Autologous decellularized extracellular matrix promotes adipogenic differentiation of adipose derived stem cells in low serum culture system by regulating the ERK1/2-PPARγ pathway

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ABSTRACT

High viability and further adipogenic differentiation of adipose-derived stem cells (ADSCs) are fundamental for engraftment and growth of the transplanted adipose tissue. It has been demonstrated that extracellular matrix (ECM) regulates cell proliferation and differentiation by interacting with ERK1/2 signalling pathway. In this study, we prepared autologous decellularized extracellular matrix (d-ECM) and explored its effect on the proliferation and adipogenic ability of ADSCs in low serum culture. We found that 2% foetal bovine serum (FBS) in growth medium inhibited cell viability and DNA replication, and decreased mRNA and protein levels of PPARγ and C/EBPβ compared with 10% FBS. Correspondingly, after 14-days adipogenic induction, cells cultured in 2% FBS possessed lower efficiency of adipogenesis and expressed less adipocyte differentiation markers ADIPOQ and aP2. On the contrary, the d-ECM-coated substrate continuously promoted the expression of PPARγ, and regulated the phosphorylation of ERK1/2 in different manners during differentiation. Pretreatment with ERK1/2 inhibitor PD98059 neutralized the effects of d-ECM, which suggested d-ECM might regulate the adipogenesis of ADSCs through ERK1/2-PPARγ pathway. In addition, d-ECM was revealed to regulate the transcription and expression of stemness-associated genes, such as OCT4, NANOG and SOX2, in the undifferentiated ADSCs, which might be related to the initiation of differentiation.

Introduction

Adipose derived stem cells (ADSCs) have been widely applied in the field of regenerative medicine because of their easy accessibility and potentials of self-renewal and multidirectional differentiation [1]. It is well known that early transplanted ADSCs will suffer from nutrition deficiency, which negatively influences the survival of stem cells and leads to an unsatisfied effect of cell-assisted lipotransfer [2]. Foetal bovine serum (FBS), full of bioactive proteins and growth factors as well as hormones, is a common medium composition for cell culture. It was demonstrated in the previous study that higher concentration of FBS was accompanied by an increase in PPARγ expression [3]. Epigenetic evidence suggests that, compared with mesenchymal stem cells (MSCs) cultured in autologous serum (AS), cells in FBS exhibit a hypomethylation of histone H3 at lysine 9 (H3K9) in the promoter of Ppary gene and a hyperacetylation of H3K9 in that of adiponectin gene [4]. Therefore, FBS may contribute to a suitable microenvironment for adipogenic gene transcription and subsequent adipogenesis. It is of great significance for clinical applications to promote the proliferation and adipogenesis of ADSCs facing serum restriction.

Extracellular matrix (ECM) is a complex architecture surrounding cells and consists of different proportions of structural proteins, adhesive proteins, and glycans. ECM from diverse sources regulates cell proliferation, migration and differentiation in different manners. Zhang et al. found that ECM secreted from ADSCs cultured in growth medium contained more fibronectin (FN) while that from cells in adipogenic medium was rich in laminin (LM). These two kinds of ECM, respectively, promoted cell migration or adipogenic differentiation of the reseeded cells [5]. Lee et al. found that ECM deposited by Mile Sven 1 endothelial cells (MS1)
delayed cell proliferation but accelerated the process of differentiation in comparison with MSCS-derived ECM [6].

There is accumulating evidence indicating that ECM components can activate the multiple differentiation potential of stem cells. Bone marrow derived mesenchymal cells (BMSCs) that were seeded onto the autologous cell-free extracellular matrix expressed more stem cell surface markers and could differentiate into multlineage cells better once cultured in the differentiation medium [7]. It was reported that autologous ECM permitted the chondrogenic differentiation of BMSCs even without any exogenous growth factors [8]. Compared to the conventional plastic culture materials, the substrates coated with ECM may possess stiffness that is more appropriate for differentiation [9]. In addition, it was reported that the reseeded cells on d-ECM expressed more integrin subunits including α5-7 on their surface, which may influence the intracellular signalling pathways mediating differentiation [5].

Extracellular signal-regulated kinase 1/2 (ERK1/2), as a member of the mitogen activated protein kinases (MAPK), plays a significant role in regulating cell proliferation, migration and differentiation. With variations of the duration and extent of MAPK activation, different stimuli will drive cell fate towards two different developments: proliferation or differentiation [10]. As a downstream target of ERK1/2, PPARγ can in turn phosphorylate ERK1/2 by binding to the EGFR receptor, which enhances the mRNA transcription of PPARγ [11]. The interaction between ERK1/2 and PPARγ may facilitate or hinder adipogenic differentiation under different experimental conditions [12,13]. Given the fact that PPARγ regulates the transcript levels of many adipogenic genes such as adiponectin [14], it is very possible that this protein attends to the ERK1/2-associated adipogenesis.

Activation of ERK1/2 and generation of ECM reinforce each other. Mechanical stress and stretch can prompt cells to secrete more matrix components including collagen (COL) I–IV and FN as well as aggregan by ERK1/2 pathway [15,16]. Recently, it has been demonstrated in a series of studies that ERK1/2 signalling pathway stimulates the production of matrix of cell species including smooth muscle cell and mesangial cell as well as nucleus pulposus cell, thus involved in the pathogenesis of many diseases such as pulmonary hypertension [17], diabetic nephropathy [18] and intervertebral disc degeneration [19]. ECM components, in turn, can influence cellular behaviour by regulating ERK1/2 pathway. A study based on a suspension culture system showed that adding ECM powder increased the ERK1/2 activity and improved the survival of mesenchymal progenitor cells in the early formed spheroids [20]. It was also reported that ECM fabricated by small intestinal submucosa promoted cell proliferation and osteoblastic differentiation of rat BMSCs through Smad1/5/8 and ERK1/2 signalling pathways [21]. Moreover, our previous study reported that autologous decellularized extracellular matrix (d-ECM) not only increased the viability but enhanced adipogenic differentiation of ADSCs exposed to H2O2 [22]. However, whether ERK1/2 pathway is involved in the adipogenic process of ADSCs reseeded on d-ECM still remains unclear.

In this study, cell viability, proliferation and morphology were examined to reveal the effects of d-ECM on ADSCs in low serum culture. Apart from Oil Red O staining, the adipocyte-specific transcription factors PPARγ and C/EPBα kept active during adipogenesis, and adiponectin (ADIPOQ) and adipocyte fatty-acid binding protein (aP2) that express robustly in the mature adipocytes were selected as indicators to evaluate the adipogenic ability of ADSCs, as described in a previous study [23]. Since stem cell status may intervene in the relation between the potential to proliferate and differentiate [24], the expression of pluripotent markers including OCT4, NANOG and SOX2 was examined simultaneously.

Materials and methods

Cell isolation and culture

Adipose tissue was obtained from the liposuction operations performed in the First Affiliated Hospital of Wenzhou Medical University in the period from November 2020 to December. The patients were randomly selected and included five adult females aged from 23 to 45. All patients signed the informed consent for scientific research and publication. Isolation of ADSCs was performed as described in our previous work [25]. Briefly, adipose tissue was fully cut with a scissor and digested using 0.1% collagenase type I (SCR103, Sigma, USA) at 37°C for 40 min. Then, the mixture was sequentially filtered using a 100 and a 200 mesh strainers. The filtrate was collected and centrifuged at room temperature at 250 g for 5 min. Finally, the pellet was resuspended in complete medium (HUXMD-90,011, Cyagen Biosciences, USA) and transferred into a plastic cell culture flask. The cells were cultured at 37°C with 5% CO₂, and observed under a CKX41 inverted phase-contrast microscope (Olympus, JPN). The medium was changed every 3 days, and cells were passaged when reaching 90% confluence. The 3rd passage ADSCs were used for cell
identification and d-ECM preparation, and the 5th passage ones were used for the subsequent experiments.

**Multi-lineage differentiation induction**

In the adipogenesis or osteogenesis assay, cells were, respectively, cultured in Human Mesenchymal Stem Adipogenic Differentiation Medium (supplemented with insulin, dexamethasone, 3-isobutyl-1-methylxanthine and indomethacin) (GUXMX-90,031, Caygen Biosciences, USA) or Osteogenic Differentiation Medium (supplemented with dexamethasone, b-glycerophosphate and L-ascorbic acid-2-phosphate) (GUXMX-90,021, ditto for supplier) for 2 weeks. Then the cells were fixed with 4% paraformaldehyde and stained with Oil Red O or Alizarin Red according to the manufacturers’ instructions.

For the chondrogenesis assay, cells were transferred into a 15-ml centrifuge tube and were centrifuged for 4 min at 250 g at room temperature. Then, the intact cell pellet was carefully transferred into Human Mesenchymal Stem Chondrogenic Differentiation Medium (supplemented with dexamethasone, ascorbate, insulin-transferrin-selenium supplement, sodium pyruvate, proline, TGF-β3) (GUXMX-90,041, Caygen Biosciences, USA). After 3 weeks, the firm cell spheroid formed was fixed and sectioned, followed by Alcian Blue staining. The samples were imaged under an OLYMPUS IX71 phase contrast microscope (Olympus Corporation, JPN).

**Flow cytometry**

The primary antibodies against human cluster of differentiation (CD) markers including CD34 (ab195013, Abcam, UK), CD44 (103,011, BioLegend, USA), CD45 (555,485, BD Biosciences USA) and CD90 (555,595, BD Biosciences, USA), and FITC-conjugated goat anti-rabbit secondary antibody were used in flow cytometry to analyze the profiles of cell surface immunophenotype. Briefly, ADSCs reaching 80–90% confluence were digested with 0.25% Trypsin and washed in PBS twice. Then, the cells were resuspended at a concentration of 1x10^6/ml and incubated in PBS containing 2% bovine serum albumin (B2064, Sigma, USA) and 0.1% sodium azide (S2002, Sigma, USA) for 30 min at 4°C. A total of 100 µL of cell suspension for each sample was incubated in primary antibodies (1:10 dilution) in the dark for 30 min at 4°C, followed by the incubation with secondary antibody (1:100 dilution) under the same condition. Then, the cells were washed in PBS twice and analysed using a FACSVersa flow cytometer (BD Biosciences, USA).

**Preparation of decellularized extracellular matrix (d-ECM)**

The extracellular matrix deposited by ADSCs was obtained according to the scheme we established before [22]. Briefly, ADSCs were collected and seeded at a density of 1 × 10^5 or 5x10^4/well in 12- or 24-well plates (Corning, USA) in complete medium. When the confluence was over 60%, 50 µM L-ascorbic acid (HY-B0166, MedchemExpress, USA) was added to stimulate matrix production [7]. The medium was changed every 3–4 days, and was discarded one week later. After washed with PBS twice, the samples were decellularized by incubating them in PBS containing 0.5% Triton X-100 (P1080, Solarbio, CHN) and 20 mM NH4OH (G1822, Solarbio, CHN) for 5 min at 37°C. Afterwards, the samples were washed gently with sterile enzyme-free water (R1600, Solarbio, CHN) three times and were treated with 100 U/ml DNase I (D8071, Solarbio, CHN) for 1 h at 37°C. Following a final three washes, the d-ECM samples were observed under a phase contrast microscope and then stored sterile at 4°C for later use.

**Immunofluorescence**

The samples treated with or without decellularization were washed with PBS twice and fixed with 4% formaldehyde for 15 min. The samples were blocked with 2% BSA (B2064, Sigma, USA) in PBS for 30 min at room temperature, and were then incubated with anti-fibronectin (250,073, ZENBIO, CHN) and anti-laminin (384,832, ZENBIO, CHN) primary antibodies diluted 1:200 in PBS overnight at 4°C. Sequentially, the samples were incubated with goat anti-mouse/rabbit secondary antibody (BYE010/BYE019, Boyun, CHN) for 1 h at room temperature. After washed with PBS three times, the samples were counterstained with 4,6-diamidino-2-phenylindole (DAPI) (C0065, Solarbio, CHN) and imaged under a DM2500 fluorescent microscope (Leica, GER).

**Cell Counting Kit-8 (CCK-8) assay and cell counting assay**

For the CCK-8 assay, ADSCs were inoculated at 5x10^3/well in 24-well plates coated with or without d-ECM in 500 µL medium containing 0% or 2% foetal bovine serum (FBS, GUXMX-90,031, Caygen Biosciences, USA), and then cultured for different time (1, 2, 3, 4 days). The cells growing on the plastic substrates in 10% FBS were used as control group. After the indicated culture time, cells in each well were washed with
PBS twice and then incubated in 500 μL serum-free medium containing 10% CCK-8 regent (CK04, Dojindo Molecular Technologies, JPN) for 4 h at 37°C. Sequentially, the plates were sufficiently shaken, and a total of 400 μL mixture for each sample was equally transferred into 4 wells of a 96-well plate. Absorbance values at 450 nm were measured using a SPECTRA max 384 microplate reader (Molecular Devices Corporation, USA) and were then normalized by control group.

For cell counting, cells were seeded at 5x10^4/well on the plastic or d-ECM substrates of 24-well plates and then cultured in 2% or 10% FBS for 3 days. Afterwards, cells were collected and counted using a Countess II Automated Cell Counter (Thermo Fisher Scientific, USA).

**EdU (5-ethynyl-2′-deoxyuridine) staining**

ADSCs were treated as described above. Cell-Light Edu Apollp567 In Vitro Kit (C10310, RiboBio, China) was used in this experiment. Briefly, cells in each well of a 24-well plate were washed with PBS three times and incubated in 200 μL serum-free medium containing 50 μM EdU for 2 h at 37°C. Then, the cells were washed three times to remove the residual EdU. After that, cells were fixed with 4% paraformaldehyde for 30 min at room temperature and then stained with 200 μL 1x Aopll67 dye in the dark for 30 min at room temperature. Sequentially, cells were treated with 0.5% TritonX-100 diluted in 100 μL PBS and were then counterstained using 200 μL 1x Hoechst33342 for 30 min. Afterwards, the samples were washed with PBS three times and observed under an ECLIPSE TI fluorescent inverted microscope (Nikon, JPN). The result was expressed as the percentage of EdU-positive cells.

**Assessment of ADSCs morphology**

The long/short axis ratio of cells was calculated to quantitatively evaluate the change of the morphology of ADSCs treated with low-serum culture or d-ECM. For each group, 10 representative cells with unmasked contour were selected at 12 h or 72 h of culture, respectively. According to a previous study [6], the ratios of long axis to short axis of the cells of each sample were calculated using ImageJ 1.8.0 software (National Institutes of Health, USA). The extreme values including the greatest and the smallest values were removed.

**Real-Time Polymerase Chain Reaction (RT-PCR)**

Gene expression was quantitatively analysed by qRT-PCR. Briefly, the total RNA was extracted from the ADSCs reseeded onto plastic or d-ECM using Trizol Reagent (15,596,018, invitrogen, USA) following the manufacturer’s instructions. RNase-Free DNase I (D8071, Solarbio, CHN) was used to remove residual DNA in the sample. Then, reverse transcription was performed using a RevertAid First Strand complementary DNA (cDNA) Synthesis Kit (1622, Thermo, USA). Real-time PCR consisting of initial denaturation (94°C, 10 min) and subsequent 40 cycles (denaturation, 94°C, 20 s; annealing, 55°C, 20 s; extension, 72°C, 20 s) was carried out with 20-fold diluted cDNA sample and 2x SybrGreen qPCR Master Mix (F-415XL, Thermo, USA) in an ABI-7500 Real-Time System (Applied Biosystems, USA). The relative expression levels of target genes were calculated by 2^(ΔΔCT) method with GAPDH as calibrator gene and were normalized to control group (10% FBS). The primer sequences used in the PCR are listed in Table 1.

**Western blotting analysis**

The protein samples used for this experiment were prepared in two different ways. For matrix component analysis, samples with or without decellularization were lysed in RIPA buffer (Solarbio, CHN) containing 1% phenylmethylsulfonyl fluoride (P0100, Solarbio, CHN) and 1% phosphatase inhibitor (P1260, Solarbio, CHN). In order to remove the cellular debris, the lysate was centrifuged at 14,000 g for 11 min and then boiled in 4x loading buffer (P1015, Solarbio, CHN) for 5 min. For protein analysis, cells were seeded at 1x10^5/well on the plastic or d-ECM substrates of 12-well plates in medium containing 2% FBS, and then cultured for 3 days. In order to inhibit ERK1/2 signaling pathway, 50 μM PD98059 (HY-12,028, Medchemexpress, USA) dissolved in 0.1% dimethyl sulfoxide (DMSO) was added at least 2 h prior to

| Table 1. Primer sequence of human genes used in this study. |
|-----------------|-----------------|------------------|
| **Gene** | **Primer sequence** | **Product size (bp)** |
| NANOG | 5′-CAGAGAAGATAGTGACAAAGGA-3′ | 165 |
| | 5′-TGGAGCTGATGATGAGTTC-3′ | |
| SOX2 | 5′-GCCAGATGCGCTCCAGCCTAC-3′ | 170 |
| | 5′-GGGCTGCGTACGGCAAGCC-3′ | |
| OCT4 | 5′-GCAGAAGGAGCGATGACCA-3′ | 174 |
| | 5′-GGAAAGGACGGAGGATAC-3′ | |
| PPARy | 5′-GAAGGGGAGGAGGATACG-3′ | 163 |
| | 5′-TGAGGCTCTTAAATGGACCA-3′ | |
| C/EPB | 5′-CCTGCGGCAAGAAGCCGAGC-3′ | 227 |
| | 5′-CCTGCGGCAAGAAGCCGAGC-3′ | |
| GAPDH | 5′-AGAAGGGGGCTGACCTTTG-3′ | 258 |
| | 5′-AGGGGCGCATCCAGTTC-3′ | |

F: forward direction; R: reverse direction.
the inoculation. Other groups were treated with the same amount of DMSO. The cells cultured in complete medium (10% FBS) were used as control group. Then, cells were harvested for protein extraction. A BCA kit (P0012, Beyotime, CHN) was used to quantify the protein concentration of samples among all groups. Sequentially, protein samples were separated by sodium salt-polyacrylamide gel electrophoresis, followed by Western blotting analysis according to a previous study [5]. Protein bands were visualized using an enhanced chemiluminescence substrate (PE0010, Solarbio, CHN) in a ChemiDoc MP imager (Bio-Rad, USA), and then analysed by Image Lab V3.0 software (Bio-Rad, USA). Equal loading of proteins was demonstrated by GAPDH, and all the band density values were normalized by control group.

The primary antibodies used in this experiment were anti-fibronectin (1:1000, 250,073, ZENBIO), antilaminin (1:1000, 384,832, ZENBIO), anti-collagen I/III/IV (1:1000, 380,760/R23957/252,404, ZENBIO), anti-OCCT (1:1000, K005554P, Solarbio), anti-Sox2 (1:1000, KO08063P, Solarbio), anti-NANOG (1:1000, KO03637P, Solarbio), anti-p-ERK1/2 (1:1000, AF1015, Affinity), anti-ERK1/2 (1:1000, AP0155, Affinity), anti-p-PPARγ (1:1000, AF3284, Affinity), anti-PPARγ (1:1000, AF6284, Affinity), anti-C/EPBβ (1:1000, AF6333, Affinity), anti-ADIPOQ (1:1000, DF7000, Affinity), anti-aF2 (1:1000, AF0535, Affinity), and anti-GAPDH (1:2000, 380,626, ZENBIO). The secondary antibody was used was HRP-conjugated goat anti-rabbit/mouse IgG (1:2000, SE134/SE131, ZENBIO).

**Oil Red O staining**

After 3-days treatment and 14-days adipogenic induction, cells were fixed with 4% paraformaldehyde and stained with 0.5% Oil Red O solution in the dark for 1 h. The samples were rinsed with 60% isopropanol and photographed under a phase contrast microscope. For each group, three samples were stained and examined. Relative stained area (Area%) and the ratio of integrated density to stained area (IntDen/Area) were calculated for analysis. Afterwards, the samples were destained in 100% isopropanol for 15 min. The supernatant was collected and optical density at 540 nm (OD540nm) was measured using a DU-800 spectrophotometer (BECKMAN, USA).

**Statistical analysis**

Each experiment in this study was repeated three times. All data, expressed as the means ± SD, were compared by one-way ANOVA with Tukey’s post hoc test using SPSS Statistics 20.0 software (IBM, USA). P-values <0.05 were considered as statistically significant.

**Results**

**Identification of ADSCs**

Morphological phenotype, cluster of differentiation markers and multilineage differentiation potential were determined to identify ADSCs. The 3rd passage cells cultured in complete medium for 3 days appeared spindle-shape and arranged in swirls (Figure 1(a)), which was regarded as a morphological signature of mesenchymal stem cells [26]. After stained with Oil Red O, Alizarin Red and Alcian Blue, the cells cultured in differentiation medium for 2 or 3 weeks were positive for lipid droplets, calcified nodules and acid mucopolysaccharides, which suggested the success of adipogenesis, osteogenesis and chondrogenesis, respectively (Figure 1(a)). Consistent with our previous study [27], the result of flow cytometry showed that the isolated cells were positive for CD44 (99.6%) and CD90 (97.7%) while negative for CD34 (1.6%) and CD45 (1.7%) (Figure 1(b)), which demonstrated these pluripotent cells were non-haematopoietic mesenchymal cells.

**Characterization of d-ECM**

In our previous work, observation of d-ECM using a scanning electron microscope revealed a fibrous structure that was made up of many cords with the same direction [22]. In this study, we also noticed a similar structure under an inverted microscopes (Figure 2(a)). Immunofluorescence results showed that the fluorescence intensity of LM and DAPI decreased significantly after decellularization, while that of FN did not change obviously. Furthermore, FN was mainly located in the intercellular matrix while LM was distributed more closely to cells (Figure 2(b)), which suggested that LM might be lost during the process of decellularization. The results of Western blotting analysis showed that compared with non-decellularized matrix, d-ECM retained almost all FN and COL I, and partial COL III, COL IV and LM. It seemed that intracellular substance was fully eliminated as GAPDH was not present in the d-ECM samples prepared (Figure 2(c)).

**Cell viability and proliferation of ADSCs in growth medium**

As showed in the CCK-8 assay, compared with control group (10% FBS), low serum concentrations (0% and
2% FBS) progressively decreased cell viability as the duration of starvation increased, and the trend became stable since day 3 post inoculation. Compared to the plastic material, d-ECM significantly increased cell viability of ADSCs cultured in 2% FBS for 3 days (from $57.9 \pm 2.27\%$ to $72.9 \pm 3.78\%$), but did not influence those in 0% FBS whose viability was decreased severely (Figure 3(a)). Consistent with the result of CCK-8 assay, cell number counting showed that the d-ECM-coated substrates provided more cells (68.6 ± 5.17%) than the plastic ones (52.2 ± 3.54%) on day 3 post treatments (Figure 3(b)). Based on the data given above, we used 2% FBS and 3-days culture as the conditions for low serum treatment in the subsequent experiments. In addition, the percentage of EdU-positive cells in 2% FBS plus d-ECM group was increased while no obvious DNA replication occurred in 2% FBS-only group (Figure 4(a) and 5), indicating an accompanying change in cell proliferation.

**Morphological change of ADSCs before and after differentiation**

Before the adipogenic induction, we found that the ratios of long/short axis of ADSCs increased on the whole with culture time increased. The long/short axis ratios of 2% FBS group were more concentrated and had no significant difference with 10% FBS group. Compared to the other two groups, ADSCs reseeded on d-ECM possessed larger morphological polarity at both 12 h and 24 h (Figure 3(d)). Although it was difficult to quantify cell morphology after adipogenesis, many ‘normal’ cells with spindle-shape could be observed in 10% FBS group and d-ECM group. However, ADSCs in 2% FBS group were more slender than the other groups, which was exactly opposite to the situation before differentiation. In addition, the cell density was significantly decreased, and the spindle shape was absent in PD98059 pretreatment group (Figure 6(a)).

**Expression of stemness-associated genes of undifferentiated ADSCs**

RT-PCR detection revealed that the transcription of NANOG, OCT4 and SOX2 genes was decreased in 10% FBS group in a time-dependent way, but was increased in 2% FBS group. Compared to 2% FBS group, a similar but weaker uptrend was observed in d-ECM treatment group (Figure 4(a)). Western blotting analysis before differentiation confirmed that the protein expression levels of NANOG, OCT4 and SOX2 were increased in 2% FBS group and fallen back by d-ECM treatment (Figure 4(b)), which was consistent with the result of RT-PCR.

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**Figure 1.** Identification of ADSCs. A. Adipogenesis, osteogenesis and chondrogenesis of the 3th passage ADSCs. The panel on the far left presented the morphology and arrangement of the uninduced ADSCs, and the other three ones showed the lipid droplets, calcified nodules and acid mucopolysaccharides in differentiated cells, respectively (marked by the arrows). Bars = 100 μm. B. ADSCs were immunophenotyped for CD34 (a), CD44 (b), CD45 (c) and CD90 (d) by flow cytometry.
Phosphorylation of ERK1/2 and expression of adipogenesis-associated genes and proteins before and after differentiation

Before adipogenic induction, as shown in (Figure 4), PPARγ and C/EPBα mRNA levels among groups did not differ significantly until day 2. Low serum culture (2% FBS) down-regulated the expression levels of mRNA over time, and d-ECM significantly relieved this decrease (Figure 5(a)). The results of Western blotting confirmed the effects of low FBS concentration on the expression of PPARγ and C/EPBα, and d-ECM treatment alleviated the 2%-FBS-inducing inhibition of PPARγ expression. As a selective inhibitor of ERK1/2, PD98059 inhibited the phosphorylation of ERK1/2, accompanied by a decrease in PPARγ expression. However, it seemed that C/EPBα protein expression...
Figure 3. Effects of serum-restriction and d-ECM on cell viability, proliferation and morphology of ADSCs. A. ADSCs at the 5th passage were cultured in medium containing 0% or 2% FBS on the plastic or d-ECM-coated surface for 1–5 days, followed by a CCK-8 assay. Cells cultured in complete medium (10% FBS) on the plastic surface were used as control group. N = 4. **, p < 0.01, 0% or 2% FBS group vs. 10% FBS group; ##, p < 0.01, vs. 2% FBS group. B. Numbers of ADSCs treated with 2% FBS or d-ECM for 3 days were counted using an automated cell counter. N = 3. **, p < 0.01, vs. 10% FBS group; #, p < 0.05, vs. 2% FBS group. C. Cells were grouped according to the ‘cell counting’ experiment, followed by EdU staining and Hoechst33342 counterstaining. Red-stained nuclei appeared in the EdU-positive cells (marked by the arrows), which represented a high level of DNA replication. N = 3. *, p < 0.05, vs. 10% FBS group; **, p < 0.01, vs. 10% FBS group; ##, p < 0.01, vs. 2% FBS group. Bars = 200 μm. D. Cells were treated as above, and observed under an invert microscope at 12 h and 72 h post treatments. Bars = 100 μm. *, p < 0.05, vs. 10% FBS group; #, p < 0.05, vs. 2% FBS group; ##, p < 0.01, vs. 2% FBS group. FBS, foetal bovine serum. EdU, 5-ethynyl-2’-deoxyuridine.
was regulated neither by d-ECM nor by PD98059 (Figure 5(b)).

Following adipogenic differentiation, Western blotting was performed again for further analysis. Compared to control group (10% FBS), the expression levels of ERK1/2 and PPARγ decreased in 2% FBS group, while the phosphorylation of both proteins increased. Reseeding cells on the substrates coated with d-ECM reversed the effects of serum-restriction. On the basis of 2% FBS and d-ECM, pretreating cells with PD98059 unexpectedly upregulated the phosphorylation of ERK1/2, accompanied by the decreased PPARγ expression. In addition, the expression of adiponectin and aP2 was highly consistent with PPARγ under aforementioned conditions (Figure 7). Given the above, we discovered an interesting phenomenon that there was an opposing relationship between the ERK1/2 phosphorylation of undifferentiated and fully differentiated ADSCs.

Figure 4. Expression of stemness-associated genes. A. ADSCs at the 5th passage were inoculated into plates with plastic or d-ECM substrates, and were then cultured in 2% FBS for 3 days in total. Cells cultured in 10% FBS served as control group. The transcription of OCT4 (a), SOX2 (b) and NANOG (c) genes was determined by RT-PCR every day. N = 3. * p < 0.05, vs. 10% FBS group; **, p < 0.01, vs. 10% FBS group; #, p < 0.05, vs. 2% FBS group; ##, p < 0.01, vs. 2% FBS group; &, p < 0.05, vs. 10% FBS group on day 1; &&, p < 0.01, vs. 10% FBS group on day 1. B. The expression of OCT4, SOX2 and NANOG proteins was determined by Western blotting on day 3. Equal loading of proteins was confirmed by GAPDH. N = 3. *, p < 0.05, vs. 10% FBS group; **, p < 0.01, vs. 10% FBS group; ##, p < 0.01, vs. 2% FBS group. OCT4, octamer-binding transcription factor-4; SOX2, SRY-related high-mobility-group-box protein-2; NANOG, Nanog homeobox protein.
Differentiation of ADSCs into adipocytes

Compared to control group (10% FBS) and d-ECM group, lipid droplets resembling refractile spheres were almost disappeared in 2% FBS group, and were less but bigger in PD98059 pretreatment group (Figure 6(a)). The result of Oil red O staining showed that there were more fat globules visible when supplemented with 10% FBS or with d-ECM (Figure 6(a)). Relative stained area, mean staining intensity (IntDen/Area) and the absorbance at 540 nm were determined to quantitatively evaluate the adipogenic ability of ADSCs. The decline of these indexes induced by low serum was ameliorated by d-ECM, but aggravated once again by the addition of PD98059 (Figure 6(b)).


Figure 6. Adipogenic induction and Oil Red O staining. A. As described above, the 5th passage ADSCs with or without PD98059 pretreatment were seeded on the plastic or d-ECM substrates and then cultured in 2% FBS for 3 days, followed by 14-days adipogenic induction and subsequent Oil Red O staining. Cells cultured in complete medium were regarded as the control group, N = 3. Bars = 100 μm. B. For each group, relative stained area (Area%), a, mean staining intensity (IntDen/Area, b) and optical density at 540 nm (ODS40nm, c) after destaining were determined to evaluate the adipogenic ability of ADSCs. **, p < 0.01, vs. 10% FBS group; #, p < 0.05, vs. 2% FBS group; ##, p < 0.01, vs. 2% FBS group; $$, p < 0.01, vs. 2% FBS + d-ECM group.

Discussion
It was demonstrated in our previous study that the d-ECM substrate could protect ADSCs against H2O2-induced senescence and stimulate cell proliferation [22]. As a natural substance secreted by autologous cells, d-ECM is very safe and has tremendous potential for usage in regeneration medicine [28]. In this study, we reproduced d-ECM by decellularization using the aforementioned method. We found that d-ECM increased viability and proliferation of the reseeded ADSCs in low serum culture (Figure 3(a-c)), which was in agreement with previous results [29]. It has been demonstrated that bioactive components in extracellular matrix can promote cell proliferation and differentiation through ERK1/2 signalling pathway [20,21,30]. In addition, the ERK1/2 pathway is proved to be required for serum-stimulated DNA synthesis [31], and sustained activation of ERK1/2 is related to the mitogenic potential of growth factors [32]. Therefore, we hypothesize that the ERK1/2 pathway may be involved in the positive effect of d-ECM on serum-restricted ADSCs.

Under the condition without any interference (10% FBS), the transcript levels of stemness-associated genes were decreased over time (Figure 4(a)). In contrast, the adipogenic genes increased during this process (Figure 5(a)). It suggested that there was a tendency of spontaneous adipogenesis in ADSCs cultured in growth medium, which was supported by a previous study where the expression of PPARγ in MSCs relied on serum rather than any differentiation inducers [3]. Moreover, we found that serum-restriction decreased this tendency while d-ECM increased it. At the beginning of adipocyte differentiation process, preadipocytes will undergo a proliferative period called mitotic clonal expansion (MCE) [33]. Yu et al. found that arresting
cell cycle and blocking MCE by inula britannica could prevent early differentiation of 3T3-L1 preadipocytes [34]. In the present study, we demonstrated that proliferation and cellular polarity of the uninduced ADSCs reseeded on d-ECM were increased (Figure 3(c,d)), which suggested a possible acceleration of MCE. However, from the perspective of McBeath et al. [35], excessive polarity in the late stage of differentiation was regarded as a feature of poor differentiation, which was consistent with what was observed in ADSCs in the 2% FBS group (Figure 6(a)).

In the present study, it was demonstrated that ADSCs reseeded on d-ECM continuously expressed PPARγ at higher level compared to those on the plastic substrates whether the cells were adipogenically induced or not (Figure 5(b), Figure 7). The expression of aP2 and adiponectin, and the adipogenic differentiation of ADSCs were also facilitated by d-ECM treatment (Figure 6, Figure 7). However, the changes of ERK1/2 phosphorylation were opposite before and after adipogenic differentiation (Figure 5(b), Figure 7). This inconsistence was reflected in the previous studies where the ERK1/2 pathway regulated cell adipogenesis differently. On the one hand, it was reported that activation of the ERK1/2 positively regulated the expression of PPAR and C/EPB and promoted adipogenic differentiation of preadipocytes or MSCs [36,37]. On the other hand, Font de Mora et al. discovered that the phosphorylation of ERK1/2 was not necessary for adipogenesis and even antagonized it [38].

The modulation effects of ERK1/2 on cell proliferation and differentiation are stage-dependent, and phosphorylation of ERK1/2 at different time points produces different adipogenic efficiency [39]. Engelman et al. discovered that activation of MAPK including ERK1/2 only occurred in the early stage of adipogenesis (PMID: 9,822,687). Up-regulation of ERK1/2 pathway at this time can increase PPARγ expression and initiate adipogenic differentiation, and vice versa [40,41]. On the contrary,
the dephosphorylation of ERK1/2 happens spontaneously in the later stages. This inactivation can be induced by dual specificity phosphatase 1 (DUSP1) [42]. Sustained phosphorylation of ERK1/2 inhibits protein expression of PPARγ through phosphorylating it at Serine-82 and 112 sites, thereby hindering adipogenic differentiation [43,44]. Based on the above findings and our data, we speculate that d-ECM may promote PPARγ expression and accelerate adipogenesis by successively increasing and decreasing ERK1/2 phosphorylation during the induction.

In addition, we noticed that PD98059 inhibited ERK1/2 activation and PPARγ expression in the uninduced ADSCs (Figure 5(b)), which was consistent with the previous study [3]. However, the phosphorylation of ERK1/2 in the terminally differentiated cells was significantly increased in PD98059 pretreatment group (Figure 7), despite the inhibited adipogenesis. Meanwhile, we found that the corresponding cells had rounder bodies, contained bigger lipid droplet (Figure 6(a)), and expressed less adiponectin and adip2 proteins (Figure 7). These phenomenon suggested the formation of hypertrophied adipocytes, whose ERK1/2 phosphorylation level was higher than the normal ones [45]. Therefore, we speculate that the early inhibition of ERK1/2 by PD98059 might result in abnormal adipogenic differentiation and a delayed compensatory increase in ERK1/2 phosphorylation.

This study has several limitations. Firstly, different from the result of RT-PCR, Western blotting analysis showed that the protein expression of C/EPBα in uninduced ADSCs was not significantly influenced by d-ECM. Also, as one of the downstream targets of ERK1/2 [46], it was not inhibited by PD98059 (Figure 5(b)). It remains to be proved that whether the post-transcriptional regulation and other signalling pathways are involved in the process. Secondly, as an active ingredient mediating cell adhesion to extracellular matrix, laminin (LM) was reported to regulate the osteogenic differentiation of dental follicle cells by targeting integrins α2 and β1 [47]. Decellularization in this study significantly reduced the LM content (Figure 2), which possibly impaired the LM-associated effect on the adipogenesis of ADSCs. In addition, it was demonstrated that other collagen species and glycoproteins as well as various growth factors existed in the decellularized adipose tissue (DAT) [48]. Mechanisms underlying the effects of these bioactive molecules need to be confirmed in the future experiments. Last but not least, we found that there were an early alternation between the transcriptions of pluripotent genes and adipogenic genes (days 1–3), and a subsequent inconsistency of ERK1/2 phosphorylation during the adipogenesis of ADSCs (day 3 vs. day 14). However, Pauken et al. discovered that many matrix components including COL1, COL4, FN and LM could obviously up-regulate ERK1/2 phosphorylation within 90 min, which wore off in the later periods [49]. Therefore, precise determination of ERK1/2 phosphorylation at more and earlier time points may help to further reveal the role of ERK1/2 pathways on the d-ECM-induced adipogenesis promotion of ADSCs.

In conclusion, we demonstrated that autologous d-ECM ameliorated adipogenic differentiation of ADSCs in low serum culture by a stage-dependent regulation on ERK1/2-PPARγ pathway. We believe this result support an idea that increasing ECM components around cells may bring the superiority of ADSCs into full play in the field of regeneration medicine.

Disclosure statement

The authors declare that they have no conflicts of interest to report regarding the present study.

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Ethics approval and consent to participate

This study was approved by the Research Ethics Committee of Wenzhou Medical University.

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