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Efficient Isolation and Long-term Red Fluorescent Nanodia-mond Labeling of Umbilical Cord Mesenchymal Stem Cells for the Effective Differentiation into Hepatocyte-like Cells

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HIGHLIGHTS

- Fluorescent nanodiamond labeling does not affect the life and growth of hUCMSC.
- FND labeling does not affect the hUCMSC differentiation into hepatocyte-like cells.
- FND labeling maintain during the procedure of hUCMSC differentiation into hepatocyte-like cells.

Abstract: Fluorescent nanodiamond (FND) has been used for long-term cell labeling and in vivo cell tracking because they have good at photostability and biocompatibility. In this study, we evaluate the effect of fluorescent nanodiamond labeling on in vitro culture and differentiation of human umbilical cord mesenchymal stem cells (hUCMSCs) into hepatocyte-like cells (HLCs). For hepatic differentiation of hUCMSCs, cells were induced with human hepatocyte growth factor, nicotinamide and Dexamethasone. FND was supplied in two experimental groups with 20 µg/mL and 100 µg/mL in 2 hours. The cell was assessed for FND uptake by laser scan microscopy and flow cytometry methods. The effect of FND on hUCMSCs was evaluated by the cell viability and growth assays as well as the differentiation throughout of morphology alterations or gene expression of anfa-fetoprotein, albumin, and hepatocyte nuclear factor 4α. The results showed that the labeling of hUCMSCs is efficient and easy and there was significant cellular uptake of FND. We did not observe any negative impacts of FND to the cell viability and growth. FND can be utilized for the long-term labeling and tracking of hUCSCs and HLCs in vivo studies.

Keywords: Differentiation; fluorescent nanodiamond; hepatocyte-like cells; in vitro culture; umbilical cord stem cells.

INTRODUCTION

Liver transplantation or hepatocyte transplantation is currently considered a promising substitute towards therapies for hepatic diseases. However, liver transplantation is crucially limited by shortage in liver donors, high transplantation costs, immune excretion of transplanted cells, and the requirement of long-term immuno-inhibition [1]. Mesenchymal stem cells or stem cell derived hepatocytes are favored research subjects due to the fact that they can be easily collected and multiplied *in vitro* to meet the needs of hepatic function [1]. For application of this potential therapy, it is essential to comply with general and reliable protocols to estimate effects of transplantation on homing and differentiation of cells as pre-clinical evaluation [2]. Tracking of the localization of transplanted cells using FND has proved to be effective in pigs and other animal models [3].

To evaluate localization of cells after transplantation, efforts have been made to monitor human mesenchymal stem cells in murine models by using polymerase chain reactions (PCR) to detect human DNA or immuno-analysis to determine human nuclear proteins [4]. However, data provided by these two methods have limited information on biological reliability and is not yet quantitative enough to evaluate safety and effectiveness of *in vivo* cell transplantation. Moreover, for application on large animals such as pigs, which are similar in body and organ scale to humans, PCR is not adequately reliable [5]. FND, which is considered non-toxic to cells and tissue, has advantages in research on localization of cells during transplantation [6]. Effects of fluorescent nanodiamond (FND) absorption has also been reported [7]. This material has been proven to be effectively absorbed by many cell types, such as embryonic stem cells [6], neural stem cells [8], and cancer stem cell lineages [4]. The previous studies have showed that all Nano-marked samples have not been changed in morphology, size, growth and the development of stem cells, but almost of reported show that it were enhanced differentiation into other cell types [9-12]. Scientists have been more deeply surveying effects of FND markers on cellular genomes and protein expression levels. The results have shown that this method is considerably safe and stable for cells. FND-labeled stem cells would be especially suitable for *in vivo* evaluation and tracking due to their optical characteristics, as previously mentioned. Moreover, for slow-growth stem cells such as quiescent cancer stem cells, conventional labeling methods are not physically and optically stable enough, which might allow *in vitro* and *in vivo* tracking of stem cells in long-term research. FND, therefore, is proven to be the most appropriate marker for that purpose.

One of the concerns of FND marker research is to evaluate its cytotoxicity and cellular stability in different cell lines or in various stages of stem cell differentiation process. And this can be conducted by surveying the absorption and maintenance of the nanodiamond particle both *in vitro* and *in vivo* [13]. Nevertheless, effect of FND labeling on multiplication and differentiation of stem cells have been reported for cancer stem cells [14] and osteoblasts [15], and neural stem cells [8]. However, umbilical cord stem cells' competence on absorption of FND and its effects on division and differentiation into hepatic-functional cells have not yet been studied. In the present study, we have conducted experiments to clarify this issue.

MATERIAL AND METHODS

Isolation and culture of human umbilical cord stem cells

The umbilical cord (UC) samples were obtained from healthy patients at the Hanoi Obstetrics and Gynecology Hospital. These donors were clearly explained about the purpose of using the specimen and they were consented providing. The UCs were transferred to the laboratory and washed in sterile phosphate buffered saline (PBS) at least 3 times to remove red blood cells. They were then immersed in 70% ethanol (Sigma, UK) for 30 seconds, immediately transferred back into PBS solution. A middle UC piece of approximately 2-3 cm was cut from the whole cord for processing. Subsequently, the UC piece was cut into explants of small pieces (1-2 mm^3) with a sterile scalpel and placed into 4 well plates (Nunc, UK) prefilled with culture Dulbecco's Modified Eagle's Medium (DMEM/F12) supplemented with fetal calf serum (FCS) (15%) (Life Sciences, UK), and 1% penicillin and streptomycin (P/S) (Life Sciences, UK) and cultured in an incubator with humidified atmosphere, 5% CO_2 at 37 ^oC. The medium was changed every 2-3 days and cells were passaged when they reached 70% confluence.

The sub-culture was done as following [16]: if cell had grown up to 70%-80% confluence, eliminated the old medium, and added 200 µl trypsin-EDTA pre-warmed at 37 °C for 5 min until the trypsinised cells detached from the disk surface. An amount of medium as much as two times of the trypsin volume was added

to inactivate, following by a centrifugation for 5 min at 1000 rpm. After removal of supernatant, cell pellet were resuspended in culture medium (400 µl/well or 2mL/35mm disk) at a concentration of about $1.9x10^4$ cell/cm^2, re-incubated at 37 °C with 5% CO_2 and humidified.

FACS determination of hUCMSCs phenotype

Flow cytometry was performed for hUCMSCs at passage 3. To determine cell surface marker expression, cell suspensions ($1×10^6$ cells) were incubated with 10 µl of each antibody of CD44, CD73, CD90, CD105 (Miltenyi Biotec Inc. USA) for 10 min at 2-8 ºC in the dark. The cells were washed three times by buffer (PBS+ 0.5% BSA+ 2 mM EDTA). Samples were sorted on a FACS Aria flow cytometer. The resulting data was analyzed using CellQuest software (BD Bioscience).

Induction of adipogenic and osteogenic differentiation

In in vitro differentiation assays, two differentiation assays were performed as follows [16]. For inducing the adipogenic differentiation, hUCMSCs from passages 3 were plated at a density of $1x10^4$ cells/well of 4 well plates (Nunc, UK) in medium for expansion which supplemented with 10 µg/L insulin, 0.5 µM IBMX and 1 mM Dexamethasone (all from Sigma). Expansion medium was changed every 3 days. After 3 weeks, cells were stained with Oil Red staining O (Bio Basic Canada Inc.) to make lipid vesicles visible.

For inducing osteogenic phenotype, hUCMSCs from passages 3 were plated at a density of $1x10^4$ cells/well of 4 well plates (Nunc, UK) in medium for expansion which supplemented with 10 µM b-glycerophosphate, 0.1 µM Desamethasone and 50 ng/mL vitamin C (all from Sigma). The culture medium was changed every 3 days. After 3 weeks, cells were subjected to Alizarin Red staining (Bio Basic Canada Inc.) for illustration of calcium deposition.

FND Preparation

Red fluorescent nanodiamonds which had nominal size of 100 nm was purchased from FND Biotech (Taiwan). For enhancing the solubility of the FND in cell culture media and facilitating cell biological analysis, FND was activated with albumin proteins (HSA: human serum albumin or BSA: bovine serum albumin) by mixture with HSA of BSA with a mass ratio of 1 FND: 1 albumin (w/w), vortexing for 2 hours . A group of non-activated FND (without coating with BSA or HAS) was also surveyed. Albumin-treated FND is re-obtained by centrifugation at 12,000 rpm for 10 minutes. The protein-FND complex was dissolved in sterile distilled water at a concentration of 1 mg/mL and stored at 4°C until use.

For FND absorption, cells were rinsed 3 times with PBS. Fresh DMEM/F12 culture medium without FBS was then added into culture wells. Finally, FND solution of an adequate amount was gently mixed and added into wells until reaching the final concentrations of 20 µg/mL or 100 µg/mL. Cells were put back to incubator of 37 ºC and 5% CO_2. After 2 hours, FND-fed cells were able to be collected. They were rinsed 3 times with PBS solution after removal of FND supplemented medium. Trypsin/EDTA 0.25% was used to detach cells as previously mentioned in cell passaging. Cell suspension could be subjected to flow cytometry directly. For laser scanning survey, cells were fixed by solution of 4% paraformaldehyde.

Cell growth assays

The hUCMSCs were cultured at a density of $1x10^6$ cells per 100-mm Petri dish in culture medium for 24 hours. Then, cells were treated with FNDs (100 µg/mL) for 2 hours, another group of non-treating disks served as control. Subsequently, FND-treated or non-treated cells were re-cultured in fresh medium. In every of a total of 8 days, total cell number of each group were recorded.

Cell viability test

The hUCMSCs were cultured in 35-mm Petri dish for 24 hours to reach a density of $1x10^5$ cells. At that point, a solution of FNDs 100 µg/mL or no FNDs were added into the disk for 2 hours. Subsequently cells were cultured in normal medium overnight. The next day, cells were detached, collected and rinsed 2 times with phosphate-buffered saline. An aliquot of $1×10^5$ cells was used for 1:1 staining with 0.4% trypan blue dye for 3 min and viable cells were counted using a hemo-cytometer. The experiments were repeated at least three times. The viability of cells was evaluated every day for 4 days.

Total RNA Isolation of Reverse Transcriptase-Polymerase Chain Reaction

All procedures followed protocol as reported on the manufacturer's instructions (QIAGEN: RNeasy® Mini Kit). Qualitative RT-PCR was performed following [18] using Quiagen RT-PCR kit (Sigma). The PCR products amplified by the primer pairs for each marker in Table 1 were then analyzed by electrophoresis using 2% agarose gel and visualization by ethidium bromide staining [18, 19].

Table 1. The oligonucleotide sequences of primers [18,19]

Name	Forward primer	Reverse primer
CD44	5-TCTCAAGGGCGTAACTCTGG-3	5-GCCAATTCTACCAGGCTTGA-3
CD73	5-CCTGCTCAGCTCTGCATAAGTA-3	5-CCCTATTTTACTGGCCAAGTGT-3
CD90	5-TTTGGCCCAAGTTTCTAAGG-3	5-AGATGCCATAAGCTGTGGTG-3
CD105	5-TCCAGCACTGGTGAACTGAG-3	5-TGTCTCCCCTGCCAGTTAGT-3
CD166	5-TGGTGTGGGAGATCAAAGGT-3	5-TGTGGCTGCCATTAAACAAG-3
Oct-1	5-GCAACCCTGTTAGCTTGGTC-3	5-CTCTCCTTTGCCCTCACAAC-3
Eras	5-GCAAGGTCCTGTAGGGAGAA-3	5-GCAGCTTTGAAACCCAAAAC-3
GATA.4	5-CCAGAGATTCTGCAACACGA-3	5-ATTTTGGAGTGAGGGGTCTG-3
HNF-4a	5'-CTGCTCGGAGCCACAAAGAGATCCATG-3'	5'-ATCATCTGCCACGTGATGCTCTGCA-3'
TAT	5'-TGAGCAGTCTGTCCACTGCCT-3'	5'-ATGTGAATGAGGAGGATCTGAG-3'
Alb	5'-TGCTTGAATGTGCTGATGACAGGG-3'	5'-AAGGCAAGTCAGGGCATCTCATC-3'
AFP	5'-TGCAGCCAAAGTGAAGAGGGAAGA-3'	5'-TGCAGCCAAAGTGAAGAGGGAAGA-3'

Hepatocytes differentiation

The hepatocytes differentiation method was according to the previous protocol [20].The hUCMSCs at passage 5 which reached 70% confluency were used for differentiation assays. Cells were serum-deprived for 2 days in DMEM/F12 supplemented with 20 ng/mL EGF and 10 ng/mL bFGF (Gibco, USA) to stop cell proliferation, prior to induction of differentiation toward a hepatic phenotype. Thereafter, a 2-phase differentiation protocol was performed, followed by sequential addition of growth factors, cytokines and hormones. Phase 1 differentiation was carried out in medium consisting of DMEM/F12 supplemented with 20 ng/mL HGF, 10 ng/mL bFGF and nicotinamide 4.9 mmol/L, for 7 days. Phase 2 differentiation was carried out in medium consisting of DMEM supplemented with 1 µmol/L Dexamethasone, 20 ng/mL Oncostatin M (OSM), and 10 µL/mL ITS + 1µM Trichostatin A (TSA) + premix (final concentration: 100 µmol/L insulin, 6.25 µg/mL transferrin, 3.6 µmol/L selenious acid, 1.25 mg/mL BSA and 190 µmol/L linoleic acid) to achieve cell maturation up to day 21. Twice weekly, media were changed by gently aspirating and re-filling. Hepatic differentiation was assessed at different time points by RT-PCR for liver-associated genes, immune-staining and PAS checking.

Periodic Acid-Schiff Staining

Medium was removed from culture disks and cells were rinsed with PBS three times. Then, 4% paraformaldehyde was used to fix the cells in 30 minutes. Oxidizing the cells by 10 g/l periodic acid for 10 minutes and washed three times with dH_2O, Schiff's reagent (Sigma-Aldrich) was added to treat the cells for 15 minutes. Afterwards, cells were rinsed in dH_2O for 10 minutes and counterstained with hematoxyline. The staining results were observed under an inverted microscope.

Fluorescence imaging expression of hepatocytes marker

The Hepatocyte-like cells (HLCs) were fixed with 4% Paraformaldehyde in 1xPBS at 37 ^oC for 15 minutes. Fixed HLCs were permeabilized with 0.25% triton X-100 for 5 mins at room temperature, and blocked for 30 mins at 37 ^oC with 10% BSA. The HLCs were incubated for 1 hour at 37 ^oC with primary antibodies (1:50, Santa Cruz) in 3% BSA. m-IgGκ BP-HRP - labeled secondary antibodies (1:100, Santa

Cruz) were incubated for 1 hour at 37 ^{o}C in the dark. The fluorescence intensity of was excited with wavelength 488 nm, and the emission was collected in 450-605 nm. The nuclei were simultaneously stained with Hoechst 33258. Fluorescence images were acquired with a Nikon Eclipse-Ti inverted microscope with Photometrics laser scanning. The Nikon NIS-Element imaging software was used to automatically acquire fluorescence images.

RESULTS

hUCMSCs isolation and valuation

The hUCMSCs were expanded in DMEM/F12 with 15% FCS supplementation, after 7~12 days of culture (Figure 1A). After 3 weeks, the cells were transplanted and regrowth to cover up to 70~90% of the culture plate surface. Cells isolated from UC tissues were maintained in an additional 15% FCS medium with fibroblast form (rhombus). This is a typical morphology of MSC (Figure 1B). In addition, the cells maintain their morphology when growing sub-cultures until the 20th transplanted stage (Figure 1C).

Figure 1. A. Migration of hUCSCs from the explants after 10–15 days. **B.** hUCSCs form a monolayer of adherent fibroblast-like cells at day 21. **C.** hUCSCs form adherent fibroblast-like cells by 20th passage.

Characterization by flow cytometry

Flow cytometry was performed for hUCMSCs at passage 3 to determine cell surface marker expression. The results indicated that expressed high levels of the mesenchymal stem cell marker: CD44 (98.5%), CD73 (99.9%), CD90 (98.2%) and CD105 (90%) (Figure 2).

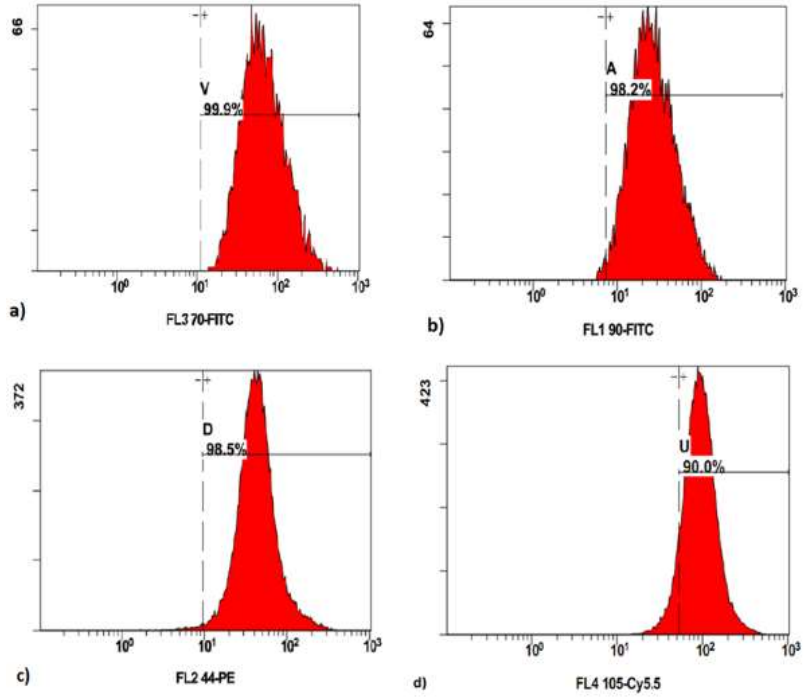

Figure 2. Flow cytometry of hUCMSCs cultured in DMEM/F12 medium. Cells positive to CD73 (**A.**), CD90 (**B.**), CD44 (**C.**), and CD105 (**D.**)

Differentiation potential

After 14 days culturing the hUCMSCs in Adipocytes-differentiated medium, there was the emergence

of larger round cells with more lipid vacuoles in the cytoplasm. Lipid droplets are positive for Oil red-O dyes (Figure 3A). In the differentiated process of osteogenic differentiation, after 2 weeks of culturing hUCMSCs cells, the appearance of colored crystals was observed when stained with Alizarin red (Figure 3B).

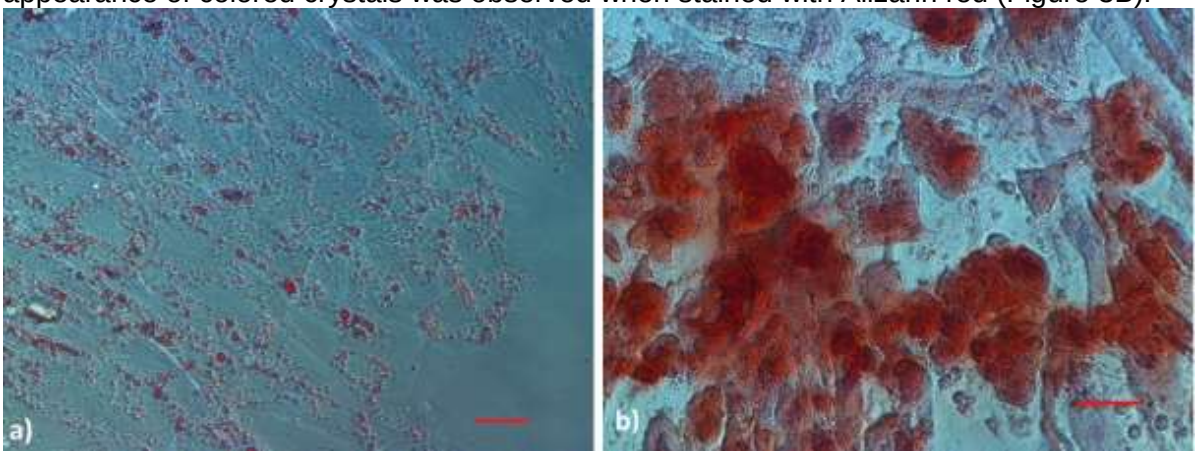

Figure 3. hUCMSCs were characterized by their competence of differentiation to adipogenic and osteogenic cells. **A.** Adipocytes positive for Oil red-O staining; **B.** Osteocytes positive for Alizarin staining

Reverse transcript-PCR analysis

The RT-PCR qualitative results of hUCSCs were shown in Figure 4. Cells exhibit all indicators of MSCs marker (CD73, CD86, CD90, and CD105), markers of embryonic stem cells (Oct-1), and transcription factors involved in the development of several mesoderm organs (GATA-4) and endoderm-derived (HNF3β). But cells were not yet shown two specific markers for hepatocyte (AFP, HNF4α).

Figure 4. Expression of several markers specific for mesenchymal stem cells of hUCMSC

Evaluation of FND uptake

Effect of nanoparticle concentration and protein coating

Figure 5A represents the cellular uptake of bare-FND by hUCMSCs after 2-hours incubation without and with FND at concentrations of 20 μg/mL and 100 μg/mL. It is clear that the fluorescence of cells is concentration- dependent. Cells fed with 20 μg/mL FND can be well separated from unstained cells (without FND feeding) but the much better result is observed for FND feeding at 100 μg/mL concentration. The cellular luminescence is clearly proportional to the concentration of nanoparticles used in the labeling (or herein we used the word “staining”) experiment. To save the nanomaterial, we suggested the use of the 100 μg/ mL concentration of FND for 2-hours treatment, the result showed that the FND concentration is sufficient to distinguish between untreated and treated experiments as observed by a conventional flow cytometer. In experiments with additional coating of BSA and HSA (Figure 5B), the analysis showed that protein coating does not significantly affect cellular uptake of FND and the fluorescence of protein-coated FND-fed cells is comparable with that of cells fed with bare-FND, after 2 hours feeding. Thus, absorption of diamond nanoparticles into hUCMSCs depends largely on particle concentration, not the protein coating.

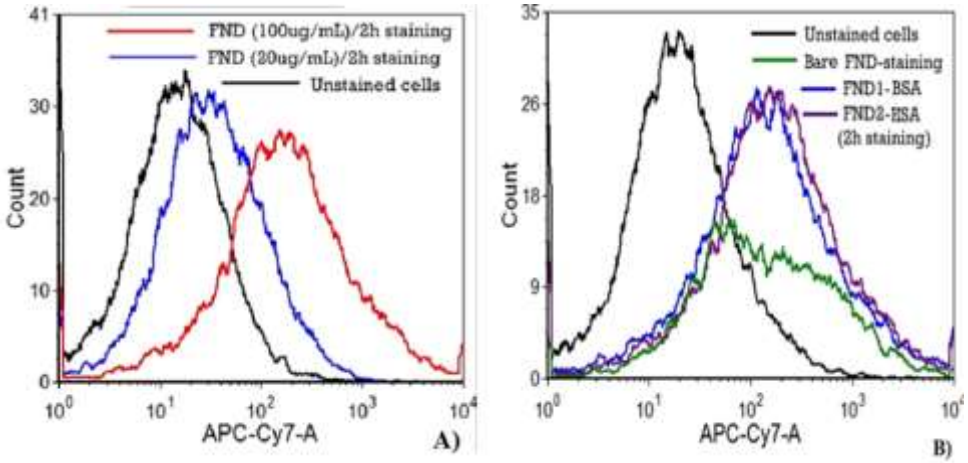

Figure 5. Fluorescence intensity of hUCSCs with and without FND labeling: **A.** in a concentration-dependent manner. **B.** a coating-dependent manner

After treatment with BSA-FND, the hUCMSCs were examined under a live cell confocal microscope imaging system. As shown in Figure 6, FND-fed cells exhibited highly bright red fluorescence of FND and the intensity is concentration-dependent. In which, cells in the group of 100 μg/mL are much brighter than that cells of 20 μg/mL group are. These results are consistent with the one observed by flow cytometry above. Moveover, we can see a significant amount of FND inside cells. However, the FND-bearing cells showed no significant difference in the fluorescence intensity in group 100 μg/mL between FND coating BSA and HSA.

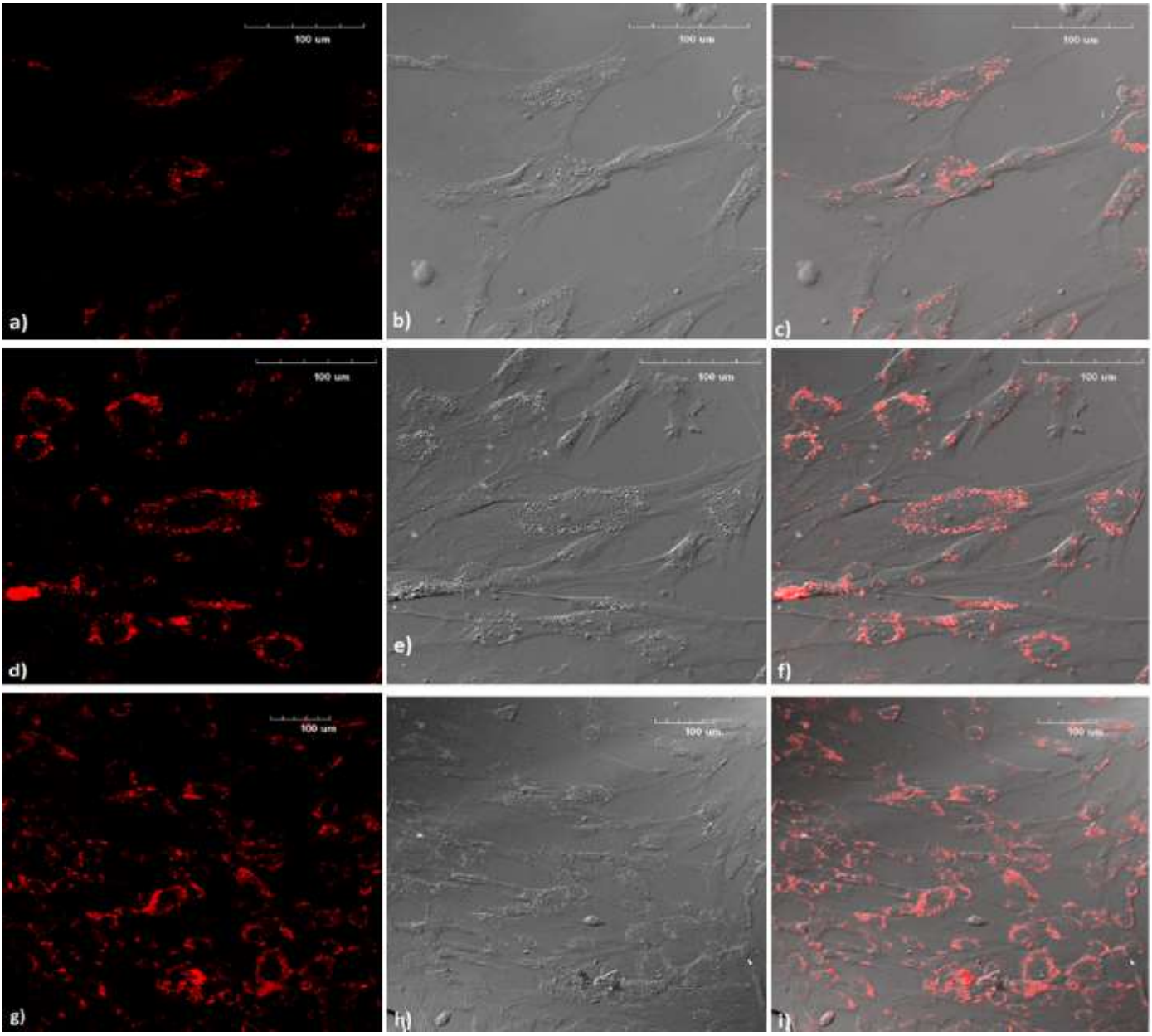

Figure 6. Cellular internalization of FNDs under confocal laser scanning: the hUCMSCs uptake in 20 □g/mL FND coating with BSA (a,b,c respectively for flourescence, trans and merge); the hUCMSCs uptake in 100 □g/mL FND coating with BSA (c,d,e respectively for flourescence, trans and merge); the hUCMSCs uptake in 100 □g/mL FND coating with HSA (f,g,h respectively for flourescence, trans and merge)

The hUCMSCs were treated with FND continuing *in vitro* culture. Cells still grow normally through passage 5. The results showed that stem cell residue in diamond nanoparticles decreased over time but still remained in cells after the 5th passage (15 days) (Figure 7). In particular, in differentiated cell groups, although there was a 21 days duration, the amount of the nanopaticle observed in the cell was still abundant.

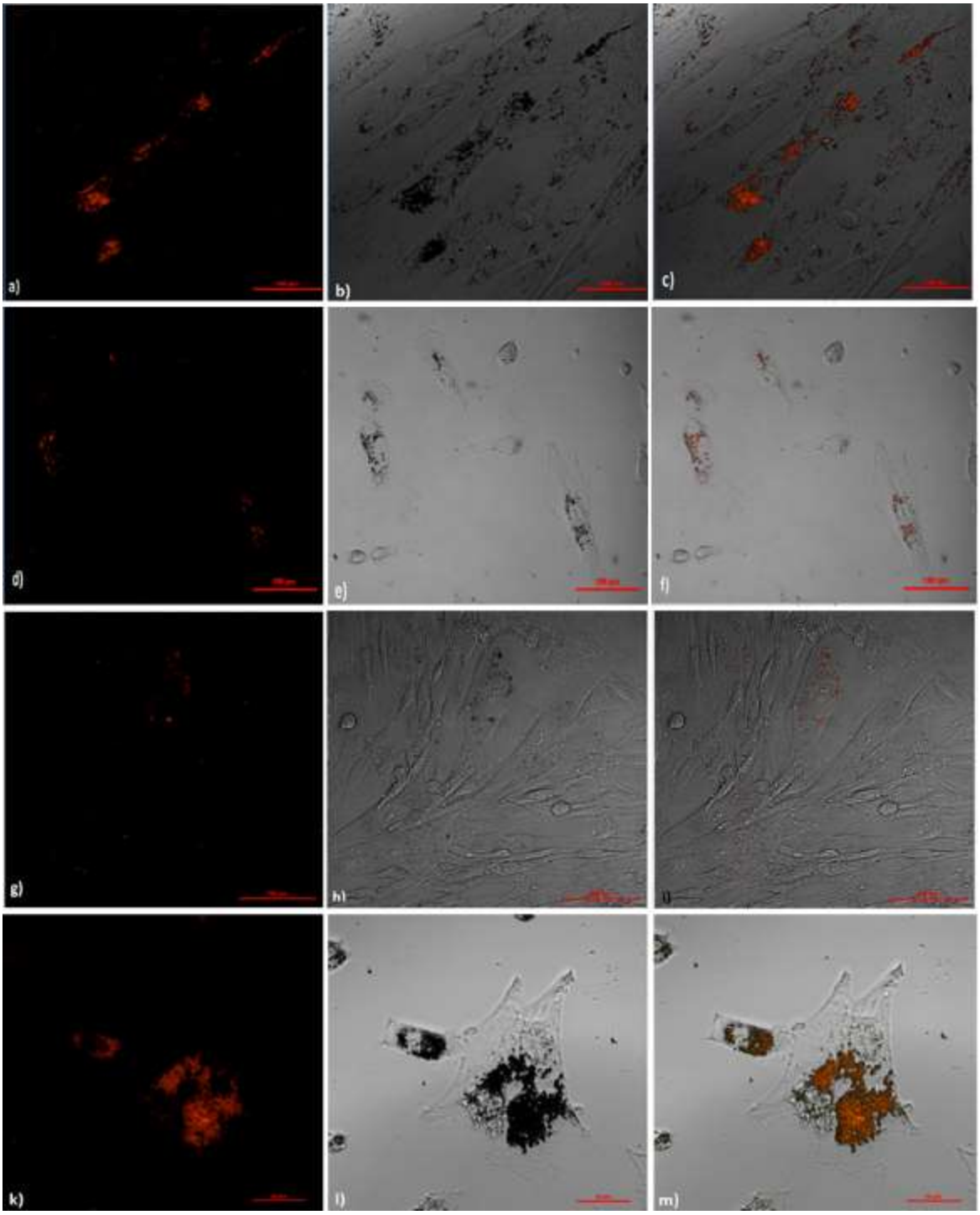

Figure 7. FND particles remain in hUCSCs cultured *in vitro* through passages. hUCSCs in passage 1 after 5 days FND treatment (a, b, c respectively for fluorescence, trans and merge); hUCSCs in passage 3 after 9 days FND treatment (d, e, f respectively for fluorescence, trans and merge); hUCSCs in passage 5 after 15 days FND treatment (g, h, i respectively for fluorescence, trans and merge); HLCs after 21 days FND treatment and differentiation (k, l, m respectively for fluorescence, trans and merge)

Effect FND labelling on the hUCMSCs viability and growth.

In the passage 8th, hUCMSCs were treated with or without FNDs (20 μg/mL and 100 μg/mL for 2h), to further examine the effect of FNDs on the cell growth. The total cell number was counted every day until day 5. The results show that, Passage 8 hUCMSCs displayed relatively higher cell viability rate in the control than the treated group in the day 1 and day 2. However, there was no significant differentiation at day 4 and day 5 (Figure 8A). The cell growth ability was analyzed after treatment with or without 20 μg/mL and 100 μg/mL FND for 2 h in hUCMSCs, and then further cultured for another 8 days. The total cell number was counted every day. Figure 8B shows that FND particles did not alter the cell growth ability in hUCMSCs.

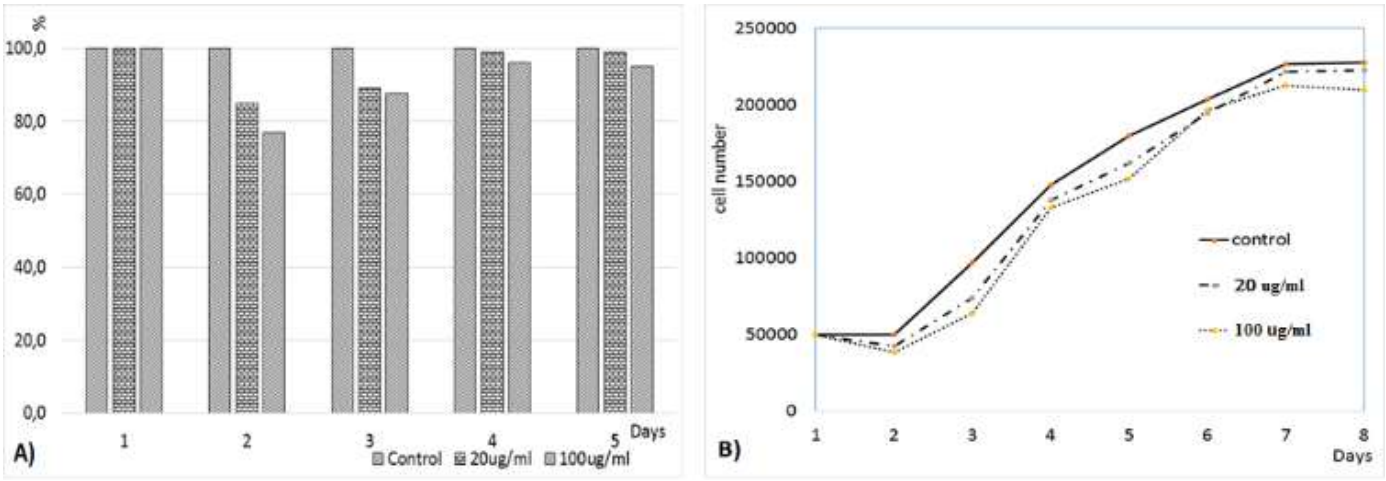

Figure 8. Effect of treatment with FND particles on cell viability and growth ability in hUCMSCs. **A.** Cell viability in untreated (Control) or treated by FND with 20 μg/mL and 100 μg/mL for 2 hours with checking at day 1,2,3,4, and 5. **B.** Cell growth rates after treatment of FND (hUCMSCs were plated at a density of 1 x 10^5 cells on 35-mm Petri disks for 24 hours, subsequently incubated with or without 20 μg/mL or 100 μg/mL of FNDs for 2 hours. Thereafter, disks were changed with fresh medium. Total cell number was recorded every day for a total of 8 days).

Evaluation of hepatocytes after differentiation

Morphological Examination

We analyzed the morphological changes of hUCSCs at the differentiation stages in order to evaluate the effect of uptake FND or not. The hUCSCs which have been obtained either from uptake sample or the control shown morphological alteration at day 7. Cells after differentiation (D7) exhibited a fibroblast-like morphology (Figure 9). Cell morphology in both groups did not change significantly during differentiation progress.

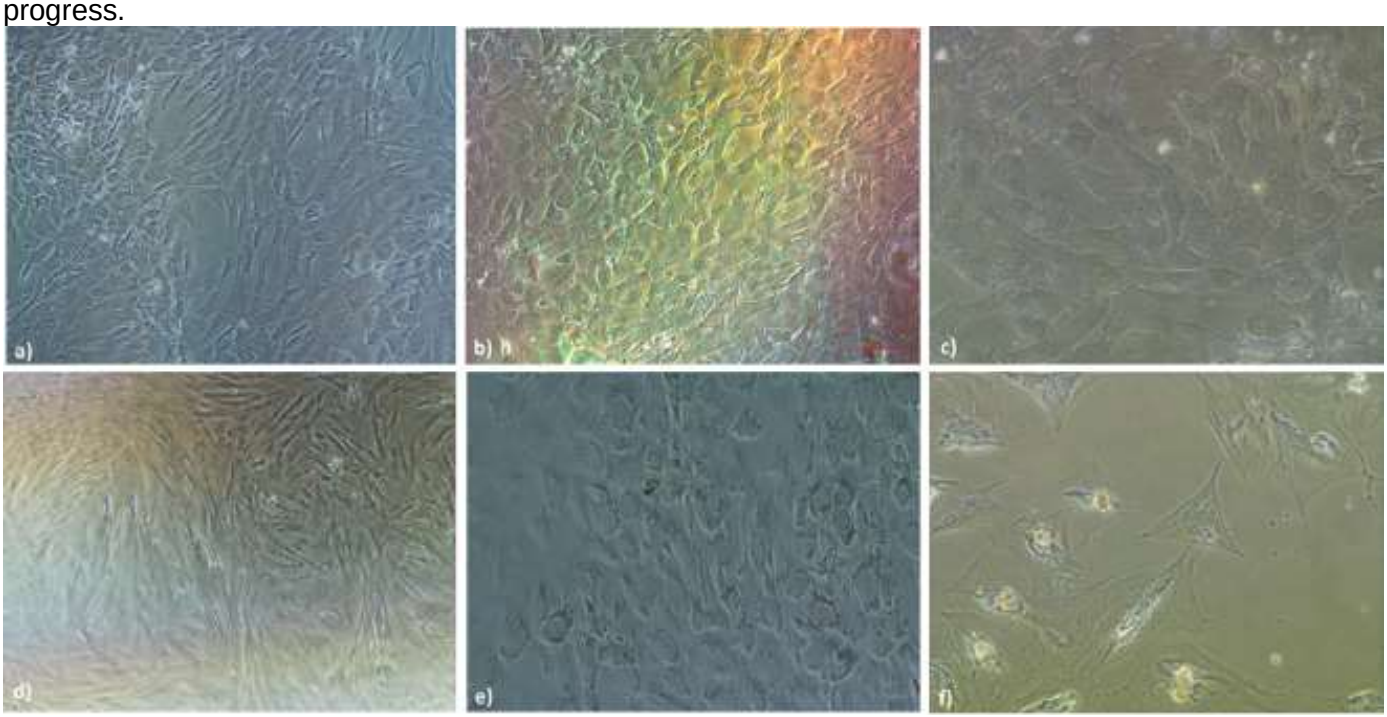

Figure 9. Changes in morphology of hUCSCs during the differentiation process. Differentiation was induced by sequential addition of growth factors, cytokines and hormones. There was no significant morphological alteration between the two groups during differentiation. Figures showed the morphology of HLCs in control group without FND uptake at day 7 (a), day 14 (b), day 21 (c) and in group with 100ng/mL of FND uptake at day 7 (d), day 14 (e) and day 21 (f).

Immunostaning with hepatocyte maker function

To further confirm the homogeneous expression of HNF4α, albumin and AFP in our cell populations, we examined differentiated hUCSCs into HCL by immunocytochemistry. This analysis showed that the differentiated cells stain positive for albumin and AFP (Figure 10) at day 21, at the end of differentiation. Our results demonstrate that the penetrative levels of hepatic protein markers show non homogeneousness in both of uptake or uptake FND experiments.

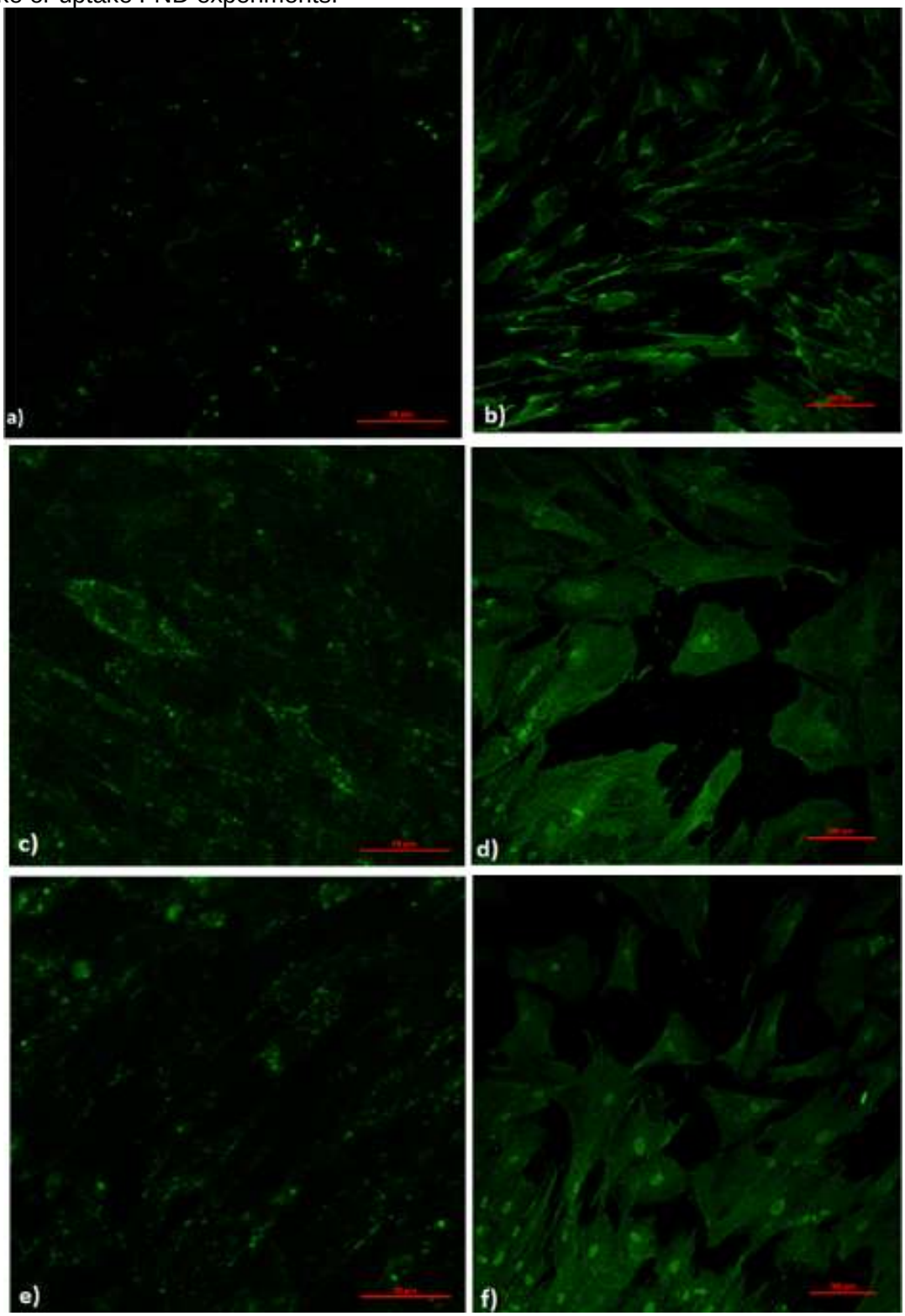

Figure 10. Expression of hepatocyte markers in MSC-derived HLCs at day 21 days differentiation are shown in green by immunofluorescence staining. For HNF4α staining in without FND (a) and with FND (b); for ALB staining in without FND (c) and with FND (d); for AFP staining in without FND (e) and with FND (f)

We then examined the functional level of hUCMSCs. Periodic acid-Schiff (PAS) staining showed that native hUCMSCs were able to specifically store glycogen. Moreover, the stain was weaker than that detected in HLCs (Figure 11). The glycogen was stored in the HLCs of negative control group, while the expression level was much higher than that observed for HLCs in differentiation at day 14 and day 21 (Figure 11). There was also no significant difference between the FND treatment and none FND treatment group. In our research, all experiments were performed at least three time and they were showed similar results.

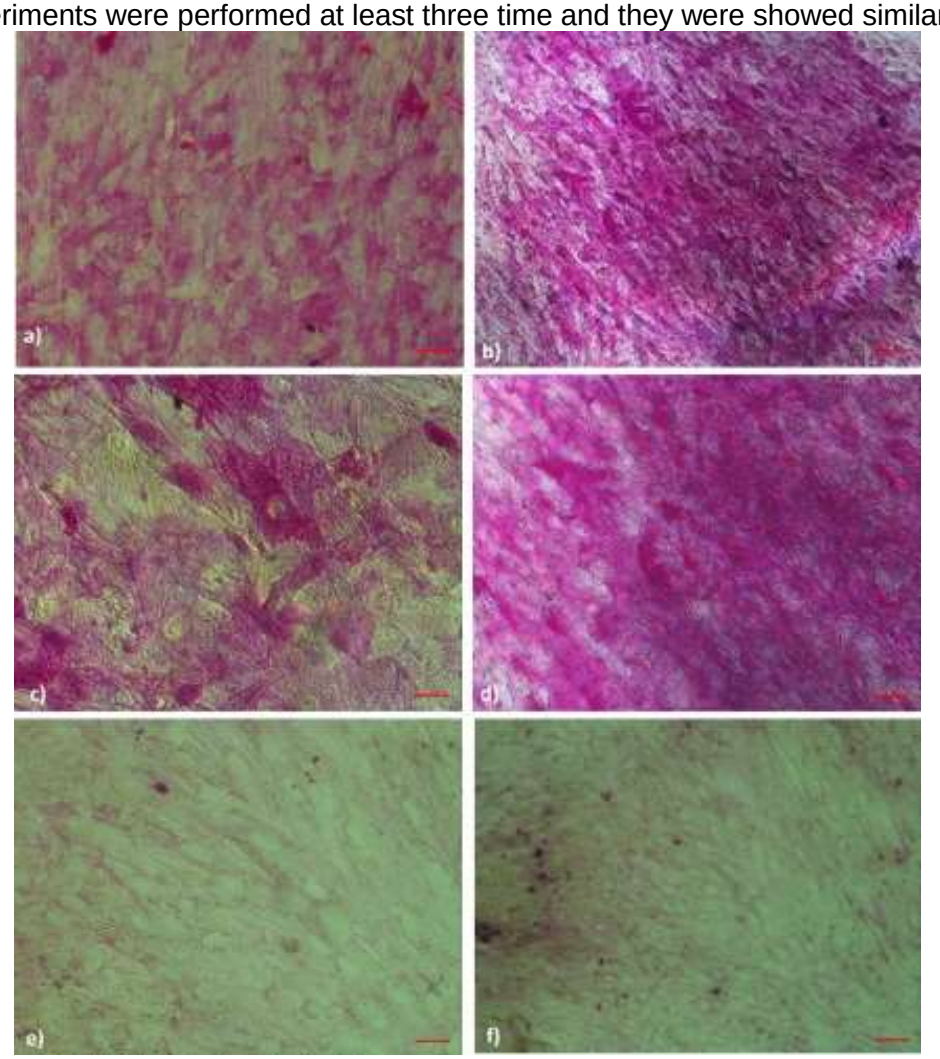

Figure 11. Functional assays (Periodic Acid-Schiff staining assay - PAS) of HLCs. PAS staining of HLCs in group with FND treatment at day 14 (a) and day 21 (b); PAS staining of HLCs in group without FND treatment at day 14 (c) and day 21 (d); staining of undifferentiated hUCMSCs in group with FND treatment at day 14 (e) and day 21 (f)

DISCUSSION

Currently, hUCMSCs have been determined to have differentiation competence into multiple cell types and considered a cell source with advantages in medical research and cell therapy. There are two popular methods to isolate hUCMSCs: isolation of cells by enzyme and cell culture from small tissue pieces [21]. Each method has its own advantages with different isolation efficiency, growth rate, as well as cell quality, through markers expression and differentiation competence [21]. In this research, we have isolated and obtained cell lines that could maintain their stable typical morphology until passage 20 with molecular marker expression specific for mesenchymal stem cells and embryonic stem cells such as Oct 4, Eras or Nanog. However, they were not expressed hepaticyte specific markers such as HNF4α and AFP. The hUCMSCs have the competence to differentiate into many cell types including HLC cells, with the efficiency not lower than that of mesenchymal stem cells [20], and the derived product has been proven to be beneficial in cirrhosis treatment [22].

Rapid development of nano-technology has led to abundant supply of many nano materials, especially metal nano particle, nano carbon, nano semiconductor, nano polymer particles, and DNA nano structure, which is very promising in regulating stem cell dynamics and tissue regeneration [12]. Nano materials have been determined to be able to enhance the competence to release medical molecule into the environment or

to promote the differentiation process of stem cells [11]. Moreover, nano materials with special characters or special complement or adjuvant could be regulated to have specific characteristics to identify stem cell types, their hardness, connectivity and some other specifications play an important role in their effect on stem cells [12]. For its complication, the mechanism that nano materials affect stem cell fate has not been well studied. Most of the published literature could not have a deep understanding in the differentiation mechanisms promoted by nano materials. Therefore, further researches are necessary to clarify mechanisms and bio-effects of nano materials on stem cell differentiation. To evaluate of stem cell to each nano material, they should be survey to have an optimal surface [23]. In the present study, we use HSA and BSA, which both reveal similar absorption efficiency.

Recent literatures showed that FND particles could be transferred into stem cells to monitor and evaluate homing competency on in vivo transplantation conditions [3]. FND does not affect cellular physiological functions, including morphology, viability, and cell growth [14]. It was also proven that FND is a nano material compatible for localization into cytoplasm, long-term appearance in the cells, therefore it could be used as a stem cell marking material applicable for monitoring stem cell differentiation into plenty of functional cell types, or could be used for other purposes [14,24]. Besides, FND absorbed cells have also enhanced cellular functions such as cellular immune response capability24. To evaluate the effects of FND on differentiation of stem cells into neural cells, Hsu and coauthors [8] has determined that FND does not affect morphology changes, it is not toxic to the cells and it does not result apoptosis during differentiation. Moreover, FND does not vary viability of cells and gene expression of neural cells, at least specific neural markers during differentiation [8]. In the meanwhile, most of the other nano materials has been determined that raise differentiation level in neural- and osteo-genesis [12]. In this research, similar results that FND does not affect viability and differentiation of hucMSCs have been achieved.

CONCLUSION

The FND uptake does not affect the viability or growth of HUCMSs and the process differentiation of it into hepatocytes and it can be used for long-term maker for further in vivo tracking and quantification of the cell lines.

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