Enhance the Osteogenic Differentiation of Human Pluripotent Stem Cells Through the Addition of Osteogenesis Peptide During Cell Type-based Periods

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Research Article

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Abstract

Background: The *in vitro* osteogenic differentiation of human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) have been one of the hot topics in bone tissue engineering. Moreover, supplement of osteogenesis chemical compounds is commonly applied to improve the differentiation efficiency. However, the differentiation process of hPSCs is much more complex than adult stem cells, and the unknown effect of material addition at different stages hamper the establish of step-wise induction systems for these human pluripotent stem cells (hPSCs). Recently, a bone forming peptide-1 (BFP-1) that derived from bone morphogenetic protein-7 (BMP-7) has confirmed its excellent performance in promoting the osteogenic induction of human stem cells mainly human mesenchymal stem cells.

Methods: In this study, we cultured hESCs and hiPSCs in the osteogenic induction medium for 28 days, and added BFP-1 at varying weeks. After differentiation for varying days (0, 7, 14, 21 and 28), we investigated the expression of related gene/protein markers and the differentiation efficiency by RT-PCR, immunofluorescence and alizarin red staining assay. Moreover, the expression of marker genes relating to germ layers and epithelial-mesenchymal transition (EMT) was investigated at day 7, aiming to study the impact of BFP1 peptide on the lineage differentiation and osteogenic induction process of hESCs and hiPSCs.

Results: Biocompatible BFP1 peptide could remarkably promote the generation of mesoderm cells and mesenchymal-like cells from hiPSCs through the enhanced epithelial-mesenchymal transition, but the effect was not found in hESCs because of cell line difference. Moreover, peptide addition could apparently up-regulate the expression of marker gene/protein in hESCs, especially the differentiation efficiency was improved by determining the optimal treatment periods.

Conclusions: Our work has a great value in improving the in vitro osteogenic differentiation efficiency of hPSCs by adding functional osteogenesis compounds at specific stages and promoting the fundamental and clinical applications of osteoblast like cells.

1. Introduction

To solve the shortcomings of current therapies such as limited autogenous source, second surgery and immunogenicity, bone tissue engineering technology has been developed to treat the significant bone defects in clinical [1, 2]. However, how to obtain enough functional seed cells of osteoblasts is still needed to be solved. Stem cells of human mesenchymal stem cells (hMSCs) and human pluripotent stem cells (hPSCs) are commonly applied to induce osteoblast-like cells *in vitro*. The latter exhibit better abilities in both long-term self-renewal and specialized differentiation, becoming an outstanding cell source in the field of regenerative medicine [3, 4].

It is well known that hPSCs must be differentiated towards osteoprogenitor cells or osteoblast-like cells before transplantation to avoid the tumorigenicity. Up to now, many studies have been performed to

achieve the osteogenic differentiation of hPSCs using the embryoid body method or monolayer induction method [5–7]. Unfortunately, the differentiation efficiencies in most published reports are relatively low [5]. In this regard, many kinds of growth factors have been applied to establish robust and efficient induction methods for the derivation of osteoblast like cells from hPSCs [8, 9]. However, they are mainly added through the whole induction process. Notably, during the *in vivo* embryonic development, mesoderm and ectoderm cells differentiate toward mesenchymal cells, which further differentiate into osteogenic precursor cells and osteoblasts [10, 11]. There is no doubt that each growth factor should be supplemented at a specific stage to treat corresponding cell types. For example, it is reported that enamel matrix derivatives (EMD) added at the early stage promote the differentiation of hPSCs into osteoblasts, but it inhibited cell maturation and mineralization at the late stage [12]. Therefore, hPSCs being treated by growth factors at specific stages of osteogenic differentiation are critical to establish a step-wise osteogenic induction system.

Bone morphogenetic proteins (BMPs), a number of TGF-β superfamily, play important roles in processes such as human embryonic development, bone formation and cartilage formation [13]. Moreover, many researchers believe BMPs are the most potent osteoinductive growth factors available [14]. Nevertheless, some drawbacks include high cost, instability, immune response and batch difference hamper their applications [15, 16]. To solve these problems, cost-effectively peptides have received much attention recently [17]. In 2012, an osteogenesis bone forming peptide-1 (BFP-1; GQGFSYPYKAVFSTQ sequence) was derived from the immature form of bone morphogenetic protein-7 (BMP-7) with enhanced osteogenesis performance [18]. Compared to BMP-7 protein, BFP-1 avoids folding and self-assembly and reduce the steric hindrance during the modification process, showing excellent prospects in tissue engineering [19]. Up to now, BFP-1 peptide has proved to promote the aggregation and osteo-differentiation of hPSCs [20, 21]. However, BFP-1 containing induction medium was applied constantly, and it is necessary to clarify the specific supplement time period to maximize its effect on osteogenic differentiation.

In this study, monolayer cultured hESCs and hiPSCs were cultured in the osteogenic induction medium for 28 days, and the effect BFP-1 addition at varying weeks was serially performed. After differentiation for varying days (0, 7, 14, 21 and 28), RT-PCR and immunofluorescence were used to study the expression of related gene/protein markers in cell samples, and the differentiation efficiency was also determined by alizarin red staining assay. Based on these results using peptide treatment throughout the induction process or not as controls, the impact of peptide addition on the differentiation process of hPSCs was discussed to confirm the optimal addition times for hESCs and hiPSCs. Moreover, the expression of marker genes relating to germ layers and epithelial-mesenchymal transition (EMT) was investigated at day 7, aiming to study the impact of BFP1 peptide on the lineage differentiation and osteogenic induction process of hESCs and hiPSCs. Our work has a great value in improving the *in vitro* osteogenic differentiation efficiency of hPSCs by adding functional osteogenesis compounds at specific stages and promoting the fundamental and clinical applications of osteoblast like cells.

2. Material And Methods

2.1 Cell culture

H9 hESCs and hNF-C1 hiPSCs were obtained as we described previously[22]. Both cells were cultured in E8 medium on the Matrigel coated cell culture plates. After growing into about 90% confluence, cells were passaged at a split ratio of 1:6 by exposure to 0.5 mM EDTA for 4–5 min at 37°C. All cells were cultured under standard culture conditions (37°C, 100% humidity and 5% CO_2) in an incubator (PHCbi, Japan).

2.2 Osteogenic differentiation of hPSCs

After growing into about 70% confluence, the medium of hPSCs was changed to be osteogenic induction medium (OM; DMEM medium containing 15% fetal bovine serum, 1% non-essential amino acids, 0.1 mM β-mercaptoethanol, 1% penicillin/streptavidin, 5 $\mu g \cdot mL^{-1}$ vitamin C, 10 mM sodium glycerophosphate and 10^{-8} M dexamethasone). To find an optimal concentration, after culturing for 2 days, BFP-1 peptide was added into the OM at varying concentrations (0 $\mu g \cdot mL^{-1}$, 1 $\mu g \cdot mL^{-1}$, 5 $\mu g \cdot mL^{-1}$ and 20 $\mu g \cdot mL^{-1}$) for another 12 days. The induction efficiency was analyzed by alizarin red staining at day 14.

Then, using a concentration of 20 $\mu g \cdot mL^{-1}$ for BFP1 peptide, 28 days of osteogenic induction of both hESCs and hiPSCs were performed. The peptide containing OM was, respectively, applied at each week. Moreover, the osteogenic differentiation in OM with or without peptide addition throughout the 28 days of induction was used as controls. Cell samples were obtained for the assays at the desired time intervals (0 day, 7 days,14 days, 21 days and 28 days). The viability of cells was investigated using a cell counting kit-8 regent as we recently reported[23]. Besides, the morphology of these cell samples was photographed using an inverted microscope with a CCD.

Finally, based on previous experiment settings, hPSCs were also treated with BFP-1 peptide at specific periods with same control group settings.

2.3 Quantitative real-time polymerase chain reaction (RT-PCR) study

In order to assess the effect of BFP-1 peptide addition at varying weeks on the gene transcription, total RNA was obtained using a trizol regent for hPSCs. Then, mRNA was extracted from the samples through a chloroform-isopropanol precipitation method. cDNA was obtained by reverse transcription of mRNA using a SuperQuick RT MasterMix. Using *ACTB* as an endogenous reference gene, the gene expression of *OCT-4*, *ALP, CD73*, *RUNX2, COL1A1* and *OPN* was detected through setting up to RT-PCR reactions utilizing a SYBR®PremixEx Taq™ Kit. Besides, germ layer marker genes relating to endoderm (*AFP*, *GATA4*), mesoderm (*T*, *MEOX1*, *MSX1*) and ectoderm (*PAX6*, *SOX1*, *FOXD3*) were measured in hPSCs after 7 days in induction. Epithelial-mesenchymal transition (EMT) marker genes of *Snail*, *Twist*, *Slug*, *E-cadherin*, β-catenin, *ZO-1*, N-*cadherin*, *Vimentin* and α-SMA were also investigated. Three parallel samples were set for each sample, and the comparative CT ($2^{-\Delta\Delta CT}$) method was employed to evaluate fold gene expression differences between groups. Primer sequences are shown in table S1.

2.4 Immunofluorescence analyses

Immunofluorescence staining was applied to detect the expression of osteogenesis-related marker proteins of RUNX2, COL1A1 and OPN during the osteogenic differentiation of hPSCs in 28 days. Cells on the cell culture plates were fixed with 4% (v/v) paraformaldehyde for 30 min, and then treated with 0. 2% Triton-100 for 30 min. After blocking in 3% BSA solution for 2 h, cells were respectively overnight incubated with primary antibodies that diluted at a ratio of 1:100. Then, cells were incubated with corresponding secondary antibodies for 1 h. Finally, cell nuclei were stained with DAPI for 5 min. All staining steps were followed by washing in DPBS buffer for 3 times. The stained signals in the cells were photographed using a confocal fluorescence microscope (Axiovert 200M; Carl Zeiss Jena, Germany). The wavelengths of DAPI, green fluorescent labeled antibody and red fluorescent labeled antibody are 405 nm, 488 nm, and 594 nm respectively. The information of these antibodies is shown in table S2.

Moreover, the expression level of each of these proteins in cell samples was quantitatively measured as determined by the fluorescence of pristine pictures using an Image J software.

2.5 Flow cytometry study

After incubation for up to 28 days, hESCs were digested into single cells and then fixed with 1% paraformaldehyde. Cells on the ice were permeated in 200 μL pre-cooled 90% methanol solution for 30 min. Subsequently, the samples were washed twice with the flow buffer (DPBS containing 2% FBS) and incubated with a Rabbit Anti-RUNX2 antibody at a dilution rate of 1:100 in flow buffer for 30 min at 37 ^{o}C. This was followed by incubation with a secondary antibody of Fluor 488-labeled goat anti-Rabbit IgG at a dilution rate of 1:500 in DPBS. Finally, all these cell samples were analyzed by a BD FACS Calibur System (BD, USA) and Flowjo software.

For hiPSCs, we constructed a RUNX2-GFP hiPSCs line from hNF-C1 hiPSCs using the CRISPR-Cas9 gene editing technology, and the endogenous promoter of the *RUNX2* gene could drive the expression of GFP. Purified GFP-RUNX2 hiPSCs were acquired through further puromycin screening and cell subcloning culture methods. As described above, GFP-RUNX2 hiPSCs were cultured in OM with or without BFP1 peptide and sent into flow cytometry analysis after induction for each 7 days for 28 days.

2.6 Alizarin red staining assay

Calcium deposits in cell samples were quantified using alizarin red (AS) staining. Briefly, fixed cells were stained with 2% (w/v) alizarin red solution (0.01 M Tris Buffer, pH = 4.2) for 20 min. Then, cells were washed with distilled water in order to remove excess AS. Finally, cells were observed under an inverted microscope with a CCD, and the whole plate was recorded using a mobile phone. In order to quantify the orange-red coloration of AS, the substrates were immersed in 1% (m/v) hexadecylpyridinium chloride solution overnight to dissolve the deposited calcium mineral. Then, 100 μL of supernatants were collected into new 96-well cell culture plates, and the OD value at 490 nm wavelength was detected using a microplate reader (Bio-Rad; USA).

2.7 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 Discussions

3.1 Survival of hPSCs in the BFP1 peptide containing osteogenic induction medium

Prior to the osteogenic induction, we have performed a simple cell experiment to select the optimal concentration for BFP-1 peptide supplement. After treating with BFP-1 peptide at varying concentrations (0 $\mu g \cdot mL^{-1}$, 1 $\mu g \cdot mL^{-1}$, 5 $\mu g \cdot mL^{-1}$ and 20 $\mu g \cdot mL^{-1}$) for 14 days, alizarin red (AS) staining results showed that BFP-1 accelerated the calcium deposition in cell samples (Fig. 1b, Fig. S1). Moreover, significant difference is only found in 20 $\mu g \cdot ml^{-1}$ group in comparison to the pristine control. Therefore, BFP-1 peptide at 20 $\mu g \cdot ml^{-1}$ was applied for the following studies.

Then, the impact of one-week supplement of BFP1 peptide on the osteogenic differentiation of both H9 hESCs and hNF-C1 hiPSCs was studied as shown in Fig. 1a. Besides, pristine OM and peptide containing OM were performed as negative and positive controls respectively. Before differentiation, hPSCs presented undifferentiated cell morphology with defined edges and large nuclei-to-cytoplasm ratios (Fig. S2-3). After induction for 7 to 14 days, much cobblestone or spindle-shaped cells appeared in all groups. For the cell viability, BFP1 peptide treatment showed slightly negative effect for both cells at day 7 (Fig. 1a-b). However, after induction for more than 14 days, the viability of hESCs that treated with peptide at the first week was remarkably lower than both negative and positive controls. Interestingly, this result was not found for hiPSCs. Both for hESCs and hiPSCs during the induction from day 14 to day 28, it is worth to note that positive control group exhibited higher cell viabilities than the negative control group. Besides, peptide addition at the second week only reduced the viability of hESCs, but not for hiPSCs. Went on differentiation for another 14 days, both cells secreted much collagen and arranged in multiple layers with inapparent cell morphology (Fig. S2-3). Consistently, after culturing for 28 days, cobblestone morphology was found due to the increased extracellular matrix. Similarly, it was found that peptide treatment at the second week and third week could promote the viability of hiPSCs, but contract results were detected for hESCs ($p < 0.01$). Besides, for both cell types, peptide treatment throughout the whole induction process accelerated the cell survival except for the first week.

3.2 The impact of BFP-1 peptide supplement on the maker expression in hPSCs

The dynamic changes in the expression of markers relating to osteogenesis are popularly performed to monitor the osteogenic differentiation of hPSCs [10]. In this study, ALP, RUNX2, COL1A1 and OPN were

analyzed using both RT-PCR and immunofluorescence technology.

Alkaline phosphatase (ALP) is considered as a marker of early differentiation and may be involved in the calcification of bone matrix [24]. For both hESCs and hiPSCs incubated in the pristine OM, cells expressed *ALP* gene at quite low levels during the 14 days of induction, and up-regulation was measured at day 21 for hESCs and day 28 for hiPSCs (Fig. 2f). For the positive control group with peptide treatment throughout the induction, apparently enhanced gene expression was found for hESCs after differentiation for more than 21 days, and the time point was day 14 for hiPSCs ($p < 0.01$). Then, for the peptide addition at each week, quite different results were measured between hESCs and hiPSCs. It was found that peptide addition at the first week only promoted the gene expression in hESCs at day 28, but much higher results were found from day 7 for hiPSCs ($p < 0.05$). Then, peptide addition during the second week enhanced the gene expression in hESCs from day 21. However, positive result, a high expression level resembles to first week treatment group, was only found at day 14 for hiPSCs. After 14 days of induction, one week peptide treatment also up-regulated the gene expression in both cells. Notably, a very high gene expression level was measured in hESCs at day 28. Finally, when the supplement period was the fourth week, positive results were observed only in hiPSCs. In summary, for ALP expression, BFP1 should be supplemented during 7–21 days for hESCs and the time period was 7–28 days for hiPSCs.

RUNX2 is an early critical marker for the osteoblastic differentiation of stem cells, and its expression can upregulate markers that associated with mineralization such as COL1A1, OCN, and bone sialoprotein (BSP) [25]. For both cells, the gene expression of *RUNX2* was peaked at day 21. Then, the application of BFP1 peptide throughout the 28 days of differentiation did not change the tendency. Notably, after induction for more than 14 days, the positive control group exhibited a much higher gene expression level for *RUNX2* at each time points for both cells ($p < 0.05$). This indicated that the addition of BFP1 peptide could promote the expression of critical RUNX2 gene in hPSCs. Then, peptide supplement at the first week remarkably upregulated the gene expression in hiPSCs after induction for 7 days, but contrast results were measured in hESCs. Fortunately, consistent positive results were found for both cells during the following inductions. When the addition period was the second week, the expression of *RUNX2* gene in hESCs at day 21 and 28 were higher than the pristine control ($p < 0.05$), but reduced results were detected for hiPSCs. For both cells after 14 days of induction in pristine OM, it is found that the gene expression levels were apparently promoted with one week of peptide treatment, even higher than the positive groups at day 21. It is surprising that third week group exhibited the highest gene expression levels at day 21 for both cells, which proved that this time period is highly recommended in terms of critical RUNX2 expression. Finally, peptide added during the final week only up-regulate the gene expression in hESCs at day 28.

COL1A1 is a matrix-mineralizing protein that regulates the bone formation through the control of morphology, differentiation, and other biological functions of cells [25–27]. Totally speaking, regardless of the values, the gene expression pattern of *COL1A1* in both hESCs and hiPSCs were quite resemble *ALP* gene after induction for more than 21 days (Fig. 1f). For hiPSCs, peptide addition at the first week especially at the second week could remarkably up-regulated the gene expression at day 14, both of

which were significantly higher than the positive control groups ($p < 0.05$). However, the promoting effect was only found at day 28 in hESCs for first and second groups. Besides, it is worth to note that the highest gene expression levels were measured for hiPSCs in positive groups after culturing for more than 21 days. This supposed that peptide treatment at the third week is also crucial for the expression of *OCN*.

OPN is a non-collagenous, phosphorylated glycoprotein localized in bone and dentin [28]. Osteoblasts can secret OPN, and thus it is regarded as a marker of osteogenesis during the induction of hPSCs [29]. For negative control groups, the expression of *OPN* in both cells was increased with the augment of induction times, and reached peak values at day 21 (Fig. 1f). Then, apparently up-regulations were detected in positive groups at day 21 and 28. Then, peptide supplemented at the first week only up-regulated the gene expression in hESCs at day 28, but the up-regulation was found in hiPSCs after induction for more than 7 days. Changing the time period to be second week, peptide addition significantly promoted the expression of *OPN* gene in hESCs at day 28, and day 14 for hiPSCs. Surprisingly, the treatment of peptide at the third week contributed highest expression levels at day 21 and day 28. However, no such huge accelerated results were found for hiPSCs. Finally, the treatment of peptide at the fourth week is not effective for hiPSCs.

Meanwhile, the protein expressions of RUNX2, COL1A1 and OPN in hESCs and hiPSCs were also detected by immunofluorescence analyses (Fig. 2–3). Consistent to the RT-PCR results, BFP1 peptide addition at each designed period could promote the expression of RUNX2 in hPSCs (Fig. 2a-b, 3a-b). Moreover, similar expression patterns were found for the RUNX2 protein. Then, cells with peptide treatment during the second week showed the best protein expression in hESCs both after induction for 14 days and 21 days. For hiPSCs, the promoting effect was not so apparent for all groups at day 14. Fortunately, contrast to the gene expression results, we found that peptide treatment during the second week could promote the expression of RUNX2 in hiPSCs after induction for more than 21 days. For the COL1A1 protein, quite different results were found for hESCs at day 28, and hiPSCs throughout the whole process (Fig. 2c-d, 3c-d). This should because COL1A1 is a kind of extracellular matrix protein that secret from the indued cells. However, it is consistent to find that peptide addition at the third week or whole 21 days could promote the protein expression in hESCs at day 21. Then, in comparison to both negative and positive groups, the addition of peptide at the second week could significantly increase the expression of COL1A1 in hiPSCs at day 21 ($p < 0.05$), the values of which were almost same to cells with peptide treatment during the third week. Finally, consistent the gene expression of *OPN*, peptide treatment at the first week or throughout the 28 days of culturing could accelerate the expression of OPN protein in both hESCs and hiPSCs (Fig. 2e-f, 3e-f). Then, unlike the remarkably increased gene expression result, no positive results were detected for the third week treatment group in hESCs. However, consistent positive results were measured for hiPSCs. Interestingly, more OPN protein was detected in hESCs with peptide treatment at the second week at day 21, and same result was also found in hiPSCs. Notably, these immunofluorescence results demonstrated that only a small number of inducted cells positively expressed all three protein markers (Fig. S4-9), proving the differentiation efficiency is needed to be improved.

3.3 BFP1 peptide promote the calcium deposition in hPSCs

In order to investigate the effect of BFP-1 peptide supplement on the osteogenic differentiation efficiency of hPSCs, alizarin red (AS) staining was conducted at each 7 days for 28 days. As shown in Fig. 3a-d, typical calcium nodules were found after 21 days of induction for both hESCs and hiPSCs in the negative control group. However, at day 14, some typical calcium nodules were found in both cells in the positive control group. Quantitative results confirmed that the application of BFP1 peptide throughout the induction process could remarkably promote the calcium deposition in hPSCs (Fig. e-f).

Then, it is found that peptide treatment at the first week could significantly promote the deposition of calcium in hESCs after induction for 14 days (Fig. 3). However, calcium depositions resemble to the negative control were found after induction for more than 21 days, which were much lower than the positive control. Moreover, it is surprise to find that this treatment exhibit nearly no impact on the calcium deposition in hiPSCs. These results proved that BPF1 peptide supplemented during the first week is unnecessary in terms of the calcium deposition in hPSCs. Changing the peptide treatment period to be the second or third week, both could remarkably promote the calcium deposition in hPSCs in the following differentiation process ($p < 0.01$), which suggest that the addition of peptide during the time period of 7–21 days show benefit to the calcium deposition. Finally, the absorbance of alizarin red stained cells after 28 days of culturing was not changed by the peptide addition at the fourth week for hiPSCs, and that of hESCs was significantly lower than positive control.

3.4 BFP1 peptide applied at defined stage promote the osteogenic induction of hESCs

As described previously, the impact of supplement period of osteogenesis BFP1 peptide on the osteogenic induction of hPSCs was investigated in detail. It is certain that the addition of BFP1 peptide can apparently change the induction process of cells. Consistent to reported results, BFP1 peptide harbor good cytocompatibility, and the changes in viability of differentiated cells should due to the selective killing effect from the OM^{30} (Fig. 1d-e). Then, using the pristine OM as controls, the critical results of marker expression and calcium nodule formation was discussed for the hESCs and iPSCs respectively.

For hESCs, peptide treatment during the first week mainly upregulated the gene expression of *RUNX2*, *ALP*, *COL1A1*, and *OPN* at day 28 (Fig. 1f). However, remarkable down-regulations were also detected during the 14–21 days of culture. Moreover, this treatment failed to significantly enhanced the protein expression of *RUNX2* and *COL1A1* during the 28 days of induction. Although much more deposited calcium was detected in first week group after 14 days in induction, the values were much less than the positive control at day 21 and 28 (Fig. 3e). Then, both RT-PCR and immunofluorescence analyses showed that peptide addition at the second week upregulated the expression of early markers of ALP and RUNX2 in hESCs after 21 days in induction, and promoted the expression of latter markers of OPN at day 28 (Fig. 1f). More important, we found that the calcium deposition in cells samples was greatly enhanced, and showed comparable performance to positive control. Afterwards, much better promoting effects on the expression of all 4 gene markers was found at day 21 for cells with peptide supplement at the third week in comparison to second week group. Furthermore, cell samples exhibited highest gene expressions

among all groups for these 4 markers at day 28. Unfortunately, the time point for RUNX2 protein was day 28, and not good OPN expression result was obtained. These results demonstrated that the period of 7–21 days is highly recommended for hESCs. Finally, the addition of BFP1 peptide at the fourth week only accelerate the gene expression of *OPN* with enhanced calcium depositions, but the levels of both are lower than other peptide treatment groups[31, 32].

For hiPSCs, it is surprise to find that the addition of BFP1 peptide at the first week significantly promote all marker expressions after induction for more than 7 days (Fig. 1f). Unfortunately, the treatment has no impact on the critical calcium depositions in cells throughout the 28 days of induction (Fig. 3f). Then, peptide supplemented at the second week also significantly up-regulated the expression of all marker genes at day 14, and upregulated gene expressions were found for ALP and COL1A1 in the following induction process. Then, enhanced protein expression was found for RUNX2, COL1A1 and OPN at day 21. Fortunately, the calcium deposition in cells was remarkably accelerated and harbor the highest values at final day 28. Changing the addition period to be the third week, all 4 marker expressions in cells was enhanced after induction for more than 21 days, especially for the expression of RUNX2 at day 21. Moreover, better calcium deposition results were also measured. Finally, peptide treatment at the fourth week was found to up-regulated the gene expression of *RUNX2* and *COL1A1*, as well as the protein expression of RUNX2 and OPN in hiPSCs. Notably, these results proved that BFP1 peptide treatment at each week could promote the osteogenic induction of hiPSCs, but the period of 7–21 days seems more efficient.

Above results demonstrated BFP1 peptide should not added throughout the osteogenic induction process of hESCs, and the supplement period exhibited great influence on the differentiation efficiency of both hESCs and hiPSCs. More important, the difference among various type of hPSCs is nonnegligible. Previously shown that adding BFP-1 peptide at 7–14 days and 14–21 days could significantly promote the efficiency of osteogenic differentiation of hPSCs, especially in hESCs line. In order to further confirm this result, BFP-1 peptide was added at 7–21 day in the following section.

It is found that the treatment of BFP-1 peptide during the period of 7–21 days could significantly promote the viability of hESCs after induction for 28 days, the values of which even higher than positive control group (Fig. 5a, Fig. S10). Then, for each investigated osteogenesis relating genes of *ALP*, *RUNX2*, *COL1A1* and *OPN* at day 21, remarkably higher gene expression levels were detected in cells with peptide addition at designed period in comparison to both negative and positive groups (Fig. 5b-e). Such promoting effect is remain apparent for genes of *RUNX2*, *COL1A1* and *OPN* at day 28. Similar results were also found for the expression of protein markers of COL1A1 and OPN in cell samples by immunofluorescence (Fig. 5i-m). However, after induction for both 21 and 28 days, no apparent difference was found for the expression of RUNX2 in cells with the peptide addition during 7–21 or 0–28 days, although lower protein expression was observed in negative control groups. Moreover, flow cytometry analyses showed that target peptide treatment during 7–21 days accelerated the generation of RUNX2 + cells after 21 days of differentiation in comparison to other two control groups (Fig. 5f-g, Fig. S12). At day 28, almost same expression rates were measured for peptide treating groups, and both were higher than the negative

group. Finally, after culturing for more than 21 days, more typical calcium nodules were found in hESCs with peptide treatment than pristine control (Fig. 5i, Fig. S11). Quantitative results confirmed that peptide treatment during 7–21 day and 0–21 days both significantly promote the calcium deposition in hESCs after 21 days in differentiation (Fig. 5h). These results proved that the addition of BFP-1 during the period of 7–21 days showed excellent performance in cell survival and osteogenesis marker expression for hESCs, even better than the group with peptide addition throughout whole induction.

Unfortunately, changing the cell type to be hiPSCs, except for similar cell viability results, none up-regulation was found for the expression of all 4 gene markers in cells with peptide treatment during 7–21 days (Fig. S13, Fig. 6a-e). Almost same number of RUNX2 + cells were measured among all groups after differentiation for both 21 and 28 days as confirmed by flow cytometry analyses (Fig. 6f-g, Fig. S15). Except for remarkably accelerated OPN protein expression at day 28, similar results were found in immunofluorescence study (Fig. 6j-m). Moreover, less deposited calcium in cells was also measured at day 28 for cells with peptide addition during 7–21 days (Fig. 6f-h, Fig. S14). Fortunately, consistent to previously mentioned results, better results were found in positive group for marker genes/proteins expression and calcium depositions.

These results indicated that 2 weeks of BFP1 peptide addition should be conducted after induction for 7 days for H9 hESCs, but a simple whole addition throughout the 28 days of induction is recommended for hNF-C1 hiPSCs. It is well known that the osteogenic differentiation of hPSCs undergoes various stages such as proliferation, differentiation, matrix deposition and mineralization, so that the expression of osteogenic-related genes is dynamically changed during the differentiation process [33, 34]. In 2021, we had summarized this intrinsic regulatory mechanism in detail[23]. Our results suggested that mesenchymal-like cells are obtained at day 7. With prolonged osteogenic culture time, cells began to express osteogenic gene of *RUNX2* at a high-level during 14–21 days. Then, pre-osteoblasts positively expressing *RUNX2* will stimulate the expression of late osteogenic differentiation marker genes of *COL1A1* and *OPN*, which further promotes the formation of mature osteoblasts [23, 35]. We speculated that mesoderm, mesenchymal and preosteoblasts were obtained after cultured for 7 days, 14 days and 21 days, respectively. Consistent to published results, apparent difference was found between the expression pattern of osteogenesis relating markers in H9 hESCs and hNF-C1 hiPSCs[7, 36]. We recently reported that hESCs and hiPSCs undergo similar expression changes for markers relating to pluripotency and osteogenic differentiation, but different expression changes were found for extracellular matrix protein markers[23]. Notably, H9 hESCs exhibited much better performance than hiPSCs in extracellular matrix synthesis. Therefore, the inherent difference among hPSCs types can be the reason why different regulation effects were detected for BFP1 addition on the osteogenic induction of hESCs and hiPSCs.

It is reported that the enhanced osteogenic differentiation of hPSCs by BFP-1 peptide treatment may due to the up-regulation of critical RUNX2 expression [18, 37]. Our results showing that peptide adding during 7–21 days could significantly promote the efficiency of osteogenic differentiation of hPSCs, especially in hESCs line. Mesenchymal cells derived from mesoderm and ectoderm cells will differentiate into

osteogenic precursor cells and osteoblasts, we preliminarily concluded that BFP-1 peptides treatment promoted the osteoblastic differentiation of mesenchymal cells by activating the RUNX2 pathway [10, 11]. After cultured for 21 days, peptide addition may promote the differentiation of preosteoblasts into osteoblasts. To verify these concludes, as well as get more knowledge about the impact of BFP1 peptide on the osteogenic differentiation process of hPSCs, the expression of marker genes relating to germ layers and epithelial-mesenchymal transition (EMT) was studied in detail at day 7. As shown in Fig. 7a-b, peptide addition at the first week has none or negative impact on the generation of mesoderm and ectoderm cells, resulting in reduced expression of mesenchymal marker of *CD73* at day 14 and 21 (Fig. 7e). These can explain why peptide addition at the first week could not promote the osteogenic induction of hESCs. However, the treatment at the first week was found to significantly up-regulated the gene expression of mesoderm markers of *T* and *MEOX1* as well as the transcription factor of *Twist* in hiPSCs ($p < 0.05$; Fig. 7c-d), suggesting BFP1 peptide addition could promote the EMT process and the generation of mesoderm cells. Consistently, the expression of CD*73* was remarkably increased during the following induction process (Fig. 7f). Besides, peptide addition at the fourth week is workless in terms of *CD73* gene expression. However, peptide addition at other three weeks all could promote the expression of CD*73* genes, suggesting BFP1 peptide should be added throughout the osteogenic induction process as mentioned above. These results also confirmed the huge difference between BFP 1 peptide induced spontaneous differentiation of hESCs and hiPSCs. Besides, the effect of peptide addition at each week on the osteogenic differentiation of hESCs and hiPSCs was summarized in Fig. 7g.

4. Conclusion

In this presented study, both hESCs and hiPSCs were induced into osteoblast like cells in common FBS containing osteogenic induction medium using a monolayer induction method. Simultaneously, one week of osteogenesis BFP1 peptide supplement was conducted after differentiation for varying days (0, 7, 14, 21 and 28). The induction process was monitored by the expression of gene/protein markers using RT-PCR and immunofluorescence analyses, and the differentiation efficiency was evaluated using the alizarin red staining. It is found that BFP1 peptide treatment could remarkably up-regulate the expression of all investigated genes/protein makers in hPSCs, but huge difference was found between H9 hESCs and hNF-C1 hiPSCs. Moreover, the supplement period is quite important to maximize the performance of BFP1 peptide in accelerating the osteogenic induction of hPSCs, because peptide added throughout the 28 days of osteogenic induction showed no good results in hESCs. Specifically speaking, the epithelial-mesenchymal transition and following mesoderm cells generation processes were significantly improved in hiPSCs with peptide addition at the first week, but not for hESCs. Then, the treatment of BFP-1 peptide during the period of 7–21 days showed even better performance than positive control group for hESCs, but this period is workless for hiPSCs. On the contrary, peptide should be added at the fourth week for hiPSCs rather than hESCs. Our study has value to maximize the osteogenic differentiation of hPSCs through the addition of osteogenesis peptide at a specific time period based on the revealed mechanisms, which is of great significance in bone tissue regeneration.

Abbreviations

hESCs: human embryonic stem cells; hiPSCs: human induced pluripotent stem cells; BFP-1: bone forming peptide-1; hPSCs: human pluripotent stem cells; BMP-7: bone morphogenetic protein-7; hMSCs: human mesenchymal stem cells; EMT: epithelial-mesenchymal transition; OM: osteogenic induction medium; AS: alizarin red; ALP: Alkaline phosphatase; BSP: bone sialoprotein.

Declarations

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Authors' contributions

PZ, YLY and BL contributed to design the study and critically revised the manuscript. PZ, YMS, FF, HJL, JMS and JL contributed to draft and review the manuscript. YMS, FF, HJL, LW, LZL, BYX, SQM, SZL and YZ performed all the experimental works. YMS contributed to perform the statistical analysis and interpret the results. All authors have read and approved the article, and do their due diligence to ensure the integrity of the manuscript. Neither the entire manuscript nor any part of its content has been published or has been accepted elsewhere.

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Availability of data and materials

The datasets used and/or analyzed in this study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

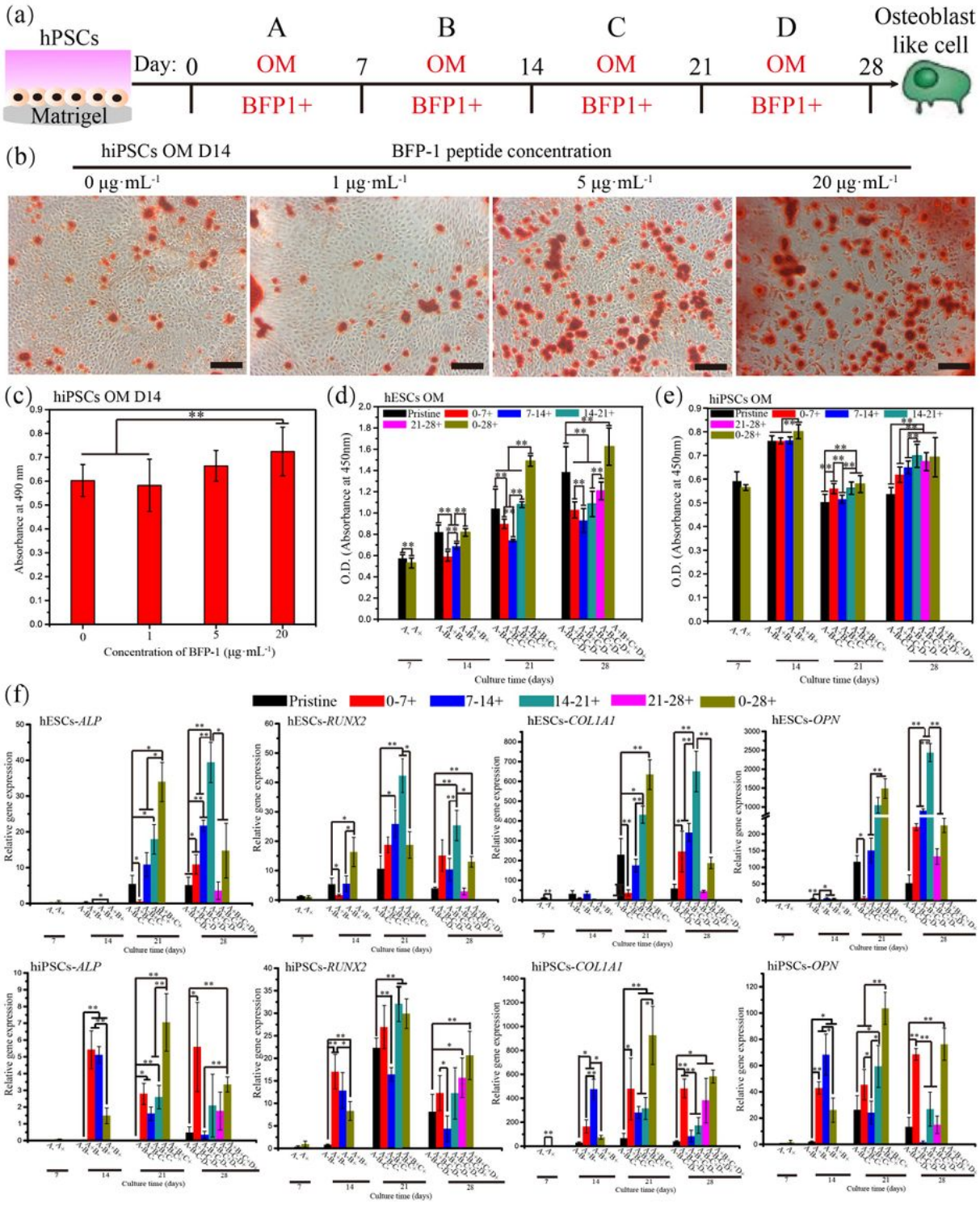

Figure 1

Accelerating the osteogenic induction of hPSCs by the treatment of BFP1 peptide. (a) Illustrate image for the addition of BFP1 peptide in osteogenic induction medium (OM) at each week for mono-layer cultured hPSCs. Besides, the whole induction process with or without BFP1 treatment were conducted as controls. (b-c) The osteogenic differentiation of hiPSCs as a function of BFP-1 peptide concentration at day 14 was evaluated by alizarin red staining. The morphology (b) as well as quantitative determination results

(c) for stained cells were shown. (d-e) For BFP-1 peptide treated hPSCS, the viabilities of H9 hESCs (d) and hNF-C1 hiPSCs (e) during osteogenic induction were detected at desired intervals (0, 7, 14, 21, and 28 days) using a CCK8 reagent. (f) After culturing for each 7 days, the gene expression of *RUNX2*, *ALP*, *COL1A1*, and *OPN* in cell samples was measured by the quantitative RT-PCR. The expression of these genes in undifferentiated hPSCs has been standardized to 1. The letter of A, B, C, D serially represents the first week to the fourth week. + indicates OM supplemented with peptide, and - indicates OM without peptide. Scale bar, 100 μm. * represents $p < 0.05$, ** represents $p < 0.01$.

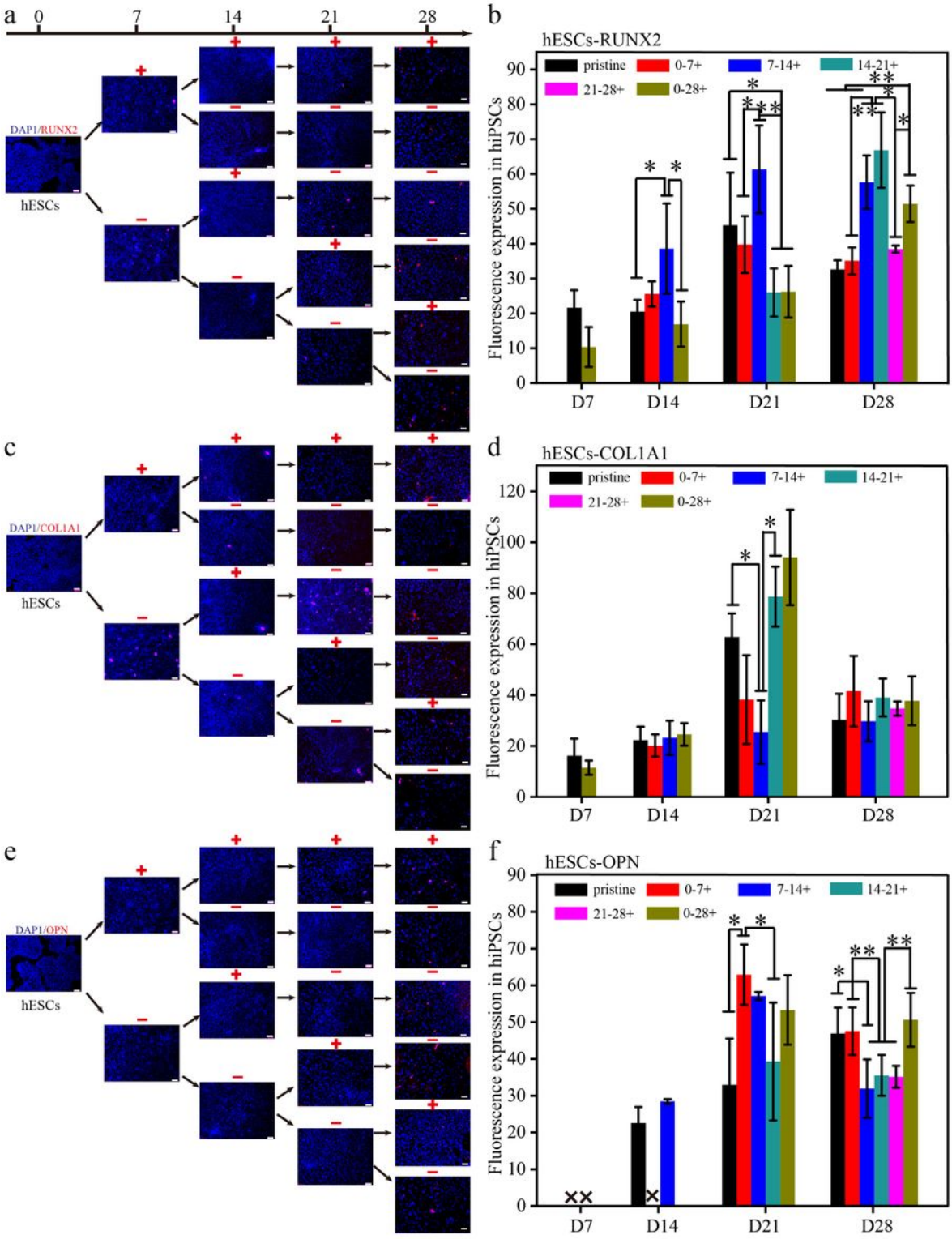

Figure 2

The expression of marker proteins in induced hESCs. (a-e) The protein expression of RUNX2 (a), COL1A1 (c) and OPN (e) in H9 hESCs were measured at each 7 days for 28 days by immunofluorescence. Cell nuclei were stained blue with DAPI. Moreover, semi-quantitative measurements were performed based on the fluorescence of stained cell samples using an image J software. Scale bars, 100 μm. * represents $p < 0.05$, ** represents $p < 0.01$.

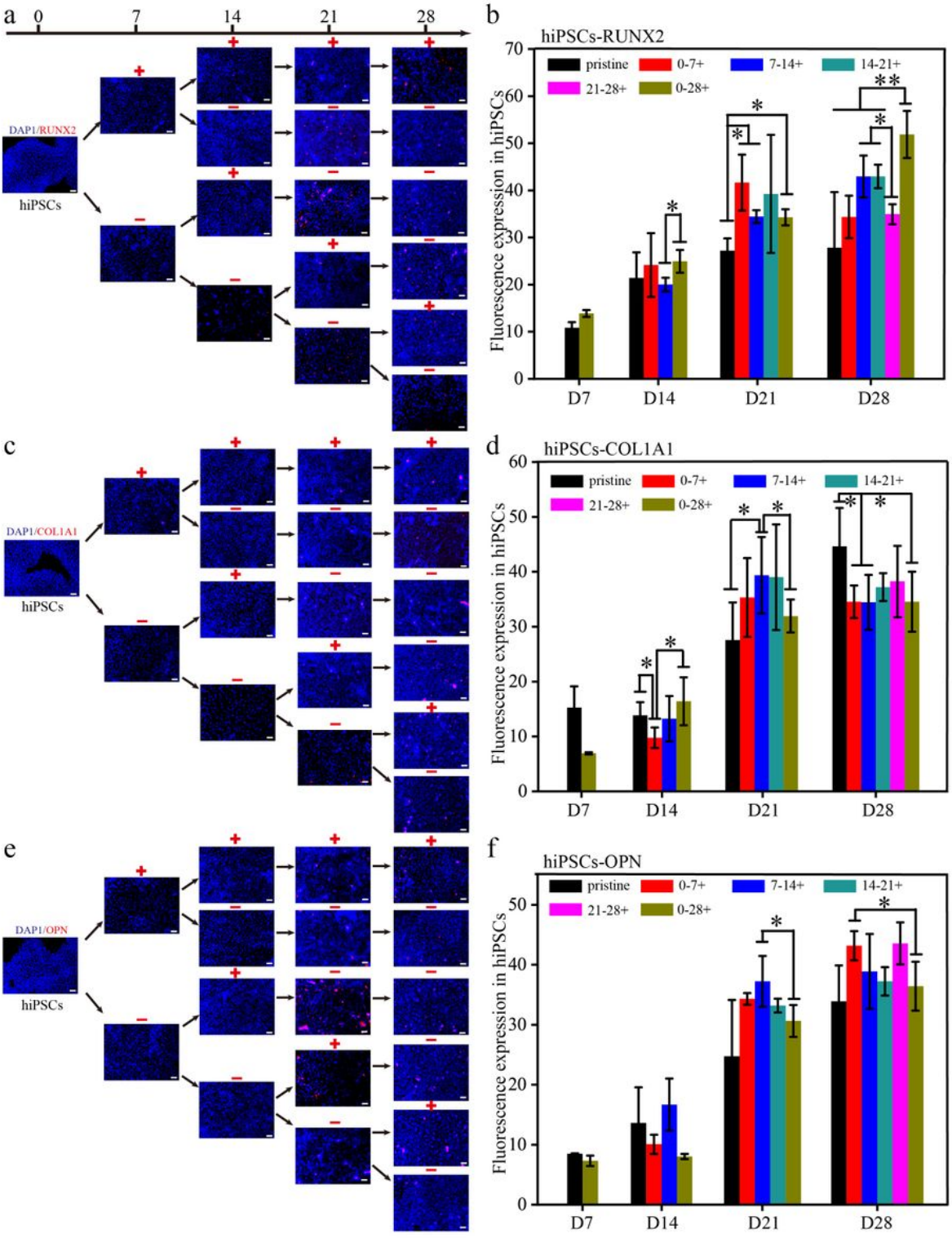

Figure 3

The marker protein in hiPSCs during the osteogenic induction. (a-e) After culturing in osteogenic induction medium for each 7 days in 28 days, the expression of protein markers includes RUNX2 (a), COL1A1 (c) and OPN (e) in hNF-C1 hiPSCs were measured by immunofluorescence. Cell nuclei were stained blue with DAPI. Moreover, semi-quantitative measurements were performed based on the fluorescence of stained cell samples using an image J software. Scale bars, 100 μm. * represents $p < 0.05$, ** represents $p < 0.01$.

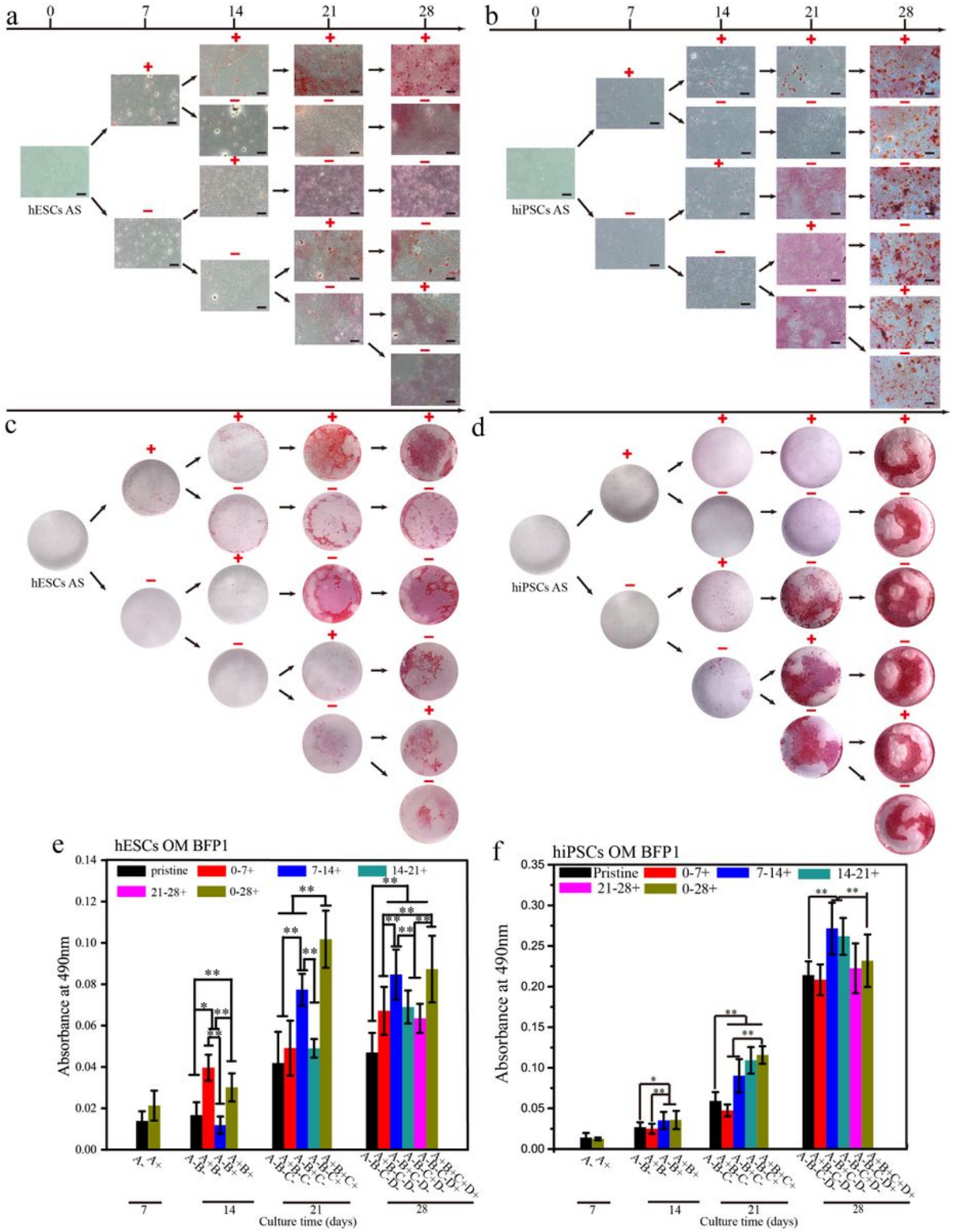

Figure 4

Analyses of alizarin red staining for induced hPSCs. (a-b) The images for the alizarin red stained hESCs (a) and hiPSCs (b) at desired intervals (0 days, 7 days, 14 days, 21 days, and 28 days). (c-d) The images of these plates with cell samples. (e-f) Cetylpyridinium bromide solution was applied to dissolve deposited alizarin red, and the absorbance at 490 nm was measured. Scale bars, 100 μm. n=3.

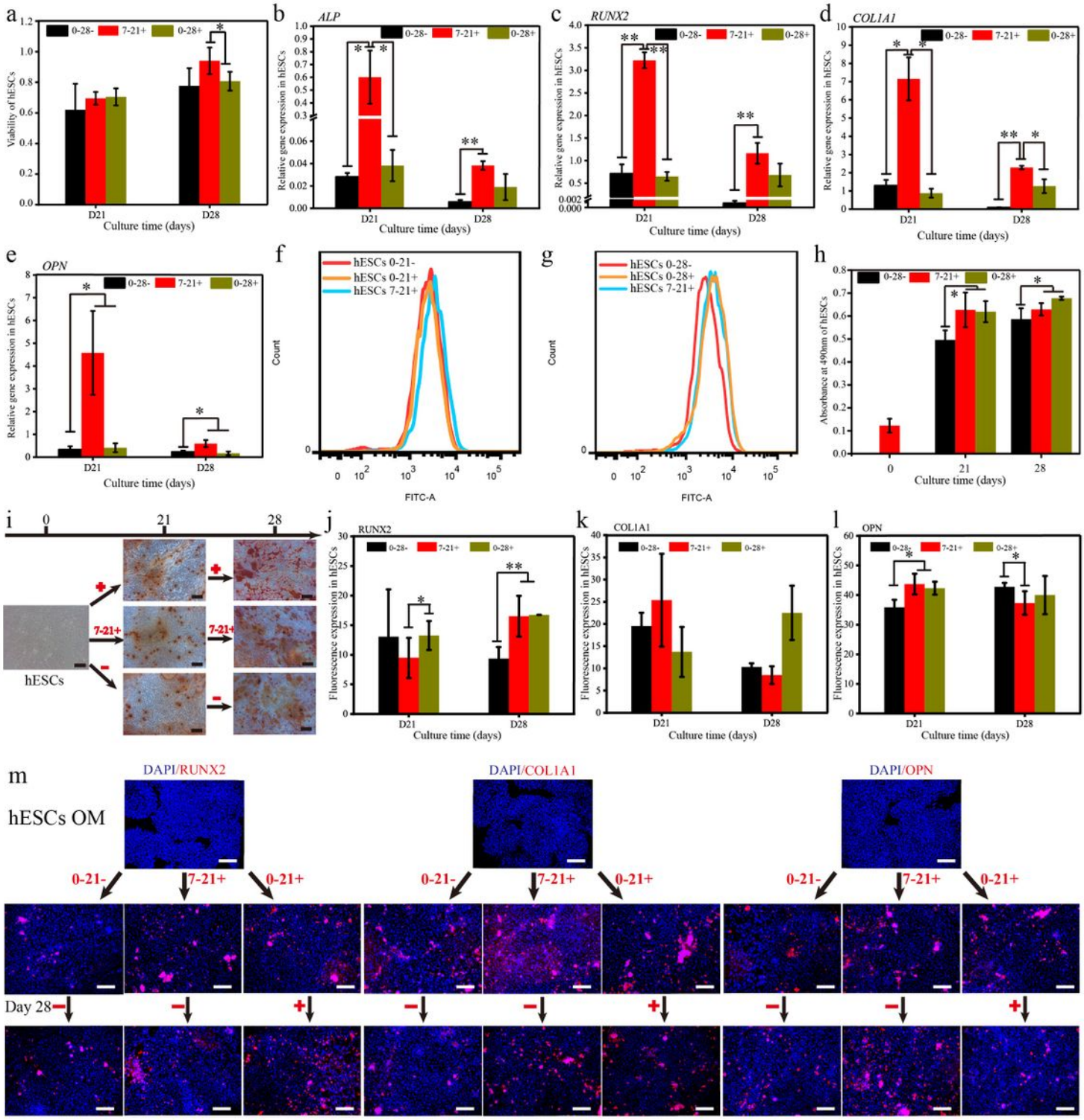

Figure 5

The osteogenic differentiation of hESCs with peptide addition during 7-21 days of induction. (a) After 7 days in induction, two weeks application of BFP1 peptide containing OM was conducted for H9 hESCs. After culturing for 21 days and 28 days, the viability of cells was measured using a CCK8 reagent. (b-e) The gene expression of *ALP* (b), *RUNX2* (c), *COL1A1* (d) and *OPN* (e) in cell samples was measured by RT-PCR. The expression of these genes in undifferentiated hPSCs has been standardized to 1. (f-g) The

positive expression of RUNX2 protein in induced cell samples at day 21 (f) and 28 (g) were measured by flow cytometry. (h-i) Cells were stained by alizarin red. Images for cell samples (i) are shown. Then, the deposited calcium was quantitative detected at 490 nm wavelength (i). (m) The protein expression of RUNX2, COL1A1 and OPN in cells was studied by immunofluorescence. * represents $p < 0.05$, ** represents $p < 0.01$. Scale bars, 100 μm.

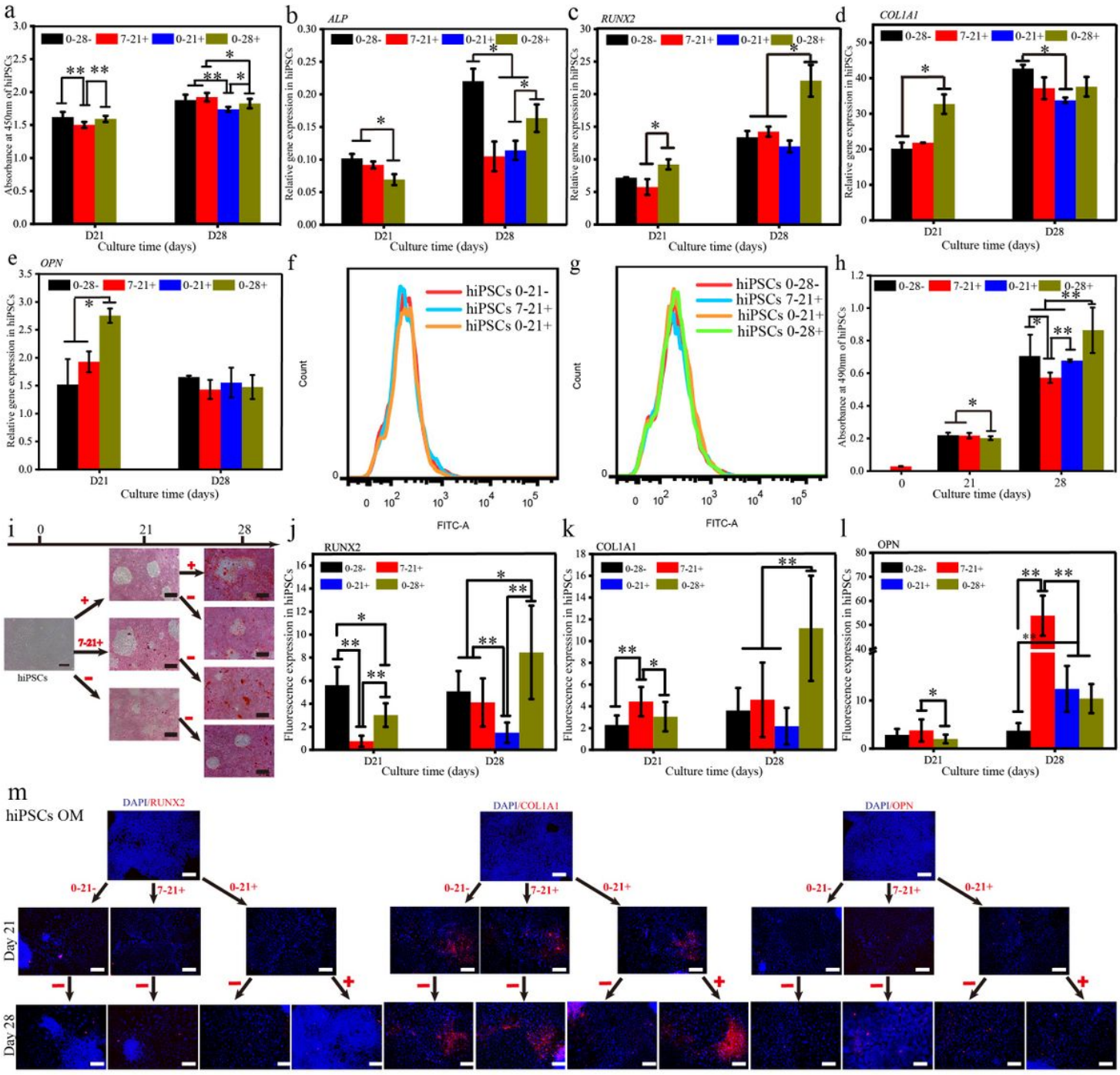

Figure 6

The osteogenic differentiation of hiPSCs with the treatment of peptide addition at special periods. (a) After 7 days in induction, two weeks application of BFP1 peptide containing OM was conducted for hNF-C1 hiPSCs. Then, both cells differentiated in peptide supplement or not OM was conducted at the final week. After culturing for 21 days and 28 days, the viability of cells was measured using a CCK8 reagent. (b-e) The gene expression of *ALP* (b), *RUNX2* (c), *COL1A1* (d) and *OPN* (e) in cell samples was measured by RT-PCR. (f-g) The expression of RUNX2 protein in cells after induction for 21 (f) or 28 (g) days as measured by flow cytometry. (h-i) Cells were stained by alizarin red. Images for cell samples (i) are shown. Then, the deposited calcium was quantitative detected at 490 nm wavelength (i). (m) The protein expression of RUNX2, COL1A1 and OPN in cells was studied by immunofluorescence. * represents $p < 0.05$, ** represents $p < 0.01$. Scale bars, 100 μm.

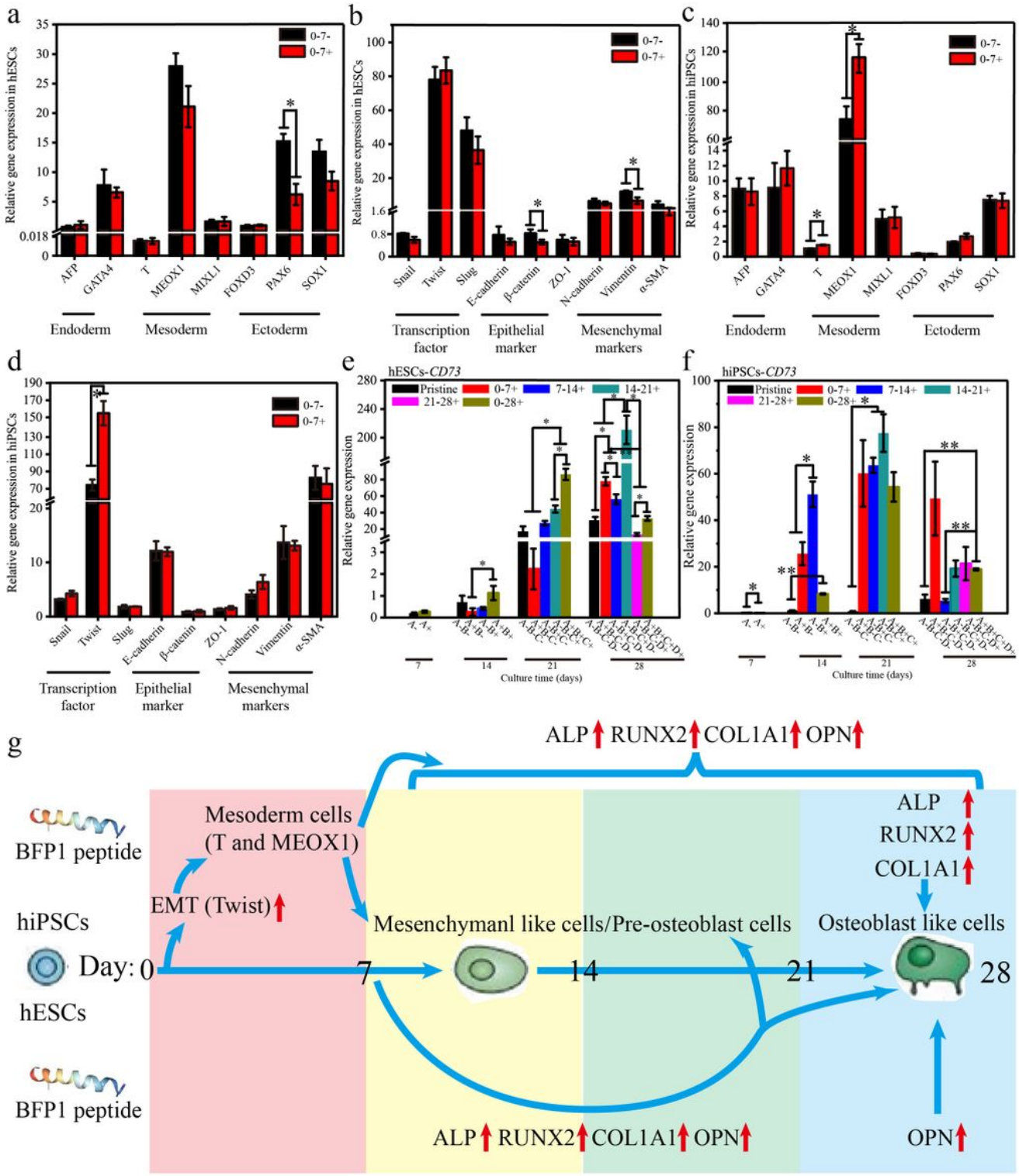

Figure 7

The mechanism underlies the effect of BFP1 peptide supplement on the osteogenic differentiation of hPSCs. (a-b) The impact of BFP1 peptide addition on the differentiation process of both H9 hESCs (a) and hNF-C1 hiPSCs (b) was investigated after 7 days in induction. The relative expression of marker genes relating to germ layer (endoderm: *AFP*, *GATA4*; mesoderm: *T*, *MEOX1* and *MSX1*; ectoderm: *PAX6*, *SOX1* and *FOXD3*) were measured using RT-PCR. (c-d) The expression of gene markers relating to

epithelial-mesenchymal transition (EMT) include *Snail*, *Twist*, *Slug*, *E-cadherin*, β-catenin, *ZO-1*, N-*cadherin*, *Vimentin* and α-SMA were also investigated. (e-f) The gene expression of mesenchymal marker of *CD73* was detected for hESCs (e) and hiPSCs (f) with peptide treatment at varying periods. The expression of these genes in hPSCs before differentiation was standardized to 1. (g) A dynamic map to summarize the impact of BFP1 peptide addition on the osteogenic differentiation of hPSCs during various weeks of induction in 28 days. * indicates $p < 0.05$, and ** indicates $p < 0.01$.

Supplementary Files

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