were used to prepare artificial ECM coats on culture plates.

3.2. Cell adhesion and proliferation on fp-151-peptide-coated surfaces

The peptide sequences of fibronectin, laminin, type IV collagen, and substance P are known biological motifs for cellular signaling related to adhesion, proliferation, spreading, and differentiation via interactions with various cellular receptors, such as integrins, receptor tyrosine kinases, and G-protein coupled receptors [5,17,18,25,39]. In particular, the RGD sequence recognized by cellular receptors stimulates cell adhesion, and has been successfully used as a biomimetic ECM coating material [26,34]. Our previous study showed that incorporation of RGD in fp-151 enhances cell adhesion and growth properties [33]. The laminin-derived YIGSR sequence also facilitates attachment, spreading, and resistance of endothelial cells against shear stress [34,35]. The peptide sequence of type IV collagen promotes adhesion and spreading of various cell types in a concentration-dependent manner [36]. Substance P stimulates migration and proliferation of skin fibroblasts through NK-1 receptors and enhances adhesion of corneal epithelial cells to the fibronectin matrix [37,38].

Efficient immobilization of functional peptides is critical in the preparation of artificial ECM environments. Our strategy involving the fusion of short peptides with a MAP is a potentially powerful solution to effectively immobilize short functional ECM peptides onto surfaces and promote cellular processes (Fig. 1A). We initially investigated the cell adhesion promotion abilities of fp-151-peptides using three mouse cell lines: pre-osteoblast MC3T3-E1, chondrocyte ATDC5, and pre-adipocyte 3T3-L1. PLL and Cell-Tak (naturally extracted MAPs) were used as positive controls, and non-coated surfaces and bare fp-151 as negative controls. Due to the

large numbers of adhesive factors present in serum-containing environments, including fibronectin and vitronectin, all cell adhesion experiments were performed under serum-free conditions. Total adhesion numbers of MC3T3-E1 cells were clearly higher on all fp-151-ECM mimic-coated surfaces than bare fp-151- and PLL-coated surfaces, and 2-fold enhanced compared to the non-coated surface (Fig. 2A). In addition, cell attachment abilities were similar to those of Cell-Tak. Analogous cell adhesion trends were also observed for ATDC5 (Fig. 2B) and 3T3-L1 (Fig. 2C) cell lines. These findings strongly suggest that the peptides conjugated with fp-151 are functional as binding motifs for various cellular receptors in an environment lacking the factors contained in serum.

Cell proliferations on surfaces coated with fp-151-peptides were investigated for 72 h using all three cell lines (Fig. 3). As expected, the proliferation levels on all fp-151-peptide-coated surfaces were significantly higher than those on non-, bare fp-151-, PLL- and Cell-Tak-coated surfaces, although the degree of proliferation was dependent on the individual cell line. Interestingly, although proliferations on fp-151-RGD-, fp-151-LN-, and fp-151-CollV-coated surfaces were significantly increased, compared with bare fp-151-coated surfaces, that on the fp-151-SP-coated surface was not generally as high. Activation of integrin signaling from ECM molecules leads to increased growth rates in various cell lines [17,18,25]. However, substance P, a neuromodulator, is not directly involved in integrin signaling [37,38]. Its positive effect on 3T3-L1 proliferation (Fig. 3C) may originate from an indirect signaling pathway involving substance P in pre-adipocytes [39]. Based on these results, we conclude that the MAP mainly enhances cell adhesion and the fused functional peptides efficiently promote cell proliferation. Moreover, we suggest that the effects of biofunctional ECM peptides on cell adhesion and proliferation vary across cell lines, which differ in terms of distribution and importance of cellular receptors for efficient adhesion and proliferation [41].

3.3. Cell spreading on fp-151-peptide-coated surfaces

Spreading is an important event in the adhesion process for many cell types, and is regulated by signaling pathways that become activated upon integrin-mediated cell attachment. ECM materials, such as fibronectin and collagen, facilitate cell spreading under serum-reduced conditions [42,43]. Communication between cells and ECM activates the signaling pathway for spreading and growth, and provides facile access of several growth factors [44]. Accordingly, we examined the morphological changes of cells on fp-151-peptide-coated surfaces in serum-depleted conditions via immunocytochemical analyses (Fig. 4). MC3T3-E1 cells displayed superior adhesion morphology on all fp-151-peptide-coated surfaces, compared to bare fp-151-coated surface (Fig. 4A). Actin filament formation in MC3T3-E1 cells on fp-151-peptide-coated surfaces was evident upon immunostaining using FITC-conjugated phalloidin whereas poor cell spreading ability was observed with other non-coated, bare fp-151-coated, and PLL-coated surfaces. Additionally, ATDC5 and 3T3-L1 cells showed similar spreading morphology in all cases (Fig. 4B and C). Interestingly, some MC3T3-E1 and ATDC5 cells on the fp-151-SP-coated surface exhibited relatively poor spreading morphology, and remained round in shape (Fig. 4A and B). However, the degree of spreading of 3T3-L1 cells on the fp-151-SP-coated surface was similar to that on other fp-151-peptide-coated surfaces (Fig. 4C), consistent with the effect of substance P on 3T3-L1 proliferation. We propose that activation of signaling pathways by ECM peptides fused with MAPs leads to enhanced proliferation and spreading of attached target cells without the aid of any of the components in serum. Thus, short functional ECM peptides are efficiently immobilized on surfaces with our fusion strategy.

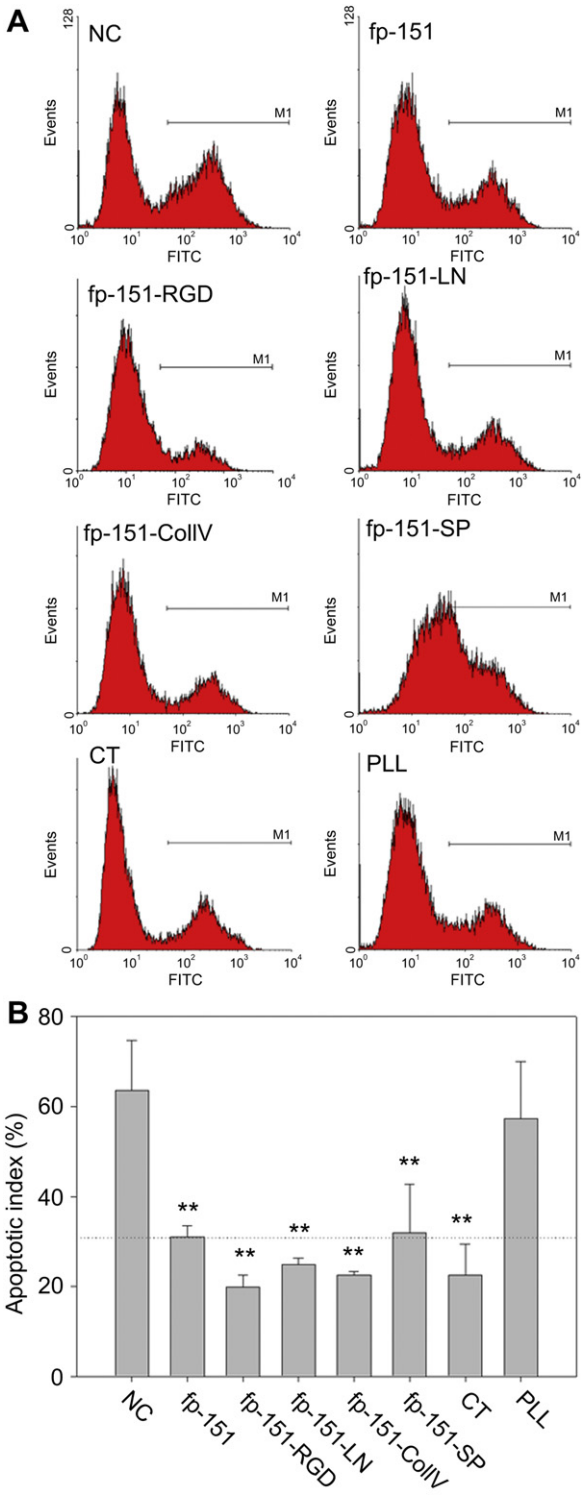

Fig. 5. Effects of fp-151-peptides on MC3T3-E1 apoptotic cell death. Flow cytometry analysis with (A) FITC-conjugated Annexin V histogram and (B) vertical bar plot of the apoptotic cell index. The FITC-conjugated Annexin V-positive cell population was calculated from the fixed M1 region drawn according to the wild-type cell population area. Values and error bars represent the means of three independent experiments with triplicate samples and standard deviations.

3.4. Apoptosis of pre-osteoblast cells on fp-151-peptide-coated surfaces

Apoptosis or programmed cell death is a well-defined self-destruction mechanism distinct from necrosis [45]. In the initial

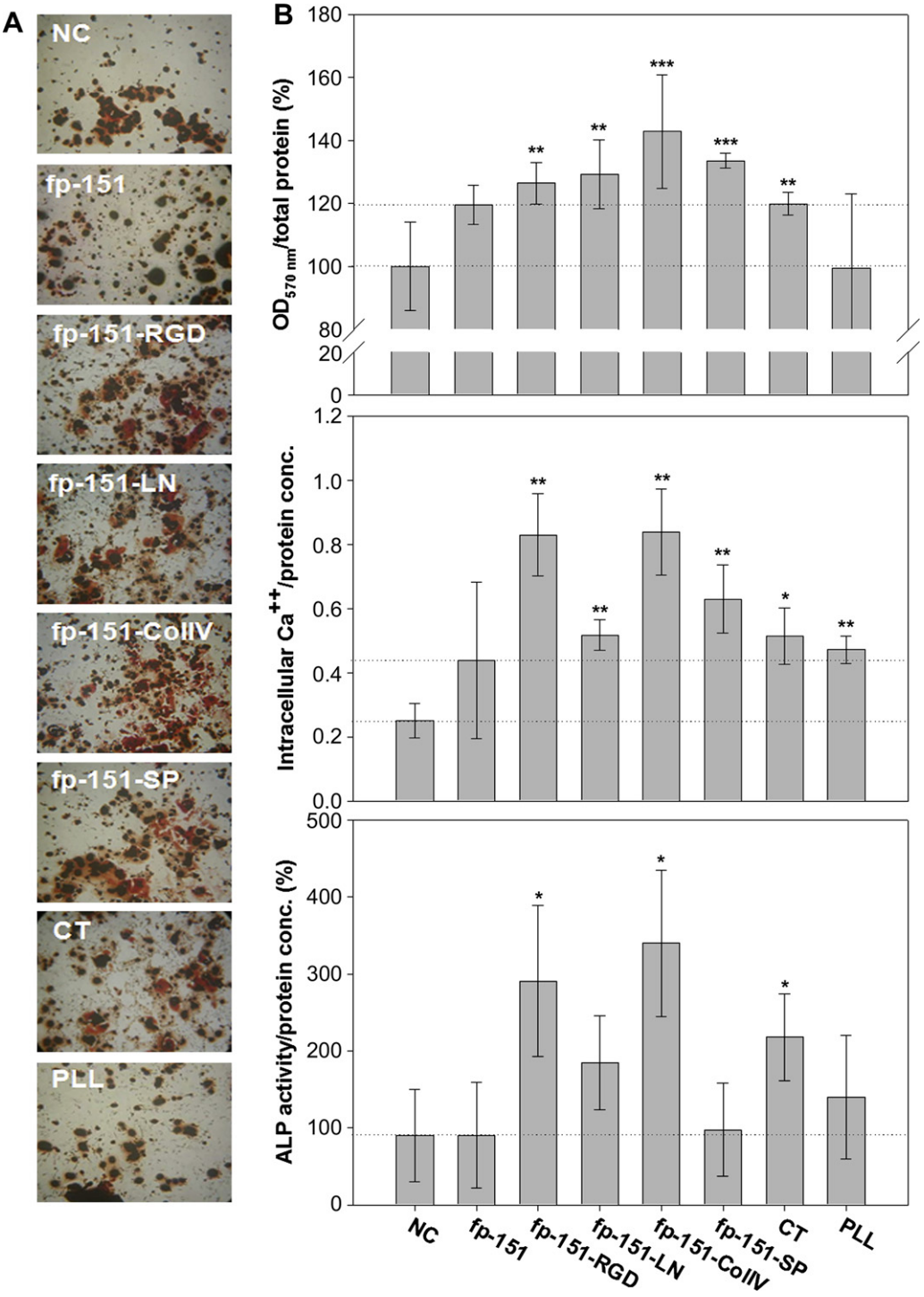

Fig. 6. Differentiation of MC3T3-E1 cells on fp-151-peptide-coated surfaces. (A) Matrix mineralization of osteoblast cells observed with Alizarin red S staining on sample-coated 6-well polystyrene culture plates after 20 days treatment with the differentiation signal and (B) vertical bar plot of the percentage of stained osteoblast cells. (C) Intracellular Ca^{++} concentration and (D) ALP activity of differentiated MC3T3-E1 cells. Values and error bars represent the means of three independent experiments with triplicate samples and standard deviations with statistical significance ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$).

stages of apoptosis, cells undergo several morphological changes, including rounding up, shrinking, and losing contact with adjacent structures. Simultaneously, chromatin condensation and DNA degradation occur within the nucleus and cell junctions disintegrate in the plasma membrane. Interactions of cells with ECM and factors contained in serum are required for enduring apoptotic cell death [46]. In particular, integrin signaling inhibits cell death of MC3T3-E1 cells subjected to apoptotic stimuli [47]. Data on attachment, proliferation, and spreading of pre-osteoblast MC3T3-E1 cells clearly indicate that fp-151-RGD, fp-151-LN, and fp-151-ColIV induce integrin signaling, while fp-151-SP does not activate the signaling pathway. In these regards, we examined the apoptosis of MC3T3-E1 cells on various fp-151-peptide-coated surfaces under serum depletion conditions. Serum depletion-induced apoptotic death on sample-coated surfaces was monitored using Annexin V staining (Fig. 5). Externalization of phosphatidylserine of the inner

cell membrane is an early event of apoptosis, and apoptotic cells can be thus detected via binding events of Annexin V to phosphatidylserine [48]. Since apoptotic cells detach from the surface in a serum-free environment, total cell numbers on the surface and within the medium were analyzed. As expected, the levels of cell death on all MAP-coated surfaces were significantly lower (approximately one-third), compared to those on non-coated and PLL-coated surfaces (Fig. 5). Notably, cells on the PLL-coated surface showed low survival ability, similar to those on the non-coated surface, although PLL slightly enhanced cell adhesion and proliferation (Figs. 2 and 3). Survival rates on fp-151-RGD-, fp-151-LN-, fp-151-ColIV- and Cell-Tak-coated surfaces were relatively higher than those on bare fp-151- and fp-151-SP-coated surfaces due to the presence of integrin signaling molecules. Cell-Tak, a naturally extracted MAP, contains ECM-like materials [49,50]. Interestingly, bare fp-151 also had inhibitory effects on apoptosis, possibly because the initial stages of cell death were impeded by the strong adherence of cells on the fp-151-coated surface.

3.5. Differentiation of pre-osteoblast cells on fp-151-peptide-coated surfaces

ECM-mediated integrin signaling is an important modulator of osteoblast differentiation [51]. We investigated the effects of biofunctional ECM peptides conjugated with a MAP on bone formation via differentiation of pre-osteoblasts. Calcium deposition of MC3T3-E1 cells on fp-151-peptide-coated surfaces was analyzed: matrix mineralization of differentiated cells was evaluated via alizarin red S staining and intracellular calcium deposition measured using a colorimetric assay. Mineralization levels of MC3T3-E1 cells on all fp-151-peptide (fp-151-RGD, fp-151-LN, fp-151-ColIV, and fp-151-SP)-coated surfaces were increased by 5%, 8%, 19%, and 11%, respectively, compared to that on the bare fp-151-coated surface (Fig. 6A and B). In addition, intracellular calcium deposition by MC3T3-E1 cells was increased by 89%, 18%, 91%, 44%, respectively, in relation to that on the bare fp-151-coated surface (Fig. 6C). Other phenotype markers related to osteoblast differentiation include ALP, which plays a role in inorganic pyrophosphate metabolism in osteoblasts [52]. ALP activity is critical in osteoblast differentiation [53]. ALP activities in MC3T3-E1 cells on fp-151-peptide-coated surfaces were detected using the colorimetric assay. Analogous to calcium deposition findings, ALP activities on fp-151-peptide-coated surfaces were significantly higher than that on the bare fp-151-coated surface (Fig. 6D). Because ALP participates in hydrolysis of phosphate esters and precipitating bone minerals [52], these results suggest that biofunctional ECM-derived peptides fused to MAPs are effective for bone mineralization and intracellular calcium deposition.

The present results collectively confirm that our fp-151-peptides significantly enhance cellular behavior in terms of initial attachment, proliferation, spreading, survival, and differentiation. Therefore, MAP-based ECM mimics can be successfully used in cell culture and tissue engineering, and possibly extended to other tissue-specific recognition motifs to allow the efficient culture of targets.

Recent attempts to develop synthetic peptide-based artificial ECM have involved co-immobilization of two or more ECM components on a scaffold [15,42,54,55]. For example, one system is related to the synergistic effects of co-immobilized laminin and nerve growth factor on neural cells [15]. Another method is associated with integrin-proteoglycan co-receptors, which are directly or indirectly affiliated with cell adhesion [42,55]. Because our ECM mimics are basically composed of MAPs, co-immobilization can be simply performed by mixing different types to prepare a valid artificial ECM environment with no protein and/or surface modifications. Thus, we propose that a mixture coat of our developed ECM mimics on various scaffolds stimulates positive physiological cell behaviors as an efficient artificial ECM environment in cell and tissue engineering.

4. Conclusion

In the present study, we functionalized a recombinant MAP, fp-151, via introduction of bioactive peptides derived from fibronectin, laminin, type IV collagen, and substance P, using a fusion strategy, to facilitate use as efficient cell and tissue-friendly biomaterials. The fp-151-peptides generated were efficiently coated on the culture surface by means of the adhesive property of the MAP without any protein and/or surface modifications. The essential cell behavior patterns of adhesion, proliferation, and spreading were significantly enhanced through interactions of fused ECM peptides with cellular receptors in three mouse cell lines of pre-osteoblast, chondrocyte, and pre-adipocyte. Furthermore, mouse pre-osteoblast survival and differentiation were substantially improved on the fp-151-peptide-coated surfaces. Based on these results, we conclude that MAP-based ECM mimics can be successfully employed in cell culture and tissue engineering, and the fusion strategy with MAPs further extended to other tissue-specific recognition motifs. In addition, mixture coats of our MAP-based ECM mimics will have potential as artificial ECM coating platforms.

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Appendix

Figures with essential colour discrimination. Figs. 1, 4–6 in this article have parts that are difficult to interpret in black and white. The full colour images can be found in the online version, at doi:10.1016/j.biomaterials.2010.08.027.

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