Proteomics reveals the potential mechanism of Tanshinone IIA in promoting the Ex Vivo expansion of human bone marrow mesenchymal stem cells

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A B S T R A C T

Introduction: Bone marrow mesenchymal stem cells (BMSCs) are a promising cell type for tissue engineering, however, the application of BMSCs is largely hampered by the limited number harvested from bone marrow cells. The methods or strategies that focused on promoting the capacity of BMSCs expansion ex vivo become more and more important. Tanshinone IIA (Tan IIA), the main active components of Danshen, has been found to promote BMSCs proliferation, but the underlying mechanism is still unclear. The aim of this study is to explore the effect and underlying mechanism of Tan IIA on the expansion capacity of hBMSCs ex vivo.

Methods: In this present study, the effect of Tan IIA on the expansion capacity of BMSCs from human was investigated, and quantitative proteome analysis was applied furtherly to identify the differentially expressed proteins (DEPs) and the molecular signaling pathways in Tan IIA-treated hBMSCs. Finally, molecular biology skills were employed to verify the proposed mechanism of Tan IIA in promoting hBMSCs expansion.

Results: The results showed that a total of 84 DEPs were identified, of which 51 proteins were upregulated and 33 proteins were downregulated. Besides, Tan IIA could promote hBMSCs proliferation by regulating the progression of S phase via increasing the release of fibroblast growth factor 2 (FGF2), FGF-mediated PI3K/AKT signaling pathways may play an important role in Tan IIA’s effect on hBMSCs expansion.

Conclusions: This study employed molecular biology skills combined with quantitative proteome analysis, to some extent, clarified the mechanism of Tan IIA’s effect on promoting hBMSCs proliferation, and will give a hint that Tan IIA may have the potential to be used for BMSCs applications in cell therapies in the future.

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Abbreviations: BMSCs, bone marrow mesenchymal stem cells; Tan IIA, Tanshinone IIA; FGF1/2, fibroblast growth factor 1/2; DEPs, differentially expressed proteins; DMSO, dimethyl sulfoxide; MTT, Three-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; EdU, 5’ Ethynyl 2’ deoxyuridine; TMT, tandem mass tags; TEAB, triethylammonium bicarbonate; HPLC, high performance liquid chromatography; LC-MS, liquid chromatography-mass spectrometry; FDR, false discovery rate; FC, Fold Change; GO, Gene Ontology; COG, Clusters of Orthologous Groups of proteins; KEGG, Encyclopedia of Genes and Genomes; PPI, protein–protein interactions; PCNA, proliferating cell nuclear antigen; FGFR, FGF receptor; CDK, cyclin-dependent kinase.

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1. Introduction

Bone marrow mesenchymal stem cells (BMSCs), one type of important non-hematopoietic stem cells, derive from the early stage of mesoderm and ectoderm in embryonic development and distributes widely throughout the body [1,2]. Due to the unique characteristics of highly self-renewal, multipotency and immune regulation, BMSCs have provided a promising future in the field of tissue engineering and regenerative medicine [3–5]. During the past two decades, a great deal of studies has reported satisfactory progresses in clinical research on BMSCs, such as for the treatment of liver diseases, immune system diseases, and even nervous system diseases [6–10]. However, the clinical application of BMSCs is largely hampered by the fact that the number of BMSCs harvested of liver diseases, immune system diseases, and even nervous system diseases past two decades, a great deal of studies has reported satisfactory characteristics of highly self-renewal, multipotency and immune tissue engineering and regenerative medicine [3–5].

Currently, a number of approaches have been developed in order to enhance the proliferation of BMSCs ex vivo, such as modulation in oxygen or glucose, modification in culture medium or regulation the culture substrate [17,18]. Recently, it is notable that certain small compounds have been found to have an expansion or ameliorate the senescence of BMSCs. For example, ZnCl2 was found to be used for extensive expansion of BMSCs in aged populations [19]. And, curcumin, the compounds from herbs, was reported to alleviate the senescence of BMSCs in vitro by activating the autophagy [20]. Moreover, it is found that A83-01, a small molecule inhibitor of transforming growth factor-β receptor, promoted proliferation of BMSCs [21]. And, the combination of A83-01 and fibroblast growth factor 2 (FGF2) was also applied to augment cell expansion, reduce cell apoptosis, and even magnify stemness makers expression in BMSCs [22]. All these results may provide new methods or novel strategies for the culture expansion of BMSCs ex vivo, indicating the potential for cell therapies and regenerative medicine in BMSCs applications in the future.

_Salvia miltiorrhiza Bunge_ (also known as Danshen), the traditional Chinese herbal medicine, has been reported to have therapeutic effects on cardiovascular, nervous system diseases, hepatitis and liver fibrosis, etc. [23–25]. In these years, the effects of Danshen on the capacity of BMSCs expansion attained promising results. Danshen injection, the aqueous extracts of _S. miltiorrhiza_, was found to enhance the cell viability and DNA synthesis in adult stem cells of mice [26]. It is also showed that the aqueous extracts of Danshen markedly improved cells viability of BMSCs, and thus improved the recovery of ischemic stroke in rats [27]. It is notable that Tanshinone IIA (Tan IIA), the main active components of Danshen, was also reported to promote the proliferation of BMSCs, but the underlying mechanism is still not very clear [28]. Quantitative proteomics is a powerful tool to explain the mechanism of complex life activities, especially in traditional medicine research [29–31]. Nowadays, the combination of proteomics methods and biological techniques provides a new way to gain insight into target mechanisms of medicine monomers.

In this present study, the effects of Tan IIA on the expansion capacity of BMSCs was investigated using BMSCs from human, and quantitative proteome analysis was applied furtherly to identify the differentially expressed proteins (DEPs) and the molecular signaling pathways in Tan IIA-treated hBMSCs. Finally, molecular biology skills were employed to verify the proposed mechanism of Tan IIA in promoting hBMSCs expansion. The aim of this study is to explore the effect and underlying mechanism of Tan IIA on the expansion capacity of hBMSCs ex vivo.

2. Materials and methods

2.1. Cell culture and treatment

The hBMSCs were kindly provided by Key Laboratory of Biotherapy and Regenerative Medicine, Gansu Province. Cells were cultured in DMEM/F12 medium supplemented with 10% fetal bovine serum (Serana, Germany) and 1% streptomycin and penicillin (Hyalone, USA), and maintained under standard conditions at 37 °C in a humidified air containing 5% CO2. For cells treatment, Tan IIA (Purity >98%, Must Bio-Technology Co., Ltd., China) and CH5183284/Debio-1347 (Purity >98%, InvivoChem, USA) was freshly diluted with dimethyl sulfoxide (DMSO), the stock solution (100 mM) was stored at −20 °C until used. To evaluate the effect of Tan IIA on hBMSCs, Tan IIA stock solution was further diluted with culture medium into the final concentration of 0.001–10 μM. To investigate the inhibitory effects of Debio-1347 on FGF receptor, hBMSCs were pretreated with Debio-1347 (0.1, 0.5, 1 or 5 μM) for 30 min [32] prior to the application of Tan IIA.

2.2. Cell viability assay

Three-(4,5-dimethylthiazol-2-yl)-2,5-diphenytetrazolium bromide (MTT) assay was performed to assess the cell viability. Cells were seeded onto 96-well plates, after incubated overnight, the cells were cultured with Tan IIA (0.001–10 μM) for 24 h. Afterwards, fresh MTT solution (0.5 mg/ml, Beyotime, China) was added and reincubated for another 4 h. Then, 150 μl DMSO was added to dissolve the formazan crystals product, and a microplate reader (wavelength, 490 nm, Thermo Fisher Scientific, USA) was used to measure the absorbance value and to record the obtained results.

2.3. 5′ Ethynyl 2′ deoxyuridine (EdU) assay

The effect of Tan IIA on the proliferative activity of hBMSCs was evaluated using BeyoClick™ EDU 488 imaging Kit (Beyotime, China). Cells were seeded onto 96-well plates, treated with Tan IIA (0.1, 1, and 10 μM) for 6, 12, 24, 36 or 48 h or Tan IIA (1 μM) plus Debio-1347 (0.1, 0.5, 1 or 5 μM) for 24 h, respectively. After that, 20 μM Edu was added to each well, incubated for 2 h at 37 °C, fixed with 4% paraformaldehyde, and permeabilized with 0.3% Triton X-100 for 15 min. Cells were washed with 3% BSA, and then incubated in click reaction solution in the dark for another 30 min. Finally, the absorbance value was measured at a maximum excitation wavelength of 495 nm with maximum emission wavelength of 519 nm.

2.4. Proteomics sample preparation

For establishing the proteomics model, hBMSCs were cultured in 60-mm dishes (triplicate of each model) and treated with Tan IIA (1 μM, experimental samples) or blank control (culture medium, control samples) for 24 h. Then, collecting cells for quantitative proteomic analysis at 48 hpi as previously described [33,34]. Briefly, the samples were added with 4 × volume cold lysis buffer (containing 1% protease inhibitor, 8 M urea) and lysed by sonication on ice. Then the lysates were centrifuged at 4 °C, 12,000 G for 10 min to remove the cellular debris. After that, the resulting supernatant was collected and BCA protein assay kit (Beyotime, China) was used to quantify the protein concentration.
2.5. Trypsin digestion, and tandem mass tags (TMT) labeling

For tryptic digestion, equal amounts of protein samples were taken and lysis solution was added to adjust the volume to uniformity. Then, 20% trichloroacetic acid (Sigma—Aldrich, Germany) was added, vortexed, mixed and precipitated at 4 °C for 2 h. Next, the samples were centrifuged at 4500 G for 5 min to discard the supernatant, and the precipitate was washed three times with acetone (Hannuo Chemical Technology, China). After drying, 200 mM triethylammonium bicarbonate (TEAB, Sigma—Aldrich, Germany) was added and the precipitate was ultrasonically broken up. Subsequently, trypsin (protease: protein = 1:50, m/m, Promega, USA) was added to digest overnight. Afterwards, dithiothreitol and iodoacetamide (Sigma—Aldrich, Germany) were added to the trypsin-digested peptides to adjust the final concentration of 11 mM, and then incubated for 45 min in the dark environment.

After digestion, a Strata X SPE (Phenomenex, USA) column was used to desalt the peptides and then the peptides were dried under vacuum freezing conditions. Subsequently, the prepared peptides were dissolved in 0.5 M TEAB and labeled with a TMT kit (ThermoFisher Scientific, USA). Briefly, the peptides were mixed with the dissolved labeling reagent and incubated for 2 h at room temperature. After that the labeled peptide mixtures were desalted, and dried under vacuum freezing conditions again.

2.6. High performance liquid chromatography (HPLC) fractionation

A high pH reverse-phase HPLC on Agilent 300Extend C18 column (250 mm length, 4.6 mm ID, 5 μm particles) was employed to fractionate the TMT-labeled peptides. Briefly, the peptides were fractionated into 54 fractions with acetonitrile (8%—32% gradient, PH 9.0) in about 64 min. Then combined peptides into 6 fractions, dried under vacuum freezing conditions for the following operation.

2.7. Liquid chromatography—mass spectrometry (LC-MS) analysis

An EASY-nLC 1200 ultra-HPLC system was employed to separate the combined peptides which were in advance dissolved with liquid chromatography mobile phase A aqueous solution (containing 0.1% formic acid and 2% acetonitrile). The gradient of the separation liquid phase was maintained at a flow rate of 500.00 nL/min and set as: 7%—11% mobile phase B (containing 0.1% formic acid and 90% acetonitrile) for 0—4 min; 11%—32% phase B for 4—54 min; 32%—80% phase B for 54—57 min; 80% B for 57—60 min. Following separated, the peptides were ionized in a NSI ion source (voltage, 2.3 kV; compensation 45 V), and then detected and analysed by a high resolution Orbitrap Exploris™ 480 MS. The scan range of MS analysis was set as: primary MS, 400—1200 m/z at a resolution of 60,000; secondary MS, fixed starting point with 110 m/z at a resolution of 15,000. A date-dependent procedure was used as date acquisition model. In addition, other parameters include: the automatic gain control, 100%; the signal threshold, 564 ions/s; the maximum injection time, 50 ms and the dynamic exclusion time of the tandem MS scans, 30 s.

2.8. Database search

The raw data of secondary MS were retrieved using tool Proteome Discoverer (Version 2.4.1.15). The Homo_sapiens_9606.SP_20201214.fasta (20,395 sequences) adding an inverse library and a standard contamination library was used as the database. Other search parameters for database search were listed in Table 1. Furthermore, the quantitation method was set to TMT-6plex, and the false discovery rate (FDR) for identification of proteins, peptides and PSM were all set to 1%.

2.9. Bioinformatics analysis of proteomics data

In consideration of the dynamic compression effect of the TMT label, and the choice of the Fold Change (FC) will be lower than that of the label free project, the DEPs were determined based on the quantification FC > 1.2 and the p value < 0.05 (Tan IIA-treated group vs control group) as previously described [35–40]. The UniProt-GOA database (http://www.ebi.ac.uk/GOA/) was selected for Gene Ontology (GO) annotation, and the eggNOG-mapper software (v 2.0) was used to conduct the GO functional classification; Clusters of Orthologous Groups of proteins (COG) database (http://www.ncbi.nlm.nih.gov/COG/) was performed for COG analysis; Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://www.genome.jp/kegg/) was selected to annotate protein pathway: WOLF PSORT software was carried out to predict subcellular localization; and the STRING database (http://string-db.org, version 11.0) was searched against for protein–protein interactions (PPI) network analysis. Moreover, a two-tailed Fisher’s exact test was employed for enrichment analysis of GO and KEGG pathway, and a corrected p value < 0.05 was considered significant.

2.10. Western blotting assay

Western blot technique was used to analyze the protein expression and validate the results of quantitative proteomics. Protein samples were extracted from cell lysates and quantified by BCA protein assay kit. Then samples were separated by 8%—12% SDS-PAGE gel, followed by transferring to PVDF membrane (Bio-Rad, USA), blocked with 5% milk or 5% BSA for 1 h, and incubated with primary antibody overnight at 4 °C. Table 2 showed the primary antibodies used in Western blot analysis. After that, the

| Table 1 |
| --- |
| Searching parameters for database search. |
| Parameters | Set Point |
| Digestion mode | Trypsin (Full) |
| Missed cut sites’ number | 2 |
| Peptides’ minimum length | 6 amino acid residues |
| Peptides modification’s maximum number | 3 |
| Primary parent ions’ quality error tolerance | 10 ppm |
| Secondary fragment ions’ quality error tolerance | 0.02 Da |
| Fixed modifications | Carbamidomethyl (C), TMT6plex (peptide N-Terminus), TMT6plex (K) |
| Variable modifications | Acetyl (protein N-Terminus), Oxidation (M) |

| Table 2 |
| --- |
| Primary antibodies used in Western blot analysis. |
| Antigen | Source | Dilution |
| Cyclin D1 | Abcam (ab40754) | 1:1000 |
| PP2A | Abcam (ab32065) | 1:500 |
| CREB | Abcam (ab32515) | 1:1000 |
| p-CREB | Abcam (ab32096) | 1:1000 |
| p27 | Abcam (ab32034) | 1:1000 |
| p-p27 | Abcam (ab62364) | 1:1000 |
| FGFR2 | Abcam (ab92337) | 1:1000 |
| FGFR | Abcam (ab10648) | 1:2000 |
| PI3K | Cell Signaling Technology (#4292) | 1:1000 |
| p-PI3K | Cell Signaling Technology (#17366) | 1:1000 |
| Akt | Cell Signaling Technology (#9272) | 1:1000 |
| p-AKT | Cell Signaling Technology (#9271) | 1:1000 |
| CD44 | PTMBD-551 | 1:2000 |
| GAPDH | Santa (SC-25778) | 1:2000 |
| PCNA | Abcam (ab29) | 1:1000 |
membrane was incubated with appropriate secondary antibodies and the immunoreactive bands were detected by an ECL kit (Milipore, USA). The intensity of bands on X-ray films were quantified by ImageJ software (NIH, USA), and all the detected bands were normalized to GAPDH (Santa Cruz, USA) level.

2.11. Elisa assay

The cell culture medium and hBMSCs were collected at 24 h exposure to Tan IIA (1 μM) for 24 h. After that, cells were fixed in 4% paraformaldehyde for 20 min, permeabilized in 0.1% TritonX-100 for 10 min, blocked with 5% BSA for 30 min, and incubated with anti-FGF2 (1:200, Abcam, UK) or anti-FGFR (1:200, Abcam, UK) overnight at 4 °C. Subsequently, the cells were washed in PBS and incubated with Alexa Fluor® 488-conjugated secondary antibody (1:500, Abcam, UK) for 1 h, and further treated with DAPI (1 mg/ml) for 5 min in the dark. Finally, the cells were rinsed and cover slipped, observed with a fluorescence microscope (OLYMPUS, Japan), the images were captured with 400 ×.

2.12. Immunocytochemistry assay

Cells were seeded in glass coverslips and treated with Tan IIA (1 μM) for 24 h. After that, cells were fixed in 4% paraformaldehyde for 20 min, permeabilized in 0.1% TritonX-100 for 10 min, blocked with 5% BSA for 30 min, and incubated with anti-FGF2 (1:200, Abcam, UK) or anti-FGFR (1:200, Abcam, UK) overnight at 4 °C. Subsequently, the cells were washed in PBS and incubated with Alexa Fluor® 488-conjugated secondary antibody (1:500, Abcam, UK) for 1 h, and further treated with DAPI (1 mg/ml) for 5 min in the dark. Finally, the cells were rinsed and cover slipped, observed with a fluorescence microscope (OLYMPUS, Japan), the images were captured with 400 ×.

2.13. Flow cytometry

Cell cycle progression was evaluated by Flow cytometry assay. Cells were cultured in 60-mm dishes and treated with Tan IIA (1 μM) for 6, 12, 24 or 36 h. Then, the cells were harvested, washed with PBS, and fixed with 75% ice-cold ethanol at −20 °C overnight. After centrifuge at 1000 G for 5 min, the cells were washed with PBS again and incubated with propidium iodide (50 μg/mL, Beyotime, China) for 30 min in the dark. Subsequently, the cell cycle distribution was evaluated by FACSVerse system (BD, USA). Flowjo software was used to quantify the cells percentage in the respective phases (G1, G2 and S) of the cell cycle.

2.14. Statistical analysis

All relevant data were presented as the mean ± standard deviation (SD) using GraphPad prism v 8.0 software (GraphPad Software, USA). Statistically significant differences between different groups were determined using student’s t-test or one-way analysis of variance, and p < 0.05 was considered as the difference was significant.

3. Results

3.1. Effect of Tan IIA on the proliferation of hBMSCs

We first investigated the proliferative effects of Tan IIA on hBMSCs. As shown in Fig. 1A, result from MTT assay showed that the cells viability in hBMSCs were significantly elevated after 24 h exposure to Tan IIA at the concentration of 0.001–10 μM (p < 0.05). Result from Edu assay was shown in Fig. 1B, the results showed that the proliferation rate in Tan IIA-treated groups (0.1–10 μM, 24 h) were markedly increased (p < 0.05), while it gradually declined after 24 h, and markedly decreased after 48 h exposure to Tan IIA (p < 0.01). Consistent well with the results of Edu assay, the protein expression of proliferating cell nuclear antigen (PCNA) in hBMSCs were significantly increased after 24 h exposure to Tan IIA at the concentration of 1 μM (p < 0.01, Fig. 1C), and the protein expression of CD44, the stemness marker was also markedly increased after 12 h and 24 h exposure to 1 μM of Tan IIA (p < 0.05, Fig. 1C). These results all indicated that Tan IIA could promote hBMSCs proliferation and maintain the stemness after 12–24 h exposure.

3.2. Quantitative proteome analysis on Tan IIA-treated hBMSCs

According to the results of proliferation assay, the concentration of Tan IIA treatment was selected as 1 μM, and 24 h was used as the culture time for quantitative proteome analysis. In order to make the analysis more accurate, we did not fill in the missing values and performed the analysis completely based on the actual MS data, and the proteins that were all missing from the three replicates in the sample group were not included in the differential analysis. As shown in Fig. 2A, the number of secondary spectra obtained by MS was 348825, after searching and comparing with the theoretical secondary spectrum, the effective number of spectra was obtained as 81364, the utilization rate was 23.5%. Moreover, 43289 peptides were identified, of which 42000 were unique. Based on peptides information, 6574 proteins were identified, of which 6519 were quantifiable, and with most of these proteins identified with below 30% sequence coverage (Fig. 2B). Furthermore, the distribution of peptide numbers was mainly concentrated in the range of 0–20, and the distribution of molecular weights of the identified proteins were in the range of 0–200 kD (Fig. 2C–D). All the peptides and proteins were identified with the FDR at 1% and p < 0.05 using Proteome Discoverer v 2.4.1.15 software (Table S1). Among the identified proteins, 51 upregulated proteins (Table S2) and 33 downregulated proteins (Table S3) were identified based on the FC > 1.2 and the p value < 0.05 (Fig. 2E–F).

3.3. Bioinformatics analysis of the DEPs in Tan IIA-treated hBMSCs

A total of 84 DEPs were annotated by the UniProt-GOA database. The subcellular localization analysis showed that the identified DEPs belong to nucleus (39.29%), cytoplasm (17.86%), extracellular (16.67%), mitochondria (14.29%), plasm membrane (9.52%) and endoplasmic reticulum (2.38%) (Fig. S1). In this study, the eggno-g-mapper software (v 2.0) was used to carry out GO functional classification of the identified DEPs based on protein sequence alignment method (Table S4). These proteins are classified into biological process, cellular components and molecular functions (Fig. S2), and the enrichment information of the DEPs were presented in Fig. 3 and Table S3. Among them, the cellular component contains 8 categories, i.e. extracellular space, keratin filament, protein phosphatase type 2A complex, cornified envelope, extracellular region, extracellular matrix, endoplasmic reticulum lumen, and extracellular organelle. The molecular functions contain 8 categories, i.e. structural constituent of skin epidermis, endopeptidase inhibitor activity, peptidase inhibitor activity, eukaryotic initiation factor 4E binding, endopeptidase regulator activity, fibroblast growth factor receptor binding, peptidase regulator activity, and ligand-gated cation channel activity. The biological process contains 14 categories, i.e. vitamin D metabolic process, morphogenesis of a branching epithelium, negative regulation of mitotic cell cycle, etc.

We also carried out COG analysis of the identified proteins. As shown in Fig. S3, the DEPs were largely involved in transcription, signal transduction mechanism, posttranslational...
modification, protein turnover, chaperones and function unknown. To elucidate the molecular pathways related to the DEPs derived from Tan IIA-treated cells, the KEGG database was employed. The enrichment analysis of KEGG revealed that the DEPs were mainly enriched in PI3K-AKT signaling pathways and MAPK signaling pathways (Fig. 4 and Table S6), which were the classical signaling pathway related to cell proliferation [41,42].

3.4. Interaction analysis of the DEPs in Tan IIA-treated hBMSCs

Based on the above results, we further searched against the STRING database to conduct the PPI analysis of the DEPs. After excluding external candidates, we fetched all interactions according to a “confidence score” (a metric defined by STRING) ≥ 0.7 (high confidence). Then, we employed R package “network D3” to visualize the DEPs interaction network. As shown in Fig. 5, the circles in the figure represented differential proteins, and different colors represented the differential expression of proteins (red was up-regulated proteins, green was down-regulated proteins), and the darker the color, the greater the difference fold. Herein, we found that in the upregulated DEPs, these proliferation-related proteins, such as FGF1 and FGF2 were significantly upregulated (Table 3), indicating that Tan IIA treatment may activate the proliferation-related biological process in hBMSCs.

3.5. Increased FGF2 production and its effect on cells proliferation in Tan IIA-treated hBMSCs

FGF2 is now recognized as one of the potent regulators in proliferation capacity of stem cells [43,44]. In this study, from the results of quantitative proteome and PPI, FGF2 level was found increased in Tan IIA-treated hBMSCs, so we further verify the alteration of FGF2 in Tan IIA-treated hBMSCs. As shown in Fig. 6A, compared to the control, the content of FGF2 level in the culture medium and cell lysates both significantly increased after 24 h exposure to Tan IIA (p < 0.01), indicating that Tan IIA may have the ability to enhance FGF2 production in hBMSCs. As shown in Fig. 6B, the nucleus staining of FGF2 was presented in hBMSCs, while FGFR expression was found located in the membrane of hBMSCs. Results from Western blot assay also indicated that the protein expression of FGF2, but not FGFR, was markedly increased in hBMSCs at 24 h exposure to Tan IIA (p < 0.05, Fig. 6C). As shown in Fig. 6D, results from EdU assay showed that Tan IIA-mediated hBMSCs proliferation was significantly and dose-dependently abolished by the preincubation of FGFR inhibitor Debio-1347 (0.5, 1 or 5 μM, p < 0.01). The role of FGF2 in cells proliferation of Tan IIA-treated hBMSCs was also confirmed by the results from Western blot assay (Fig. 6E), which showed that the increased protein expression of PCNA and CD44 in Tan IIA-treated hBMSCs were significantly reversed in the presence of Debio-1347 (p < 0.05). All these results

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**Fig. 1.** Effect of Tan IIA on the proliferation of hBMSCs. (A) MTT assay for cells viability, hBMSCs were exposed on Tan IIA (0.001–10 μM, 24 h, mean ± SD, n = 4). (B) EdU assay for proliferation rate evaluation, hBMSCs were treated with Tan IIA (0.1–10 μM) for 6, 12, 24, 36 or 48 h, respectively (mean ± SD, n = 5). (C) Western blot technique detecting the protein expression of PCNA and CD44 of hBMSCs treated with Tan IIA (1 μM) for 12 or 24 h (mean ± SD, n = 3). *p < 0.05, **p < 0.01 vs the control.
indicated that FGF2 played an important role in Tan IIA-mediated hBMSCs proliferation.

3.6. Alteration of FGF2-mediated PI3K/AKT signaling pathway in Tan IIA-treated hBMSCs

To further investigate the signaling pathways that involved in Tan IIA-treated hBMSCs, the global proteome data in Tan IIA-treated cells were mapped in the KEGG mapper (https://www.genome.jp/kegg/mapper.html). Results from KEGG mapper indicated that FGF2 mediated PI3K/AKT signaling pathway played an important role in the mechanism of action of Tan IIA in enhancing hBMSCs proliferation (Fig. 7A). To further confirm the effect of Tan IIA on hBMSCs, the proteins that related to FGF2 mediated PI3K/AKT signaling pathway were further evaluated by Western blot technique. As shown in Fig. 7B, compared to the control, the expression of phosphorylated CREB (p-CREB) was significantly increased (p < 0.05), while the expression of PP2A was markedly decreased.

Fig. 2. Basic information on the TMT based proteomics analysis. (A) Total spectrums, matched spectrums, peptides, unique peptides, identified proteins and quantifiable proteins from TMT based proteomics analysis; (B) Distribution of protein sequence coverage; (C) The number of peptides per protein; (D) Identified protein mass distribution; (E) Distribution of DEPs (Tan IIA vs Control); Up represents upregulated proteins in Tan IIA-treated hBMSCs compared with those in control group; Down represents downregulated proteins; The middle section represents unchanged proteins (significance p < 0.05); (F) Volcano plot of DEPs in Tan IIA-treated; Orange dots indicate the upregulated proteins; Green dots show the downregulated proteins. DEPs with at least 1.2-FC and a p value less than 0.05 are shown in orange or green color.
after Tan IIA treatment (p < 0.05). Results from Fig. 7C showed that the protein expression of p-PI3K and p-AKT were significantly increased after Tan IIA treatment (p < 0.05), while the total protein expression of PI3K and AKT did not show alteration (p > 0.05). Moreover, the expression of p-p27 and cyclinD1, the key proteins downstream of PI3K/AKT pathway, were also significantly increased after Tan IIA treatment (p < 0.05, Fig. 7C). All these results indicated that Tan IIA may promote hBMSCs proliferation via activating FGF2 mediated PI3K/AKT signaling pathway.

3.7. Effect of Tan IIA on cell cycle progression in hBMSCs

To evaluate the effects of Tan IIA on cell cycle progression, flow cytometry detection was applied to investigate the proportion of hBMSCs in cell cycle. As shown in Fig. 8, after exposed to Tan IIA (1 μM) for 6, 12, 24 or 36 h, the proportion of hBMSCs in S phase was gradually increased from 6 h to 24 h, the percentage of S phase cells was significantly increased after 12 h and 24 h exposure to Tan IIA (p < 0.05). It is notable that the cells in S phase were markedly decreased (p < 0.05), while the cells in G0/G1 phase were significantly increased (p < 0.05), after 36 h exposure to Tan IIA, indicating that Tan IIA might enhance cell proliferation capacity by regulating the progression of S phase.

4. Discussion

Nowadays, BMSCs have been considered as a promising cell type for tissue engineering, however, the research and clinical application of BMSCs are largely hampered by the limited number harvested from bone marrow cells. Therefore, the strategies aimed at
promoting the capacity of BMSCs expansion become more and more important, and a number of small compounds have been found to enhance the culture expansion of BMSCs ex vivo. This present work studied the effect and underlying mechanism of Tan II on hBMSCs expansion capacity ex vivo, the results showed that Tan II promoted the proliferation of hBMSCs via increasing FGF2 production, and FGF2-mediated PI3K/AKT signaling pathway was contributed to the effect of Tan II on hBMSCs (Fig. 9).
### Table 3
The up-and-down regulated DEPs of the PPI networks in Tan IIA-treated hBMSCs.

| Accession no. | Protein | Fold change | p value | Functions |
|---------------|---------|-------------|---------|-----------|
| P05230        | FGF1    | 1.61        | 0.00580 | Transcription |
| Q95182        | NDUF8A7 | 1.32        | 0.02996 | Energy production and conversion |
| P01024        | C3      | 1.27        | 0.01463 | Posttranslational modification, protein turnover, chaperones |
| Q95946        | RNF2    | 1.26        | 0.00318 | Posttranslational modification, protein turnover, chaperones |
| Q99999        | CYCS    | 1.22        | 0.00049 | Energy production and conversion |
| P15336        | ATF2    | 1.21        | 0.03575 | Transcription |
| P90038        | FGF2    | 1.20        | 0.03472 | Transcription |
| Q14186        | TDP1    | 0.83        | 0.04234 | Transcription |
| P67775        | PPP2CA  | 0.82        | 0.00016 | Signal transduction mechanisms |
| Q8N4Q1        | CHCHD4  | 0.64        | 0.00727 | Function unknown |
| P04004        | VTN     | 0.56        | 0.00391 | Posttranslational modification, protein turnover, chaperones; Extracellular structures |

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**Fig. 6.** Increased FGF2 production and its effect on cells proliferation in Tan IIA-treated hBMSCs. (A) The concentration of FGF2 in culture medium and cell lysates of hBMSCs (mean ± SD, n = 3). (B) Immunocytochemistry showing the location of FGF2 and FGFR in hBMSCs (Nuclei were stained with DAPI. Scale bar: 50 μm). (C) Protein expression of FGF2 and FGFR in Tan IIA (1 μM, 24 h) treated hBMSCs (mean ± SD, n = 3). (D) EdU assay for proliferation rate evaluation, hBMSCs were treated with Tan IIA (1 μM) for 24 h in the absence or presence of Debio-1347 (0.1, 0.5, 1 or 5 μM, mean ± SD, n = 5). (E) Protein expression of PCNA or CD44, hBMSCs were treated with Tan IIA (1 μM) for 24 h in the absence or presence of Debio-1347 (1 μM, mean ± SD, n = 2). *p < 0.05, **p < 0.01, ***p < 0.001 vs the control; #p < 0.05, ##p < 0.01, ###p < 0.001 vs the Tan IIA group.
Fig. 7. Alteration of FGF2-mediated PI3K/AKT signaling pathway in Tan IIA-treated hBMSCs. (A) The PI3K-AKT signaling pathway obtained from the global proteome data by KEGG pathway analysis in Tan IIA-treated hBMSCs. The proteins in green are down-regulated, proteins in red are up-regulated. (B) Protein expression and statistical analysis of p-CREB and PP2A in Tan IIA-treated hBMSCs. (C) Protein expression and statistical analysis of p-AKT, p-p27 and cyclinD in Tan IIA-treated hBMSCs. Data were expressed as mean ± SD (n = 3). *p < 0.05 compared to the control.
Based on the results of MTT assay and Edu assay, we found that Tan IIA (0.001–10 μM) could enhance the expansion capacity of hBMSCs within 24 h exposure, and the proliferative ability of hBMSCs was gradually decreased after 24 h exposure to Tan IIA. It is notable that the protein expression of PCNA and CD44 in hBMSCs were also markedly increased at 24 h exposure to Tan IIA, indicating that Tan IIA could promote hBMSCs proliferation and maintain the stemness within 24 h. The cell cycle is a cyclical physiological process

\[ \text{Fig. 8.} \text{ Effect of Tan IIA on cell cycle progression in hBMSCs. The hBMSCs were collected after exposure to Tan IIA (1 μM) for 6, 12, 24 or 36 h, and then analyzed by flow cytometry.}^* \text{p < 0.05 vs the control.} \]

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\[ \text{Fig. 9.} \text{ Mechanism of Tan IIA in regulating the expansion of hBMSCs ex vivo. Tan IIA may promote hBMSCs proliferation by regulating the progression of S phase via stimulating the release of FGF2, and thus activate FGFR-mediated downstream PI3K/AKT signaling pathways to promote cells proliferation. On the other hand, the upregulated p-CREB in FGF-mediated PI3K/AKT signal pathway may also contribute to the increase of cell number of hBMSCs induced by Tan IIA via reducing cell apoptosis.} \]
can be divided into G0/G1, S and G2/M phase, and DNA synthesis phase, that is S phase, playing an important role during cell proliferation process [45,46]. Based on above evidence, it seems that Tan IIA may promote the progression of cell cycle from G1 to S phase, and thus enhance the expansion capacity of BMSCs. Consistent well with our findings, previous studies also reported that Tan IIA sulfonate promoted the proliferation of the BMSCs that derived from rats, and the BMSCs proliferation rate became highest after 24 h drug exposure [28], indicating that 24 h Tan IIA treatment was enough to promote BMSCs proliferation. However, currently, the mechanism about Tan IIA’s effect on promoting BMSCs proliferation is still unclear.

Knowing that proteomics is one of the powerful tools to explore the mechanism of complex life activities, proteomics analysis was further applied in this study in order to elucidate the mechanism of Tan IIA on hBMSCs proliferation. From the results, we found that a total of 51 proteins were upregulated markedly, among them, FGF1 and FGF2 were significantly upregulated. FGF is a family of multifunctional proteins, it is reported that FGF can regulate essential biological processes, such as survival, migration and proliferation of tissue-specific stem cells [47,48]. In view of the important role of FGF2 in regulating the self-renewal and proliferation of human stem cells [43,44,49], we selected FGF2 for our further research. Results from ELISA assay showed that the level of FGF2 in hBMSCs culture medium and cell lysates were both markedly increased after Tan IIA treatment. Western blot assay also showed the increased FGF2 protein expression in Tan IIA-treated hBMSCs. It is notable that the results from immunocytochemistry showed that FGF2 and FGRF were both expressed in hBMSCs, and the pre-incipubation of FGFRI inhibitor markedly eliminated Tan IIA-induced hBMSCs proliferation and protein expression of PCNA and CD44, indicating that FGF2 played an important role in Tan IIA-mediated hBMSCs proliferation. Based on above evidence, it seems that Tan IIA induced FGF2 autocrine may be responsible for its effect on promoting hBMSCs proliferation. As previous studies showed that stem cells may have an autocrine manner to enhance cell proliferation [50–53], we hypothesized that Tan IIA may stimulate hBMSCs cells secret endogenous growth factors FGF2, and thus activate FGF2-mediated downstream signaling pathways to promote cells proliferation.

Results from KEGG mapper indicated that FGF-mediated PI3K/AKT signaling pathway may be involved in Tan IIA’s effect on hBMSCs. In this study, Western blot technique was applied to confirm the alteration of the key proteins of FGF-PI3K/AKT pathway. We found that the expression of PP2A, the inhibitory regulator of AKT phosphorylation [54,55], was markedly decreased in Tan IIA-treated hBMSCs, this finding was consistent well with the increased expression of p-PI3K, p-AKT, and the protein p27 and cyclin D1 downstream of PI3K/AKT. It is well known that p27, the cyclin-dependent kinase (CDK) inhibitor, could impede the progression of cell cycle and hinder cell proliferation [56]. Indeed, recent studies reported that p27 could also functioned as an activator of CDKs activity [57,58]. Previous studies also showed that growth-promoting factors could upregulate the expression of cyclin D1, and thus facilitate the cell cycle progression from G1 to S phase [59,60]. All these evidences indicated that decreased PP2A expression induced by Tan IIA may enhance the effect of FGF-PI3K/AKT signal on hBMSCs proliferation, which were coincide with the promoted progression of cell cycle from G1 to S phase in Tan IIA-treated hBMSCs. Moreover, CREB, the downstream molecule of PI3K/AKT signal, was recently found to protect rat BMSCs against apoptosis [61]. In this study, we also found increased expression of p-CREB in Tan IIA-treated hBMSCs, suggesting that the upregulated p-CREB in FGF-mediated PI3K/AKT signal pathway may also contribute to the increase of cell number of hBMSCs induced by Tan IIA via reducing cell apoptosis.

Danshen is commonly used as a traditional herbal medicine to exert its therapeutic effects on cardiovascular diseases, liver diseases and nervous system diseases in China [23,62,63]. Evidences from previous studies indicated that Danshen as well as its aqueous extracts could enhance the capacity of BMSCs expansion, which may contribute to improving the recovery of ischemic stroke in rats [27]. As one of the main active components of Danshen, Tan IIA was also discovered to promote the proliferation of endogenous liver stem cells and BMSCs, but the underlying mechanism is still unclear [28,64]. In this present study, we found that Tan IIA enhanced hBMSCs proliferation by increasing FGF2 autocrine, FGF-mediated PI3K/AKT signaling pathways may involved in Tan IIA’s effect on hBMSCs. This study, to some extent, clarified the mechanism of Tan IIA’s effect on promoting hBMSCs proliferation. The limitation of this study is that we did not confirm our findings using an animal model, namely, the effect of Tan IIA on the in vivo expansion capacity of BMSCs is not confirmed. Further studies are still needed to investigate the effect of Tan IIA on BMSCs transplantation in vivo. Even though, the results from this study will still give a hint that Tan IIA may have the potential to be used for BMSCs applications in cell therapies in the future. It is notable that Tan IIA was also reported to have multiple pharmacological activities, such as immune-regulation, anti-inflammation, antioxidation, and anti-fibrosis activities [65–67]. Therefore, it still leaves the open possibility of the involvement of other regulators or mediators induced by Tan IIA treatment in regulating BMSCs proliferation, more studies are still needed in the future in order to enrich the mechanism of action about Tan IIA on BMSCs.

5. Conclusion

In summary, this study shows that Tan IIA can promote hBMSCs proliferation by regulating the progression of S phase via increasing the release of FGF2, FGF-mediated PI3K/AKT signaling pathways may play an important role in Tan IIA’s effect on hBMSCs expansion. The results of this study may provide further evidence about the potential of Tan IIA to be used for the research of hBMSCs therapies.

Date availability

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium using the PRIDE partner repository with the dataset identifier PXD036093 (http://www.ebi.ac.uk/pride/archive/).

Author contributions

P.Y. performed experiments and analyzed data and wrote this manuscript. H.Y.Q. designed the study. J.Y.W, and G.S.Y.C. assisted with experiments and analyzed data. H.Y.Q., and X.L. contributed to completing the revision and submission of the article. All authors have given approval to the final version of the manuscript.

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