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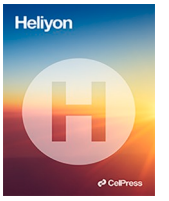

Research article

Fluorescent monitoring osteogenic differentiation of osteosarcoma cells with an aggregation-induced emission probe

Junxiong Zhang [a,1], Zhongming Lai [a,1], Zhongmin Zhang [a,**], Shuai Zheng [a,*]

[a] *Department of Orthopaedics, The First Affiliated Hospital, Southern Medical University, Academy of Orthopaedics, Guangdong Province, Guangzhou, 510515, China*

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ABSTRACT

Osteosarcoma is widely believed to be an osteogenic differentiation disorder. In recent years, to further understand this disease, a lot resources were poured into the potential link between differentiation defects and tumorigenesis. Long-term monitoring of the differentiation progress of osteosarcoma cells is of great importance. In order to better promote the research, we have developed a novel fluorescent probe called PTB-EDTA, which exhibits remarkable bio-compatibility and demonstrates high selectivity towards osteosarcoma cells. Not only is the PTB-EDTA is capable of live cell imaging while conventional histology requires to kill the cells, its fluorescence is also enhanced as the osteogenic differentiation proceeding. These properties make PTB-EDTA a promising tool for monitoring osteosarcoma cells.

1. Introduction

Osteosarcoma is the most common primary malignant bone cancer, causing severe disability and high medical expenditure for patients worldwide [1]. It is widely accepted that osteosarcoma originates from mesenchymal stem cells (MSCs) at early stage of osteogenic differentiation [2]. Research has found that osteosarcoma cells can be induced into a mature osteoblast-like phenotype [3], which may leads to irreversible loss of tumor phenotype [4]. To monitor the process of osteogenic differentiation in osteosarcoma cells, conventional techniques including immunohistochemistry, immunofluorescence, Western blot (WB) and polymerase chain reaction (PCR) are often used. However, these methods are cumbersome and steps such as immobilization, homogenization or purification kill cells, which significantly compromise the native structural and natural functions of the cells.

Fluorescence imaging has many advantages such as high bio-compatibility, easy operation, and low cost [5]. Various fluorescent materials have been used for long-term tracking of live cell behaviors such as cell differentiation [6–8]. Recently, a new class of fluorescent materials named aggregation-induced emission (AIE) materials has emerged, offering several advantages such as excellent photo-stability and large Stokes shifts [9]. Due to the outstanding optics properties, AIE materials were widely used in long-term cell tracking [10].

Although osteosarcoma lack of specific biomarkers. However, In previous study, researchers found out that osteoblast are enriched with an specific organelle called matrix vesicles(MVs). MVs are originated from plasma membrane according to endocytosis. Based on

* Corresponding author.
** Second corresponding author
E-mail addresses: zzmzzc@smu.edu.cn (Z. Zhang), allen@smu.edu.cn (S. Zheng).
[1] These authors contributed equally to this work.

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this property, we have developed an nanoparticle AIE materials, named PTB-EDTA(Fig. 1), which is capable of imaging osteoblast cell through MVs biogenesis pathway [11]. Similar to osteoblast, studies also found that osteosarcoma cells have a similar phenotype with osteoblast and can also produce MVs [12,13]. It is also widely believed that osteosarcoma originates from committed osteoblast and can be induced into a mature osteoblast-like phenotype [14–16]. Therefore, we believed that PTB-EDTA is also suitable for fluorescent tracing of osteosarcoma cells.

2. Results and discussion

2.1. Cytotoxicity of PTB-EDTA

To assess the cell toxicity of PTB-EDTA, osteosarcoma cell line MG-63 cells were used. Fig. 2 demonstrates that cell viability was unaffected by different concentrations of PTB-EDTA. This result indicated that PTB-EDTA has negligible cytotoxicity to osteosarcoma cell. In order to avoid the interference of the PTB-EDTA absorption peak (429 nm) on the results, the absorption value of 490 nm was adopted in this experiment.

2.2. Fluorescence labeling of osteosarcoma cells with PTB-EDTA

To verify whether PTB-EDTA can fluoresce osteosarcoma cell selectively, we adopted three osteosarcoma cell lines MG-63 cells, U2OS cells and Saos-2 cells as the experimental group and fibroblast cells (HFF-1), human skeletal muscle cells (HSMC) and human endothelial cells (HUVEC) as the control group [17]. As shown in Fig. 3, after incubated with PTB-EDTA (10 μM) for 6h, MG-63 cells (Fig. 3A–C), U2OS cells(Fig. 3D–F) and Saos-2 cells(Fig. 3G–I) emitted bright fluorescence. While the HUVEC cells(Fig. 3J-L), HSMC cells(Fig. 3M − O) and HFF-1 cells(Fig. 3P-R) exhibited no obvious fluorescence signals (Fig. 3K, N, 3Q). This result conveys promising foregroud of PTB-EDTA and prompt us to further researches.

2.3. Selective fluorescent labeling of osteosarcoma cells with PTB-EDTA

To further verify whether PTB-EDTA are selective to osteosarcoma cells, SVHUC1 cells were used as a contrast and were co-cultured

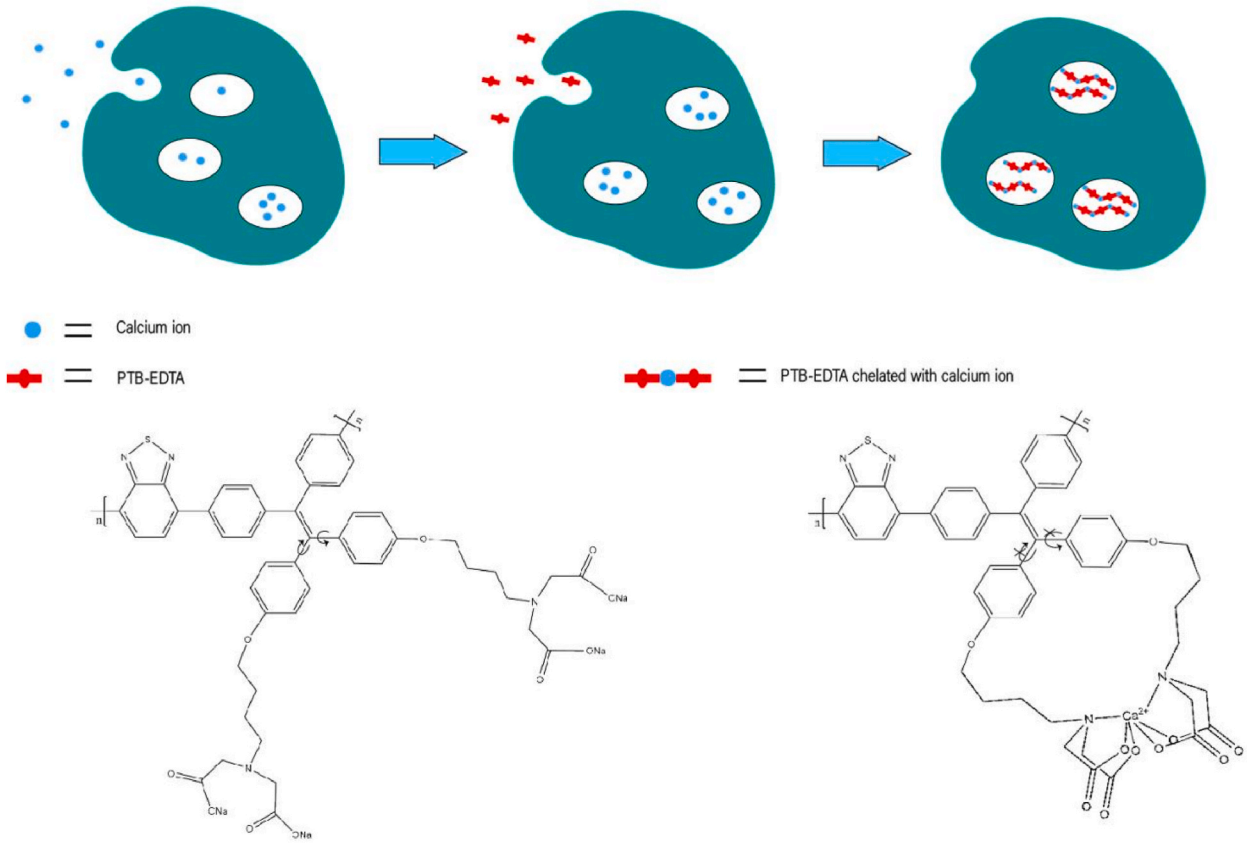

Fig. 1. Schematic illustration of the structure of PTB-EDTA and its staining process in osteosarcoma cells.

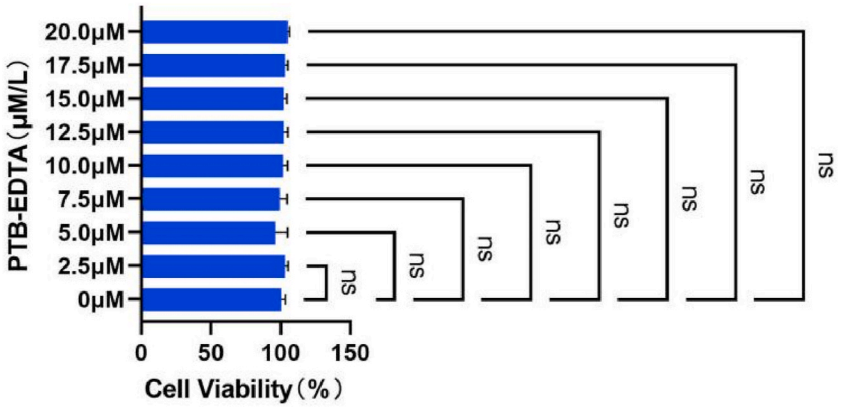

Fig. 2. Cytotoxicity of PTB-EDTA. Viability of MG-63 cells assessed by CCK-8 assay after incubation with different concentrations of PTB-EDTA for 48h.

with osteosarcoma cell lines MG-63(Fig. 4A–C), Saos-2(Fig. 4D–F), U–2OS(Fig. 4G–I) respectively. Each dish was incubated with PTB-EDTA (10 μM) for 6h. Interestingly, only osteosarcoma cells exhibited bright fluorescence, while the small size SVHUC1 cells (Fig. 4B–E and H blue arrow) hardly emitted any signals. This result indicate that the resolution of PTB-EDTA are precise to cell level. In order to avoid errors caused by different levels of CLSM images, fluorescence microscopic imaging was adopted in supplemental experiment. As shown in the supplemental data(S Fig. 10), PTB-EDTA could selectively fluorescently label MG-63 cells, but had no obvious labeling effect on other cells.

2.4. Relative photo-stability of PTB-EDTA

Photo-stability is the key factor of long-term fluorescent tracing. Thus, we adopted conventional cell fluorescent probe Fluo-4 AM as a contrast [18]. U2OS cells were stained with Fluo-4 AM (5 μM) for 15min and PTB-EDTA (10 μM) for 6h respectively, followed by continuous excitation light irradiation for 5min. As shown in Fig. 5A, the signal loss of PTB-EDTA was less than 20 % after irradiation for 5 min. While in Fig. 5B, the signal of Fluo-4 AM attenuated markedly over time. Fluorescence video recording seen in supplemental document(Video data 1). The results show that the light stability of PTB-EDTA is much higher than that of common fluorescent dyes, which is more suitable for long-term monitoring.

2.5. Mechanism of selective fluorescent labeling of osteosarcoma cells by PTB-EDTA

Unlike familiar fluorescent probe that are usually small single molecule, PTB-EDTA are nanoparticles which can not pass cell membrane freely. Although PTB-EDTA demonstrates remarkable selectivity for osteosarcoma cells, the mechanism remains unproven. Bone biology studies have shown that osteosarcoma cell form matrix vesicles (MVs) intracellularly, which are rich in calcium ions and osteogenic proteins to form bone matrix exactly like osteoblast do [19]. Therefore, we believed that the accompanying membrane transport effect of MVs formation is likely to be the pathway for PTB-EDTA to enter cells. Previous research has reviewed that Pit-1, a sodium-phosphate cotransporter, is abundant on MVs membrane, contributing to the formation of MVs [20]. In order to find out whether PTB-EDTA staining mechanism is accompany with MVs(through endocytosis), in this study, we performed immunofluorescence of Pit-1. As shown in Fig. 6, PTB-EDTA(Fig. 6A) was highly co-localized with Pit-1(Fig. 6B), indicating precise localization of PTB-EDTA in MVs(Fig. 6C). To further verify the MVs biogenesis(endocytosis) mechanism of PTB-EDTA staining, we also performed endocytosis blocking experiment(Supplemental data Fig. 5) and the result review that PTB-EDTA staining osteosarcoma cell was absolutely rely on endocytosis(MVs biogenesis).

2.6. Fluorescence labeling of osteosarcoma cells enhanced with osteogenic differentiation proceeding

Many studies have indicated that the MVs biogenesis metabolism is improved with the progression of osteogenic differentiation [19]. Similar to osteoblast, osteosarcoma cells have an elevated metabolic level of calcium ions and osteogenesis. Therefore, it is wondering whether the fluorescent labeling effect of PTB-EDTA is affected by osteogenic differentiation of osteosarcoma cells. In this study, PTB-EDTA was given to osteosarcoma cells at different stages of osteogenic differentiation. Dexamethasone (100 nM), ascorbic acid (0.05 mM) and β-sodium glycerophosphate (10 mM) were added in the cell culture medium to induce osteogenic differentiation. As shown in Fig. 7, after 3 weeks of osteogenic induction, the osteosarcoma cell acquired highly mature osteoblastic phenotype, indicated by ALP staining((Fig. 7A and B)). With the increase of pit-1(MVs) expression(Fig. 7C and D), the fluorescent labeling intensity of PTB-EDTA also increased(Fig. 7E and F). This result has indicated, with the continuous osteogenic differentiation of osteosarcoma cells, the fluorescent labeling intensity of PTB-EDTA also increased, which provided a promising tool for long term monitoring osteogenic differentiation of osteosarcoma cell.

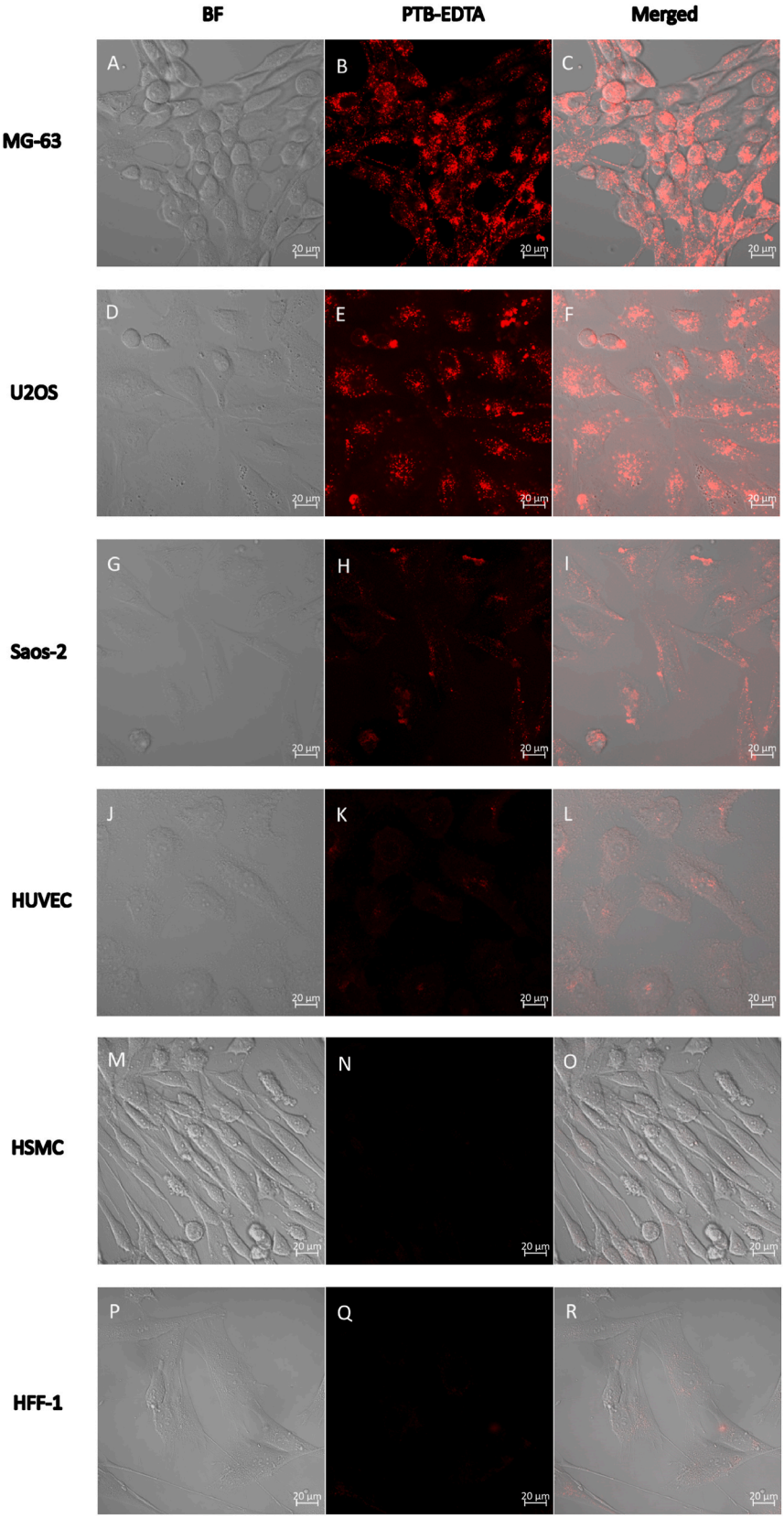

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Fig. 3. Selective fluorescent labeling of osteosarcoma cells. CLSM images of MG-63 cells (A, B, C), U2OS cells (D, E, F), Saos-2 cells (G, H, I), HUVEC cells (J, K, L), HSMC cells (M, N, O), HFF-1 cells (P, Q, R) under 40 × objective after incubation with PTB-EDTA (10 μM) for 6h. Scale bar = 20 μm. Imaging scale = 40 × .

3. Conclusion

In summary, we have successfully monitored osteogenic differentiation of osteosarcoma cells with a fluorescent probe PTB-EDTA. Mechanistically, PTB-EDTA is targeting to osteoblastic organelle MVs to achieve osteogenic differentiation recognition of osteosarcoma cells and enhanced fluorescence. This results make PTB-EDTA an promising agent for long-term cell monitoring and provide new data for the imaging of osteosarcoma.

Methods and materials

PTB-EDTA was synthesized according to our previous study.

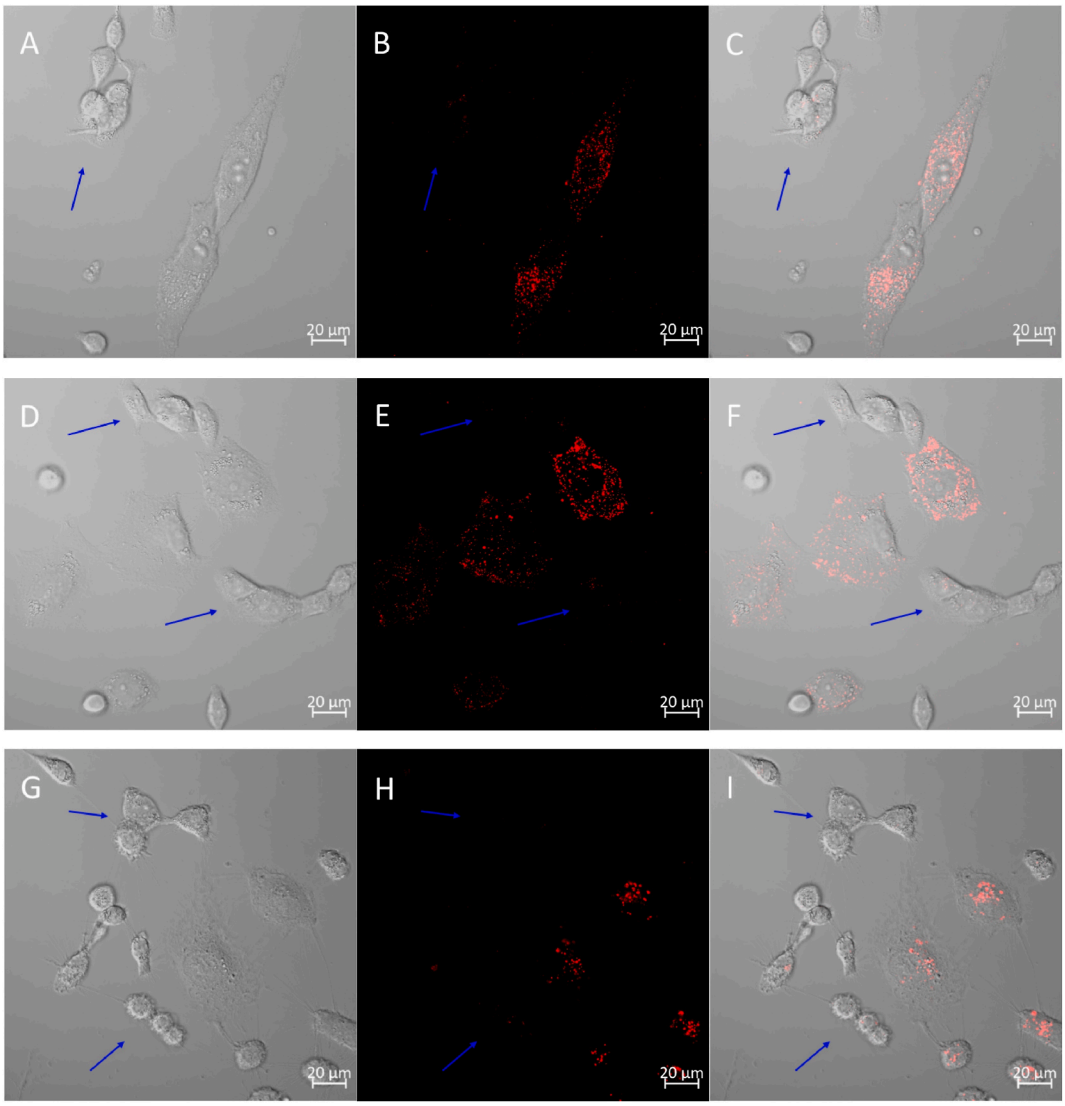

Fig. 4. Selective fluorescent labeling of osteosarcoma cells in co-culture system. CLSM images of MG-63 cells and SVHUC1 cells (A, B, C), Saos-2 cells and SVHUC1 cells (D, E, F), U–2OS cells and SVHUC1 cells (G, H, I) after incubation with PTB-EDTA (10 μM) for 6h. Scale bar = 20 μm. Imaging scale = 40 × .

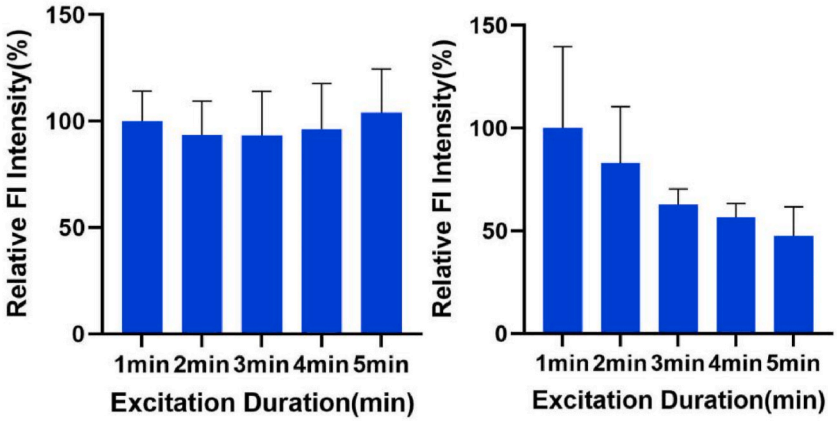

Fig. 5. Photo-stability of PTB-EDTA and Fluo-4 AM. (A) Relative fluorescence intensity of MG-63 cells incubated with PTB-EDTA (10 μM) under continuous irradiation for 5min. (B) Relative fluorescence intensity of MG-63 cells stained with Fluo-4 AM (5 μM) under continuous light irradiation for 5min. Imaging scale = 20 × . Excitation light source system: X-Cite® 120Q. Fluorescence filter: U-FBWA.

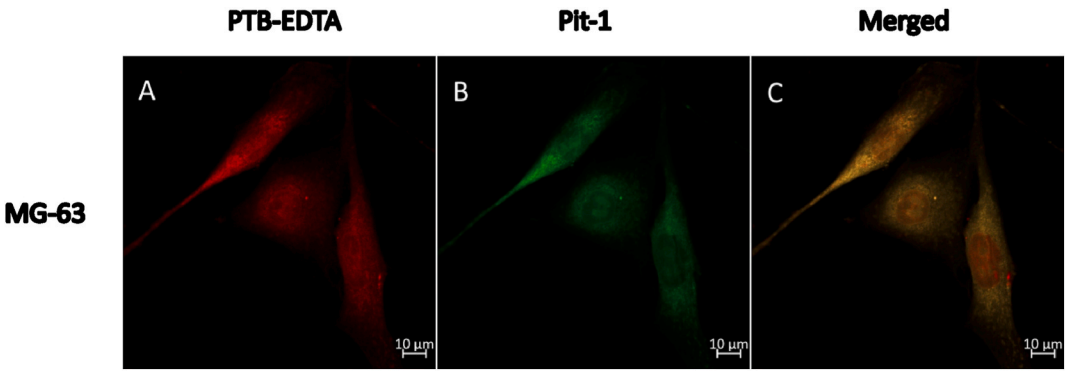

Fig. 6. Fluorescence co-localization of PTB-EDTA and Pit-1. CLSM images of MG-63 cells. (A) PTB-EDTA image of MG-63 cells. (B) Pit-1 immunofluorescence image of MG-63 cells. (C) Merged image of PTB-EDTA and Pit-1. Scale bar = 10 μM. Image scale = 63 × .

Equipment and methods

Fluorescent images were obtained using a confocal laser scanning fluorescence microscopy (Zeiss LSM 980) under the excitation laser at 405 nm for PTB-EDTA and at 543 nm for Lyso-Tracker BL766A. Photostability images were obtained by an inverted fluorescence microscope IX73 (Olympus) under constant irradiation of UV excitation laser (130W) for 5min. The cell toxicity of PTB-EDTA was detected by SpectraMax ix3 multifunctional microplate reader (Molecular Devices) for CCK-8 assay. PTB-EDTA fluorescence intensity was measured by SpectraMax i3x (Molecular Devices). Images were statistically analyzed by image processing software (ImageJ, National Institutes of Health, United States). All data were statistically analyzed by data processing software (Prism, GraphPad Software).

Materials and chemicals

The cell Counting Kit-8, phosphate buffered saline (PBS), penicillin and streptomycin, and Lyso-tracker BL766A were purchased from Biosharp. MG-63, Saos-2, U–2OS, CNE2, HUVEC, SVHUC1, Mccoys'5A medium, calcium-free Mccoy's 5A medium were purchased from CELL RESEARCH. IFF-1 was purchased from CELL RESEARCH. HSMC was purchased from iCell. Fetal bovine serum (FBS) was purchased from CORNING. Chlorpromazine and dynasore were purchased from MACKLIN. BCIP/NBT Alkaline Phosphatase Color Development Kit was purchased from Beyotime. Calcium chloride was purchased from Mecklin. Fluo-4 AM was purchased from YEASEN. Pit-1 fluorescent antibody was purchased from Santa Cruz Biotechnology.

Cell culture

MG-63, Saos-2, U–2OS, CNE2, HUVEC, SVHUC1 cells were cultured in Mccoy's 5A medium. For the calcium dependent experiment, the cells were cultured in Mccoy's 5A medium and calcium-free Mccoy's 5A medium, respectively. All the cells were cultured in

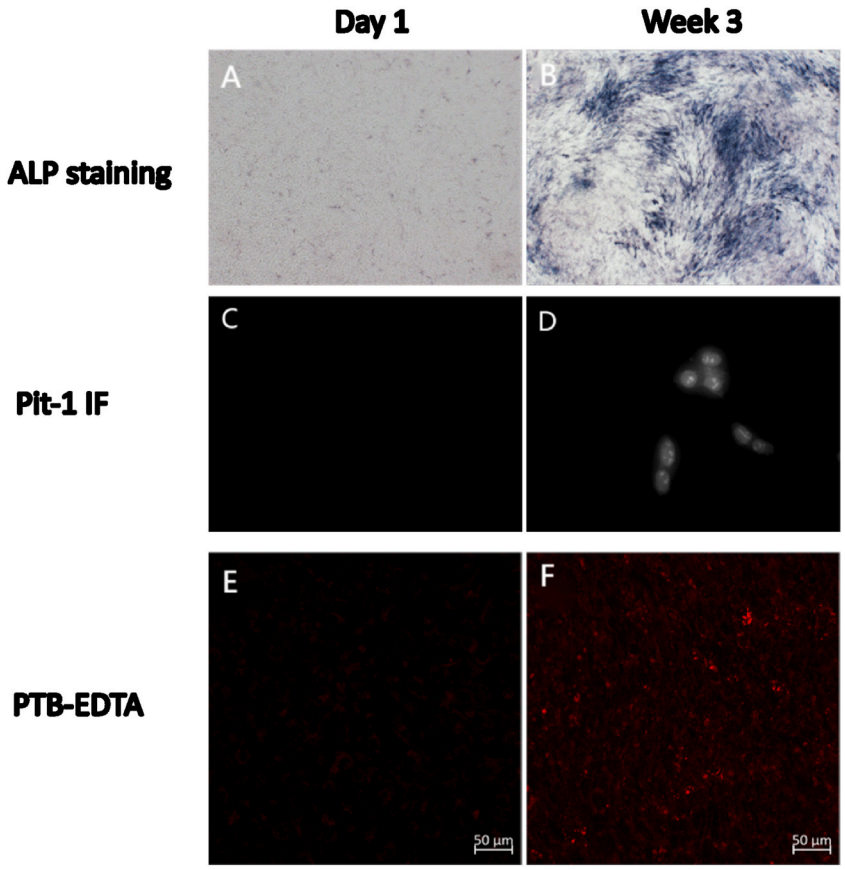

Fig. 7. Fluorescence imaging of osteosarcoma cells with different degrees of osteogenic differentiation. (Ã D) ALP staining after different osteogenic induction duration under 10 × objective. (E ~ H) PTB-EDTA staining after different osteogenic induction durations under 20 × objective. Scale bar = 50 μm. Image scale = Ã D in 10 × , E ~ H in 20 × .

medium supplemented with 10 % heat-inactivated FBS, 100 units/mL penicillin and 100 μg/mL streptomycin in a humidity incubator with 5 % CO2 at 37 °C. Before the experiment, the cells were pre-cultured until confluence was reached. All cells were cultured overnight inside a 60 mm Petri dish.

Confocal laser scanning fluorescence microscopy

For cells staining experiment, live cells were incubated with 10 μM PTB-EDTA for 6h, and then imaged using confocal laser scanning fluorescence microscopy (Zeiss LSM 980). For fluorescence colocalization experiments, the cells were co-stained with PTB-EDTA (10 μM) for 6h and a commercial biomarker (Lyso-Tracker BL766A 1 μM) for 15min. For the co-cultured experiment, the cells were co-cultured for 24h followed by incubation with 10 μM PTB-EDTA for 6h. The cells were imaged using a confocal laser scanning fluorescence microscopy (Zeiss LSM 980) with excitation at 405 nm for PTB-EDTA and 543 nm for Lyso-Tracker BL766A.

Photostability of PTB-EDTA

Fluorescent images were obtained using the IX73 fluorescence microscope (Olympus) under excitation of UV laser at 130W for a prolonged time (5min) and statistically analyzed using image processing software (ImageJ, National Institutes of Health, United States).

Cytotoxicity of PTB-EDTA

Cytotoxicity of PTB-EDTA was measured by the CCK-8 assay according to the manufacturer's method (n = 5). A suspension of 5000 MG-63 cells per well was added to a 96-well plate (100 μL). After 24h of culture, the medium was replaced with PTB-EDTA of different concentrations. Following an additional 24h of culture, the medium was replaced again, and 10 μL CCK-8 working solution was added to each well. The plate was then incubated at 37 °C for 1h, and the absorption was measured at 450 nm using a microplate reader (SpectraMax i3x). All data were statistically analyzed using One-way ANOVA (Prism, GraphPad Software).

Ethical approval and consent to participate

This is a non-clinical study.

Consent for publication

All authors consented this work to be published.

Availability of data and materials

No data were generated or analyzed in the presented research.

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Data availability statement

Data availability is not applicable to this article as no new data were created or analyzed in this study.

CRediT authorship contribution statement

Junxiong Zhang: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Zhongming Lai:** Supervision, Software. **Zhongmin Zhang:** Project administration, Funding acquisition. **Shuai Zheng:** Visualization, Validation, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

ABBREVIATIONS

AIE	aggregation-induced emission
PTB-EDTA	poly-tetraphenylthene-benzothiadiazole-ethylenediaminetetraacetic acid
WB	western blot
PCR	polymerase chain reaction
ACQ	aggregation-caused quenching
MVs	matrix vesicles
CLSM	confocal laser scanning microscope
PBS	phosphate buffered saline
FBS	Fetal bovine serum

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e31664.

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