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RESEARCH PAPER

Immobilized Laminin-derived Peptide Can Enhance Expression of Stemness Markers in Mesenchymal Stem Cells

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Abstract Due to number of reasons such as ease of isolation and their broad differentiation capability, adult human mesenchymal stem cells (MSCs) are widely considered as of the most promising cells for regenerative medicine and tissue engineering. Nevertheless quick decrease in expression of transcription factors associated with stemness and self-renewal during *ex vivo* expansion of MSCs is an impediment against their therapeutic applications. Since the influence of extra cellular matrix (ECM) proteins on the fate of stem cells is well documented, the culture of MSCs on ECM-derived synthetic biomolecules is worth investigating. In the present study, a laminin-derived peptide, YIGSR was covalently immobilized on the chitosan film surface using carbodiimide chemistry and confirmed by fluorometry. The results obtained from surface characterization by atomic force microscopy (AFM) and contact angle measurement, showed no significant difference in topological features and hydrophilicity after peptide immobilization. Employment of these surfaces for culture of human adipose-derived mesenchymal stem cells demonstrated that the immobilized YIGSR peptide has a favorable effect on adhesion and maintaining viability of the MSCs as well as on the expression of stemness markers (Nanog, Oct-4, and Sox-2) in these cells.

Keywords: chitosan, immobilization, mesenchymal stem cells, stemness markers, YIGSR peptide

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1. Introduction

Due to plenty reasons such as ease of isolation [1], immune evasiveness [2], and broad differentiation potency toward mesodermal lineages including osteocytes, chondrocytes, and adipocytes [1] and even non-mesodermal ones such as neural cells [3], hepatocytes [4], and pneumocytes [5], mesenchymal stem cells (MSCs) also referred as mesenchymal stromal cells are one of the most promising cells for regenerative medicine and tissue engineering [6-8]. Nevertheless, decrease in stemness properties and cell viability during *ex vivo* expansion is considered as a challenging problem in their medical applications [9-11]. Different approaches have been yet proposed to preserve stemness in MSCs. Fotia *et al.* showed that the hypoxic condition considerably increased MSCs proliferation whereas the expression of stemness markers, Nanog and Sox-2 were not significantly altered [12]. According to the results obtained by Cheng *et al.*, prior to conventional monolayer culture, short-term culture of adipose-derived mesenchymal stem cells on chitosan film which results spheroid formation, enhances expression of pluripotency

markers such as Oct-4, Nanog, and Sox-2 compared to the control group [13]. The incremental effect of chitosan membrane on expression of aforementioned stemness markers in MSCs is also reported by Li *et al.* [14]. However the statistical analysis of their results is not clear. Spheroid culture of mesenchymal stem cells under non-adherent culture conditions and its impact on maintaining stemness was also studied by Yu *et al*. They documented a crosstalk between spheroid formation of human mesenchymal stem cells cultured on agarose coated plates, CD49f, and pluripotency markers Oct-4 and Sox-2 [15]. In another study, Al-Habib *et al.* demonstrated that treatment of human dental pulp-derived mesenchymal stem cells by some small molecules increases the expression of Nanog, Oct-4, and Sox-2 as stemness markers [16]. It is also well documented that extracellular matrix (ECM) proteins are momentous regulators determining mesenchymal stem cells fate and behavior [17]. For example, Rakian *et al*. demonstrated that proliferation of MSCs cultured on bone marrow-extracted extracellular matrix (ECM) was higher than those grown on commercially available matrix (CELLstart™) or tissue culture plastic (TCP). Moreover, they showed that the number of cells positive for MSC markers was significantly increased when they were cultured on ECM for 7 days [18]. The use of isolated ECM proteins to retain MSCs stemness is also investigated. For instance, Han *et al.* utilized collagen as a three-dimensional scaffold to improve expression of stemness markers (Oct-4, Sox-2, Rex-1, and Nanog) in rat bone marrow-derived mesenchymal stem cells [19]. Eventually, exploiting ECM proteins-derived peptides rather than the whole proteins has been regarded in many works trying to build an ECM-mimicking microenvironment for stem cell culture with the lower cost and more defined architecture [20,21]. For example, Chien *et al.* demonstrated that conjugation of poly(carboxybetaine) hydrogel with low amounts of RGD peptide can promote stemness of human mesenchymal stem cells [22]. Ra'em *et al.* studied the influence of immobilized RGD peptide in macroporous alginate scaffolds on chondrogenic differentiation of human mesenchymal stem cells [23]. Lee *at al.* utilized two cell-binding domains from FGF-2 (fibroblast growth factor-2) containing reverse RGD sequence (DGR) to increase cell attachment and osteoblastic differentiation of mesenchymal stem cells [24]. In another work, synergistic effect of co-immobilized RGDSP and TYRSRKY peptides on elevating MSCs adhesion in the absence of serum was documented [25]. Among the synthetic peptides which are mainly inspired from the integrin binding domains of ECM proteins, a pentapeptide one (*i.e.* YIGSR) derived from the laminin b1 chain, is well-known as a supporting motif for cells adhesion and proliferation [26-28]. Nevertheless, to the best of our knowledge, the effect of this sequence on stemness of mesenchymal stem cell was not already studied.

In the present study, among the natural and synthetic polymers containing primary amine groups such as chitosan, poly-L-lysine, and poly-L-ornithine, chitosan was employed to immobilize laminin-derived peptide, YIGSR, due to more literature reporting its biocompatibility as well as its potency to maintain stem cells stemness [14,29,30]. The peptide was covalently attached to the chitosan surface via carbodiimide chemistry. Following the confirmation of immobilization process by the fluorometry and then characterization of surfaces using atomic force microscopy and water contact angle measurement, they were used for the culture of human adipose-derived mesenchymal stem cells. The viability of the cells as well as expression level of stemness transcripts were analyzed to investigate the influence of immobilized peptide on MSCs fate. According to the results, we concluded that the immobilized laminin-inspired peptide (YIGSR) can improve viability of the seeded MSCs and increases expression of stemness genes including Oct-4, Nanog, and Sox-2 in the basal culture medium.

2. Materials and Methods

2.1. Preparation of chitosan film

Chitosan film was fabricated as described previously [31]. Briefly, 1 wt% chitosan solution was prepared by dissolving calculated amount of medium molecular weight chitosan powder (Sigma-Aldrich) in 2% v/v aqueous acetic acid. After 48 h magnetic stirring, the undissolved visible particles were excluded from the solution by 20 min centrifugation followed by filtration using the sintered glass funnel (with G0 degree of porosity). The filtrated solution was added to the culture plates and incubated overnight at 45°C for evaporation of the solvent. The remained acetic acid on formed chitosan films was then neutralized by 2 h alkaline treatment (NaOH, 0.5 M) followed by multiple washing by deionized distillated water.

2.2. Peptide immobilization on chitosan film

The peptide with the sequence Ac-EGGYIGSR-NH2 (Biomatik Corporation, Canada) which was acetylated and amidated in N-terminal and C-terminal respectively was dissolved in 4 M solution of aqueous urea for making a stock solution. The working solutions of peptide with the concentrations of 100 and 200 μg/mL was prepared in 0.1 M MES buffer (containing 0.5 M NaCl, pH 5.7). As briefly depicted in Fig. 1, the carboxyl group of glutamate residue was activated by freshly prepared solutions of 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide, EDC (Acros), and N-Hydroxysuccinimide, NHS (Acros) whereas the

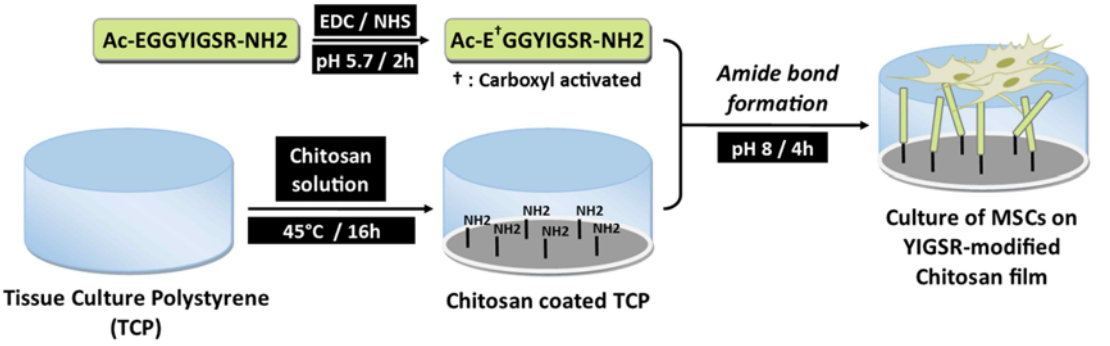

Fig. 1. Schematic illustration of chitosan film preparation and YIGSR peptide immobilization as described in methods.

molar ratios of EDC and NHS to the peptide were 20 and 40 respectively. After 2 h incubation at room temperature, the pH was increased to 8 using saturated solution of sodium bicarbonate. Eventually, the peptide solution was added to chitosan coated culture plates followed by 4 h incubation at room temperature. The non-covalently attached peptides were removed by multiple washing with the urea solution and deionized distillated water. To confirm the covalent attachment of peptides, the FITC-conjugated kind of peptide having the sequence Ac-EGGYIGSRK (FITC)-NH2 was employed with the same molar concentration. Serving this purpose, a control group was also studied in which neither EDC nor NHS was added to the FITC-peptide solution. After accomplishment of aforementioned procedures the fluorescence intensity of treated microwells were measured by the fluorescent microplate reader (Cytation3, Biotek).

2.3. Surface analysis by atomic force microscopy (AFM)

The chitosan coated surfaces including pristine chitosan film and those modified by the peptide solutions (100 and 200 μg/mL) were analyzed by an atomic force microscopy (Ara Pajuhesh) at the contact mode using silicon cantilever with a spring constant of 1–5 N/m. The scanning was performed at 24ºC and the humidity of 25%. Finally, Topo (height) images were analyzed by the imager software to measure average roughness parameter (Ra).

2.4. Water contact angle measurement

To investigate the effect of peptide immobilization on wettability of chitosan coated surfaces, the static water contact angles of the surfaces were studied at 25ºC by the drop method. Measurements were implemented at 3 different positions on each sample and averaged.

2.5. Cell adhesion assay

Adipose-derived human mesenchymal stem cells (h-MSCs) at passage 2 were provided from Stem Cell Technology Research Center (Tehran, Iran). After trypsin-based detachment of the cells from tissue culture polystyrene plates, they were resuspended in DMEM- high glucose culture medium (Gibco) containing 10% FBS (Gibco). The cells (at a density about 1.0×10^4/well) were seeded in 96 well chitosan coated plate which were pre-sterilized by U.V. radiation for 1 h. To investigate the influence of immobilized peptide on cell attachment, the Hoechst staining was performed as described previously [31]. Briefly, After 24 h incubation at 37°C in 5% CO_2 atmosphere, the medium was removed and the cells were washed by PBS. Afterwards, 150 μL of Hoechst 33342 (Thermo Scientific) solution (2 μg/mL in PBS) was added to each microwell. Finally, after 15 min incubation at 37°C in dark, the fluorescence intensity of microwells was measured at 461 nm following the excitation at 352 nm.

2.6. Cell viability assay

The effect of immobilized YIGSR peptide on survival and viability of the cells cultured on chitosan coated plates was studied by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay (Merck) as described by the manufacturer. Briefly the cell seeding in 96 well chitosan coated plates was carried out in quintuple as mentioned in section 2.5 and incubation of the cells at 37°C in 5% CO_2 with daily medium exchange was performed. In days 1, 3, and 5 after cell seeding, the media were completely replaced by the fresh ones containing 0.1 mg/well MTT reagent. Following the 4 h incubation at 37°C, the media were gently removed and the formazan crystals were dissolved in 150 μL sterile dimethyl sulfoxide, DMSO (Sigma-Aldrich). The absorbance at 570 nm was finally measured with an ELISA plate reader (Biotek).

2.7. Microscopy based studies

The morphology of the cells cultured on chitosan coated surfaces was studied using an inverted microscope (Motic, AE31) three days after seeding with no pre-imaging preparation.

Table 1. Sequence of the primers used for Real-time PCR

Gene of interest	Primers	
	Forward	Reverse
Oct-4	GAAGCTGGAGAAGGAGAAGCTGG	CAAGGGCCGCAGCTTACACAT
Nanog	CTCCTTCCATGGATCTGCTTATTC	AGGTCTTCACCTGTTTGTAGCTGAG
Sox-2	GGCAGCTACAGCATGATGCAG	GCTCTGGTAGTGCTGGGACATG
GAPDH	GACAAGCTTCCCGTTCTCAG	GAGTCAACGGAT-TTGGTCGT

2.8. Real-time quantitative PCR

Total RNAs from Adipose-derived human mesenchymal stem cells cultured on tissue culture polystyrene based plates (TCP) as well as those cultured on chitosan coated surfaces including pristine one and chitosan film modified by the peptide solution (200 μg/mL) were extracted after 5 days from seeding by RX-BON extraction reagent (Tehran, Iran) following the manufacturer's instructions. Concentration of the obtained RNA in each case was estimated with optical density (OD) measurement by Biophotometer (Eppendorf). Afterwards, the synthesis of complementary DNA (cDNA) was performed by Thermo Scientific cDNA synthesis kit as described by the manufacturer. Eventually, the expression of stemness markers including Oct-4, Nanog, and Sox-2 in three biological replicates of each group of cells was investigated by real-time PCR using Takara Bio real-time qPCR kit. Employing the GAPDH as the reference gene, the expression of aforementioned genes in the cells cultured on chitosan coated surfaces was normalized to that of ones cultured on TCP using the $2^{-\Delta\Delta Ct}$ method. Sequences of the primers used for this study are listed in Table 1.

2.9. Statistical analysis

All data are expressed as the average ± standard deviation (SD). Statistical comparisons were performed by GraphPad Prism8 using unpaired t-test, one-way ANOVA, and two-way ANOVA as required.

3. Results and Discussion

3.1. Peptide immobilization on chitosan film

In the present study, chitosan, a natural polymer with the versatile biological applications [32] containing plenty reactive primary amine groups, was chosen as the cell culture substrate. To make it possible to covalently attach the YIGSR peptide to the chitosan surface with proper orientation, a glutamate residue was added to the N-terminus of the main sequence with a two glycine spacer. To investigate covalent attachment of peptide to the chitosan surface, the peptide-FITC conjugate was employed. As shown in Fig. 2, fluorescence intensity was drastically higher ($p < 0.0001$) when EDC/NHS was used indicating covalent attachment of the peptide. The less fluorescence seen in the absence of EDC/NHS was due to non-covalent attachment of the peptides in addition to the non-specific binding of their FITC residues to the chitosan and polystyrene surfaces. Furthermore, it was evident from the results that increase in concentration of the peptide solution resulted in further surface density of the immobilized peptide as reported before [31].

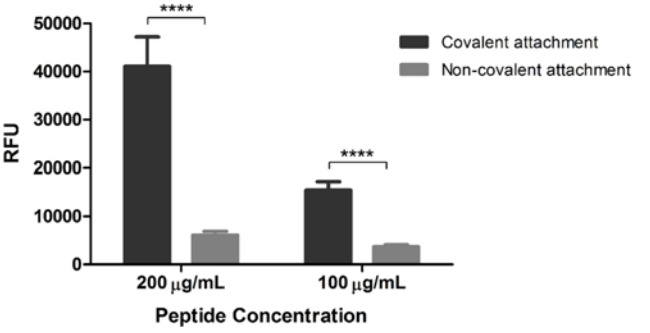

Fig. 2. Fluorescence intensity obtained from peptide-FITC molecules attached upon chitosan surface covalently (with using ECD/NHS) or non-covalently (without using EDC/NHS) versus the concentration of peptide-FITC primary solutions. ****: p-value < 0.0001.

3.2. Surface analysis by AFM

Since it was proven that the topological cues affect MSCs behavior [33], the nano-topography as well as roughness parameter of pristine and peptide-modified chitosan surfaces was obtained by using atomic force microscopy (AFM) at the contact mode to gain more details. According to the Fig. 3A and Fig. 3B, the covalent attachment of the peptide had no significant effect on topographical features and roughness of the chitosan film surfaces. It was in agreement with our previous report wherein the covalently peptide attachment on chitosan film even through a partly long linker was not reflected in AFM results [31]. Thus, we could conclude that the surface modification by the YIGSR peptide would have no influence on the cells behavior through topography-based characteristics.

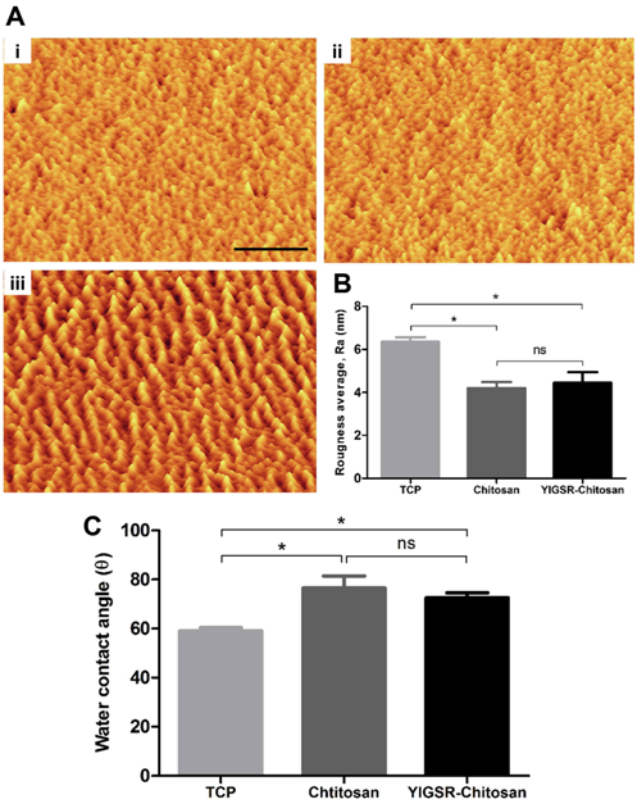

Fig. 3. (A) Height (TOPO) AFM images of pristine chitosan (i), YIGSR modified chitosan (ii), and TCP (iii) surfaces. Scale bar represents 300 nm. (B) Roughness average of TCP, pristine chitosan, and YIGSR modified chitosan surfaces. (C) Water contact angle of TCP, pristine chitosan, and YIGSR modified chitosan surfaces. *: p-value < 0.05, ns: non-significant (p-value > 0.05).

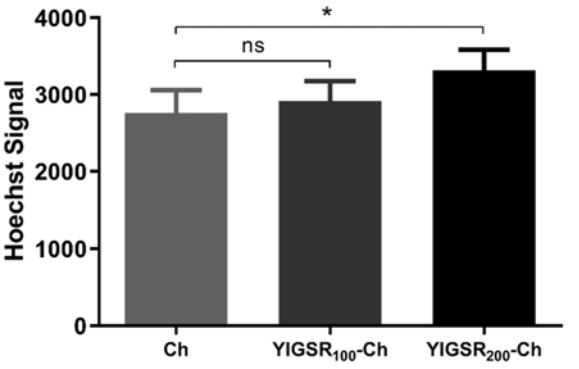

Fig. 4. Comparative Hoechst staining of MSCs after 24 h culture on pristine chitosan surface (Ch) and chitosan surfaces modified by 100 μg/mL and 200 μg/mL YIGSR peptide solutions. *: p-value < 0.05, ns: non-significant (p-value > 0.05).

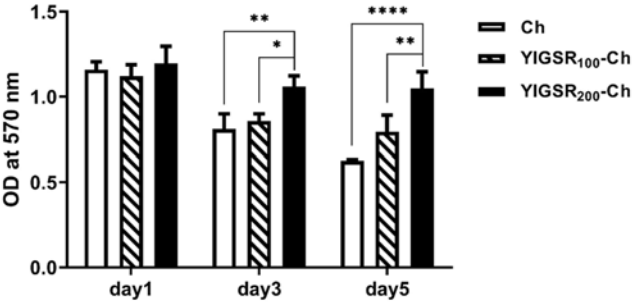

Fig. 5. Effect of immobilized YIGSR peptide on MSCs viability. The results were analyzed statistically by two-way ANOVA followed by Benferroni post-test.*: p-value < 0.05, **: p-value < 0.01, ****: p-value < 0.0001.

3.3. Surface wettability

The study of surface wettability as a critical factor affecting both cell adhesion and spreading [34] by the water contact angle test indicated that chitosan surface modification by the YIGSR peptide could not alter its hydrophilicity meaningfully (see Fig. 3C). Hence in the present study, the difference in cell spreading and attachment toward the chitosan coated surfaces would not be relevant to the different hydrophilicity.

3.4. Cell adhesion assay

Cell adhesion to the chitosan based surfaces was studied by the cell staining with Hoechst 33342 dye which binds to the double-stranded DNA particularly at AT-rich regions. Fluorometry assay showed that though the modification of the chitosan film by the 100 ng/mL peptide solution had no effect on cell adhesion toward the chitosan surface, using 200 ng/mL peptide solution was efficient in significant improving the adhesion of mesenchymal stem cells to these surfaces (see Fig. 4). This result was in agreement with those reported in previous studies introducing YIGSR peptide as an integrin binding motif which enhances cell adhesion [27,28]. It is noteworthy that according to a previous report, integrins α4β1 and α6β1 are the main integrins responsible for the MSCs-YIGSR sequence interaction [35].

3.5. Cell viability assay

The viability of the cells seeded on pristine chitosan film (Ch) as well as chitosan surfaces modified by 100 μg/mL ($YIGSR_{100}$-Ch) and 200 μg/mL ($YIGSR_{200}$-Ch) was evaluated by MTT assay as described. As shown in Fig. 5, on day 3 of culture, a significant difference ($p < 0.01$) was observed between the viability of MSCs cultured on pristine chitosan and those cultured on $YIGSR_{200}$-Ch surfaces. In addition, $YIGSR_{200}$-Ch was more prosperous in maintaining viability of the cells than $YIGSR_{100}$-Ch ($p < 0.05$). These differences were more remarkable on day 5 so that MSCs seeded on $YIGSR_{200}$-Ch showed more viability compared to those cultured on pristine chitosan ($p < 0.0001$) and $YIGSR_{100}$-Ch ($p < 0.01$) surfaces. Nevertheless

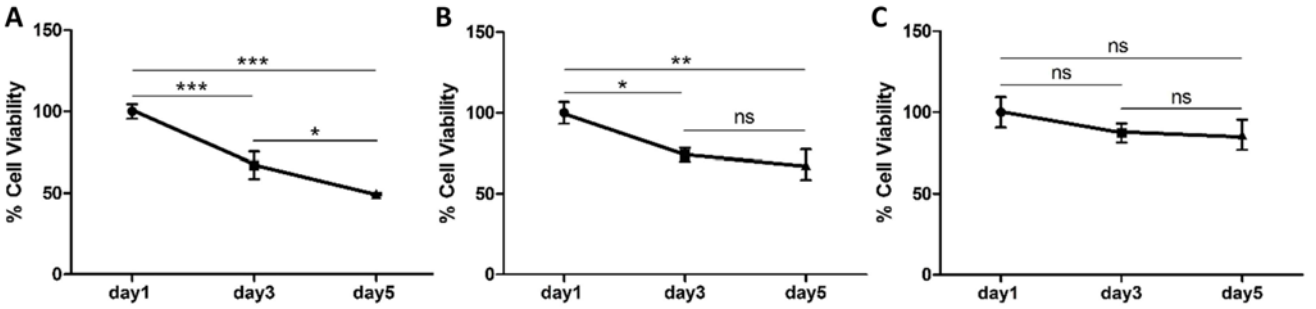

Fig. 6. Viability of MSCs cultured on (A) Pristine chitosan, (B) Chitosan modified by 100 μg/mL YIGSR peptide solution ($YIGSR_{100}$-Ch), and (C) Chitosan modified by 200 μg/mL YIGSR peptide solution ($YIGSR_{200}$-Ch). The results were analyzed statistically by one-way ANOVA followed by Tukey's post test.*: p-value < 0.05, **: p-value < 0.01, ***: p-value < 0.001, ns: non-significant (p-value > 0.05).

according to this results analyzed by two-way ANOVA, no statistically significant difference was observed between viability of the cells on pristine chitosan and $YIGSR_{100}$-Ch surfaces at this time period. To further analysis of MTT results, the viability of the cells cultured on aforementioned surfaces was evaluated individually on intended days. For this purpose, the percentage of cell viability in each group was determined by normalization of the optical densities of days 3 and 5 to those obtained from the 1st day. Herein, the results of each group were analyzed using one-way ANOVA. As shown in Fig. 6A, the viability percentage of the MSCs cultured on pristine chitosan film was decreased to ~ 67% and ~ 50% in days 3 and 5 respectively compared to the 1st day. These results was contrary to what reported previously by Li *et al.* wherein the bone marrow-derived mesenchymal stem cells maintained their viability on chitosan film at least for three days followed by decrease in their survival percentage after 7 days [14]. The differences in tissues of origin, seeding density, and percentage of fetal bovine serum (FBS) used in culture medium as well as difference in techniques utilized to determine cell viability can be considered as some possible causes of this contradiction. In another work, Huang *et al.* demonstrated that the number of human adipose-derived mesenchymal stem cells were maintained on chitosan film with a significant increase after 7 days [29]. This discrepancy in the results, can also be justified regarding the difference in methods used for chitosan film preparation resulting different surface features. In the present study as shown in Fig. 6B, covalent modification of chitosan surface by the peptide solution (100 μg/mL) improved the cell viability so that the difference in viability of the cells in days 3 and 5 (~ 74% and ~ 68% respectively) were statistically insignificant ($p > 0.05$). Likewise, employment of the peptide solution with higher concentration (200 μg/mL) resulting further surface density of the immobilized peptide, maintained the preliminary viability of the cells in days 3 and 5 (see Fig. 6C). Altogether it can be considered that the surface density of the immobilized peptide has a momentous role in maintenance of MSCs viability.

3.6. Inverted microscopy based studies

The morphology of MSCs cultured on the chitosan based surfaces was investigated by inverted microscopy (Fig. 7). While the cells cultured on pristine chitosan film formed spheroids as reported before [29] indicating poor attachment to the surface, those cultured on YIGSR-modified chitosan surfaces represented more spreading and elongation as

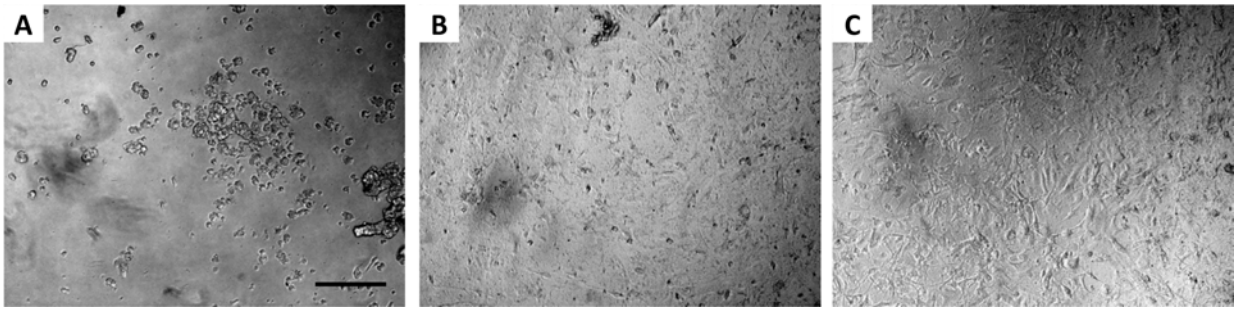

Fig. 7. Morphology of MSCs cultured on chitosan based surfaces. (A) Pristine chitosan. (B) Chitosan modified by 100 μg/mL YIGSR peptide solution ($YIGSR_{100}$-Ch). (C) Chitosan modified by 200 μg/mL YIGSR peptide solution ($YIGSR_{200}$-Ch). Scale bar represents 200 μm.

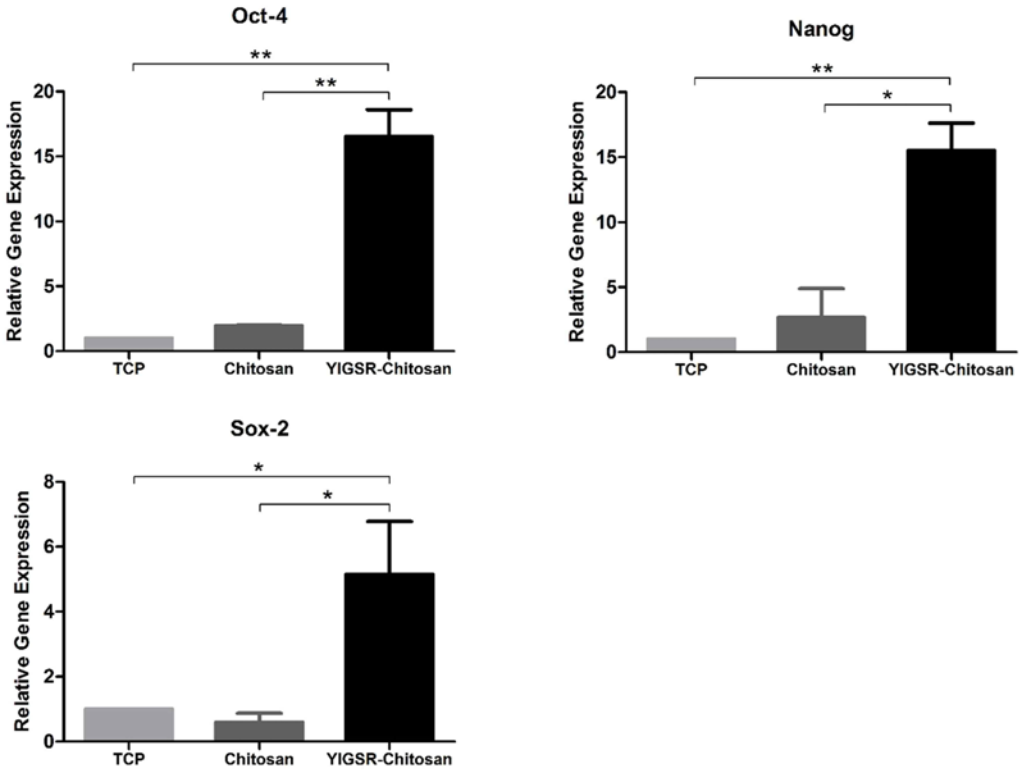

Fig. 8. Relative gene expression of stemness markers (Oct-4, Nanog, Sox-2) in MSCs cultured on TCP, pristine chitosan, and YIGSR modified chitosan surfaces. *: p-value < 0.05, **: p-value < 0.01.

spindle-shaped morphology. These results were in contrast with what reported previously by Frith *et al*. in which human mesenchymal stem cells cultured on immobilized YIGSR peptide did not spread and remained as rounded cells with some membrane protrusion [35]. This contradiction may be justifiable according to this fact that the peptide concentrations used in our work (*i.e.* 100 and 200 μg/mL) were much more than what was used in the previous work (*i.e.* 20 μg/mL). Therefore we could expect that despite the difference in immobilization mechanisms, the surface density of the immobilized peptide was further in our study which could support improved cell adhesion and spreading.

3.7. The expression of stemness markers

The relative expression of stemness markers in human adipose-derived MSCs cultured on tissue culture polystyrene plates (TCP), pristine chitosan, and YIGSR-modified chitosan surfaces were evaluated after 5 days by Real-time PCR technique and statistically analyzed by one-way ANOVA method (Fig. 8). According to the results, the expression level of Oct-4, Sox-2, and Nanog were all elevated significantly (with the values of $p < 0.01$, $p < 0.05$, and $p < 0.05$ respectively) in MSCs cultured on YIGSR-modified chitosan film compared to those cultured on pristine chitosan film. Likewise, this statistically significant increase in expression of stemness genes in MSCs cultured on YIGSR-modified chitosan was observed compared to those seeded on TCP (with the values of $p < 0.01$, $p < 0.05$, and $p < 0.01$ for Oct-4, Sox-2, and Nanog respectively). No significant difference was observed in expression level of aforementioned genes in cells grown on TCP and those cultured on pristine chitosan film. The effect of ECM-derived peptides on cellular events is mediated basically by integrins which are responsible for focal adhesion formation and mechano/chemo transduction [36,37]. Although the members of integrin superfamily are well characterized, their downstream signaling pathways are not completely identified. As aforementioned, α4β1 and α6β1 are the main integrins playing role in MSCs-YIGSR interaction [35]. Hence, understanding the underlying molecular mechanisms involved in YIGSR peptide-mediated phenomenon including enhanced expression of stemness markers by MSCs needs to more studies on downstream signaling triggered by these integrins.

4. Conclusion

We successfully prepared a novel substrate for culture of mesenchymal stem cells through covalent immobilization of laminin-derived peptide, YIGSR on the surface of chitosan film. Collectively, our results demonstrated that

the immobilized YIGSR peptide, not only promotes the adhesion and the viability of human mesenchymal stem cells but significantly enhances the expression of stemness markers in these cells at least in the short-term culture. Our findings have the potential to be inspired for developing commercial substrates for culture of human mesenchymal stem cells.

Declaration of Interests

The authors declare no conflict of interest.

References

1. Pittenger, M. F., A. M. Mackay, S. C. Beck, R. K. Jaiswal, R. Douglas, J. D. Mosca, M. A. Moorman, D. W. Simonetti, S. Craig, and D. R. Marshak (1999) Multilineage potential of adult human mesenchymal stem cells. *Science*. 284: 143-147.
2. Ankrum, J. A., J. F. Ong, and J. M. Karp (2014) Mesenchymal stem cells: immune evasive, not immune privileged. *Nat. Biotechnol.* 32: 252-260.
3. Scuteri, A., M. Miloso, D. Foudah, M. Orciani, G. Cavaletti, and G. Tredici (2011) Mesenchymal stem cells neuronal differentiation ability: a real perspective for nervous system repair? *Curr. Stem Cell Res. Ther.* 6: 82-92.
4. Wu, X. B. and R. Tao (2012) Hepatocyte differentiation of mesenchymal stem cells. *Hepatobiliary Pancreat. Dis. Int.* 11: 360-371.
5. Huang, K., X. Kang, X. Wang, S. Wu, J. Xiao, Z. Li, X. Wu, and W. Zhang (2015) Conversion of bone marrow mesenchymal stem cells into type II alveolar epithelial cells reduces pulmonary fibrosis by decreasing oxidative stress in rats. *Mol. Med. Rep.* 11: 1685-1692.
6. Kharaziha, P., P. M. Hellström, B. Noorinayer, F. Farzaneh, K. Aghajani, F. Jafari, M. Telkabadi, A. Atashi, M. Honardoost, M. R. Zali, and M. Soleimani (2009) Improvement of liver function in liver cirrhosis patients after autologous mesenchymal stem cell injection: a phase I–II clinical trial. *Eur. J. Gastroenterol. Hepatol.* 21: 1199-1205.
7. Kazem-Arki, M., M. Kabiri, I. Rad, N. H. Roodbari, H. Hosseinpoor, S. Mirzaei, K. Parivar, and H. Hanaee-Ahvaz (2018) Enhancement of osteogenic differentiation of adipose-derived stem cells by PRP modified nanofibrous scaffold. *Cytotechnology*. 70: 1487-1498.
8. Chamberlain, G., J. Fox, B. Ashton, and J. Middleton (2007) Concise review: mesenchymal stem cells: their phenotype, differentiation capacity, immunological features, and potential for homing. *Stem Cells*. 25: 2739-2749.
9. Halfon, S., N. Abramov, B. Grinblat, and I. Ginis (2010) Markers distinguishing mesenchymal stem cells from fibroblasts are downregulated with passaging. *Stem Cells Dev.* 20: 53-66.
10. Yang, Y. H. K., C. R. Ogando, C. W. See, T. Y. Chang, and G. A. Barabino (2018) Changes in phenotype and differentiation potential of human mesenchymal stem cells aging *in vitro*. *Stem Cell. Res. Ther.* 9: 131.
11. Atsma, D. E., W. E. Fibbe, and T. J. Rabelink (2007) Opportunities and challenges for mesenchymal stem cell-mediated heart repair. *Curr. Opin. Lipidol.* 18: 645-649.
12. Fotia, C., A. Massa, F. Boriani, N. Baldini, and D. Granchi (2015) Hypoxia enhances proliferation and stemness of human adipose-derived mesenchymal stem cells. *Cytotechnology*. 67: 1073-1084.
13. Cheng, N. C., S. Y. Chen, J. R. Li, and T. H. Young (2013) Short-term spheroid formation enhances the regenerative capacity of adipose-derived stem cells by promoting stemness, angiogenesis, and chemotaxis. *Stem Cells Transl. Med.* 2: 584-594.
14. Li, Z., X. Tian, Y. Yuan, Z. Song, L. Zhang, X. Wang, and T. Li (2013) Effect of cell culture using chitosan membranes on stemness marker genes in mesenchymal stem cells. *Mol. Med. Rep*. 7: 1945-1949.
15. Yu, K. R., S. R. Yang, J. W. Jung, H. Kim, K. Ko, D. W. Han, S. B. Park, S. W. Choi, S. K. Kang, H. Schöler, and K. S. Kang (2012) CD49f enhances multipotency and maintains stemness through the direct regulation of OCT4 and SOX2. *Stem Cells*. 30: 876-887.
16. Al-Habib, M., Z. Yu, and G. T. Huang (2013) Small molecules affect human dental pulp stem cell properties via multiple signaling pathways. *Stem Cells Dev.* 22: 2402-2413.
17. Hashimoto, J., Y. Kariya, and K. Miyazaki (2006) Regulation of proliferation and chondrogenic differentiation of human mesenchymal stem cells by laminin-5 (laminin-332). *Stem Cells*. 24: 2346-2354.
18. Rakian, R., T. J. Block, S. M. Johnson, M. Marinkovic, J. Wu, Q. Dai, D. D. Dean, and X. D. Chen (2015) Native extracellular matrix preserves mesenchymal stem cell "stemness" and differentiation potential under serum-free culture conditions. *Stem Cell. Res. Ther.* 6: 235.
19. Han, S., Y. Zhao, Z. Xiao, J. Han, B. Chen, L. Chen, and J. Dai (2012) The three-dimensional collagen scaffold improves the stemness of rat bone marrow mesenchymal stem cells. *J. Genet. Genomics*. 39: 633-641.
20. Bellis, S. L. (2011) Advantages of RGD peptides for directing cell association with biomaterials. *Biomaterials*. 32: 4205-4210.
21. Ali, S., J. E. Saik, D. J. Gould, M. E. Dickinson, and J. L. West (2013) Immobilization of cell-adhesive laminin peptides in degradable PEGDA hydrogels influences endothelial cell tubulogenesis. *Biores Open Access*. 2: 241-249.
22. Chien, H. W., S. W. Fu, A. Y. Shih, and W. B. Tsai (2014) Modulation of the stemness and osteogenic differentiation of human mesenchymal stem cells by controlling RGD concentrations of poly (carboxybetaine) hydrogel. *Biotechnol J*. 9: 1613-1623.
23. Re'em, T., O. Tsur-Gang, and S. Cohen (2010) The effect of immobilized RGD peptide in macroporous alginate scaffolds on TGFβ1-induced chondrogenesis of human mesenchymal stem cells. *Biomaterials*. 31: 6746-6755.
24. Lee, J. Y., J. E. Choo, Y. S. Choi, I. K. Shim, S. J. Lee, Y. J. Seol, C. P. Chung, and Y. J. Park (2009) Effect of immobilized cell-binding peptides on chitosan membranes for osteoblastic differentiation of mesenchymal stem cells. *Biotechnol. Appl. Biochem*. 52: 69-77.
25. Hudalla, G. A. and W. L. Murphy (2010) Immobilization of peptides with distinct biological activities onto stem cell culture substrates using orthogonal chemistries. *Langmuir*. 26: 6449-6456.
26. Graf, J., R. C. Ogle, F. A. Robey, M. Sasaki, G. R. Martin, Y. Yamada, and H. K. Kleinman (1987) A pentapeptide from the laminin B1 chain mediates cell adhesion and binds to 67000 laminin receptor. *Biochemistry*. 26: 6896-6900.
27. Zheng, J., D. Kontoveros, F. Lin, G. Hua, D. H. Reneker, M. L. Becker, and R. K. Willits (2014) Enhanced Schwann cell attachment and alignment using one-pot "Dual Click" GRGDS and YIGSR derivatized nanofibers. *Biomacromolecules*. 16: 357-363.
28. Boateng, S. Y., S. S. Lateef, W. Mosley, T. J. Hartman, L. Hanley, and B. Russell (2005) RGD and YIGSR synthetic peptides

facilitate cellular adhesion identical to that of laminin and fibronectin but alter the physiology of neonatal cardiac myocytes. *Am. J. Physiol. Cell Physiol*. 288: C30-C38.
29. Huang, G. S., L. G. Dai, B. L. Yen, and S. H. Hsu (2011) Spheroid formation of mesenchymal stem cells on chitosan and chitosan-hyaluronan membranes. *Biomaterials*. 32: 6929-6945.
30. Cheng, N. C., S. Wang, and T. H. Young (2012) The influence of spheroid formation of human adipose-derived stem cells on chitosan films on stemness and differentiation capabilities. *Biomaterials*. 33: 1748-1758.
31. Sohi, A. N., H. Naderi-Manesh, M. Soleimani, E. R. Yasaghi, H. K. Manjili, S. Tavaddod, and S. Nojehdehi (2018) Synergistic effect of co-immobilized FGF-2 and vitronectin-derived peptide on feeder-free expansion of induced pluripotent stem cells. *Mater. Sci. Eng. C Mater. Biol. Appl.* 93: 157-169.
32. Sohi, A. N., H. Naderi-Manesh, M. Soleimani, S. Mirzaei, M. Delbari, and M. Dodel (2018) Influence of chitosan molecular weight and poly (ethylene oxide): Chitosan proportion on fabrication of chitosan based electrospun nanofibers. *Polym. Sci. Ser. A*. 60: 471-482.
33. Anderson, H. J., J. K. Sahoo, R. V. Ulijn, and M. J. Dalby (2016) Mesenchymal stem cell fate: applying biomaterials for control of stem cell behavior. *Front Bioeng. Biotechnol*. 4: 38.
34. Dowling, D. P., I. S. Miller, M. Ardhaoui, and W. M. Gallagher (2011) Effect of surface wettability and topography on the adhesion of osteosarcoma cells on plasma-modified polystyrene. *J. Biomater. Appl.* 26: 327-347.
35. Frith, J. E., R. J. Mills, J. E. Hudson, and J. J. Cooper-White (2012) Tailored integrin–extracellular matrix interactions to direct human mesenchymal stem cell differentiation. *Stem Cells Dev.* 21: 2442-2456.
36. Mobasseri, R., L. Tian, M. Soleimani, S. Ramakrishna, and H. Naderi-Manesh (2018) Peptide modified nanofibrous scaffold promotes human mesenchymal stem cell proliferation and long-term passaging. *Mater. Sci. Eng. C Mater. Biol. Appl*. 84: 80-89.
37. Arnaout, M. A., S. L. Goodman, and J. P. Xiong (2007) Structure and mechanics of integrin-based cell adhesion. *Curr. Opin. Cell Biol.* 19: 495-507.