Establish deeper understanding on the osteogenic differentiation of monolayer cultured human pluripotent stem cells using novel and detail analyses

Ping Zhou
School and hospital of Stomatology, Lanzhou University

Jia-Min Shi
College of Life Science, Lanzhou University

Jing-E Song
School and Hospital of Stomatolog, Lanzhou university

Yu Han
School and Hospital of Stomatolog, Lanzhou University

Hong-Jiao Li
School and Hospital of Stomatolog, Lanzhou University

Ya-Meng Song
School and Hospital of Stomatolog, Lanzhou University

Fang Feng
School and Hospital of Stomatolog, Lanzhou University

Jian-Lin Wang
Collage of Life Science, Lanzhou University

Rui Zhang
School and Hospital of Stomatolog, Collage of Life Science, Lanzhou University

Feng Lan (✉ fenglan@ccmu.edu.cn)
Capital Medical University affiliated Being Anzhen Hospital

Research

Keywords: Osteogenic differentiation, Human embryonic stem cells, Human induced pluripotent stem cells, Marker expression.

DOI: https://doi.org/10.21203/rs.3.rs-45401/v1

License: Creative Commons Attribution 4.0 International License.

Read Full License
Abstract

**Background:** Derivation of the osteoblast-like cell from human pluripotent stem cell becomes a hot topic in bone tissue engineering. Although many improvements have been achieved in this field, low induction efficiency because of the non-directed differentiation process hampers their application in bone regeneration. We think lack of detailed understanding on the osteogenic differentiation process should be the main reason.

**Methods:** Monolayer cultured human embryonic stem cells and human induced pluripotent stem cells were inducted in traditional serum-containing osteogenic medium for 35 days. Except for traditional assays such as cell viability detection, reverse transcription-polymerase chain reaction, immunofluorescence, and alizarin red staining, we also applied cell counting, cell telomerase activity, cell cycle and quantitative expression of runt-related transcription factor 2 as essential indicators to analyze the cell type changes during the differentiation process.

**Results:** The population of differentiated cells are quite heterogenous throughout 35 days of induction. Then, cell telomerase activity and cell cycle analyses have value in evaluating the cell type changes and tumorigenicity of obtained cells. Moreover, nuclear staining should be a recommended method to evaluate the cell number, because, it is still a great challenge to dissociate cells with varying differentiation times into single cells with high survival rate. Finally, a dynamic map was made to integrated analysis of these results, and the cell types at defined stages of osteogenic differentiation of human pluripotent stem cells was concluded.

**Conclusions:** This study lay foundation to improve the *in vitro* osteogenic differentiation efficiency of human pluripotent stem cells by supplementing functional compounds at each stage according to a time-frame, then establish a step-wised induction system in the future.

1. **Background**

Owing to the low regenerative capability of bone tissue, together with limitations such as immunity risk, surgical trauma and ethical confusion existed for current treatments, tissue engineering technology harbor a promising perspective for the repair of large-area bone defects in clinical [1]. To our knowledge, how to obtain a large number of functional seed cells is the present main barrier to achieve good animal results of large bone regeneration using tissue engineering technology, because the other two compositions of growth factor and scaffold have been well characterized [2]. Mesenchymal stem cells (MSCs) are popularly used to derive osteoblast-like cells. However, MSCs has many vulnerabilities, such as limited source, easily influenced by the donor and easy to aging [3]. Compared to MSCs, human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) harbor long-term self-renewal and multi-directional differentiation potential. Consequently, the latter is the preferred seed cell origin for bone tissue engineering [4].
Unfortunately, although many researches have been performed for the derivation of osteoblast-like cells from human pluripotent stem cells (hPSCs), step by step induce mesoderm and ectoderm cells from hPSCs, and then mesenchymal-like cells from mesoderm cells were not realized. This result in the current efficiency of osteogenic differentiation is much lower than that of hPSCs induction into cells such as cardiomyocyte-like cells, neuron-like cells and hepatocytes [5]. Up to now, the medium consisting of fetal bovine serum (FBS), dexamethasone, β-glycerophosphate and vitamin A is still commonly used in the osteogenic differentiation of hPSCs. There is no doubt that this medium alone can't fulfill the establishment of an in vitro directed induction system, and many functional chemical compounds should be supplied at specific differentiation stages according to the induction system development process for the above-mentioned cells. An important reason should be the lack of a clear understanding of the osteogenic differentiation process of hPSCs. Therefore, it is necessary to understand the dynamic changes of markers and cell types during the osteogenic differentiation process of hPSCs, which helps to judge the optimal supplementary period for osteogenic induction factors and enhance the efficiency of osteogenesis.

Many analyses have been explored to identify the osteogenic differentiation process of hPSCs. Commonly used methods include staining and detection of the expression of marker genes and proteins. Explicitly speaking, methods of alizarin red staining and von Kossa staining and even Raman spectroscopy was used to detect the deposition of calcium nodules in cells. Then, alkaline phosphatase (ALP) staining and BCIP/NBT colorimetry were applied to evaluate the ALP activity of cells. More critical, pluripotency related markers of OCT-4, NANOG and many osteogenesis-related makers such as ALP, runt-related transcription factor 2 (RUNX2), osterix (OSX), type I collagen (COL1A1), osteocalcin (OCN), bone sialoprotein (BSP), osteopontin (OPN) were detected by molecular biology techniques. At present, it is agreed that hPSCs develops into osteoblasts gradually undergoing proliferation, differentiation, deposition of extracellular matrix and mineralization [6]. Unfortunately, the osteogenic differentiation process of hPSCs has been studied using almost the same method that reported for MSCs, as mentioned above. Moreover, few systematic kinds of research have been performed to analyses the cell type changes at each defined osteogenic induction stage of hPSCs. Additional experiments should be involved in analyses the differentiation process of hPSCs.

As we know, during the in vivo embryonic development, the mesoderm and ectoderm cells formed from hESCs will differentiate into mesenchymal cells, which can further differentiate into osteogenic precursor cells and osteoblasts by intramembranous or endochondral ossification [7, 8]. Thus, the in vitro differentiation of hPSCs into osteoblasts should also undergo similar changes in multiple germ layers and cell types. It is reported that cell telomerase activity and cell cycle highly correlate to cell fate regulation throughout the differentiation process [9, 10]. At the same time, osteogenic differentiation of stem cells is accompanied by the regulation of early osteogenic marker proteins of RUNX2. Therefore, it has great value to perform quantitative measurements for these important indicators during the osteogenic differentiation of hPSCs.
In this present study, H9 hESCs and hNF-C1 hiPSCs on the Matrigel surface were differentiated into osteoblast-like cells in osteogenic induction medium for 35 days. In order to clarify the differentiation process, the dynamic expression changes of the related markers were monitored by commonly used reverse transcription-polymerase chain reaction (RT-PCR) and immunofluorescence. Calcium nodule content and ALP activity were separately determined using alizarin red staining and ALP staining. At the same time, more sensitive flow cytometry was used to detect the expression of marker protein RUNX2 and the changes of cell cycle. Besides, nuclear staining was used for cell counting. Furthermore, cell telomerase activity experiment was performed as a potential indicator to analyze cell types. Finally, we established a schematic representation for characterizing the change of makers and cell type during osteogenic differentiation of hPSCs. This study could contribute valuable knowledge on the osteogenic differentiation of monolayer cultured hPSCs, accelerating the development of better in vitro osteogenic differentiation systems.

2. Materials And Methods

2.1 Materials

Ethylene diamine tetraacetic acid (EDTA), ascorbic acid, sodium glycerophosphate, dexamethasone, cetylpyridinium bromide, alizarin red S and Triton-X100 were obtained from Sigma-Aldrich (USA). Fetal bovine serum (FBS), αMEM medium, non-essential amino acid (NEAA), β-mercaptoethanol, L-glutamax and penicillin/streptavidin were purchased from Gibco (USA). Cell culture plates and Matrigel were bought from Corning (USA). Methanol, absolute ethanol, chloroform, hydrochloric acid and isopropanol were obtained from BCIGC (China). Bovine serum albumin (BSA), phosphate buffer, N-hydroxysulfosuccinimide sodium salt (NHSS) and 1-Ethyl-3-(3-dimethylamino propyl)carbodiimide (EDC) were purchased from Aladdin (China). BCIP/NBT alkaline phosphatase coloring kit and ALP quantitative detection kit was acquired from CWBIO (China). SYBR Green I and trizol were bought from Takara (Japan). Paraformaldehyde was obtained from Solarbio (China). Quartz crystal microbalance chips were obtained from HRbio (China). Cell counting kit-8 (CCK8) was purchased from Dojindo (Japan). RevertAid™ First Stand cDNA Synthesis Kit was gained from Thermofisher (USA). Cell cycle assay reagent was obtained from KoradBio (China). E8 medium was acquired from Cellapy (China). DAPI stain was purchased from Roche (Switzerland). Carboxyl functionalized QCM chips were provided by Dongwei BiologicalTechnology Co., LTD (China).

2.2 Cell culture in vitro

H9 hESCs and hNF-C1 hiPSCs were provided as gifts as described previously [11]. Both cells were cultured on the 6-well cell culture plates after coating with Matrigel at a dilution rate of 1:80. The medium used to maintain pluripotency in the experiment was a well-defined E8 medium, and it was changed every day. After grown into about 90% confluence, cells were passaged at a split ratio of 1:4 by exposure to 0.5 mM EDTA for 4 ~ 5 min at 37 °C.

2.3 Osteogenic differentiation
When grown into 80% confluence, hPSCs on Matrigel surface were transferred into osteogenic medium (OM) that composed of αMEM medium, 15% FBS, 1% NEAA, 0.1 mM β-mercaptoethanol, 1% penicillin/streptavidin, 5 µg·mL⁻¹ ascorbic acid, 10 mM sodium glycerophosphate and 10⁻⁸ M dexamethasone. The OM was changed freshly every 2 days for 35 days. After induction for different times (0 day, 3 days, 7 days, 14 days, 21 days, 28 days, 35 days), cells were observed using a phase-contrast microscope (CKX31SF, Olympus, Japan) with a CCD camera (MP3.3-RTV, Olympus, Japan), and their viability was detected using a cell counting kit-8 reagent.

2.4 Cell telomerase activity measurement

The telomerase activity of cell samples throughout the osteogenic differentiation was quantitatively measured using a previously reported method based on quartz crystal microbalance (QCM) [12]. Briefly, each of 1 million single cells was lysed in 150 µL CHAPS lysis buffer for 30 min on ice. Centrifuging at 12000 r·min⁻¹ and 4 °C for 20 min was performed to extract the supernatant containing telomerase. Subsequently, the protein content was measured using a BCA protein concentration determination kit according to the manufacturer’s instructions [13]. The protein concentration of the sample was adjusted by DPBS with the minimum protein concentration as a reference. To measure the cell telomerase activity, NHSS/EDC activated QCM chip was immediately incubated with primers (5'-NH2(CH2)6 T T T T T ∀ TC GTCGAGCAGAG T - 3’) and DNA assembly solution for 3 h. The pre-treated chips were placed into a QCM reactor and then undergo the same processes to detect the frequency changes relating to cell telomerase activity as we described [12].

2.5 Quantitative real-time RT-PCR

Cell samples were extracted using TRIzol reagent and total RNA was extracted through the chloroform-isopropanol precipitation method. The total RNA was inverted into cDNA using a RevertAid™ First Stand cDNA Synthesis Kit. The mRNA of samples was detected through quantitative real-time polymerase chain reaction (RT-PCR) using SYBR Green I via an ABI 7500 RT-PCR machine (Applied Biosystems, USA). Three parallel samples were set for each sample, and each replicate was tested in three independent replicates. Quantitatively detected genes contain the internal control gene (ACTB), pluripotency marker genes (OCT-4, NANOG), osteogenic differentiation related genes (RUNX2, ALP, COL1A1, OCN) and the telomerase related gene (TRET). The primer sequences of these genes are shown in table S1.

2.6 Immunofluorescence

After osteogenic differentiation for varying times (0 day, 3 days, 7 days, 14 days, 21 days, 28 days, 35 days), hPSCs on 12-well cell culture plates were fixed in 4% paraformaldehyde for 30 min at room temperature (RT). Fixed samples were used to detect the protein expression of OCT-4, RUNX2, COL1A1 and OCN by immunofluorescence. Briefly, cell samples were permeated for 30 min with 0.2% Triton-X100 and blocked with 3% BSA for 2 h at RT. The samples were, respectively, incubated overnight with primary antibodies at 4 °C. After washed with DPBS for 3 times, cells were incubated with corresponding fluorescently labeled secondary antibodies in the dark for 1 h. Finally, the samples were stained for 5 min at RT with DAPI that diluted in DPBS at 1:5000. All stained cell samples were observed and photographed.
using a confocal fluorescence microscope (Axiovert 200M; Carl Zeiss Jena, Germany). Meanwhile, cell numbers were counted using Image J software according to DAPI staining. Primary antibodies and corresponding secondary antibodies are shown in table S2.

2.7 Flow cytometry study

After incubation for up to 35 days, hPSCs were digested into single cells and fixed by 1% paraformaldehyde. The cells were permeated for 30 min in 200 µL pre-cooled 90% methanol solution on the ice. Subsequently, the sample was washed twice with the flow buffer (DPBS containing 2% FBS) and incubated with mouse anti-human RUNX2 monoclonal antibody at a dilution rate of 1:200 in flow buffer for 30 min at 37 °C. This was followed by secondary antibodies of Fluor 488-labeled goat anti-mouse IgG at a dilution rate of 1:500 in DPBS. Besides, for cell cycle analysis, single cells were fixed in pre-cooled 75% ethanol at 4 °C for 24 h. Before the flow cytometry study, the cells were incubated in 500 µL cell cycle assay reagent for 30 min at 4 °C in the dark. Finally, all these cell samples were analyzed by the BD FACS Calibur System (BD, USA) and Flowjo software.

2.8 Alkaline phosphatase assay

Cell samples were fixed in absolute ethanol for 30 min, and then stained using a BCIP/NBT alkaline phosphatase coloring kit according to the instructions. After washing with distilled water for 3 times, stained samples were observed by an inverted microscope containing a CCD (Olympus, Japan). Besides, the plates were photographed using a mobile phone. Moreover, the ALP activity of these cell samples was detected using an ALP quantitative detection kit according to the instructions as we previously described [13].

2.9 Determination of a calcium nodules content

hPSCs cultured on 12-well cell culture plate were fixed in 4% paraformaldehyde for 30 min. After washing 3 times with DPBS, they were incubated with 500 µL 2% alizarin red (0.01 M Tris buffer, pH = 4.2) for 20 min at room temperature. After repeatedly rinsing with distilled water until the solution was clarified, cells and plates were photographed as previously mentioned. To quantitative measure the deposited alizarin red S, 500 µL 1% (m/v) cetylpyridinium bromide solution added into each well of the plate. After the overnight reaction, 100 µL solution of each well was transferred into new 96-well plates and the absorbance at 490 nm was measured using a Bio-Rad full-wavelength microplate reader (Bio-Rad, USA). Three replicate wells were set for each experimental group, and the absorbance value of each well was measured 3 times.

2.10 Statistical analysis

All data were statistically analyzed using Student’s t-test and expressed as mean ± standard deviation. The difference was considered significant when \( p < 0.05 \). Each data was obtained by performing three independent replicates.

3. Results And Discussion
3.1 Analysis of cell morphology and cell viability

When H9 hESCs and hNF-C1 hiPSCs on the Matrigel surface grown into about 80% confluence, E8 medium was changed to be widely used OM containing FBS, ascorbic acid, glycerophosphate and dexamethasone for 35 days, and this differentiation was investigated in detail (Fig. 1). Before differentiation, both hESCs and hiPSCs exhibited typical undifferentiated morphologies with clear clone edge and high nucleo-cytoplasmic ratio (Fig. S1a-b). After incubation in OM for 3 days, cell colonies of hPSCs became loose with a large number of dead cells appeared in the medium, resulting in a decreased cell activity that confirmed by CCK8 assay (Fig. 1b-e). This may be due to the apoptosis of undifferentiated hPSCs and initially differentiated cells. Besides, the cell activities were slightly increased from this time point throughout 35 days of culture (Fig. 1b-c). Then, many cobblestones or spindle-shaped cells were observed after differentiation for 7 days and 14 days (Fig. S1a-b). With the increasing of differentiation time in 35 days, more and more cells showed irregular cell morphology (Fig. S1). In summary, due to a relatively high initial differentiation density of 80% was applied, similar cell activity with no apparent cell morphology change were found for both hESCs and hiPSCs during the differentiation process in 35 days.

3.2 The cell telomerase activity changes during osteogenic differentiation of hPSCs

The changes in cell telomerase activity were measured for hESCs and hiPSCs using a quantitative method based on QCM, as we recently reported [11]. In this method, frequency changes (Δf) show a positive correlation with cell telomerase activity. It is well known that cell telomerase activity plays a key role in the self-renewal ability of each type of cell, and it is gradually down-regulated during embryonic development [9]. Germ cells harbor high telomerase activity, while disappeared interminably differentiated cells [12]. Consistently, our previous results also confirmed that the telomerase activities of hPSCs, human bone marrow mesenchymal stem cells (hBMSCs) and MG63 osteoblasts decreased successively [12]. Therefore, cell telomerase activity can be applied as one of the important quantitative markers to monitor the in vitro osteogenic differentiation process of hPSCs.

As shown in Fig. 1d-e, the frequency changes (Δf) of cells decreased with the augment of differentiation time in 7 days, revealing that both hiPSCs and hESCs were stepwise differentiated towards osteoblast-like cells with reduced cell telomerase activity. Surprisingly, consistent cell telomerase activity results were measured for hESCs after differentiation for 7 ~ 28 days, and the telomerase activity of hiPSCs after culturing for 14 days (80 ± 10 HZ) was slightly higher than cells with culture time of 7 days (65 ± 15 HZ) (Fig. 1e). These may because popularly applied induction medium containing FBS, ascorbic acid, sodium glycerophosphate and dexamethasone resulting in heterogeneous cells throughout the osteogenic differentiation of hPSCs.

3.3 The cell cycle changes of hPSCs during osteogenic differentiation
To our knowledge, the growth and development of hPSCs depend on the regulation of the cell cycle [10]. Cell fate switches correlation with cell cycle transition in dividing cells, whereas terminal differentiation is frequently associated with cell cycle exit. It is reported that the rapid cell division supports the self-renewal of hESCs because of the shortened G1 cell cycle [14]. Cell cycle length and rate are determining factors for both self-renewal and differentiation of stem cells, so cell cycle analyses have value to determine the differentiation progress of hPSCs [15].

In this study, a cell cycle detection reagent and flow cytometry were applied to investigate the cell cycle changes in hPSCs during 35 days of osteogenic differentiation. hPSCs incubation in the induction medium activates the developmental process and reshape the cell cycle, prolonging the G1 phase and whole cell division time [16]. Although both cells were grown into about 80% confluence before differentiation, the percent of cells in the S phase stage for hESCs (56.6%) was higher than hiPSCs (34.9%), suggesting hESCs harbor better proliferation ability than hiPSCs in our study (Fig. 2). However, similar results were measured for cells in the G2/M phase stage. Then, the percent of cells in G2/M and S phase stage for both hESCs and hiPSCs were decreased with the augment of induction time in 35 days, resulting in more cells at the G0/G1 phase stage. Consistent with the cell viability assay, many hPSCs remain in the S/G2/M phase stage after 3 days of culture resulted in an increase in cell viability from 3 days to 7 days. Moreover, decreased proliferation rate combined with the medium selectively calls killing effect, which can explain previous results showing that only slightly higher cell viability was detected during 35 days of differentiation (Fig. 2a). In summary, cell cycle analysis indicated that more cells were arrested in the G0/G1 phase, and decreased the percentage in G2/M and S phase with the development of osteogenic induction differentiation.

As we all know, chromosomes are replicated during the S phase and then segregated to daughter cells during the M phase for cell proliferation, but exit from the cell cycle in the G1 phase is frequently required for terminal differentiation of cells during development [14, 17]. The trend of diminishing in the proportion of S phase cells was also consistent with the decreased cell telomerase activities measured in hPSCs during the osteogenic induction process (Fig. 1d). It is reported that cell telomerase activity highly relevant to cell cycle regulation, and the highest levels of cell telomerase activity occur during the S phase [18, 19]. In fact, in vivo bone development is a process during that the pluripotency and proliferative ability decrease gradually [20]. As we all know, the in vitro self-renewal and proliferation ability for hPSCs, human mesenchymal stem cells, osteoblasts and osteocytes are precipitous decline. Therefore, we think the assay of cell telomerase activity and cell cycle play essential roles in understanding the osteogenic differentiation process of hPSCs.

3.4 Expression of gene and protein markers in induced hPSCs

As we know, in vivo bone development is a process consisting of multiple developmental stages, along with the dynamic changes in the expression of related gene/protein markers at each stage [21]. In this study, after osteogenic differentiation for varying times (3, 7, 14, 21, 28 and 35 days), we analyzed the
expression of the pluripotent gene of OCT-4 and NANOG, telomerase gene of TRET as well as osteogenesis related genes of RUNX2, ALP, COL1A1 and OCN in hESCs and hiPSCs. At the same time, immunofluorescence was used to detect the protein expression of OCT-4, RUNX2, COL1A1 and OCN in these cell samples. In addition, for the critical marker of RUNX2 protein, its expression in hPSCs during the induction process was further detected using flow cytometry.

As shown in Fig. 3, the expression of OCT-4, NANOG and TRET decreased rapidly after the replacement of osteogenic induction medium on the 3 day (Fig. 3a-c). Unbelievably, repeated experiments found that the expression of the TRET gene in hESCs was not reduced after differentiation for 3 days, which may because of these initially differentiated cells remained high self-renewal ability. Then, OCT-4 and NANOG genes were not expressed virtually after 7 days of osteogenic differentiation, and TRET was expressed barely after 14 days of culture (Fig. 3a-c). Consistently, immunofluorescence detection showed similar results of OCT-4 expression. Both hESCs and hiPSCs positively expressed OCT-4 before differentiation, and the number of positive expression cells was remarkably decreased after transferred into the induct medium, and almost disappeared after 14 days of culturing (Fig. 4). These results further confirmed that the osteogenic differentiation of hPSCs is a process of pluripotency reduction [22].

For the osteogenic markers, RUNX2 is a significant multifunctional transcription factor during the osteogenic differentiation of stem cells and can regulate the transcription of other osteoblast-related genes like COL1A1 and OCN by binding with enhancer or promoter core site [23, 24]. Analysis of RT-PCR showed that the expression of RUNX2 gene in both hESCs and hiPSCs began to rise steadily after 7 days of culture, and reached peak values at 21 days (Fig. 3d). Then, several cells positively expressing RUNX2 were found in both cells after 14 days and 21 days of induction, as confirmed by immunofluorescence (Fig. 4). Moreover, we overcome the hardness existing in cell number and cell dissociation at the latter stage of osteogenic differentiation, and successes to obtain enough cells for the flow cytometry assay. As we know, flow cytometry assay plays a very important role in quantitative evaluate protein expression and differentiation efficiency. Compared to immunofluorescence results, although consistent tendency was found for flow cytometry results as shown in Fig. 5, the expression level was quite different between them. Specifically, the expression of RUNX2 protein in both cells was increased with the augment of culture time in 21 days, and reached peak values of 49.9% and 43.1% for hESCs and hiPSCs respectively (Fig. S2). Apparently, a much higher expression level was detected for flow cytometry assay in comparison to immunofluorescence analyses, which may due to much differentiated cells expressed limited RUNX2 protein and flow cytometry assay harbor better sensitivity. Besides, after induction times for 14 days, 28 days and 35 days, 12.7 ~ 22.1% hESCs positively expressed RUNX2. However, except the time point of 21 days, nearly negatively results were detected for hiPSCs. These results proved that flow cytometry assay is a very important quantitative analysis to investigate the osteogenic induction of hPSCs, and the difference in cell line and cell state would affect the expression of RUNX2.

Then, the expression of another osteogenic differentiation maker of ALP, COL1A1 and OCN was also analyzed by RT-PCR and immunofluorescence. ALP is one of the alkaline phosphatase isozymes that ubiquitously expressed in bone-forming cells, and plays a critical role in early osteogenesis and
hydrolyzes various types of phosphates to promote cell maturation and calcification [25]. Thus, ALP is considered as an early osteogenic differentiation marker. For both hESCs and hiPSCs, our results showed that the expression of ALP gene peaked after 3 days of induction, and then rapidly decreased into a quite low expression level from the 14th day (Fig. 3e). These results may suggest that hPSCs undergo early differentiation process towards osteoblasts during 3 ~ 7 days.

As shown in Fig. 3f, the late osteogenic differentiation marker gene COL1A1 in hPSCs was up-regulated from day 14, peaked at day 21, and then down-regulated till to 35 days. These results were similar to reported studies [26, 27]. To our surprise, although the expression trend of the two cell lines was almost consistent, the expression of COL1A1 gene in hiPSCs with more than 14 days differentiation times was much higher than that in hESCs (Fig. 3f). Similarly, a significant difference was found for the gene expression of OCN, a marker of osteoblast formation, between the two cell lines. For hESCs, after a slight decrease at initially 3 days, the expression of OCN gene was increased with the augment of culture time in 35 days except for the time point of 21 days (Fig. 3g). Interestingly, hiPSCs remain a low gene expression level for OCN, and up-regulation was found at 21 days. Besides, the expression of COL1A1 and OCN protein in these cell samples was detected at the late stage of osteogenic induction (21 days, 28 days and 35 days) using immunofluorescence technique (Fig. 6). We found that both protein expression in hPSCs were gradually increased from 21 to 35 days. It is reported that the apparent down-regulation of OCN was associated with the accumulation of low levels of hydroxyapatite in the later stages [28]. In addition, previous studies reported that OCN inhibits mineralization, but is highly expressed at the end of maturation of the extracellular matrix, and undergoes rapid down-regulation before mineralization, and then gradually increases [29–31]. Therefore, the results may suggest that hPSCs form mature extracellular matrix during the culturing period of 21 ~ 28 days. In summary, our results preliminary indicated that hESCs and hiPSCs undergo similar expression changes for markers relating to pluripotency and osteogenic differentiation, but not for extracellular matrix protein markers.

As confirmed by previously results, apparently heterogenous differentiated cells were obtained throughout 35 days of induction, which is the reason why CCK8 assay cannot reflect the cell numbers (Fig. 1b-c). Moreover, dissociate cells into single cells using trypsin is a quite difficult process with a low survival rate. Therefore, DAPI staining was applied to accurately measure the number of cells after culturing for varying days (Fig. S3). When the culturing times was more than 7 days, quite different cell number results were detected for hPSCs in comparison to CCK8 assay. The cell number were remarkably decreased for both cells after culturing for 14 days, but they exhibited similar cell viability. This possibly because of increased cell size and cellular metabolic level change. Besides, analyses of cell telomerase activity and cell cycle proved that cells at this stage harbor not bad cell division ability (Fig. 1d-e and Fig. 3a-b). We could conclude that much cells were died at this period due to the selective killing effect of OM. Then, the cell number of hESCs went on reduce after 21 days of induction, but contrast results were found for hiPSCs (Fig. S3). This is consistent to previously results showing that hiPSCs at day 14 harbor much higher cell telomerase activity than hESCs (Fig. 1d-e). Finally, the number of hPSCs were increased with the argument of induction time in 35 days, suggesting very few cells were died since cells have limited proliferation ability during this period as confirmed by cell telomerase activity and cell cycle
results. These results proved that nuclear staining has value in analyzing the cell number changes as well as the killing effect of induction medium during the osteogenic differentiation of hPSCs.

### 3.5 ALP and alizarin red staining analysis

ALP staining is commonly applied to identify reprogrammed hPSCs and its osteogenic differentiation process. Both hESCs and hiPSCs highly expressed ALP before differentiation (Fig. S4). After culturing in induction medium for 3 days, many stained cells were found in hiPSCs, but not for hESCs. Then, the ALP expression in both cells decreased rapidly and then almost disappeared at day 14. With the prolongation of osteogenic differentiation time, the ALP activity of cells switched to increase until 28 days of culture (Fig. S5). This trend is similar to the results of previous studies [13, 32].

In addition, alizarin red staining (AS) was applied to study the calcium-containing nodule formation of hPSCs during the osteogenic differentiation in 35 days (Fig. 7). As shown by both qualitative and quantitative results, more deposited alizarin red was detected with the increase of culture time, especially at the time point of 28 ~ 35 days. Typical calcium nodules were found after induction for 14 days for hESCs, but the time point is 28 days for hiPSCs (Fig. 7a-b). This is the reason why quantitative results showed that the calcium salt deposition of hESCs was about 2 times higher than that of hiPSCs during the osteogenic differentiation in 35 days (Fig. 7c-d). Interestingly, we observed a slight down-regulation of calcium nodules in hESCs after induction for 28 days. All these were consistent with the OCN gene expression results as confirmed by RT-PCR, which may because the expression level of OCN is closely associated with both the production and maturation of mineral species in cells [33]. Results of RT-PCR and AS staining demonstrated that H9 hESCs harbor much better performance than hiPSCs in extracellular matrix synthesis, and this difference should be considered in evaluating the osteogenic differentiation among researches using different hPSCs cell lines.

### 3.6 Summarize the changes of researched markers during the osteogenic induction of hPSCs

Osteogenic differentiation of hPSCs is a process in which pluripotency gene expression is gradually reduced and osteogenic-related genes are dynamically changed [34]. As we all know, the mesoderm and ectoderm cells that derived from hPSCs are the primary source of MSCs, which can further differentiate into pre-osteoblasts and osteoblasts [21, 35, 36]. In this progress, RUNX2 expressing pre-osteoblasts will soon switch to cells expressing osterix, ALP and COL1A1 [37]. In addition, mature osteoblasts can also synthesize a variety of extracellular matrix proteins such as OCN, BSP and OPN, and the expression of OCN is generally regarded as a marker of osteoblasts [37].

Combined with the expression of the above-related genes and proteins, we preliminarily drew a dynamic map for the osteogenic differentiation of hPSCs (Fig. 8). The expression of pluripotent markers of OCT-4, NANOG and TRET in cells decreased gradually after osteogenic differentiation, and the expression level has been very low after induction for 7 days (Fig. 3a-c). At the same time, the cell telomerase activity and the number of cells at the S stage both at moderate levels (Fig. 1d-e). According to the reported periods
for the derivation of MSCs from hPSCs using monolayer method and MSCs culture medium [38], as well as the negative expression of osteogenic markers, we speculated mesenchymal-like cells were obtained at day 7 (Fig. 3–6). After induction for 14 days, Cells stared to express osteogenic markers of RUNX2, OCN and COL1A1, which suggested that MSCs began to differentiate into osteoblasts (Fig. 3–6). Moreover, their expression levels were increased as the osteogenic induction continued (Fig. 3–5). At the same time, cell cycle analysis indicated that more cells were arrested in the G0/G1 phase, and decreased the percentage in G2/M and S phase with the development of osteogenic induction differentiation (Fig. 2). Corresponding to the cell cycle analysis, the results of telomerase activity showed that it was stable at a lower level after 14 days of induction (Fig. 1d-e). Besides, typical calcium nodules were found in cell samples after induction for 21 days, and large alizarin red staining area was found on day 35. According to these experimental results, we speculated that there was a pre-osteoblast-like stage during 14~21 days of osteogenic differentiation, and osteoblast-like cells were induced at day 28 and 35. Moreover, our results proved that the differentiation efficiency using traditional FBS containing osteogenic induction medium is quite low, and a step by step directed induced differentiation system is highly required [39].

In this study, similar expression trends were found for almost all pluripotency and osteogenesis related markers between hESCs and hiPSCs during the osteogenic differentiation. However, it was not difficult to find that the expression of the same gene varied in different cell lines. We speculated that the difference in cell line and cell state would affect the osteogenic differentiation efficiency and gene expression changes.

In a word, hPSCs have been successfully differentiated into osteoblast-like cells using traditional FBS and osteogenic differentiation factors containing medium, but the differentiation efficiency was still quite low as confirmed by AS staining. It is urgent to further optimize the process of osteogenic differentiation of hPSCs so as to improve the efficiency of osteogenic differentiation. This presented study improves the understanding of the osteogenic differentiation process of hPSCs, but an accurate definition of various intermediate cells is still a problem because of a remarkably heterogeneous population of differentiated cells. Subsequently, more specific expression markers will be applied using MSCs and osteoblasts extracted from the human body as controls, aiming to define the osteogenic differentiation process of hPSCs more clearly. More importantly, we start to involve an effort to develop a chemically defined in vitro induction system for the stepwise osteogenic differentiation of hPSCs.

4. Conclusions

In this presented study, the osteogenic differentiation process of monolayer cultured hESCs and hiPSCs were analyzed in detail. The expression of pluripotency makers was reduced, and dynamically changes with the extension of differentiation time was found for the osteogenic-related markers. Moreover, it was confirmed that cell telomerase activity, cell cycle, quantitative protein expression of RUNX2 and nucleus staining could be used as valuable evidences to determine the cell differentiation processes. Although hPSCs were successfully induced into osteoblast-like cells in traditional serum-containing osteogenic
medium, low expression level of osteogenic-related markers and few calcium nodules were detected throughout the 35 days of induction. The low differentiation efficiency is mainly because remarkably heterogenous population of differentiated cells were obtained using a too simple induction method. Therefore, the osteogenic differentiation medium of hPSCs should be optimized by supplementing functional compounds at defined stages in the future study. Our study has achieved better understanding on the osteogenic differentiation process of hPSCs, which has value to both optimize the differentiation system and obtain target cells of mesenchymal like cells and osteoblast-like cells.

**Abbreviations**

**hPSCs**: human pluripotent stem cells; **hESCs**: human embryonic stem cells; **hiPSCs**: human induced pluripotent stem cells; **RT-PCR**: reverse transcription-polymerase chain reaction; **MSCs**: Mesenchymal stem cells; **FBS**: fetal bovine serum; **ALP**: alkaline phosphatase; **RUNX2**: runt-related transcription factor 2; **OSX**: osterix; **COL1A1**: type I collagen; **OCN**: osteocalcin; **BSP**: bone sialoprotein; **OPN**: osteopontin; **EDTA**: ethylene diamine tetraacetic acid; **NEAA**: non-essential amino acid; **BSA**: bovine serum albumin; **NHSS**: N-hydroxysulfosuccinimide sodium salt; **EDC**: 1-Ethyl-3-(3-dimethylamino propyl)carbodiimide; **CCK8**: cell counting kit-8; **OM**: osteogenic medium; **QCM**: quartz crystal microbalance; **RT**: room temperature; **hBMSCs**: human bone marrow mesenchymal stem cells; **AS**: alizarin red staining.

**Declarations**

**Funding**

This work was funded by the National Natural Science Foundation of China (No. 81571824), Gansu Province Science Foundation for Youths (18JR3RA295), Young Elite Scientist Sponsorship Program by CSA (No.2018QNRC001), Chengguan District Science and Technology Project (2018-7-6), Fundamental Research Funds for the Central Universities (lzujbky-2015-295, lzujbky-2018-27), Lanzhou University Hospital of Stomatology Research Support Fund (LZUKQKY-2019-Y10, lzukqky-2019-t9).

**Authors’ Contributions**

FL and RZ contributed to design the study and critically revised the manuscript; PZ, JM S, JES, YH, HJL, YMS and FF performed all the experimental works; PZ, JMS, JES and YH contributed to perform the statistical analysis, interpret the results and draft the manuscript; JLW contributed to review the manuscript. All authors read and approved the final manuscript.

**Availability of data and materials**

The data and materials used and/or analysed during the current study are not publicly available but available from the corresponding author on reasonable request.

**Competing interests**
The authors declare that they have no competing interests.

Acknowledgement
Not applicable.

Consent for publication
Not applicable.

Ethics approval and consent to participate
Not applicable.

Supplementary information
Additional file of supporting information.

References

1. Bran GM, Stern-Straeter J, Hörmann K, Riedel F, Goessler UR. Apoptosis in bone for tissue engineering. Arch Med Res. 2008;39(5):467–82. doi:10.1016/j.arcmed.2008.02.007.

2. El Tamer MK, Reis RL. Progenitor and stem cells for bone and cartilage regeneration. J Tissue Eng Regen Med. 2009;3(5):327–37. doi:10.1002/term.173.

3. Siegel G, Kluba T, Hermanutz-Klein U, Bieback K, Northoff H, Schäfer R. Phenotype, donor age and gender affect function of human bone marrow-derived mesenchymal stromal cells. BMC Med. 2013;11:146. doi:10.1186/1741-7015-11-146.

4. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell. 2006;126(4):663–76. doi:10.1016/j.cell.2006.07.024.

5. Brafman DA. Generation, Expansion, and Differentiation of Human Pluripotent Stem Cell (hPSC) Derived Neural Progenitor Cells (NPCs). Methods Mol Biol. 2015;1212:87–102. doi:10.1007/7651_2014_90.

6. Aubin JE. Regulation of osteoblast formation and function. Rev Endocr Metab Disord. 2001;2(1):81–94. doi:10.1023/a:1010011209064.

7. Long F. Building strong bones: molecular regulation of the osteoblast lineage. Nat Rev Mol Cell Biol. 2011;13(1):27–38. doi:10.1038/nrm3254.

8. Matsushita Y, Ono W, Ono N. Growth plate skeletal stem cells and their transition from cartilage to bone. Bone. 2020;136:115359. doi:10.1016/j.bone.2020.115359.

9. Heins N, Englund MC, Sjöblom C, et al. Derivation, characterization, and differentiation of human embryonic stem cells. Stem Cells. 2004;22(3):367–76. doi:10.1634/stemcells.22-3-367.
10. Becker KA, Stein JL, Lian JB, van Wijnen AJ, Stein GS. Establishment of histone gene regulation and cell cycle checkpoint control in human embryonic stem cells. J Cell Physiol. 2007;210(2):517–26. doi:10.1002/jcp.20903.

11. Zhou P, Wu F, Zhou T, et al. Simple and versatile synthetic polydopamine-based surface supports reprogramming of human somatic cells and long-term self-renewal of human pluripotent stem cells under defined conditions. Biomaterials. 2016;87:1–17. doi:10.1016/j.biomaterials.2016.02.012.

12. Zhou Y, Zhou P, Xin Y, et al. Trend of telomerase activity change during human iPSC self-renewal and differentiation revealed by a quartz crystal microbalance based assay. Sci Rep. 2014;4:6978. doi:10.1038/srep06978[13] Zhang S, Sun Y, Sui Y, et al. Determining Osteogenic Differentiation Efficacy of Pluripotent Stem Cells by Telomerase Activity. Tissue Eng Regen Med. 2018;15(6):751–760. doi:10.1007/s13770-018-0138-6.

13. Soufi A, Dalton S. Cycling through developmental decisions: how cell cycle dynamics control pluripotency, differentiation and reprogramming. Development. 2016;143(23):4301–11. doi:10.1242/dev.142075.

14. Kareta MS, Sage J, Wernig M. Crosstalk between stem cell and cell cycle machineries. Curr Opin Cell Biol. 2015;37:68–74. doi:10.1016/j.ceb.2015.10.001.

15. Dalton S. Linking the Cell Cycle to Cell Fate Decisions. Trends Cell Biol. 2015;25(10):592–600. doi:10.1016/j.tcb.2015.07.007.

16. Morgan DO. Principles of CDK regulation. Nature. 1995;374(6518):131–4. doi:10.1038/374131a0.

17. Zvereva MI, Shcherbakova DM, Dontsova OA. Telomerase: structure, functions, and activity regulation. Biochemistry. 2010;75(13):1563–83. doi:10.1134/s0006297910130055.

18. Taji F, Kouchesfahani HM, Sheikholeslami F, et al. Autophagy induction reduces telomerase activity in HeLa cells. Mech Aging Dev. 2017;163:40–5. doi:10.1016/j.mad.2016.12.011.

19. Neganova I, Lako M. G1 to S phase cell cycle transition in somatic and embryonic stem cells. J Anat. 2008;213(1):30–44. doi:10.1111/j.1469-7580.2008.00931.x.

20. Long F. Building strong bones: molecular regulation of the osteoblast lineage. Nat Rev Mol Cell Biol. 2011;13(1):27–38. doi:10.1038/nrm3254. Published 2011 Dec 22.

21. Ishiy FA, Fanganiello RD, Griesi-Oliveira K, et al. Improvement of In Vitro Osteogenic Potential through Differentiation of Induced Pluripotent Stem Cells from Human Exfoliated Dental Tissue towards Mesenchymal-Like Stem Cells. Stem Cells Int. 2015;2015:249098. doi:10.1155/2015/249098.

22. Wu H, Whitfield TW, Gordon JA, et al. Genomic occupancy of RUNX2 with global expression profiling identifies a novel dimension to control of osteoblastogenesis. Genome Biol. 2014;15(3):R52. doi:10.1186/gb-2014-15-3-r52.

23. Park SJ, Jung SH, Jogeswar G, et al. The transcription factor snail regulates osteogenic differentiation by repressing RUNX2 expression. Bone. 2010;46(6):1498–507. doi:10.1016/j.bone.2010.02.027.
24. Narisawa S, Yadav MC, Millán JL. In vivo overexpression of tissue-nonspecific alkaline phosphatase increases skeletal mineralization and affects the phosphorylation status of osteopontin. J Bone Miner Res. 2013;28(7):1587–98. doi:10.1002/jbmr.1901.

25. Liu J, Chen W, Zhao Z, Xu HHK. Effect of NELL1 gene overexpression in iPSC-MSCs seeded on calcium phosphate cement. Acta Biomater. 2014;10(12):5128–38. doi:10.1016/j.actbio.2014.08.016.

26. Liu J, Chen W, Zhao Z, Xu HH. Reprogramming of mesenchymal stem cells derived from iPSCs seeded on biofunctionalized calcium phosphate scaffold for bone engineering. Biomaterials. 2013;34(32):7862–72. doi:10.1016/j.biomaterials.2013.07.029.

27. Karp JM, Ferreira LS, Khademhosseini A, Kwon AH, Yeh J, Langer RS. Cultivation of human embryonic stem cells without the embryoid body step enhances osteogenesis in vitro. Stem Cells. 2006;24(4):835–43. doi:10.1634/stemcells.2005-0383.

28. Romberg RW, Werness PG, Riggs BL, Mann KG. Inhibition of hydroxyapatite crystal growth by bone-specific and other calcium-binding proteins. Biochemistry. 1986;25(5):1176–80. doi:10.1021/bi00353a035.

29. Hauschka PV, Wians FH Jr. Osteocalcin-hydroxyapatite interaction in the extracellular organic matrix of bone. Anat Rec. 1989;224(2):180–8. doi:10.1002/ar.1092240208.

30. Kärner E, Bäckesjö CM, Cedervall J, Sugars RV, Ahrlund-Richter L, Wendel M. Dynamics of gene expression during bone matrix formation in osteogenic cultures derived from human embryonic stem cells in vitro. Biochim Biophys Acta. 2009;1790(2):110–8. doi:10.1016/j.bbagen.2008.10.004.

31. Wang M, Deng Y, Zhou P, et al. In vitro culture and directed osteogenic differentiation of human pluripotent stem cells on peptides-decorated two-dimensional microenvironment. ACS Appl Mater Interfaces. 2015;7(8):4560–72. doi:10.1021/acsami.5b00188.

32. Tsao YT, Huang YJ, Wu HH, Liu YA, Liu YS, Lee OK. Osteocalcin Mediates Biomineralization during Osteogenic Maturation in Human Mesenchymal Stromal Cells. Int J Mol Sci. 2017;18(1):159. doi:10.3390/ijms18010159.

33. Kärner E, Bäckesjö CM, Cedervall J, Sugars RV, Ahrlund-Richter L, Wendel M. Dynamics of gene expression during bone matrix formation in osteogenic cultures derived from human embryonic stem cells in vitro. Biochim Biophys Acta. 2009;1790(2):110–8. doi:10.1016/j.bbagen.2008.10.004.

34. Prockop DJ. Marrow stromal cells as stem cells for nonhematopoietic tissues. Science. 1997;276(5309):71–4. doi:10.1126/science.276.5309.71.

35. Takashima Y, Era T, Nakao K, et al. Neuroepithelial cells supply an initial transient wave of MSC differentiation. Cell. 2007;129(7):1377–88. doi:10.1016/j.cell.2007.04.028.

36. Zhang C. Transcriptional regulation of bone formation by the osteoblast-specific transcription factor Osx. J Orthop Surg Res. 2010;5:37. doi:10.1186/1749-799X-5-37. Published 2010 Jun 15.

37. Arpornaemaeklong P, Brown SE, Wang Z, Krebsbach PH. Phenotypic characterization, osteoblastic differentiation, and bone regeneration capacity of human embryonic stem cell-derived mesenchymal stem cells. Stem Cells Dev. 2009;18(7):955–68. doi:10.1089/scd.2008.0310.
Figures

![Diagram of experimental protocol](image)

**Figure 1**

Analyses of cell viability and telomerase activity for H9 hESC and hNF-C1 hiPSCs during 35 days of osteogenic differentiation. (a) A schematic diagram of experimental protocol. (b-c) After osteogenic induction for various days (0, 3, 7, 14, 21, 28 and 35), the cell viability of hESCs (b) and hiPSCs (c) were detected using a CCK8 reagent. (d-e) The telomerase activity of hESCs (d) and hiPSCs (e) were measured by a quantitative method based on quartz crystal microbalance (QCM).
Figure 2

Analyses of the cell cycle for H9 hESCs and hNF-C1 hiPSCs during 35 days of osteogenic differentiation. (a-b) The cell cycle changes of hESCs (a) and hiPSCs (b) after induction for different times (0 day, 3 days, 7 days, 14 days, 21 days, 28 days and 35 days) were studied using flow cytometry.
Figure 3

The expression of marker genes in H9 hESCs and hNF-C1 hiPSCs during osteogenic differentiation. (a-g) After osteogenic induction for up to 35 days, the expression of marker genes such as OCT-4 (a), NANOG (b), TRET (c), ALP (d), RUNX2 (e), COL1A1 (f) and OCN (g) in cell samples was measured by RT-PCR.
Figure 4

The expression of OCT-4 and RUNX2 in hPSCs samples during osteogenic differentiation. The expression of OCT-4 (green) and RUNX2 (green) in H9 hESCs and hNF-C1 hiPSCs after osteogenic induction for indicated days were detected by immunofluorescence. The nucleus was shown to be blue by DAPI staining. Scale bars, 100 μm.
Figure 5

The measurements for RUNX2 positive expression in hPSCs during osteogenic differentiation. After osteogenic induction for different days (0, 3, 7, 14, 21, 28 and 35), the expression of RUNX2 in cells was measured by flow cytometry, and undifferentiated hPSCs were conducted as control.
Figure 6

The expression of COL1A1 and OCN in induced hESCs and hiPSCs. After osteogenic induction 21 days, 28 days or 35 days, the expression of COL1A1 (red) and OCN (red) in cell samples were detected by immunofluorescence. The nucleus was shown to be blue by DAPI staining. Scale bars, 100 μm.
Figure 7

The alizarin red staining analyses for hPSCs during osteogenic differentiation. (a-b) Cell morphology and culture plate photograph for alizarin red staining hESCs (a) and hiPSCs (b) after culturing in induction medium for up to 35 days. Scale bars, 200 μm. (c-d) Cetylpyridinium bromide solution was applied to dissolve deposited alizarin red and the absorbance at 490 nm was measured.
**Figure 8**

A dynamic map for the osteogenic differentiation of hPSCs. Expression change for pluripotency markers of OCT-4, NANOG, TRET, ALP, RUNX2, COL1A1 and OCN and cell telomerase activity in hPSCs during 35 days of osteogenic differentiation. The panels represent (from left to right) hPSCs were induced for 0 days, 3 days, 7 days, 14 days, 21 days, 28 days or 35 days, which cover the various stages of osteoblastic lineage development.

**Supplementary Files**

This is a list of supplementary files associated with this preprint. Click to download.

- SupportingInformationforhPSCsOM20200702.docx