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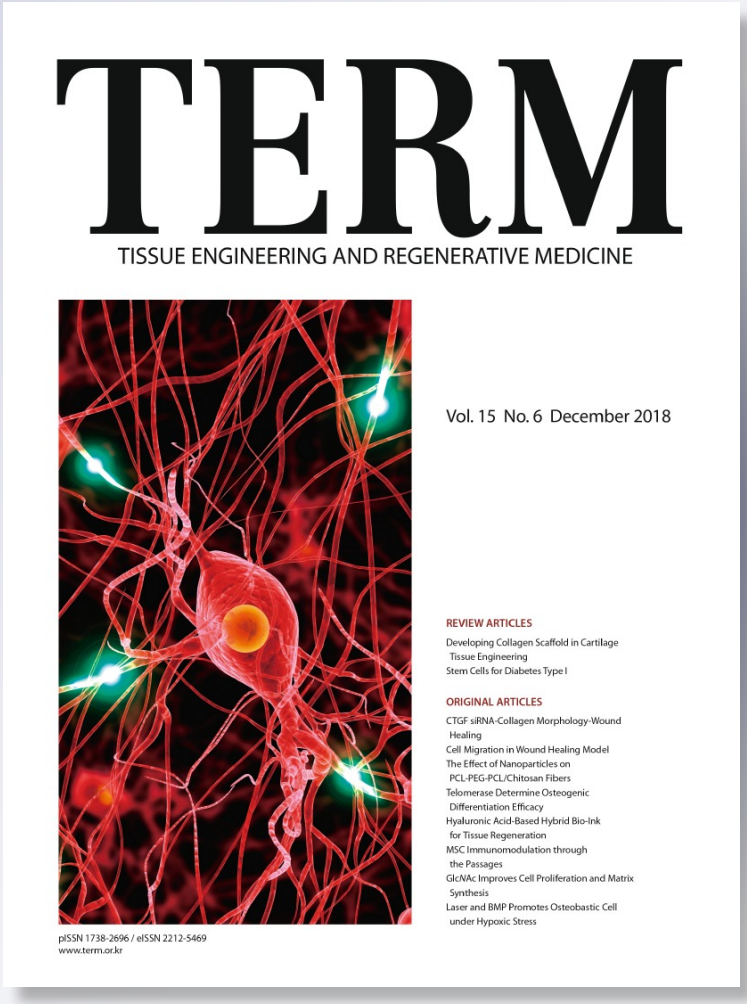
Tissue Engineering and Regenerative Medicine

ISSN 1738-2696
Volume 15
Number 6

Tissue Eng Regen Med (2018)
15:751-760
DOI 10.1007/s13770-018-0138-6



Springer

Tissue Eng Regen Med (2018) 15(6):751–760
https://doi.org/10.1007/s13770-018-0138-6

Online ISSN 2212-5469
Print ISSN 1738-2696

ORIGINAL ARTICLE

Determining Osteogenic Differentiation Efficacy of Pluripotent Stem Cells by Telomerase Activity

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Received: 26 March 2018 / Revised: 14 June 2018 / Accepted: 18 June 2018 / Published online: 24 July 2018

Abstract

BACKGROUND: Bone tissue engineering based on pluripotent stem cells (PSCs) is a new approach to deal with bone defects. Protocols have been developed to generate osteoblasts from PSCs. However, the low efficiency of this process is still an important issue that needs to be resolved. Many studies have aimed to improve efficiency, but developing accurate methods to determine efficacy is also critical. Studies using pluripotency to estimate efficacy are rare. Telomerase is highly associated with pluripotency.

METHODS: We have described a quantitative method to measure telomerase activity, telomeric repeat elongation assay based on quartz crystal microbalance (QCM). To investigate whether this method could be used to determine the efficiency of *in vitro* osteogenic differentiation based on pluripotency, we measured the pluripotency pattern of cultures through stemness gene expression, proliferation ability and telomerase activity, measured by QCM.

RESULTS: We showed that the pluripotency pattern determined by QCM was similar to the patterns of proliferation ability and gene expression, which showed a slight upregulation at the late stages, within the context of the general downregulation tendency during differentiation. Additionally, a comprehensive gene expression pattern covering nearly every stage of differentiation was identified.

CONCLUSION: Therefore, this assay may be powerful tools for determining the efficiency of differentiation systems based on pluripotency. In this study, we not only introduce a new method for determining efficiency based on pluripotency, but also provide more information about the characteristics of osteogenic differentiation which help facilitate future development of more efficient protocols.

Keywords Mouse embryonic stem cells · Pluripotency · Telomerase activity · Osteogenic differentiation · Efficiency

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1 Introduction

With an increasing number of bone injuries and diseases, the task of finding efficient methods for bone defect repair has become urgent [1]. Autologous bone grafts and allogenic bone implants have been used previously; however, shortcomings with respect to immunogenicity and the rarity of bone sources limit their application.

Pluripotent stem cells (PSCs) possess unique ability to proliferate and differentiate into all types of cells, holding great promise in the field of regenerative medicine [2]. Currently, it is easy to generate osteoblast-like cells from PSCs in the presence of three exogenous factors: dexamethasone, ascorbic acid and β-glycerophosphate [3, 4]. Although this protocol seems ideal for bone tissue engineering, low differentiation efficacy results in a large gap between basic research and clinical application [5, 6]. Therefore, many studies have endeavored to improve the efficiency of osteogenic differentiation from PSCs, including research into promotion of mineralization and osteoblast relative gene expression through introducing some new factors into differentiation system [7–11]. However, there is a lack of research on how to assess differentiation efficiency in the first instance.

It is currently impossible to obtain pure mature osteoblasts from PSCs *in vitro*, which is a basic requirement for clinical application. Undifferentiated PSCs still exist in induced systems [7]; therefore, pluripotency is an important characteristic of differentiation cultures that could be used to estimate efficacy. However, few studies have examined pluripotency patterns of PSCs in differentiated cultures [12]. Theoretically, the pluripotent capacity of PSCs should decrease during differentiation. Several parameters are used to analyze PSCs, such as stem cell marker expression (Oct-4, Nanog, Sox2, et al.), and proliferation ability. Additionally, another stem cell marker that has been neglected to date is the telomere.

The telomere is a long repeat fragment located at the terminal of the chromosome protecting the genome from degradation [13]. Telomerase is a ribo-nucleoprotein complex that can add absent telomere fragments at the chromosome terminal [14]. Cells possessing a long telomere and high telomerase activity hold great promise of proliferation and differentiation [15]. Traditional assays used to measure telomerase activity, such as telomerase repeat amplification protocol (TRAP), have shortcomings [16]. Our research group developed a new method, which we termed telomeric repeat elongation assay based on quartz crystal microbalance (QCM), to detect telomerase activity. We successfully applied this label-free, quantitative and high-throughput method to detect and quantify the pluripotency of PSCs during passage [17, 18].

In this study, we investigate whether telomerase and QCM could be used to determine the efficiency of *in vitro* osteogenic differentiation. We analyzed stemness patterns by monitoring telomerase activity using QCM. For comparison, we also analyzed stemness patterns by marker gene expression and proliferation ability to determine the viability of using telomerase as a stem cell marker. Additionally, we studied the characteristics of pre-osteoblasts and osteoblasts during osteogenic differentiation to obtain more information about *in vitro* differentiation.

2 Materials and methods

2.1 Culturing mouse embryonic stem cells (mESCs)

We cultured mESCs on plates coated with Matrigel (Corning, New York, USA). Cells were cultured using maintenance medium (MM) under standard conditions (37 °C, 5% CO_2): high-glucose Dulbecco's modified Eagle's medium (Hyclone China, Beijing, China), 15% (v/v) fetal bovine serum (FBS; Invitrogen, Life Technologies, California, USA), 10 ng/ml LIF (Novoprotein, Shanghai, China), 1 mM sodium pyruvate (Sigma-Aldrich China, Shanghai, China), 0.1 mM nonessential amino acids (Sigma-Aldrich China, Shanghai, China), 1% β-mercaptoethanol (Invitrogen, Life Technologies, California, USA), 1% L-glutamine (Hyclone China, Beijing, China), and 1% penicillin/streptomycin (Hyclone China, Beijing, China). Media were changed daily, and cells were passaged once 80–90% confluence was attained.

2.2 Osteogenic differentiation

Cells were removed from the plates by treatment with 0.25% trypsin–EDTA (Invitrogen, Life Technologies, California, USA) for 2 min at 37 °C once 80–90% confluence was attained. At equivalent doses, approximately 2.5×10^5 cells were seeded on six-well plates and 1×10^5 cells on 12-well plates; all plates were coated with 0.1% (v/v) gelatin (Sigma-Aldrich China, Shanghai, China). To initiate differentiation, we used osteogenic medium (OM) composed of KO-DMEM (Invitrogen, Life Technologies, California, USA), 20% (v/v) FBS (Invitrogen, Life Technologies, California, USA), 1% (v/v) GlutaMAX (Invitrogen, Life Technologies, California, USA), 1% penicillin/streptomycin (Hyclone China, Beijing, China), 1% β-mercaptoethanol (Invitrogen, Life Technologies, California, USA), 1% (v/v) ascorbic acid (Invitrogen, Life Technologies, California, USA), 10 mM β-glycerophosphate (Sigma-Aldrich China, Shanghai, China), and 1 μM dexamethasone (Sigma-Aldrich China, Shanghai, China). Fresh media were changed every 2 days.

2.3 Cell proliferation analysis

Cell proliferation was determined using CCK-8 assay (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. We both measured proliferation ability of mESCs and OM cultures. They were seeded at same density and mESCs were measured every day while OM cultures were at days 3, 7, 14, 21, and 28. Briefly, 600 μl of reaction reagent (10% v/v CCK-8 reagent in KO-DMEM) was added to the cells, which were then incubated for 2 h at 37 °C in darkness. Colored products were qualified by measuring absorbance at 450 nm in a microplate reader (model 680; Bio-Rad, Ontario, Canada). The activity of OM-treated cells was normalized to that of pure KO-DMEM, which is considered non-active, and three parallel wells were used for each time point.

2.4 Quartz crystal microbalance (QCM) analysis

The process of preparing QCM chips and cell extracts was based on a previously described protocol [17]. After measuring the total protein concentration of the samples using a bicinchoninic acid (BCA) kit (Invitrogen, Life Technologies, California, USA), the concentrations were adjusted to the same level. In this experiment, we performed on-line detection assay according to previously described methods [17]. To obtain a more stable reaction system, a telomerase buffer composed of 48 μl cell extracts, 12 μl 10 μM dNTP mix (Invitrogen, Life Technologies, California, USA) and 540 μl reaction buffer was used.

2.5 Alkaline phosphatase (ALP) activity

We used both qualitative and quantitative methods to determine the ALP activity of the OM cultures. For the qualitative assays, we used an ALP staining kit (CW-BIO, Nanjing, China). After fixing in absolute ethylalcohol for 30 min, the cells were washed with distilled water three times. The reaction solution was added to each well and the cells were incubated for 10 min in darkness at room temperature, and then washed three times with distilled water. We recorded ALP staining using a phase contrast inverted microscope (Eclipse TE2000-U; Nikon, Tokyo, Japan). To quantify ALP activity, we used an AKP detection kit (NJJC-BIO, Nanjing, China) according to the manufacturer's instructions. All unknown samples were normalized to a standard sample as a positive control and phosphate-buffered saline as a negative control; three wells were measured for each sample.

2.6 Alizarin red staining and quantification

Cultures were fixed in 4% (w/v) paraformaldehyde for 30 min before staining. After washing with deionized water, cells were stained with 2% (w/v) Alizarin Red S (Sigma-Aldrich China, Shanghai, China) solution, pH 4.2, for 20 min and washed three times with deionized water. To quantify the formation of calcium nodules, 500 μl of 1% (w/v) cetylpyridinum chloride (Sigma-Aldrich China, Shanghai, China) was added to the stained wells and then incubation was conducted at 37 °C for 20 min. The colored product was then transferred to 96-well plates and the absorbance was measured at 490 nm in a microplate reader (model 680; Bio-Rad, Ontario, Canada).

2.7 Immunofluorescence

After being fixed with 4% (w/v) paraformaldehyde for 30 min, the samples were permeabilized in 0.2% (v/v) Triton X-100 (Sigma-Aldrich China, Shanghai, China) for 30 min, and blocked in 3% (w/v) bovine serum albumin (Sigma-Aldrich China, Shanghai, China) for 2 h at room temperature. Cells were then incubated with primary antibody overnight at 4 °C at the following dilutions: OCT-4 (1:200; Abcam, Massachusetts, USA), RUNX2 (1:100; Abcam, Massachusetts, USA), osteocalcin (OCN) (1:200; Abcam, Massachusetts, USA), and COL1A1 (1:300; Abcam, Massachusetts, USA). Anti-mouse and anti-rabbit second antibody (1:500; Invitrogen, Life Technologies, California, USA) treatment was applied for 1 h at room temperature in darkness. Finally, 4′,6-diamidino-2-phenylindole (DAPI) (1:5000; Invitrogen, Life Technologies, California, USA) staining was performed for 5 min to identify the cell nucleus.

2.8 Gene expression analysis

Total RNA was isolated from cells in triplicate using Trizol reagent (Invitrogen, Life Technologies, California, USA). First-strand cDNA was reverse-transcribed using a RevertAid First Strand cDNA Synthesis Kit (Invitrogen, Life Technologies, California, USA), as described by the manufacturer. We used cDNA and SYBR green (Roche, China, Shanghai, China) to perform quantitative real-time polymerase chain reaction (qRT-PCR) analysis, using an ABI 7500 RT-PCR machine (Invitrogen, Life Technologies, California, USA). The reactions were run at 95 °C predenaturation for 10 min, followed by 40 cycles at 95 °C for 5 s and 60 °C for 30 s. For PCR, cDNA was amplified in a 20 μl system using Taq DNA polymerase (TOYOBO, Osaka, Japan). The conditions were as follows: 2 min at 94 °C, followed by 35 cycles of 10 s denaturation at 98 °C, 30 s annealing at 51 °C, 30 s extension at 68 °C, and a

final elongation at 68 °C for 5 min. Products were analyzed on 1% agarose gel.

2.9 Statistical analysis

All quantitative values are expressed as mean ± standard deviation (SD), unless otherwise stated. Mineralized nodule formation, ALP assay results, and gene expression (from three independent experiments) were compared between differentiation times by analysis of variance (ANOVA) and $p < 0.05$ was taken as a significant difference.

3 Results

3.1 Pluripotency pattern of mESCs and OM cultures

The mESCs exhibited characteristics of undifferentiated cells, in terms of cell morphology and Oct-4 expression (Figs. 1A, 2). The cultures were condensed and showed stem cell-like morphology. RT-PCR and immunofluorescence of Oct-4 revealed high levels of expression in mESCs.

To stimulate osteogenic differentiation, cells were resuspended in new wells and cultured in OM. RT-PCR and immunofluorescence revealed lower expression levels of Oct-4. The morphology of cells in OM conditions began

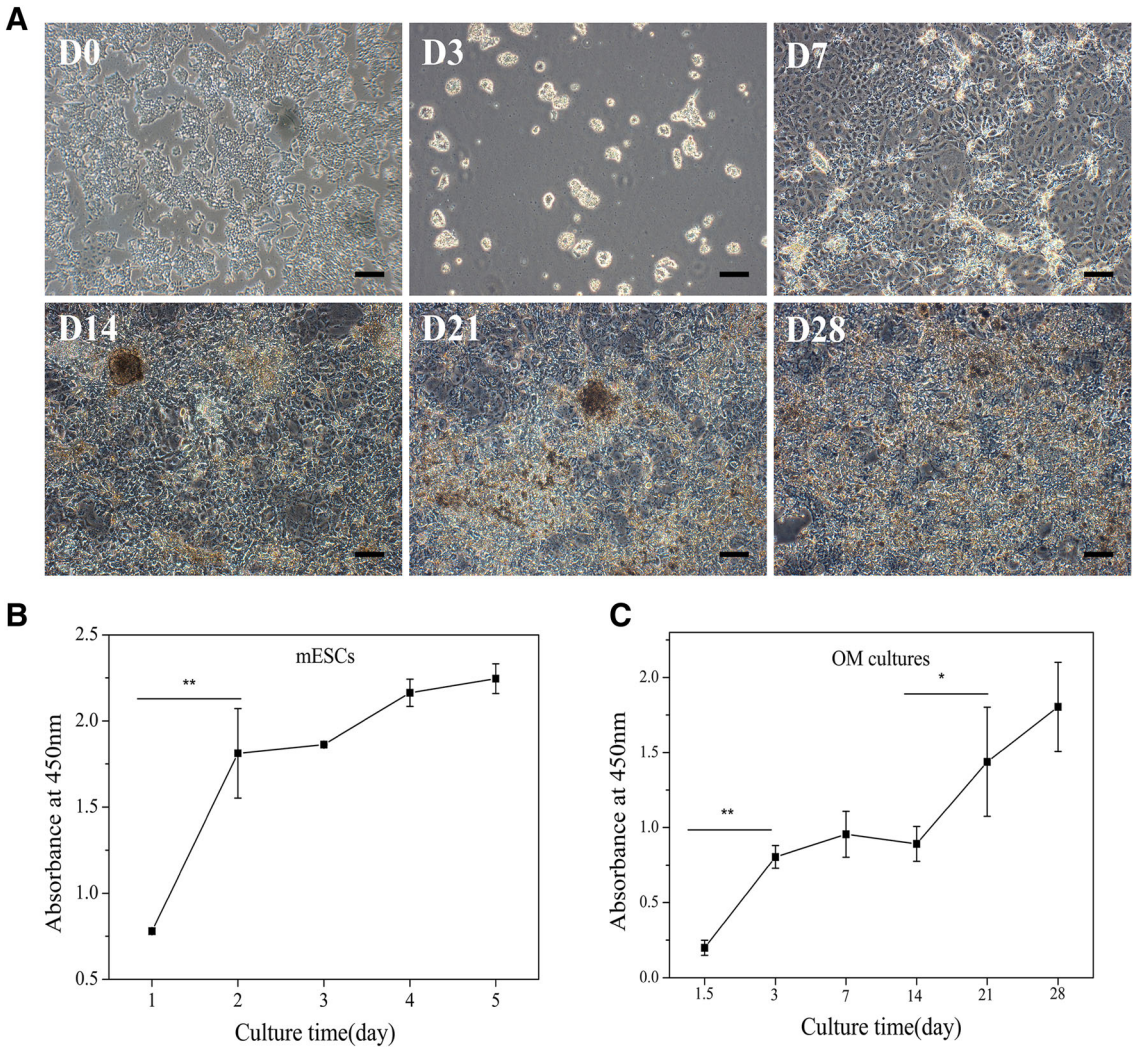

Fig. 1 Proliferation and morphology of osteogenic medium (OM) cultures. **A** Morphology of osteogenic medium cultures (D0 indicates undifferentiated mESCs). **B** CCK-8 assay results for cells in OM. Scale bar indicates 200 μm. Data are mean ± standard deviation (SD) of three independent experiments. *$p < 0.05$, **$p < 0.01$. Values differ significantly from each other

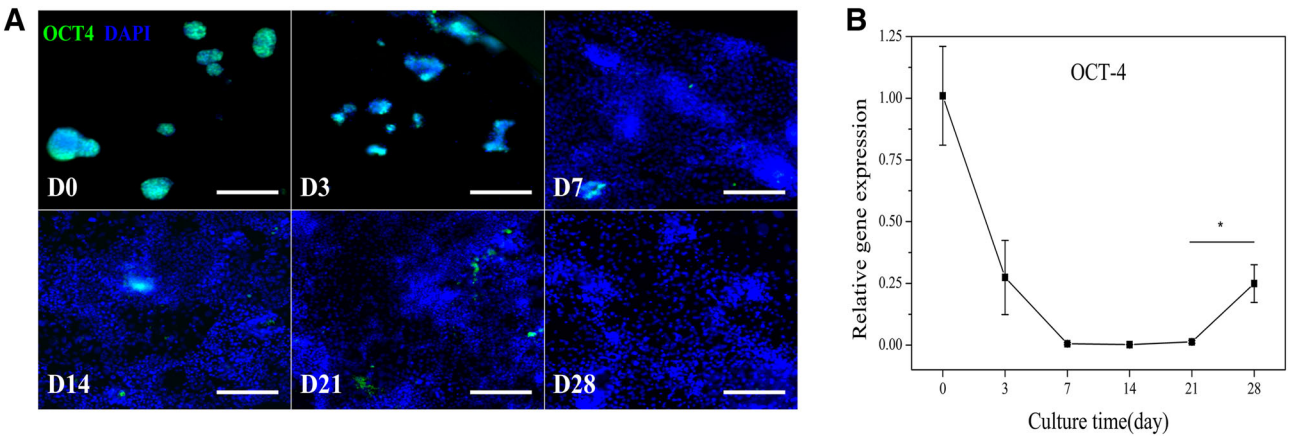

Fig. 2 OCT4 expression of mESCs and cells cultured with OM. **A**, **B** Immunofluorescence staining and real-time polymerase chain reaction (RT-PCR) analysis for OCT-4, (D0 indicates undifferentiated mESCs). Scale bar indicates 200 μm. Data are mean ± standard deviation (SD) of three independent experiments. $*p < 0.05$, $**p < 0.01$. Values differ significantly from each other

to change at day 7, with more cells changing to show a fibroblast-like morphology thereafter (Fig. 1A). Proliferation significantly increased from day 0 to day 7, at which time the cells exhibited a monolayer state. After day 14, rapid proliferation occurred, and multilayer morphology was observed. The proliferation increase prior to day 14 was caused by the limited number of cells per well during the early stages of the experiment (Fig. 1C). However, the trend after day 14 differed somewhat, showing that proliferation capacity is downregulated during differentiation. We also measured the proliferation ability of mESCs cultured in maintain medium using CCK8. Under the same seeding density, mESCs experienced more rapid proliferation than OM cultures (Fig. 1B), which demonstrated that the proliferation ability of mESCs was reduced after treatment with OM. After comparing Oct-4 expression (Fig. 2), we found that there were Oct-4 positive cells in the OM cultures at days 14 and 21, while RT-PCR results revealed that at day 28, Oct-4 showed a slight upregulation, exhibiting a similar pattern to that of the proliferation ability.

3.2 Monitoring telomerase activity during mESC differentiation

We used QCM to monitor trends of telomerase activity. Telomerase activity was defined according to frequency shifts from the beginning to the end of each independent experiment (Fig. 3A). The results (Fig. 3B, C) indicate that at day 0, telomerase activity in the mESCs was high (F_T of around 130 Hz). At the start of OM culturing, telomerase activity began to decrease. We observed a 50-Hz decrease in telomerase activity at day 21 and an 80-Hz decrease at day 28, consistent with downregulation of telomerase activity during differentiation. Additionally, our results showed that there was a small increase in telomerase activity at day 14, and telomerase activity at day 21 was higher than that at day 7. We found that the telomerase activity profiles determined by QCM were remarkably similar to the pluripotency trend described above, with both markers showing little upregulation during the late stages of OM culture. The marginal upregulation of telomerase activity at day 14 may have been caused by undifferentiated mESCs. We also measured expression of the telomerase reverse transcriptase (TERT) coding gene using RT-PCR (Fig. 3D), and observed a constant decrease; this was slightly different to the QCM results, possibly due to the different principles employed by each assays.

3.3 ALP activity and calcium nodule formation

We measured two specific properties of OM cultures: ALP activity and calcium nodule formation. ALP, a protein that may affect mineralization, is expressed in both embryonic stem cells and osteoblasts. Our results showed that undifferentiated mESCs had high ALP activity, which was downregulated prior to day 3, and then upregulated before reaching a peak at day 14 (Fig. 4A, C); this trend is consistent with previous reports [19, 20].

Calcium nodules were analyzed by ARS staining. As shown in Fig. 4C–D, positive staining areas were observed at day 21, and more appeared at day 28. The results indicated that the number of calcium nodules (bone-like structures) increased during OM culture, before peaking at day 21 (Fig. 4B, D). However, we observed a downregulation of calcium nodules by the end of the differentiation period, similar to the trend in expression observed for OCN mRNA. This result may indicate that there is an optimum time-point for osteogenic differentiation under the conditions we used in this study. There was a slight difference

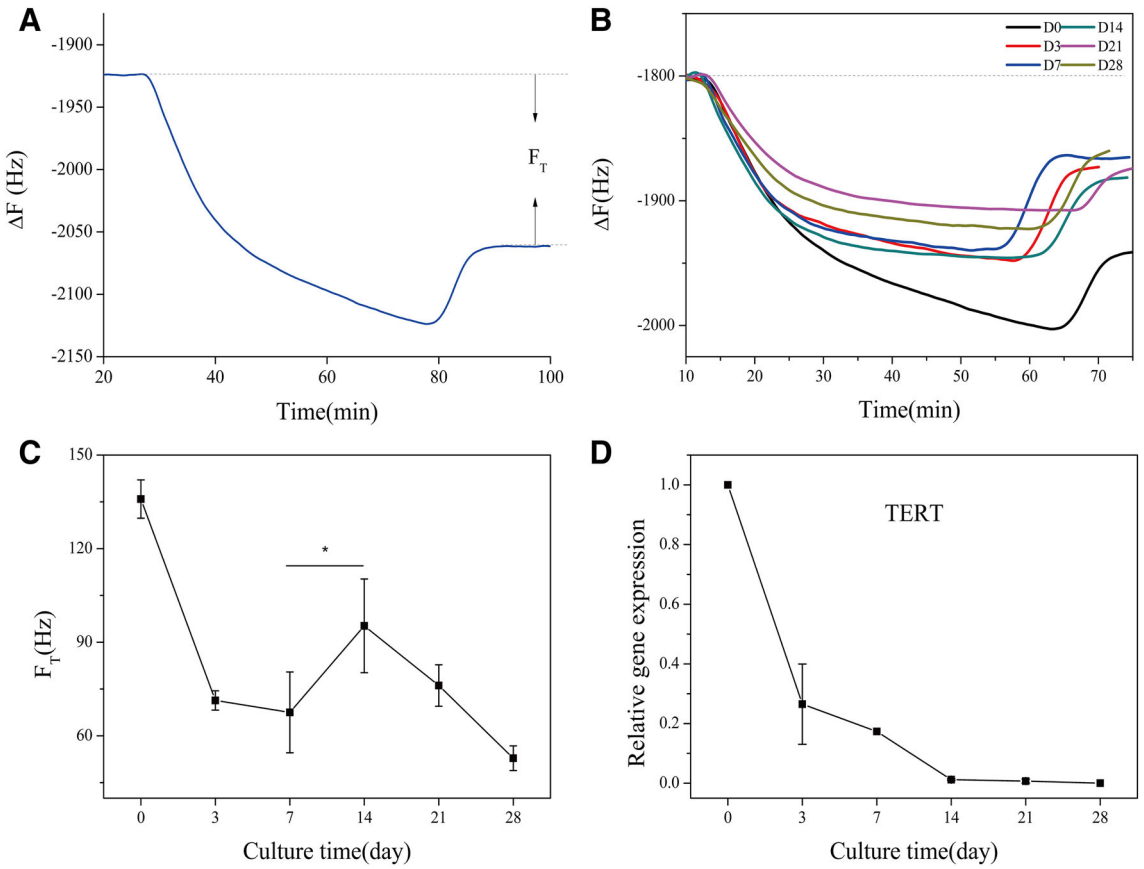

Fig. 3 Telomerase activity for osteogenic differentiation of mouse embryonic stem cells (mESCs). **A** A schematic of measuring telomerase activity by QCM. **B, C** F_T of cells cultured in OM at various time points. mESCs were undifferentiated on Day 0. **D** RT-PCR of TERT gene expression. **D** RT-PCR of telomerase reverse transcriptase (TERT) mRNA in OM cultures. Data are mean ± SD of three independent experiments. $*p < 0.05$, $**p < 0.01$. Values differ significantly from D0 (undifferentiated mESCs)

between the staining and quantification results, possibly due to the instability of the staining assays. These results show that we successfully initiated relatively efficient osteogenic differentiation.

3.4 Relative marker expression

Markers characteristic of the initiation and progenitor stages of differentiation were analyzed by semi-quantitative PCR (Fig. 5A). Brachyury, specifically expressed in the mesoderm [21], increased to a peak at day 7 and was subsequently downregulated. Meox1 and pax1 [22, 23] play roles in development of somites and sclerotomes, and are considered to be markers of a further stage of bone development. The expression of these two genes was upregulated, and then downregulated, as differentiation progressed. Moreover, the absence of meox1 expression occurred earlier compared with pax1. Sox9, a gene specific to mesenchymal condensation during bone development [24], began to be expressed at high levels at day 14; this trend was maintained until day 28. Osterix [25], an osteoprogenitor marker, was highly expressed at day 14, and then showed decreased tendency.

Collagenous and non-collagenous proteins are major constituents in the extracellular matrix and perform a calcifying function. To estimate the differentiation level of the cells and advancement to the mature differentiation stage, we measured the expression of these markers (Fig. 5B–E). Runx2 [26], a transcription factor involved in osteoblast formation, was upregulated from day 14 until the end of the OM culture period. Col1A1 [27] showed increasing upregulation during induction. OCN, a marker present during the latter stages of bone formation, was upregulated at the end of the differentiation period. Osteopontin (OPN), a transcript factor involved in matrix mineralization, was upregulated during differentiation [28]. The protein expression level displayed a profile nearly identical to that of mRNA (Fig. 6).

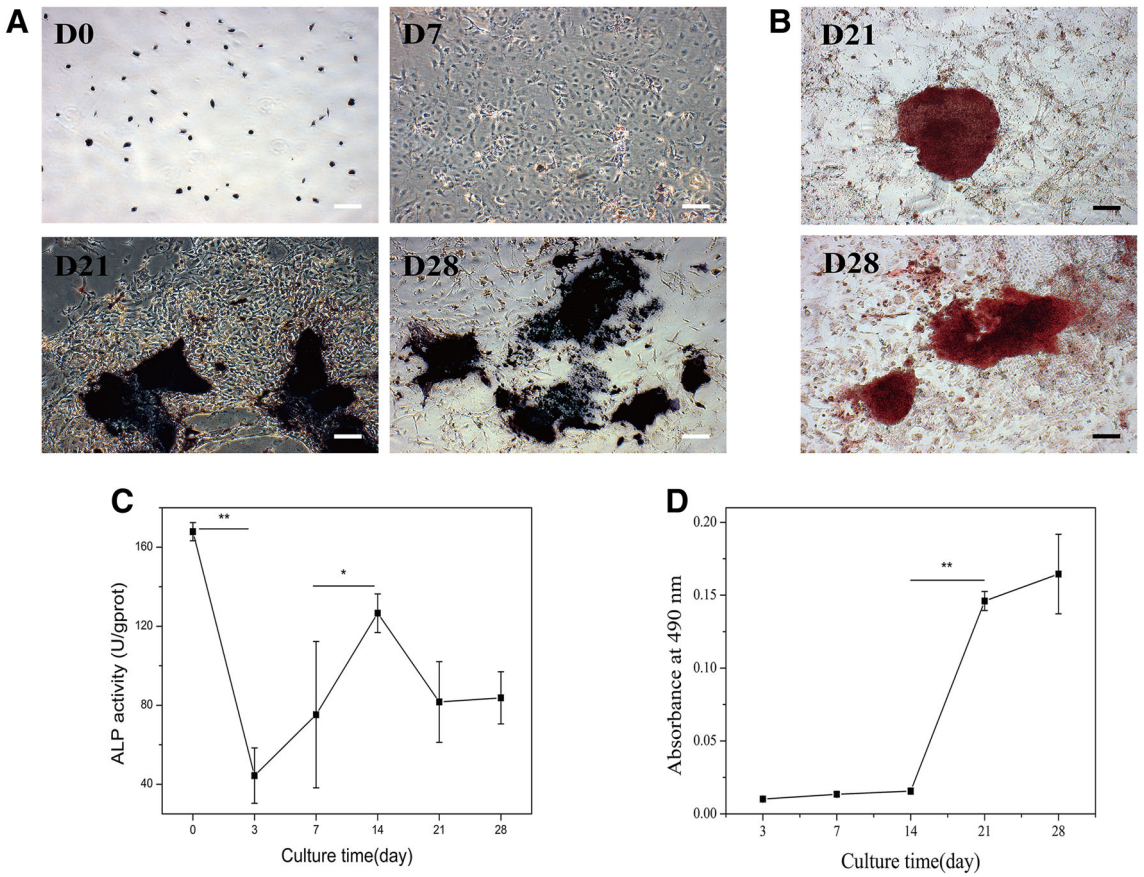

Fig. 4 Alkaline phosphatase (ALP) expression and calcium nodules of cells cultured in OM. **A** ALP staining. **B** Alizarin Red staining (ARS). **C** ALP activity quantification assay. **D** ARS quantification assay. Scale bar indicates 200 μm. Data are mean ± SD of three independent experiments. $*p < 0.05$, $**p < 0.01$. Values differ significantly from each other

4 Discussion

PSCs have become a powerful tool, having applications in regenerative medicine. In theory, PSCs can develop into any type of somatic cell, provided a suitable culture microenvironment. The pluripotency of PSC is an important factor that could affect the differentiation efficiency. In the present study, we showed that mESCs held great proliferation ability. After treatment with OM, the proliferation ability was generally reduced. However, we observed relatively increased Oct-4 expression and proliferation ability of OM cultures in the differentiation process. Some researchers have found that stem cell markers continue to be expressed after induction, possibly as a result of a number of undifferentiated cells or the unique function of some genes, which is in agreement with our results [7, 29]. These findings suggest a limited efficiency of *in vitro* differentiation protocol.

Previous studies have proven that cancer cells and PSCs hold higher telomerase activity than somatic cells. Telomerase activity is closely associated with proliferation and differentiation of PSCs [30]. Due to the limitations of traditional telomerase activity detection assays, we used a non-labeling, quantitative and high-throughput method, termed QCM, to measure telomerase activity. The results showed that although telomerase activity was generally downregulated in OM cultures, some upregulation was observed at later stages, similar to the pattern of Oct-4 expression and proliferation ability. We concluded that QCM was able to evaluate the differentiation efficacy of OM cultures, because the measured telomerase profile was similar to the patterns of other markers previously accepted in this field. Furthermore, this quantitative assay not only provides accurate results in this specific case, but may also potentially have further applications for determining other indexes associated with *in vitro* differentiation. For example, QCM may allow accurate determination of the number of undifferentiated cells in an induced system, by establishing a standard curve using a known number of PSCs and mature osteoblasts.

Some differences in telomerase activity were observed between the RT-PCR and QCM results, possibly owing to the different principles of each assay; RT-PCR determines telomerase activity by gene expression but QCM

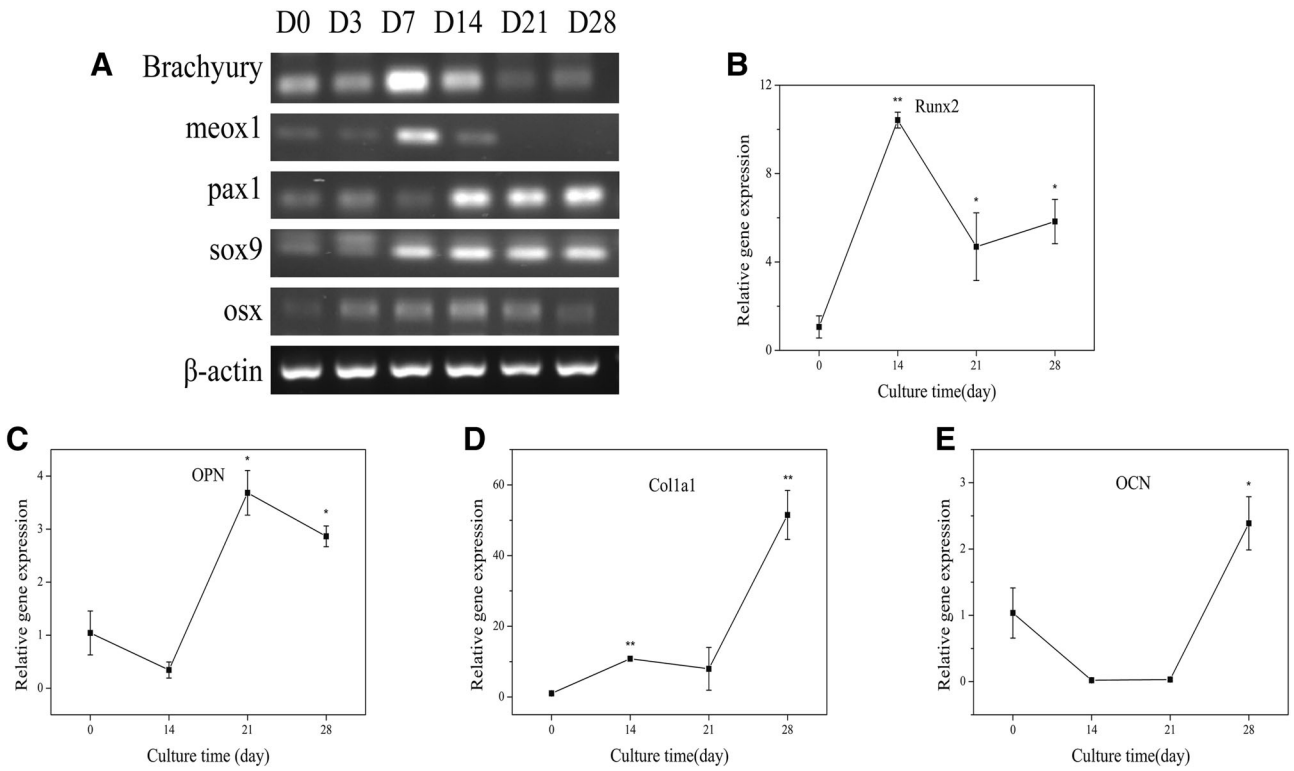

Fig. 5 Osteoblast relative marker expression of mESC cultures in OM at different stages of induction. **A** PCR analysis of early stage markers. **B–E** RT-PCR of late stage markers. Scale bar indicates 200 μm. Data are mean ± SD of three independent experiments. $*p < 0.05$, $**p < 0.01$. Values differ significantly from D0 (undifferentiated mESCs)

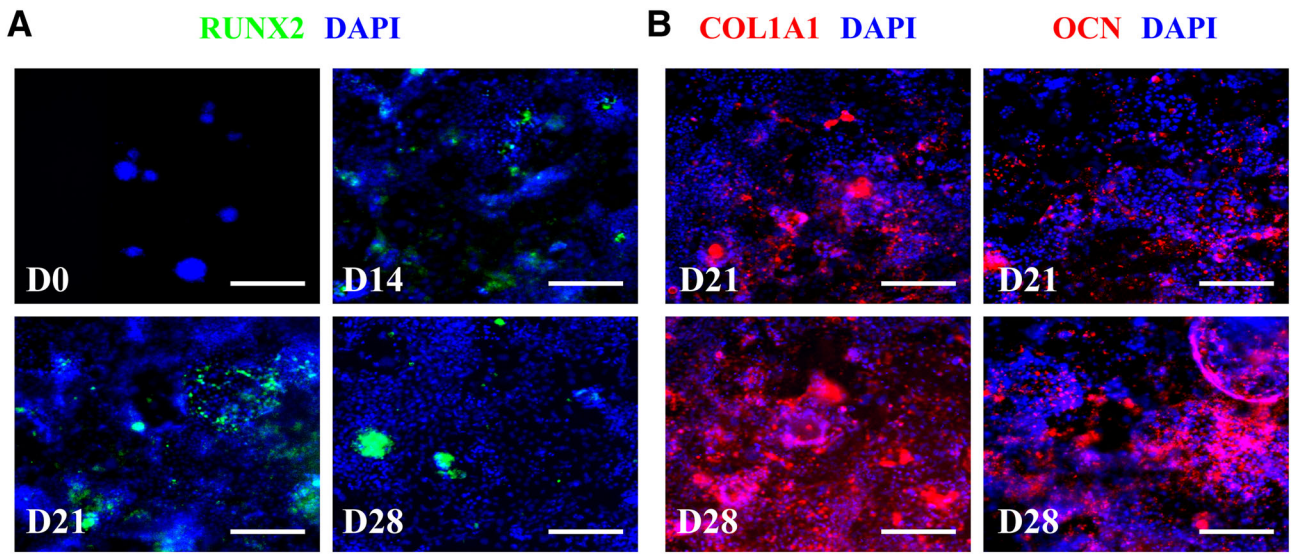

Fig. 6 Immunofluorescence staining of OM cultures for Runx2, Col1A1, and osteocalcin (OCN). Scale bar indicates 200 μm

determines it by protein phenotype. In accordance with the observation above, we suggest that QCM may be an appropriate and novel method to measure the expression of proteins at the functional level. The unique innovation that characterizes this method may provide opportunities to discover information that cannot be gleaned via conventional methods. Compared to traditional telomerase detection methods, this new assay avoids the problems associated with PCR and labeling.

In vitro osteogenic differentiation mimics the process of natural bone development. PSCs first develop into mesoderm-like cells, then show early stage bone development,

characterized by osteo-progenitors; the late stage is characterized by extracellular matrix formation and mineralization [31]. We measured markers specific to the early and late stages of bone development. The order of gene expression profiles specific to osteogenic differentiation in OM cultures was similar to that of bone development *in vivo*. Although late stage markers were all expressed after day 14, OCN and OPN showed downregulation before day 14. We speculated that the undifferentiated mESCs still existed in OM cultures may impact on the efficacy of osteogenic lineage induction. According to these results, we comprehensively established the gene expression pattern of osteogenic differentiated cultures *in vitro*.

In summary, the similar dynamic patterns of stem cell marker expression, proliferation ability and telomerase activity shown in this study not only demonstrate the extent of *in vitro* osteogenic differentiation, but also support the feasibility of QCM as a method to determine the efficiency of a certain differentiation system in a quantitative, high throughput and label-free way, by allowing identification of the number of undifferentiated PSCs. In addition, we comprehensively established the dynamic pattern of osteogenic differentiated cultures *in vitro*, taking into account the characteristics of osteo-progenitors and osteoblasts, and thus enhancing our understanding of this field. The ability to accurately determine differentiation efficacy provides a firm foundation to support the discovery of new differentiation mechanisms and aid in the development of better protocols to improve the efficiency of osteoblast differentiation, potentially bridging the gap between basic research in the laboratory and clinical applications.

Acknowledgements This work was afforded by the National Natural Science Foundation of China (No. 81571824), Natural Science Foundation of Jiangsu Province (BK20141141) and Fundamental Research Funds for the Central Universities (lzujbky-2018-27). The authors thank Dr. Ma Hongwei of the division of Nanobiomedicine, Suzhou Institute of Nano-Tech and Nano-Bionics, Chinese Academy of Sciences for the instruments and Materials required for the QCM assay. The mESCs were kindly provided by Dr. Tang Fuchou at Peking University.

Compliance with ethical standards

Conflict of interest All the authors declared that they have no conflict of interest.

Ethical statement There are no animal experiments carried out for this article.

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