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The Effect of Blended Chitosan Substrates on OP9 Cells Adipogenesis

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ABSTRACT

Background: OP9 cells were a kind of mouse Mesenchymal stem cells (MSC). Collagen, gelatin, and chitosan substrates are biocompatible extracellular matrices (ECM) for cell seeding and cultivating. Adipogenesis via blended chitosan substrates and preadipocytes OP9 cells has never been reported. Before clinical application in adipogenic differentiation, the safety, efficiency, and possible interference in differentiation due to the substrates must be verified.
Methods: OP9 cells was seeded on tissue culture polystyrene (TCPS), chitosan and blended chitosan substrates, such as chitosan-collagen (CC), chitosan-gelatin (CG), chitosan-gelatin-collagen (CGC). After confluence, serum replacement (SR) induction was adapted for adipogenic differentiation. Cell proliferation on all substrates was tested by MTT assay. Adipogenic differentiation was evaluated by adipogenesis-related genes expression. The differentiation capability determined by fat vacuoles amount and Oil Red O (ORO) stain.
Results: OP9 cells cultured on chitosan coated well have higher mRNA and protein expression on most adipogenic genes compared with TCPS. The expression of Glucose transporter type 4 (GLUT-4) increased progressively in all chitosan and blended chitosan substrates throughout the culture time and SR induction. OP9 cells seeded on CGC substrates efficiently and sufficiently differentiated to fat vacuoles. Furthermore, SR induction combined with blended chitosan substrates provides synergic effect for adipogenesis.
Conclusions: Blended chitosan substrates are biocompatible and have synergy effect on adipogenic differentiation of MSC. This model may provide adequate mature adipocytes for cell therapy and may delineate signaling pathway of adipogenesis to control metabolic disease in the future.
KEYWORDS: adipogenic differentiation, chitosan, collagen, gelatin, MSC

INTRODUCTION

MSCs have multipotent potential and they can differentiate to osteoblasts, adipocytes, myocytes, tenocytes, and so on under appropriate induction conditions. Therefore, MSC can be applied in immune modulation, cell therapy and regenerative medicine extensively. The previous study had characterized OP9 as mouse authentic MSC line.[1] After any one of three adipogenic stimuli (SR, Insulin oleate, and Adipogenic cocktail methods) to OP9 cells, they will express adipocyte late marker proteins, produce triacylglycerol and form adipocyte morphology rapidly. OP9 cells differentiation is not diminished after long term culture or culture with high density. Therefore, this stable cell line has adipogenic potential.[2]

Chitin-based materials are important in biotechnology and medical science fields. These biocompatible materials have been accepted and proved for clinical use.[3] Some of the advantages arise from its cationic nature.[4-5] This unique property is pronounced in tissue regeneration by controlling cell behavior. Besides, cationic chitosan is main ECM in our body and may easily interact with anionic proteoglycans and glycosaminoglycans (GAG). Plenty of biochemical factors are controlled and adhered to them. Therefore, the interaction between chitin-based materials ECM may regulate these substantial factors for tissue regeneration.[6]

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Trauma related soft tissue defect, breast reconstruction surgery, and burn injury still have some problems and innovative adipose engineering is necessary. Two methods about adipogenic engineering are postulated. The first one is to assume adipose tissue via cells with proliferation and differentiation potentials. We can send cells into any site of our body where adipose tissue formation is favored. Some studies suggested to inject preadipocytes into rat subcutaneous layer to produce adipocytes.[7-8] The second one is to use MSC, such as our own preadipocyte to induce adipose tissue formation in vivo. If these MSC can proliferate and differentiate in designed condition, adipogeneiss is possible and transplantation additional cells are unnecessary.

It is meaningful to make a suitable and bioactive environment for regeneration medicine. Previous studies have suggested using a biocompatible material to improve preadipocyte differentiation.[9-10] Collagen, gelatin, and laminin are common ECM, which applied as modified scaffolds for assisting adipogenic differentiation widely.[11–12] In our study, this principles were used to the adipogenic differentiation of preadipocyte. The feasibility of creating a biocompatible and blended ECM scaffold to promote OP9 cells adipogenic differentiation was evaluated. By defining the culture environment and specific induction factor, a modified bioscaffold contributed to adipogenesis in tissue engineering principle was done.

MATERIALS AND METHODS

Preparation of blended chitosan, collagen, and gelatin scaffolds

The fabrication of blended chitosan films coated on TCPS using methods described in previous studies, with some modifications. [13-14].

We dissolved chitosan (C-3646, Sigma, USA; degree of deacetylation>75%) in 1M acetic acid to prepare chitosan solution (1% (W/V). 0.5 ml chitosan solution was poured in each well of 24-well TCPS (Costar, USA) to prepare chitosan coated wells. The chitosan coated wells were placed in a pumping cabinet until solution was evaporated completely to form a solid chitosan layer. All wells were neutralized by 0.2N NaOH aqueous solution for 30min and washed thoroughly with ultrapu re water. All blended wells were sterilized by immersing within 70% alcohol overnight, followed by an extensive wash with phosphate buffered saline and UV irradiation overnight prior cell culture.

For control groups, TCPS were processed by the same procedure described above without chitosan. For chitosan-collagen (CC) coated wells, collagen solution (Cat. No. 354236, Corning, USA) was added into chitosan solution to the final concentration of 50 μg/ml before coating. Similarly, for chitosan-gelatin (CG) coated wells, 0.5 ml 2% (W/V) chitosan was added into 0.5 ml 0.5% (W/V) gelatin solution (G1890, Sigma, USA). For chitosan-gelatin-collagen (CGC) coated wells, collagen was loaded into CG solution to the final concentration of 50 μg/ml before coating.

Cell lines and reagents

Taiwan Bioresource Collection and Research Center provided the OP9 cells. The culture medium was α-MEM (Invitrogen, Carlsbad, Ca) combined with 10% fetal bovine serum (Invitrogen, Carlsbad, Ca).

Real-time polymerase chain reaction (RT-PCR) analysis

Total RNA was prepared from cell lines using TRIZOL (Invitrogen, Carlsbad, Ca) accordig to the man ufacturer's protocol. First-strand cDNA was prepared from 5μg of each total RNA sample using the SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, Ca) according to the user manual. One μl of each first-strand reaction was subsequently amplified by PCR reaction using HotStar PCR Super Mix (GeneDireX, Vegas, Nevada, USA) for gene-specific cDNA fragments. PCR products were separated by agarose gel electrophoresis, and signal was detected, captured and analyzed by a high-resolution microscope imaging system (SPOT imaging, Toulouse, France). Oligonucleotide primers used in PCR include: mouse C/EBP alpha: 5′-AGCTTACAACAGGCCAGGTTTC-3′, 5′-CGGCTGGCGACATACAGTAC-3′; mouse C/EBP beta: 5′-ATCGACTTCAGCCCCTACCT-3′, 5′-GGCTCACGTAACCGTAGTCG-3′; mouse PPAR gamma: 5′-GTCACGTTCTGACAGGACTGTGTGAC-3′, 5′-GGGTCAGCTCTTGTGAATGGAATG-3′; mouse FABP4: 5′-AAATGTGTGATGCCTTTGTG-3′, 5′-AAATCCCCATTTACGCTGAT-3′, mouse SREBP-1: 5′-CCGGGGAACTTTTCCTTAAC-3′, 5′-TCACTGTCTTGGTTGTTGAT-3′; mouse adipsin: 5′-TTAAGCTATCCCAGAATGCC-3′, 5′-TGCACACATCATGTTAATGG-3′; mouse adiponectin 5′-CAGATAACGTCAACGACTCT-3′, 5′-TCGACTGTTCCATGATTCTC-3′; mouse GLUT-4: 5′-CTCAAGCGGGTCTCACTAGAT-3′, 5′-AGAATACAGCTAGGACCAGTG-3′; mouse GAPDH: 5′-ACGGCCGCATCTTCTTGTGCA-3′, 5′-ACGGCCAAATCCGTTCACACC-3′.

Western blotting

Cells were rinsed with phosphate buffered saline (PBS) and protein was extracted by RIPA lysis buffer (89901, Thermo Fisher scientific, USA) supplemented with protease inhibitors and phosphatase inhibitors (79429, 78426, Thermo Fisher scientific, USA). Protein concentration was determined with DC protein assay kit (5000116, BioRad, USA) according to the instruction manual. Equal amount of total protein was mixed with Laemmli buffer following heating at 95°C for 5 minutes and separated by sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE), then transferred by mini trans-blot® electrophoretic transfer cell (BioRad, USA). Membranes were blocking with 2% BSA in Tris-buffered saline supplemented with 0.1% tween-20 (TBST) and incubated with primary antibody of interests overnight, followed by thoroughly wash and incubation of secondary antibody. Signal was developed by an enhanced chemiluminescence (ECL) kit (BioRad, USA) and detected by a CCD camera.

MTT assay

Cell proliferation was calculated by MTT assay. OP9 was seeded on TCPS and other materials of interest. At indicated time, MTT (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; Sigma-Aldrich, Darmstadt, Germany.) was diluted in complete medium (0.5mg/ml) and incubated with cells for 1 hour. Next, the medium was discarded and cell proliferation was calculated by dissolving produced formazan by DMSO. Absorbance (O.D. 570nm) was measured by a microplate spectrophotometer (BioTek, Winooski, Vermont, USA). Three hours after seeding was counted as beginning point and was expressed as 100%.

Induction of cell differentiation in preadipocyte OP9 cells

To induce adipocyte differentiation in OP9 cells, cells were first seeded on TCPS and other materials in the complete growth medium. Medium containing 15% SR (Serum replacement, Invitrogen, Carlsbad, Ca) was applied when cells of TCPS reached confluence. Complete growth medium was used as control. Three days post of SR, all RNA was extracted from the cells and analyzed by RT-PCR

ORO staining

ORO staining was performed following the protocol described by others research.[15] OP9 cells were rinsed with PBS and fixed in 4% paraformaldehyde solution (Sigma-Aldrich, Darmstadt, Germany.). The rinsed and fixed cells were washed thoroughly by deionized water and they were incubated in ORO solution (300mg in 36% triethyl phosphate (Sigma-Aldrich, Darmstadt, Germany.)) under room temperature for 30 minutes. Extensive wash with deionized water was done and images were captured by a conventional microscopy. DMSO was used as solvent, and O.D. 500nm was measured for quantification by microplate .spectrophotometer (Biotec, Winooski, VT, USA)

Statistical analysis

All data were analyzed using SPSS 24.0 statistical software and expressed as the mean ± standard deviation (SD). We used Student's t-test to compare two groups. Statistical comparisons between multiple groups were performed using one-way analysis of variance (ANOVA), and multiple time points were analyzed by two-way ANOVA with Bonferroni's correction. The significance level was set at 0.05 and $P<0.05$ was considered to indicate a statistically significant difference.

RESULTS

Chitosan substrates support OP9 cells adipogenic differentiation

We cultured OP9 cells onto chitosan coated wells and TCPS without induction medium. After 7 days culture, some OP9 cells were found to be induced to differentiate to adipocyte-like cells (Fig. 1A). After 10 days culture, some fat vacuoles floated away (Fig. 1A). To elucidate the adipogenic differentiation effect of chitosan substrates on OP9 cells, the adipocyte-related gene mRNA expressions were analyzed by RT-PCR. C/EBP beta and PPAR gamma expressed gradually after 7 days to 10 days culture on chitosan substrates (Fig. 1B). Besides, the PPAR gamma, C/EBP beta, and C/EBP alpha mRNA expressed higher after 7 days to 10 days culture on TCPS than on chitosan coated wells (Fig. 1B). In western blot, the expression level of perilipin A and adiponectin was progressively increasing during the culture time when OP9 cells were cultured on chitosan coating well. In contrast, there was no any expression of perilipin A and adiponectin when OP9 cells were cultured on TCPS (Fig 1C). We suggest chitosan substrates not only can induce adipogenic differentiation of OP9 cells but also support cytoplasmic fat vacuoles accumulation.

Figure 1A

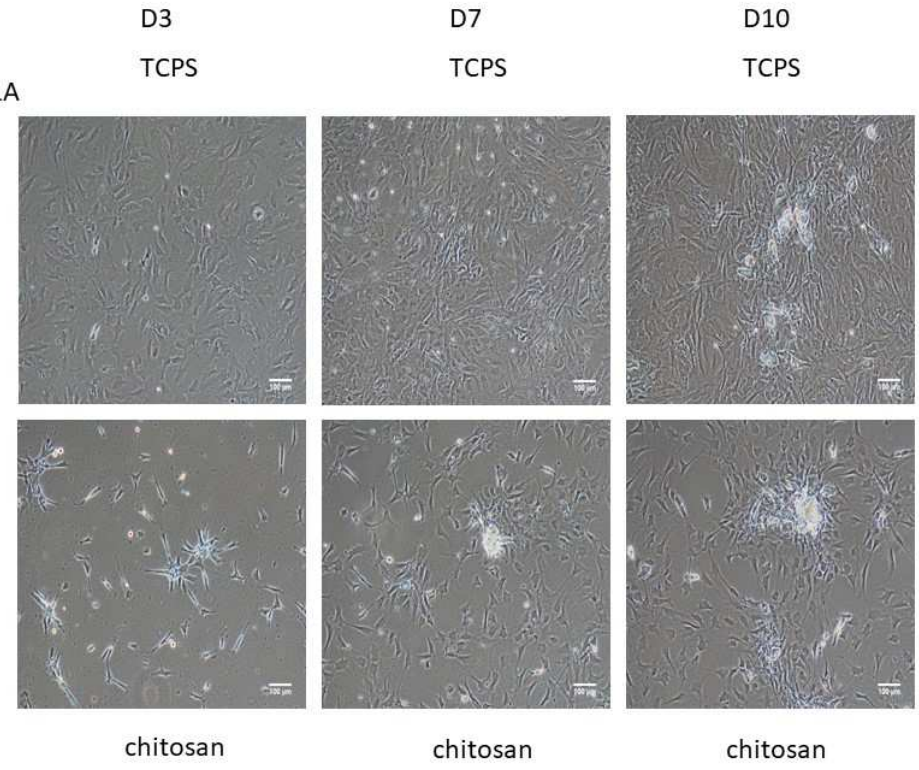

Figure 1A

After 7 days culture onto chitosan coated well, some OP9 cells aggregated and were found to be induced to differentiate to adipocyte-like cells. After 10 days culture, some fat vacuoles floated away.

Figure 1B

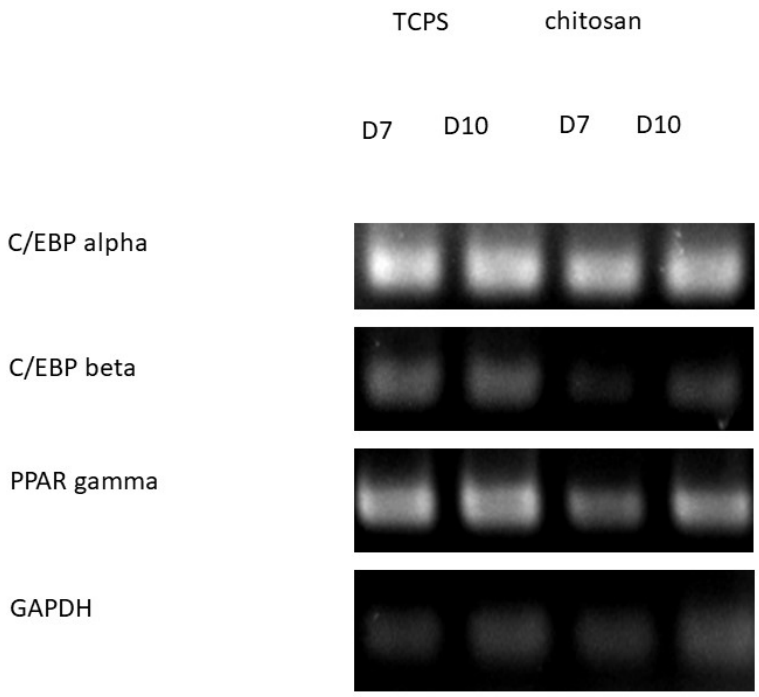

Figure 1B

The adipocyte-related gene expressions were analysis by RT-PCR. CEBP beta and PPAR gamma expressed increasing from day 7 to day 10 culture on chitosan substrates, but the expression level is less than on TCPS.

SR enhances OP9 cells adipogenic differentiation

When OP9 cells were seeded on TCPS and chitosan scaffold until confluence, we choose to add or not add SR to induce adipocyte differentiation. When the OP9 cells are cultured on both wells without SR induction for 3 days, we find that OP9 cells on chitosan change their morphology and more adipocyte-like cells formed than on TCPS. We also can observe focal aggregation on chitosan, but not on TCPS. It seems that chitosan can induce the MSC OP9 to adipocyte differentiation compared with TCPS (Fig. 2A). When the OP9 cells are cultured on both wells with SR induction for 3 days, we do not find that OP9 cells have different morphology change and the different number of adipocyte-like cells on both wells. It seems that SR has a higher ability to induce OP9 cells on both TCPS and chitosan coated well to differentiate to adipocyte-like cells (Fig. 2A). We use ORO staining to quantify the number of matured adipocytes formed on both wells (Fig. 2B). When the OP9 cells are cultured on both wells without SR induction for 3 days, we use ORO staining to check if there any adipocyte forms. We find that bigger and mature adipocytes noted on chitosan coated well than on TCPS. Furthermore, when the Op9 cells are cultured on both wells with SR induction for 3 days, we also find more and bigger ORO stained mature adipocytes form on chitosan coated well compared with TCPS. It seems that chitosan has a synergic effect on SR to induce MSC OP9 to differentiate to adipocyte.

We then evaluate adiogenesis-related genes mRNA expression via RT-PCR, such as C/EBP alpha and so on (Fig. 2C). We compared the gene expression about the confluence, control (without SR induction), and 15% SR induction groups 3 days after culture. In GLUT-4, we can observe the adipogenic effect of SR induction on both groups. After further 3 days, GLUT-4 expressed higher in SR induction group than control. C/EBP alpha, PPAR gamma, adiposin and adiponectin had similar trend in SR induction group than control, but no significant difference. We also observe that OP9 cells cultured on chitosan coated well have higher mRNA expression on all adipogenic genes except SREBP1 compared with TCPS. It seems that chitosan indeed has higher adipogenic differentiation ability compare with TCPS. We also use western blot to evaluate protein level on adipogenesis-related genes, such as GLUT-4, perilipin A, and adiponectin (Fig. 2D) In control group, only when OP9 cells cultured on chitosan coated well expressed adpigenesis genes during culture time, which is similar to the results of Fig. 1C. After SR induction for 3 days, the expression level of all adipogenesis genes were higher on chitosan coated well than on TCPS. We also found that the expression level of adiponectin was increasing gradually and obviously, which proved the synergic effect of SR and chitosan on adipogenic differentiation.

Figure 1C

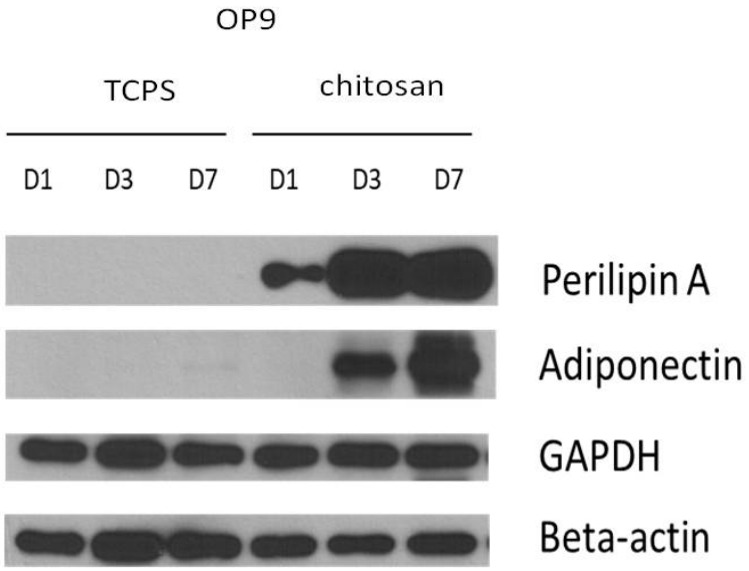

Figure 1C

In western blot, perilipin A and adiponectin was progressively increasing during the culture time when OP9 cells were cultured on chitosan coating well. In contrast, there was no any expression of perilipin A and adiponectin when OP9 cells on TCPS.

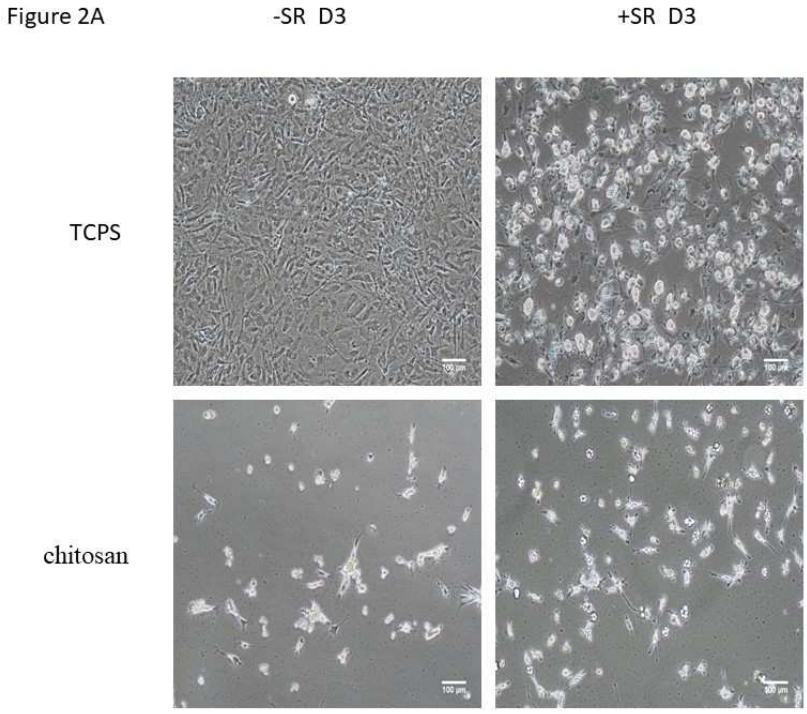

Figure 2A

When the OP9 cells on both wells without SR induction for 3 days, we find that OP9 cells on chitosan coated well change their morphology and more adipocyte-like cells formed than on TCPS. We also can observe focal aggregation on chitosan, but not on TCPS. When the OP9 cells on both wells with SR induction for 3 days, we do not find different morphology change and different number of adipocyte-like cells on both wells.

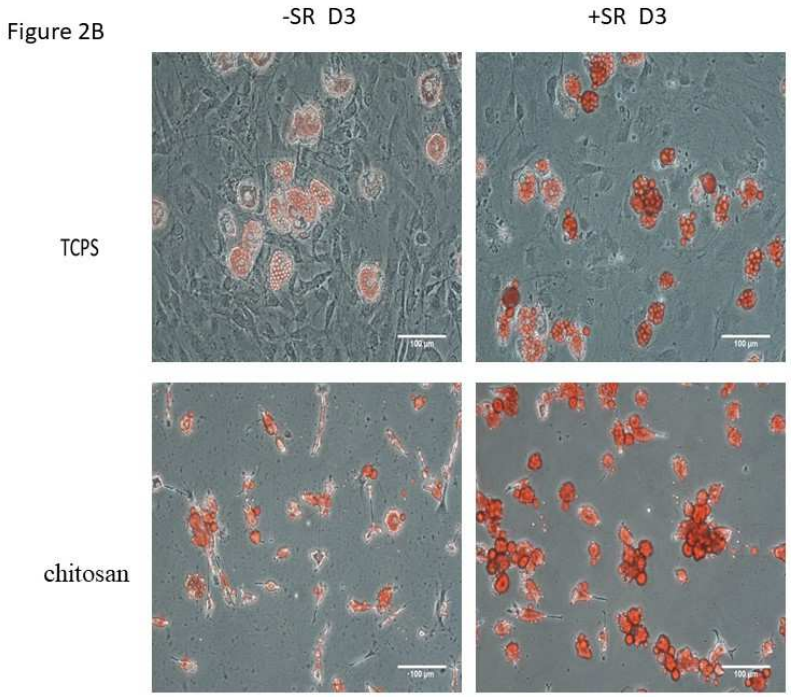

Figure 2B

We use ORO staining to quantify the number of mature adipocytes formed on both wells. When the OP9 cells are on both wells without SR induction for 3 days, we find that bigger and more mature adipocytes noted on chitosan coated well than on TCPS. Furthermore, when the OP9 cells are cultured with SR induction, we also find bigger and more ORO stained mature adipocytes on chitosan coated well.

Figure 2C

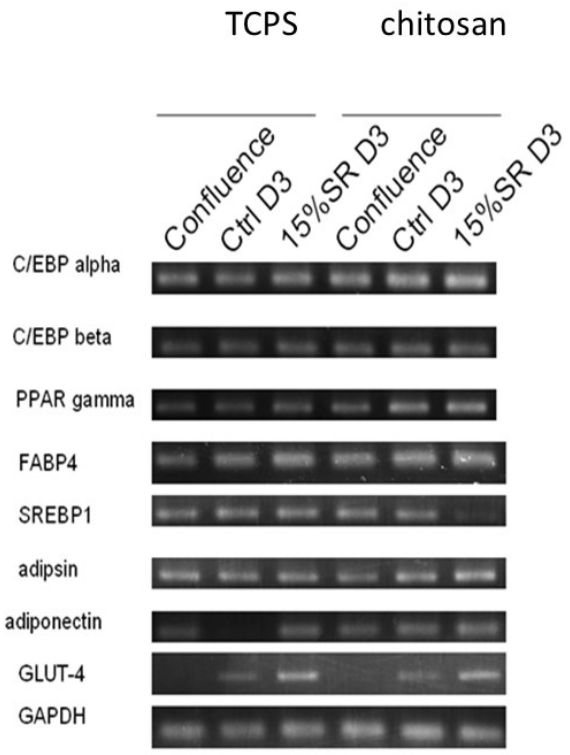

Figure 2C

In RT-PCR, GLUT-4 expression increases gradually from initial confluence and becomes higher after SR induction on both groups. We also observe that OP9 cells cultured on chitosan coated well have higher mRNA expression on all adipogenic genes except SREBP1.

Figure 2D

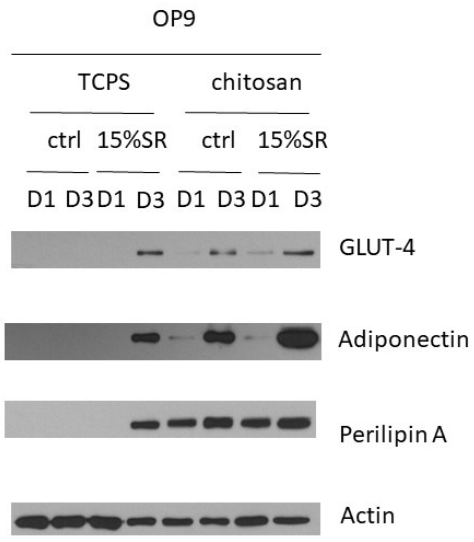

Figure 2D

We also use western blot to evaluate protein level on adipogenesis-related genes, such as GLUT-4, perilipin A, and adiponectin. In control group, only when OP9 cells cultured on chitosan coated well expressed adpigenesis genes during culture time. After SR induction for 3 days, the expression level of all adipogenesis genes were higher on chitosan coated well. The expression level of adiponectin was increasing gradually and obviously.

Fabrication and modification of chitosan, CC, CG, and CGC substrates

Although the chitosan substrates can support the adipogenic differentiation of OP9 cells, the amounts of differentiated adipocyte-like cells are sparse and easily detached. This shortage does not meet the demand for future application of stem cell-based therapy. This may because that these differentiated adipocye-like cells cannot anchor to the chitosan substrates tightly and finally die. Consequently, we obtained the relative low number of survived cells. Therefore, assume that if we could provide a suitable substrate, which can not only support adipogenic differentiation of OP9 cells but also support cell adhesion, proliferation, and survival, we can enrich the adipogenic differentiation of OP9 cells theoretically. Hence, to improve the anchoring of these differentiating adipocytes directly onto the chitosan substrates, chitosan substrates were blended with two commonly used ECM, gelatin or/and collagen, to enhance the cell adhesion and viability on the chitosan substrates.

CC and CGC substrates enhance cell adhesion and proliferation

To investigate the adhesion ability of OP9 cells on the substrates we prepared in this study, OP9 cells were collected and plated onto TCPS, chitosan, CC, CG, and CGC substrates in the α-MEM containing 10% FBS. After confluence for 3 days, it is observed that more OP9 can attach onto the CC, CG and CGC substrates than chitosan. The more OP9 cells attach, the more adipocyte-like cells form (Figure 3A). To ensure if the substrates we prepared in this study are biocompatible for OP9 cells culture, the MTT cell proliferation assay was performed (Figure 3B). As the results indicated, although the proliferation of OP9 cells on CC and CGC are not higher than that on control TCPS wells after 3 days culture, they are significantly higher than that on CG and non-blended chitosan substrates. After 7 days, the OP9 cells on CC and CGC proliferate significantly more than that on TCPS, CG and non-blended chitosan substrates. These data suggest that chitosan substrates blended with ECM such as CC and CGC not only improve cell adhesion but also increase cell proliferation.

We also use SR induction to test the effect of adepogenic differentiation of OP9 cells on chitosan coated well, CC, CG, CGC and TCPS. Only 1-day SR induction, more adipocyte-like cells are formed than no SR induction (Figure 3 C). ORO stain also confirms that many adipocytes are formed on TCPS, chitosan, CC, CG, and CGC coated wells. It seems SR, chotosan, and other ECM have a synergic effect on adipogenic differentiation.

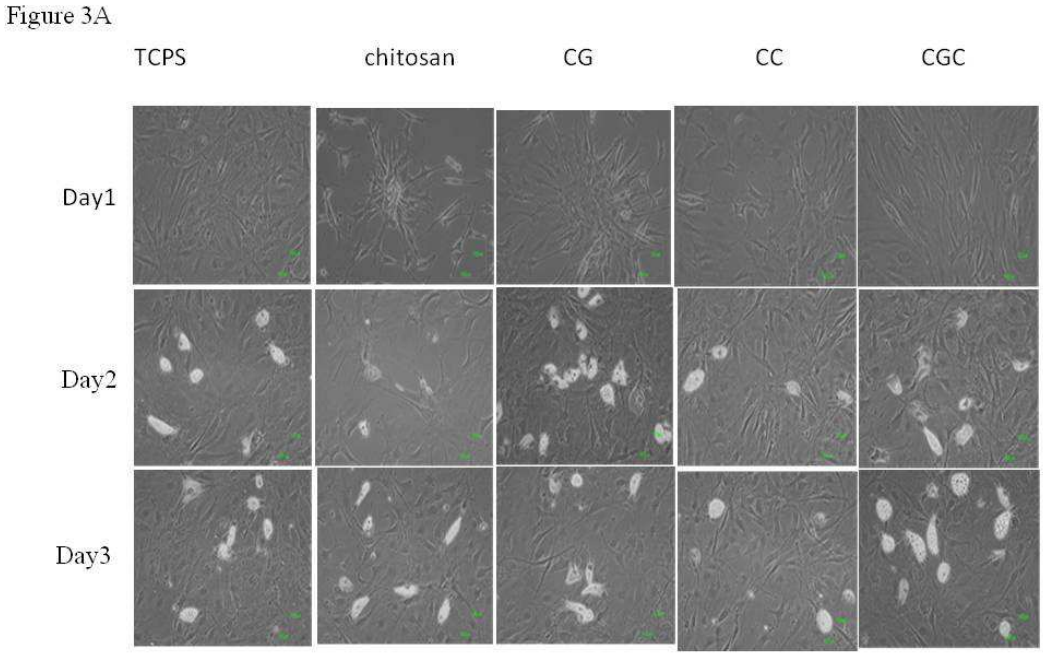

Figure 3A

After confluence for 3 days, more OP9 cells can attach onto the CC, CG and CGC coated wells than on chitosan coated well.

Figure 3B

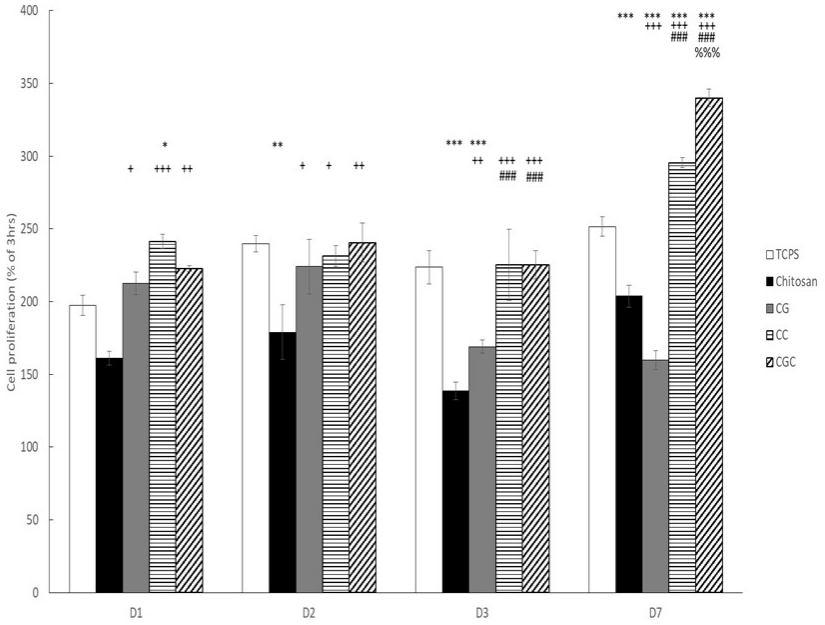

Figure 3B

MTT assay shows that although OP9 cell proliferation on CC and CGC coated wells are not higher than that on control TCPS after 3 days culture, they are significantly higher than on CG and chitosan. After 7 days, OP9 cells proliferates on CC and CGC significantly more than that on TCPS, CG and non-blended chitosan substrates. All data are showed as the mean ± standard deviation. The statistic significance level is presented as $*p<0.05$, $**p<0.01$ and $***p<0.001$ when chitosan, CC, CG, and CGC vs. TCPS; $^{+}p<0.05$, $^{++}p<0.01$ and $^{+++}p<0.001$ when CC, CG, and CGC vs. the chitosan; $\#p<0.05$, $\#\#p<0.01$ and $\#\#\#p<0.001$ when CG, and CGC vs. CC; $\%p<0.05$, $\%\%p<0.01$ and $\%\%\%p<0.001$ when CG vs. CGC.

Figure 3C

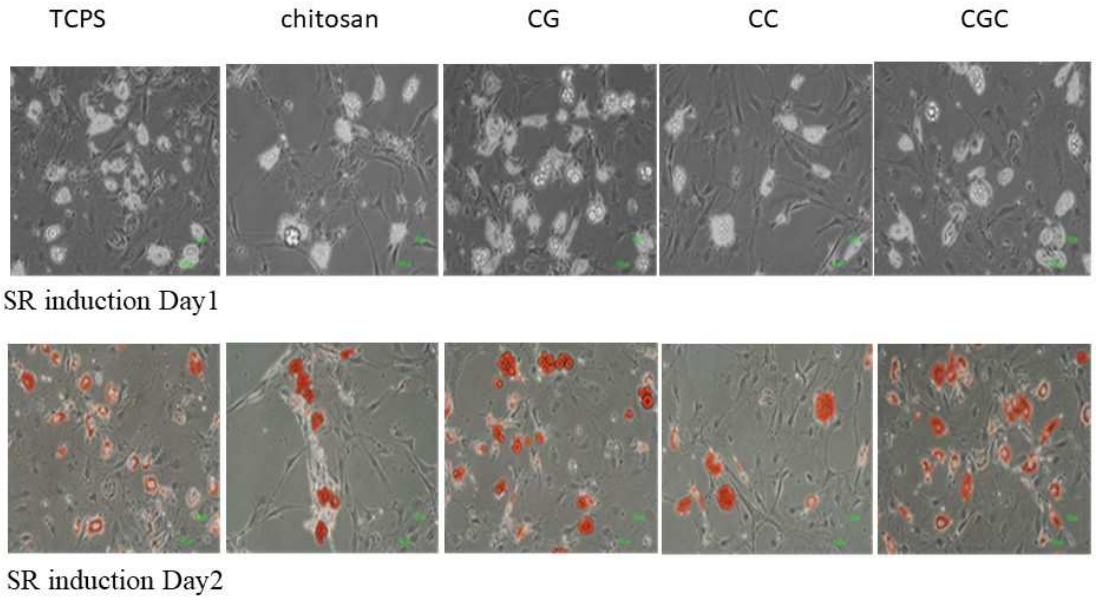

Figure 3C

We also use SR induction to test the effect of adipogenic differentiation of OP9 cells on TCPS, chitosan, CC, CG, and CGC coated wells. Only 1 day SR induction, more adipocyte-like cells are formed than no SR induction. ORO stain also confirms that many matured adipocytes are formed on TCPS, chitosan, CC, CG, and CGC coated wells.

CGC substrates with SR induction enhance adipogenic differentiation

When the OP9 cells are cultured on all four wells without SR induction for 3 days, only OP9 cells on chitosan coated well have obvious morphology change of cell aggregation (Fig. 4A). Besides, we find there are more adipocyte-like cells on CGC coated well than on chitosan, CG, and CC coated wells. When the OP9 cells are cultured on all four wells with SR induction for 3 days, we find there are significantly more adipocytes-like cells on CGC coated well than on chitosan, CG, and CC coated wells. It suggests after surface modification with ECM on chitosan coated well, the efficiency on adipogenic differentiation increases, especially on CGC coated well. We postulate that chitosan combined with ECM leads to the proliferation of OP9 cells and enforces differentiation to more adipocyte-like cells. We also use ORO staining to check the mature adipocytes number formed on all four wells (Fig. 4A). When the OP9 cells are cultured on all wells without SR induction for 3 days, we use ORO staining to check if there any adipocyte forms. We find more adipocytes form on CGC coated well and there is no noticeable difference about the ORO stained adipocytes on chitosan, CG, and CC coated wells. When the OP9 cells are cultured on all wells with SR induction for 3 days, we find more ORO stained adipocytes form on CGC coated well compared with chitosan, CG, and CG coated wells. We quantify the total amount of ORO of four wells by microplate spectrophotometer (Fig. 4B). There is no difference between four wells when culture without SR induction. But, all blended chitosan substrates, such as CG, CC, and CGC have higher ORO amount than chitosan coated well. CG and CGC substrates also have higher ORO amount than CC. It seems that chitosan blended with both gelatin and collagen has synergic effect on SR to induce MSC OP9 to differentiate to adipocyte. After ECM modification, the efficiency to differentiate OP9 cells to adipocytes is also increase.

Figure 4A

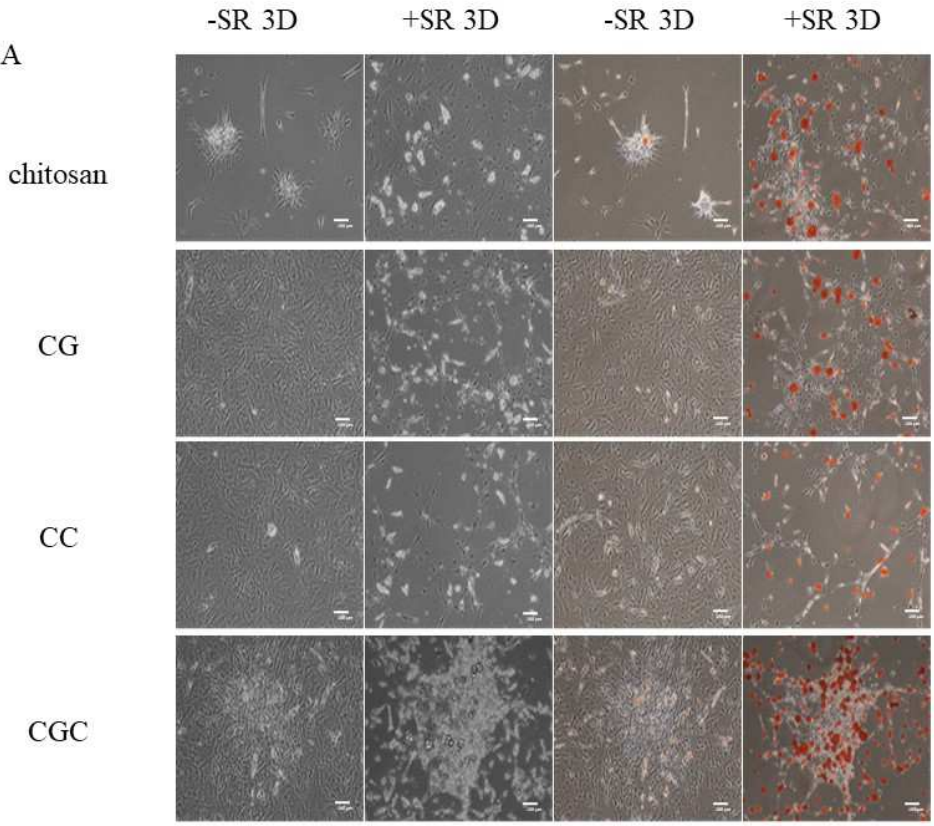

Figure 4A

When the OP9 cells are cultured on all four wells without SR induction for 3 days, only OP9 cells on chitosan coated well have obvious morphology change of cell aggregation. Besides, we find there are more adipocyte like cells on CGC coated well than on chitosan, CG, and CC coated wells. When the OP9 cells are cultured on all four wells with SR induction for 3 days, we find there are significantly more adipocyte-like cells on CGC well than on chitosan, CG, and CC coated wells.

To know the effect of ECM blended chitosan on adipogenic differentiation, we evaluate adiogenesis-related genes mRNA expression via RT–PCR, such as C/EBP alpha and so on. After OP9 cells become confluence on the four wells, we divide each group into two ways, including SR induction or not. Finally, we compared the gene expression about the confluence, control (without SR induction), and 15% SR induction groups after culture for 3 days (Fig. 4C). In GLUT-4, we can observe the synergic effect of SR induction on all wells. After further 3 days culture, GLUT-4 was increasing gradually in all wells throughout the culture time, especially after SR induction. GLUT-4 expression is higher in SR induction group than in control group. In other genes, such as adiposin and adiponectin, we observe similar trend both in SR induction and control groups. However, the difference is not significant. We also observe that OP9 cells cultured on chitosan coated well have higher mRNA expression on all adipogenic genes except SREBP1 compared with other wells. It seems that chitosan indeed has higher adipogenic differentiation ability compare with other blended wells. When OP9 cells culture on chitosan blended with collagen and/or gelatin wells, the mRNA expression of the adipogenic gene is different. On CC and CG coated wells, PPAR gamma, C/EBP beta and C/EBP alpha express lesser in SR induction group than in control group. On CGC coated well, PPAR gamma, C/EBP beta and C/EBP alpha express higher in SR induction group than control. The result is consistent with OP9 cells cultured on CGC wells formed more adipocytes via ORO staining and had higher ORO amount via absorbance meter.

Figure 4B

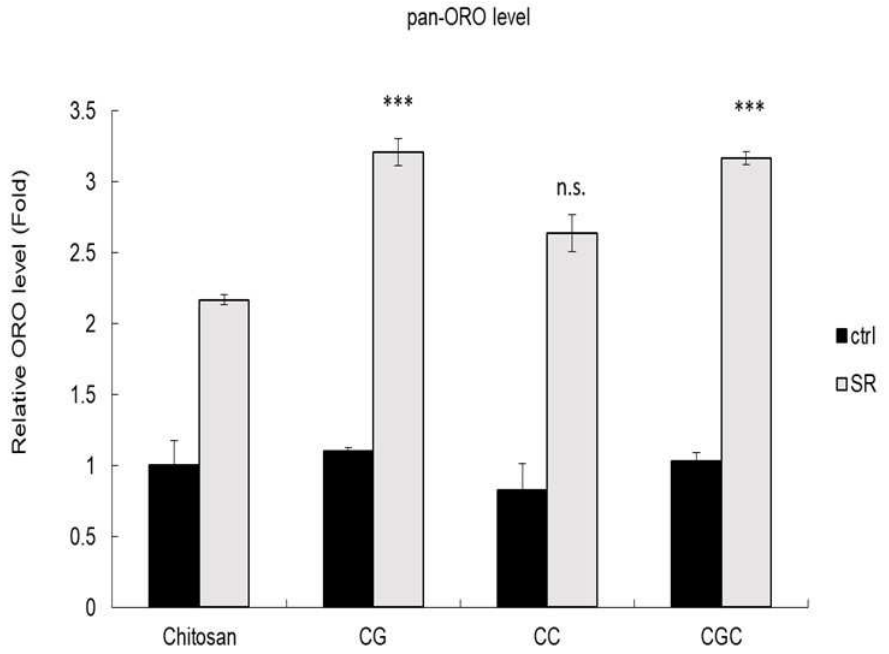

Figure 4B

We quantify the total amount of ORO of four wells by microplate spectrophotometer. There is no significant difference between four wells when culture without SR induction. But, all blended chitosan substrates, such as CG, CC, and CGC have higher ORO amount than chitosan coated well. CG and CGC substrates also have higher ORO amount than CC. All data are showed as the mean ± standard deviation. The statistic significance level is presented as *p<0.05, **p<0.01 and ***p<0.001 when CC, CG, and CGC SR induction groups vs. chitosan SR induction group.

Figure 4C

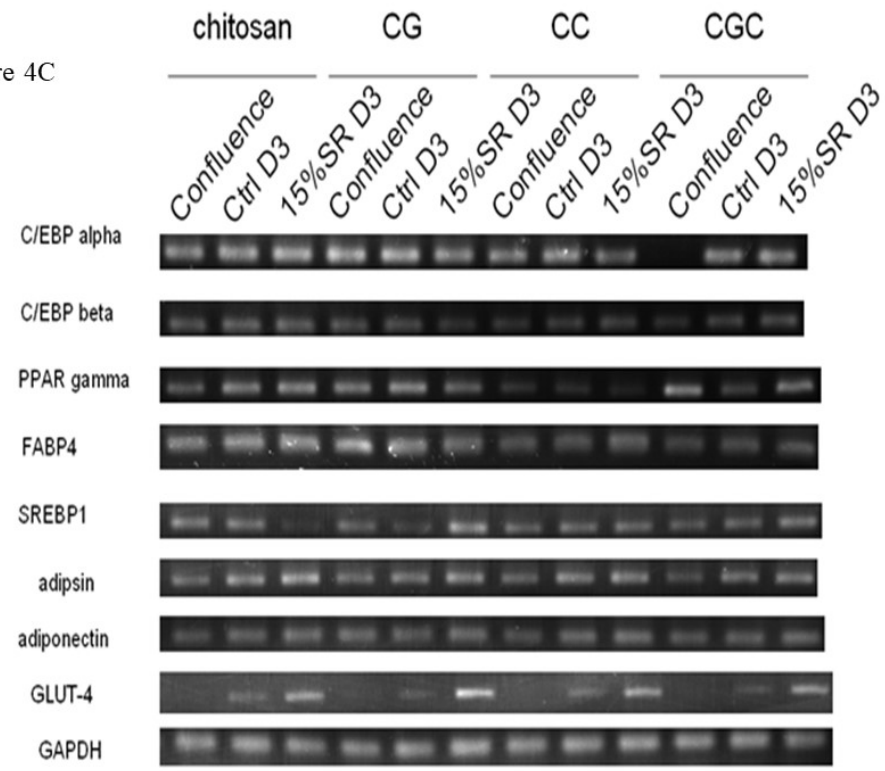

Figure 4C

The expression of GLUT-4 was increasing gradually in all wells throughout the culture time, especially after SR induction. In other genes, such as adiposin and adiponectin, we observe similar trend both in SR induction and control groups. We also observe that OP9 cells cultured on chitosan coated well have higher mRNA expression on all adipogenic genes except SREBP1 compared with other wells. In contrast to CC and CG substrates, when cultured on CGC coated wells, PPAR gamma, C/EBP beta, and C/EBP alpha express higher in SR group than in control.

DISCUSSION

In this study, we find chitosan affects adipogenic differentiation of OP9 cells, which noted on the expression of adipogenic representative genes in mRNA and protein level. Compare with SR induction, chitosan has lesser adipogenic effect showed from ORO stain, RT-PCR and western blot. After the chitosan blends with different ECM, including collagen or/and gelatin, the MTT assay shows all blended chitosan are biocompatible and the adipogenesis is better than chitosan alone. The adipogenic differentiation of all blended chitosan is not as good as SR induction, too. However, blended chitosan combines with SR induction provide a synergic effect on adipogenic differentiation of OP9 cell, especially the CGC substrates.

Chitosan has biodegradable and biocompatible properties. It has a structure of cationic polysaccharide part, which can interact with many anionic molecules. These unique characteristics make it easier for gene manipulation, cell cultivation and tissue regeneration.[16-18] Besides, it have been proved to alleviate immune responses[19-20] and also can immobilize and increase the surrounding biofactors, which let them to interact with cells meticulously and facilitate regeneration.[21-22]

Oral supplementation with chitosan in mice may decrease lipid metabolism dysfunction via downregulated adipogenesis and inflammation related gene expression.[23] Another study suggested that chitosan oral supplementation in rats inhibited adipogenesis via JAK2-STAT3 signaling pathway.[24] There are some studies showed that chitosan can inhibit adipogenesis via different concentration, which controlled by downregulated adipogenic transcription factors and by epigenetic manipulation.[25-28] However, all these studies used 3T3-L1 cells as their study model. Previous studies have showed that it was low efficiency to differentiate 3T3-L1 cells. Different preadipocytes and different cell sources had a significant difference on effects and results of adipogenesis. RT-PCR of different cell lines and cell sources during adipogenesis also showed different nuclear receptors expression.[29] Sheng et al suggested that the high degree of media was unproportionate to lipid

content in mature adipocytes during adipogenesis.[30] Therefore, different cells and differentiation ways may impact the results of adipogenesis.

Herein, we used preadipocyte cell line OP9 and blended chitosan to study the adipogenic differentiation, which has never done before. Chitosan has some basic properties, such as, renewability, a hydrating agent, film forming, absorption, nontoxicity, the deacetylation degree, anti-thrombogenic and viscosities that contribute to a broad set of applications in biochemical study.[31] The film forming feature with specific physicochemical and biological properties may influence OP9 cells adipogenic differentiation. In our study, we culture OP9 cells on chitosan coating wells and some cells differentiated to fat vacuoles after 7 days and adipogenic gene expression also supported the results. The scenario is totally different from chitosan supplementations in 3T3-L1 cells with inhibition of adipogenesis. Different form chitosan substrates and different cell lines may lead to different biologic effect in adipogenic differentiation. Whether new tissues regenerate depends on the interaction between the scaffold surface and cells. Fine manipulate the scaffold surface with appropriate size, mechanical stability, surface roughness and porosity to provide a suitable microenvironment may increases cell adhesion, proliferation and change cell behaviors [32]

To increase the efficiency of adipogenic differentiation in our study, we fabricate our chitosan substrates with ECM. The unique and extensive characters of the ECM are useful biomaterials and scaffold resources. Collagen and gelatin are essential ECM to provide various signals for maintaining the homeostasis of living organisms and cellular mechanical properties, and are reasonable to contribute to adipogenesis.[33-35] Gelatin is a kind of denatured type collagen made of high level glycine, hydroxyproline, and proline. If chitosan is added in gelatin, polyelectrolytic complexes can form with different gelated level.[36] Collagen is a widely used scaffold for adipose tissue engineering with its per se ECM component. It is noted that the cells differentiating towards the adipogenic lineage secrete collagen.[35] which is an indication to adipogenesis and this emphasis on the role of collagen in adipose tissue formation. Other study also suggested that collagen would promote and favor cells attachment and thereby their differentiation in vitro/in vivo, simultaneously filling the defect tissue.[37] We fabricate our chitosan substrates with gelatin or/and collagen in different doses. CGC consists of chitosan, gelatin, and collagen substrates that supports more OP cells after long term culture (Fig. 3B) and facilitate adpiogenic differentiation compared with CC and CG (Fig. 4).

A three-dimension culture environment can be done by use of porous scaffolds, cellular aggregates, and hydrogels. Many cells have been successfully implanted on them.[38-40] Among them, cellular aggregates can avoid unwanted and unpredictable response from exogenous biomaterials upon cell transplantation.[41-44] When OP9 cells culture on chitosan coated well in our study, cell aggregation like spheroid was observed (Fig 1A, 2A, and 4A). Previous studies suggested that adipose derived stem cells (ASC), cornea melanocytes, keratocytes, and monocytes cultured on chitosan substrates lead to cell aggregation and detachment.[45-48] Cheng et al suggested that ASC can form spheroids on chitosan films spontaneously with pluripotency. ASC dissociated from spheroids had higher proliferation and aggregation activity.[45] In our study, the OP9 cells formed spheroid and underwent adipogenic differentiation on chitosan coated wall showed on gene expression (Fig. 1B, 1C, 2C and 2D). However, the quantity and quality of fat vacuoles formed on chitosan coated well were not better in comparison with control TCPS (Fig. 2B).

To increase the efficiency of adipogenic differentiation, we added other ECM such as collagen or/and gelatin in mixture to fabricate different blended chitosan CC, CG, and CGC. When chitosan blends with gelatin, its mechanical properties increased.[13] Chen et al suggested that chitosan/gelatin scaffolds can release gelatin gradually, causing increased chitosan content in the scaffolds, which may lead to ASC agrregation.[49] Hydrophobicity or hydrophilicit of the scaffold surfaces affects cell behaviors and hydrophilicity favors cell growth and adhesion.[50-51] The limit between hydrophilicity and hydrophobicity as a water contact angle was 65°.[52-53] The chitosan film's contact angle was about 86°±2°, showing hydrophobicity, but the one of collagen/chitosan film was about 64°±2°. The blended chitosan film's contact angle was significantly smaller than chitosan film and decrease hydrophobicity.[54] Although cell adhesion is influenced by many factors, the scaffold surface with suitable contact angle seems most important.[55] OP9 cells cultured our CG, CC, and CGC coated wells did not show cell aggregation and form spheroid which shown on our chitosan coated well. However, OP9 cells cultured on CGC differentiated to more fat vacuoles than CC and CG. It may suggest that adding ECM for material surface fabrication will decrease the water contact angle and increase hydrophilicity. Different dosage and type of ECM, fabrication method, and cell type may lead to different cellular behavior, function and fate.

The process of adipogenesis starts from proliferation and differentiation of preadipocytes, then becoming of immature adipocytes, and finally formation of lipid filling fat vesicles, which is concomitant with early and late genes expression.[56] During adipogenesis process in our study, the

early gene FABP4 expression is higher in chitosan coated well but not in CG, CC, and CGC coated wells even after SR induction (Fig 4C). We suggest that the blended chitosan substrates may provide higher adipogenic differentiation ability and let preadipocyte become mature adipocyte and fat vacuoles quickly no more than 3 days. The PPAR gamma gene is a kind of transcription factor and is important for adipogenesis. It expressed higher in chitosan, CG and CGC coated wells. Previous studies reported the expression of PPAR c 2 increased initially and followed by a constant one during adipogenesis.[57-58] This study postulated that some blended chitosan substrates may have more adipogenic potential with PPAR gamma expression in the constant stage. However, late marker GLUT-4 expressed increasing gradually and SR induction in chitosan, CG, CG, and CGC coated wells (Fig 2C and 4C). We suggest GLUT-4 is a critical marker in our study to present dynamic change of the preadipocyte differentiation to mature fat vacuoles. GLUT-4 facilitate glucose metabolism at cell surface.[59] Mutations or over expression of GLUT-4 genes in adipocytes contribute to excess glucose uptake and fat mass formation.[60] Therefore, we suggest GLUT-4 may play a important role in metabolic disease study in the future.

The usage of autologous fat grafts as filling materials for soft tissue defects reconstruction are common in plastic and reconstructive surgery fields.[61-63] Besides, the synthetic and tissue transfer are also alternative choices for soft tissue reconstruction and augmentation.[61-63] Three are some disadvantages about current autologous fat transplantation, including the need of additional surgical procedures and the graft volume reduction later.[61-63] Therefore, MSC may provide a potentially unrestrictive cells for regeneration and adipogenesis with MSC has been proved feasible and accessible. [64] Furthermore, autologous cells can lessen rejection and biocompatible drawbacks.[65] If we could identify the signaling pathway that control the adipogenic differentiation on blended chitosan, we would have more ability to produce adipocytes from our own MSC for soft tissue defects, or we would inhibit adipocyte production for some metabolic disease, such as morbid obesity.

CONCLUSIONS

Chitosan is a cheap and biocompatible substrate and suitable for OP9 cells adipogenic differentiation. It can be blended with different ECM, which lead to different degree of adipogenic differentiation. Furthermore, SR induction has synergic effect with blended chitosan which might provide sufficient adipocytes and would allow implantation in an animal model in the future. Further studies related its signaling pathway needed to be delineated. It may have a therapeutic role in metabolic diseases or graft donation.

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