Boron Accelerates Cultured Osteoblastic Cell Activity through Calcium Flux

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Abstract

A low concentration of boron (B) accelerates the proliferation and differentiation of mammalian osteoblasts. The aim of this study was to investigate the effects of 0.1 mM of B on the membrane function of osteoblastic cells *in vitro*. Genes involved in cell activity were investigated using gene expression microarray analyses. The Ca^{2+} influx and efflux were evaluated to demonstrate the activation of L-type Ca^{2+} channel for the Ca^{2+} influx, and that of Na^{+}/K^{+}-ATPase for the Ca^{2+} efflux. A real-time PCR analysis revealed that the mRNA expression of four mineralization-related genes was clearly increased after 3 days of culture with a B-supplemented culture medium. Using microarray analyses, five genes involved in cell proliferation and differentiation were up-regulated compared to the control group. Regarding the Ca^{2+} influx, in the nifedipine-pretreated group, the relative fluorescence intensity for one min after adding B solution did not increase compared with that for one min before addition. In the control group, the relative fluorescence intensity was significantly increased compared with the experimental group ($P<0.05$). Regarding the Ca^{2+} efflux, in the experimental group cultured in 0.1 mM of B-supplemented medium, the relative fluorescence intensity for 10-min after ouabain treatment revealed a significantly lower slope value compared with the control group ($P<0.01$). This is the first study to demonstrate the acceleration of Ca^{2+} flux by B supplementation in osteoblastic cells. Cell membrane stability is related to the mechanism by which a very low concentration of B promotes the proliferation and differentiation of mammalian osteoblastic cells *in vitro*.

Key words Boron · Odontoblast · Cell proliferetion · Differentiation · Ca^{2+} flux

Introduction

A previous report that boron (B) can improve bone mineralization in chicks with dietary deficiency [1] first demonstrated that B is beneficial and essential for higher animals. Analyses of both human and animal data suggested that an acceptable safe range of B intake for adults could be 1-13 mg/d [2]. Furthermore, B was identified as a nutritionally important element throughout the life cycle [3]. Dietary B may be beneficial for optimal calcium metabolism and, as a consequence, optimal bone metabolism [4, 5]. Boron supplementation in rats and chicks has been shown to increase bone strength, and may also play a role in improving arthritis, plasma lipid profiles, and brain function [6]. Moreover, dietary B deprivation in mice has been shown to alter periodontal alveolar bone modeling and remodeling by inhibiting bone formation [7].

Ca^{2+} signaling and homeostasis related to B supplementation or deprivation have been widely investigated in plants [8, 9] compared with mammals. The activity of Ca^{2+} channels, pumps and transporters is responsible for generating the influx and efflux of Ca^{2+}, which regulate its homeostasis. Cyclic nucleotide-gated ion channels, autoinhibited Ca^{2+}-ATPase and cation/H^+ exchanger proteins are involved in this process [10-12]. Furthermore, elevated intracellular Ca^{2+} has been reported to stimulate MAPK activity in several mammalian cell lines [13-15].

A very low concentration of B accelerates the proliferation and differentiation in mammalian osteoblastic cells *in vitro* [16, 17]. However, the mechanisms of these phenomena are still not well understood. The aim of this study was to investigate the effects of B on the membrane function of

osteoblastic cells *in vitro*. After the expression of calcification-related genes associated with Ca^{2+} metabolism in the hard tissue was analyzed using real-time PCR in osteoblastic cells under B supplementation, the genes involved in cell activity were investigated using gene expression microarray analyses. Finally, the Ca^{2+} influx and efflux were evaluated to demonstrate the activation of the L-type Ca^{2+} channel for the Ca^{2+} influx, and that of Na^{+}/K^{+}-ATPase for the Ca^{2+} efflux.

Materials and Methods

Cell Culture for Passage

Osteoblastic cells (NOS-1 cells [18]) derived from human osteosarcoma were used in the present study. They were seeded in a 60-mm culture dish at a density of 5×10^5 cells in α-MEM (Gibco, Palo Alto, CA, USA) supplemented with 10% fetal bovine serum (FBS) (MP Biomedicals, Santa Ana, CA, USA) and 0.01% penicillin-streptomycin (Life Technologies), and then were cultured in a humidified incubator at 37°C in an atmosphere of 5% CO_2 and air. The medium was changed every 3 days.

Preparation of Boron Solution

Boric acid was purchased from Wako (Osaka, Japan). A 100 mM solution of B was prepared by dissolving powdered boric acid in α-MEM without FBS.

The completely dissolved solution was filtered using a 0.2 μm filter as a stock solution.

Total RNA Isolation for Real-time PCR and Microarray

NOS-1cells were seeded in a 60-mm culture dish at a density of 7×10^4 cells/cm^2 in α-MEM without FBS. A 0.1 mM B was used for all experiments in this study, because our basic experiments indicated that a 0.1 mM of B was suitable for the proliferation of NOS-1 cells [19]. After 3 days of culture with or without a 0.1 mM of B, total RNA from cells were isolated using TRIzol reagent (1 mL/10cm^2) (Invitrogen, Carlsbad, CA, USA), and purified using the SV Total RNA Isolation System (Promega, Madison, WI, USA) according to the manufacturer's instructions.

Real-time PCR for Mineralization-related Genes

Real-time PCR was used to detect the expression of four mineralization-related genes, alkaline phosphatase (ALP), osteopontin (OPN), osteocalcin (OCN), and bone sialoprotein (BSP). cDNA synthesis and amplification via PCR were performed using the SuperScript™ First-Strand Synthesis System (Invitrogen) and an oligo dT primer. cDNA obtained from 400 ng of total RNA was used for one PCR. For each real-time PCR sample, a master mix of the following reaction components was prepared at the indicated quantities: 9.1 μl of water, 0.3 μl of diluted reference dye, 0.2 μl of forward and reverse

primers (Table 1) and 10 µl of Brilliant® SYBR® Green QPCR master mix (Stratagene, La Jolla, CA, USA). The above-mentioned reaction was placed in a 0.2 ml tube and 0.2 µl of cDNA was added as the PCR template. The PCR sequence protocol was as follows: denaturation (94°C, 1 min) followed by 40 cycles of amplification and quantification (56°C, 1 min) and a final extension (72°C, 1 min). Amplification and data acquisition were performed using an Mx3000P QPCR machine (Stratagene).

Quantification was accomplished by determining the threshold cycle (the second derivative of the resulting fluorescence curve) at which the amplicon is detected during PCR and then comparing this to the standard curve calculated from the parallel quantification reactions. These calculations were performed with the comparative quantification method using the Stratagene form. These data were normalized by glyceraldehyde phosphate dehydrogenase (GAPDH).

Gene Expression Microarray for Proliferation and Differentiation

The cRNA was amplified, labeled using GeneChip® WT Terminal Labeling and Control Kit (Affymetrix, Santa Clara, CA, USA), and hybridized to an Affinity Human Genome U133 Plus 2.0 array (for 47,000 transcriptional products) according to the manufacturer's instructions. All hybridized microarrays were scanned by an Affymetrix scanner. Relative hybridization intensities and background hybridization values were calculated using the Affymetrix Expression Console™.

Data Analysis and Filter Criteria

The raw signal intensities for each probe were calculated from hybridization intensities. The raw signal intensities of two samples were $\log_2$-transformed and normalized by RMA and quantile algorithm [20] with Affymetrix® Expression Console™ 1.1 software. To identify up-regulated genes, Z-scores [21] from ratios (non-log scaled original fold-change) were calculated from the normalized signal intensities of each probe for comparison between control and experiment samples. Then, we established the criterion for the up-regulated genes (Z-score > 2.0).

Real-time PCR after Microarray

The NOS-1 cells were seeded and cultured under the same conditions as those in the experiment for calcification-related genes. The protocols for RNA extraction, cDNA synthesis, and PCR were similar to the above-mentioned methods. Primer's sequences of the target genes were listed in Table 2.

Cell Culture for Ca^{2+} Flux Studies

In the Ca^{2+} influx study, the NOS-1 cells were cultured in a 35 mm glass-bottom culture dish (FluoroDish, World Precision Instruments, Sarasota, FL, USA) at a density of 3×10^5 cells in α-MEM without FBS and B for 3 days.

For the L-type Ca^{2+} channel blocker-applied group, nifedipine (final concentration of 10 μM) was added to α-MEM and cultured for 30 min before Fluo 4-AM loading. The control group was incubated in α-MEM containing solvent, ethanol at a final concentration of 0.05%. In the Ca^{2+} efflux study, the NOS-1 cells were cultured in a 35 mm glass-bottom culture dish (FluoroDish) at a density of 6×10^5 cells in α-MEM without FBS for 3 days. In the Na^+/K^+-ATPase-activated group, B solution at a final concentration of 0.1 mM was added to α-MEM. The control group was incubated in only α-MEM.

Fluo 4-AM Loading

The cells were loaded with Fluo 4-AM (DOJINDO LABORATORIES, Kumamoto, Japan) by incubation with a low sodium HEPES assay buffer (5 mM NaCl, 135 mM choline chloride, 10 mM HEPES, 5 mM KCl, 1 mM $MgCl_2$, 10 mM glucose), 0.5% Fluo 4-AM in DEMSO, 1.25 mM probenecid, and 0.04% Pluronic F-127 (BASF SE, Ludwigshafen, Germany) in a 5% CO_2 incubator for 15 min.

Confocal Laser Microscopy

In the Ca^{2+} influx experiment, Fluo 4-AM loaded cells in a 35 mm glass-bottom culture dish were covered with 990 μL of recording buffer (115 mM NaCl, 20 mM HEPES, 5.4 mM KCl, 0.8 mM $MgCl_2$, 1.8 mM $CaCl_2$, 13.8 mM

glucose) and 1.25 mM probenecid for fluorescence observation of Ca^{2+} influx using a confocal laser microscope (TCS SL, Leica Microsystems GmbH, Wetzlar, Germany). The approximate fluorescence excitation/emission maximum for imaging of intracellular Ca^{2+} was 495/518 nm. One min after setting on the microscopic stage, activation of the Ca^{2+} channel was investigated by adding 10 μL of B solution at a final concentration of 0.1 mM into the recording buffer. Fluorescence was scanned every 10 s and recorded on about 5-6 cells for a total of 1 min (n=3). The effect of B on the Ca^{2+} influx was examined by comparing the coefficient (slope) of a regression line calculated using the least-squares method in both the experimental and control groups. In the Ca^{2+} efflux experiment, Fluo 4-AM loading cells were incubated in a 5% CO_2 microscopic stage incubator (H301-TC1-HMTC, Okolab S.r.L., NA, Italy) for 15 min with recording buffer (low sodium HEPES assay buffer, 1.25 mM probenecid, and 0.5 mM ouabain) for fluorescence observation of the Ca^{2+} efflux using a confocal laser microscope. Fluorescence was scanned every 10 s and recorded on about 20 cells for a total of 10 min (n=3). The comparison between the experimental and control groups was similarly analyzed using the method described in the Ca^{2+} influx study.

Statistical Analysis

The data were expressed as the means $\pm$ standard deviation. The differences between the two groups were assessed using the one-tailed Student's *t*-test.

Significance was set at a level of $P<0.05$.

Results

Gene Expression for Mineralization

The real-time PCR analysis revealed that the mRNA expression of four mineralization-related genes, ALP, OPN, OCN, and BSP, was clearly increased after 3 days of culture with 0.1 mM of boron-supplemented culture medium, with average balanced score ratios of 7.1, 8.4, 1.7, and 1.6, respectively, compared with the control groups (Fig. 1).

Gene Expression for Proliferation and Differentiation

Using a microarray analysis, 119 of the up-regulated genes (Z-score > 2.0) were confirmed. Among them, five genes craniofacial development protein 1 (CFDP), stromal cell derived factor 4 (SDF4), mitogen-activated protein kinase kinase 1 (MAP2K1), catenin alpha 1 isoform (CTNNA1), and collagen type I alpha 1 (COL1A1), involved in osteoblastic cell proliferation and differentiation processes were furthermore compared with the control group using a real-time PCR (Table 3). Although 96 of the down-regulated genes (Z-scores<-2) were confirmed, they were not found to be related to the osteoblastic cell proliferation or differentiation processes.

The PCR analysis revealed that the mRNAs expression of CFDP, SDF4, MAP2K1, CTNNA1, and COL1A1 was clearly increased after 3 days of culture with 0.1 mM B, with average balanced score ratios of >1.3, compared with the control group (Fig. 2).

Acceleration of the Ca^{2+} flux

Regarding the Ca^{2+} influx, in the nifedipine-pretreated group, the relative fluorescence intensity for one min after the addition of 10 μL of B solution was not increased compared with that for one min before the addition of 10 μL of B solution, and the calculated slope was judged to be zero, including negative values. In the control group, the relative fluorescence intensity for one min after addition of 10 μL of B solution increased compared with that for one min before the addition of 10 μL of B solution, and the calculated slope was 3 ± 1.7 (Figs. 3 and 4). There was a significant difference between the experimental and control groups ($P<0.05$).

Regarding the Ca^{2+} efflux, in the experimental group cultured in 0.1 mM of B supplemented medium, the relative fluorescence intensity for 10-min after ouabain treatment revealed a significantly lower slope value (3.3 ± 0.6) compared with the control group (5.3 ± 0.6) ($P<0.01$) (Figs. 5 and 6).

Discussion

In the present study, NOS-1 cells accelerated the osteoblastic function after 3-day culture in 0.1 mM of B supplemented medium, which was demonstrated by the increased expression of calcification-related genes. Bone ALP is an early stage marker essential for osteoblast proliferation [22]. OPN, OCN, and BSP are frequently used as markers for osteoblast differentiation [22-24]. These scientific data indicated that the NOS-1 cell line preserves osteoblastic cell characteristics, and a low concentration of B directly stimulates both the proliferation and differentiation pathways in osteoblastic NOS-1 cells.

Microarray and real-time PCR analyses for detecting genes involved in proliferation and differentiation in NOS-1 cells revealed that the z-score data using standard deviations more clearly demonstrated the effect of B on NOS-1 cells compared with the original ratio data. Five genes, including one of the intracellular signal transducer genes (MAPK), demonstrated increased gene expressions. To the best of our knowledge, this finding is the first to indicate that B stimulates the intracellular signal pathway through MAPKs. As the intimate relationship between intracellular Ca^{2+} and MAPKs has been already reported in several cell lines [13-15], we evaluated the effect of B on the intracellular Ca^{2+} flux to determine the mechanism by which B activates osteoblasts in this study.

The L-type Ca^{2+} channel has reported to exist in the cell membrane of osteoblasts [25]. Osteoblast L-type Ca^{2+} channels are activated by numerous bone regulatory factors, including parathyroid hormone [26-28], vitamin D3 [27, 29, 30], mechanical stimuli [31, 32], ascorbic acid [33], and ALP [34]. Whether B accelerates the L-type Ca^{2+} channel in osteoblasts has not yet been

determined. This is the first report to identify the effect of B on the L-type Ca^{2+} channel in osteoblasts. For this purpose, nifedipine, a specific inhibitor of the L-type Ca channel, was used in this study. Nifedipine slows Ca^{2+} entry into cardiac and smooth muscle cells by blocking the L-type Ca^{2+} channel, thereby disrupting the excitation and contraction processes [35, 36]. Ca^{2+} channel blockers prevent Ca^{2+} influx, and promote osteoblastic differentiation through high membrane retaining ability [37]. In the present study, although B significantly promoted the Ca^{2+} influx in NOS-1 cells pre-treated without nifedipine, it did not accelerate the Ca^{2+} influx in NOS-1 cell pre-treated with nifedipine. These findings demonstrate that the L-type Ca^{2+} channel exists in the NOS-1 cell membrane, and a low concentration of B rapidly stimulates this channel. This B effect on osteoblastic activity most likely promotes cell membrane stability.

According to the effect of B on cell membrane activity, the Ca^{2+} efflux was evaluated as osteoblastic cells promote calcification. Ouabain (cardiac glycoside), a commonly used inhibitor of Na^{+}/K^{+}-ATPase, was used to investigate the effect of B. This enzyme catalyzes the coupled active transport of Na^{+} and K^{+} across the plasma membrane as a sodium pump [38, 39]. The effect of ouabain is due to the partial inhibition of Na^{+}/K^{+}-ATPase, which causes an increase in intracellular Na^{+}, which in turn affects the sarcolemmal Na^{+}/Ca^{2+} exchanger and leads to the increase in intracellular Ca^{2+} as the cause of positive inotropic action [40, 41]. Our study is also the first to identify the effect of B on Na^{+}/K^{+}-ATPase activity in osteoblasts. In the present study, Ca^{2+} fluorescence intensity in NOS-1 cells cultured with

0.1 mM B supplemented medium was significantly lower than that without B. B suppressed the effect of ouabain. These findings clearly demonstrate that NOS-1 cells contains Na^+/K^+-ATPase in their cell membranes, and B stimulates the activity of Na^+/K^+-ATPase.

Conclusion

To the best of our knowledge, this is the first report to demonstrate the acceleration of the Ca^{2+} influx and efflux by B supplementation in cultured osteoblastic cells. Moreover, the membrane stabilizing activity of B was addressed by our findings. Cell membrane stability may be related to the mechanism by which a very low concentration of B promotes the proliferation and differentiation in mammalian osteoblastic cells in vitro.

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Conflict of Interest: Author, Yoshihiko Hayashi has received research grants from the JSPS. The authors certify that there are no conflicts of interest to declare.

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Figure Captions

Fig. 1 Real-time PCR reactions for 4 mineralization-related genes, ALP, OPN, OCN, and BSP. Each bar represents the standard deviation of triplicate samples.

Fig. 2 Real-time PCR reactions for 5 genes related to proliferation and differentiation, CFDP1, SDF4, MAP2K1, CTNNA1, and COL1A1. Each bar represents the standard deviation of triplicate samples.

Fig. 3 A typical example of the Ca^{2+} influx after pretreatment with nifedipine in the experimental group. (a) Fluo 4-AM fluorescence from the cells, 1 min before B supplementation [red arrow in (d)]. (b) Fluorescence from the same cells, 0 min after B supplementation [yellow arrow in (d)]. (c) Fluorescence from the same cells, 1 min after B supplementation [green arrow in (d)]. Scale bar = 20 μm. (d) The curve of fluorescent signal intensity with time (calculated slope after B supplementation: 0).

Fig. 4 A typical example of the Ca^{2+} influx without pretreatment of nifedipine (control group). (a) Fluo 4-AM fluorescence from the cells, 1 min before B supplementation [red arrow in (d)]. (b) Fluorescence from the same cells 0 min after B supplementation [yellow arrow in (d)]. (c) Fluorescence from the same cells, 1 min after B supplementation [green arrow in (d)]. Scale bar = 20 μm. (d) The curve of fluorescent signal intensity with time (calculated slope after B supplementation: 2).

Fig. 5 A typical example of the Ca^{2+} efflux after culture with B in the experimental group. (a) Fluo 4-AM fluorescence from the cells, 0 min after ouabain pretreatment. (b) Fluorescence from same cells after 10 min. Scale

bar = 20 μm. (c) The curve of fluorescent signal intensity with time (calculated slope for 10 min: 3).

Fig. 6 A typical example of the Ca^{2+} efflux after culture without B in the control group. (a) Fluo 4-AM fluorescence from the cells, 0 min after ouabain pretreatment. (b) Fluorescence from the same cells after 10 min. Scale bar = 20 μm. (c) The curve of fluorescent signal intensity with time (calculated slope for 10 min: 5).

Table 1

Gene name		Oligonucleotides
Alkaline phosphatase (ALP)	Forward	CAACCCTGGGGAGGAGAC
	Reverse	GCATTGGTGTTGTACGTCTTG
Osteopontin (OSP)	Forward	TCACCTGTGCCATACCAGTTAA
	Reverse	TGAGATGGGTCAGGGTTTAGC
Osteocalcin (OCN)	Forward	GCAAAGGTGCAGCCTTTGTG
	Reverse	GGCTCCCAGCCATTGATACAG
Bone sialoprotein (BSP)	Forward	TTCCAGTTCAGGGCAGTAGTG
	Reverse	CCATAGCCCAGTGTTGTAGCA
GAPDH	Forward	TCAGCAATGCCTCCTGCAC
	Reverse	TCTGGGTGGCAGTGATGGC

Table 2

Gene name		Oligonucleotides
Craniofacial development protein 1 (CFDP1)	Forward	GCTTCCTCAATGATGTGG
	Reverse	CTTCAGTCTCCTCTCCTTT
Stromal cell derived factor 4 (SDF4)	Forward	GGAGAGCAAGACACACTT
	Reverse	TTCACCTTATACTCGTCCC
Mitogen-activated protein kinase kinase 1 (MAP2K1)	Forward	GGATACTTAGTGGTATGTCTCTT
	Reverse	ACAGCATTGGTACTTGGATA
Catenin alpha 1 isoform (CTNNA1)	Forward	GTTAGACTGCCGATTCTG
	Reverse	TGTTAAATCAGCACTTCAT
Collagen type 1 alpha 1 (COL1A1)	Forward	GCTGTCTTATGGCTATGATGAG
	Reverse	CACGAGGACCAGAGGGAC
GAPDH	Forward	CCATGGAGAAGGCTGGGG
	Reverse	CAAAGTTGTCATGGATGACC

Table 3

Gene name	Z score	Ratio[a]	Confirmation[b]
Craniofacial development protein 1 (CFDP1)	2.5	1.4	2.1
Stromal cell derived factor 4 (SDF4)	3.5	1.3	1.5
Mitogen-activated protein kinase kinase 1 (MAP2K1)	2.7	1.1	1.9
Catenin alpha 1 isoform (CTNNA1)	2.1	1.1	1.8
Collagen type 1 alpha 1 (COL1A1)	3.6	1.1	1.3

[a] The fold-change in the raw signal intensities

[b] Confirmed by the quantitative polymerase chain reaction data (data from triplicate samples)

Fig. 1

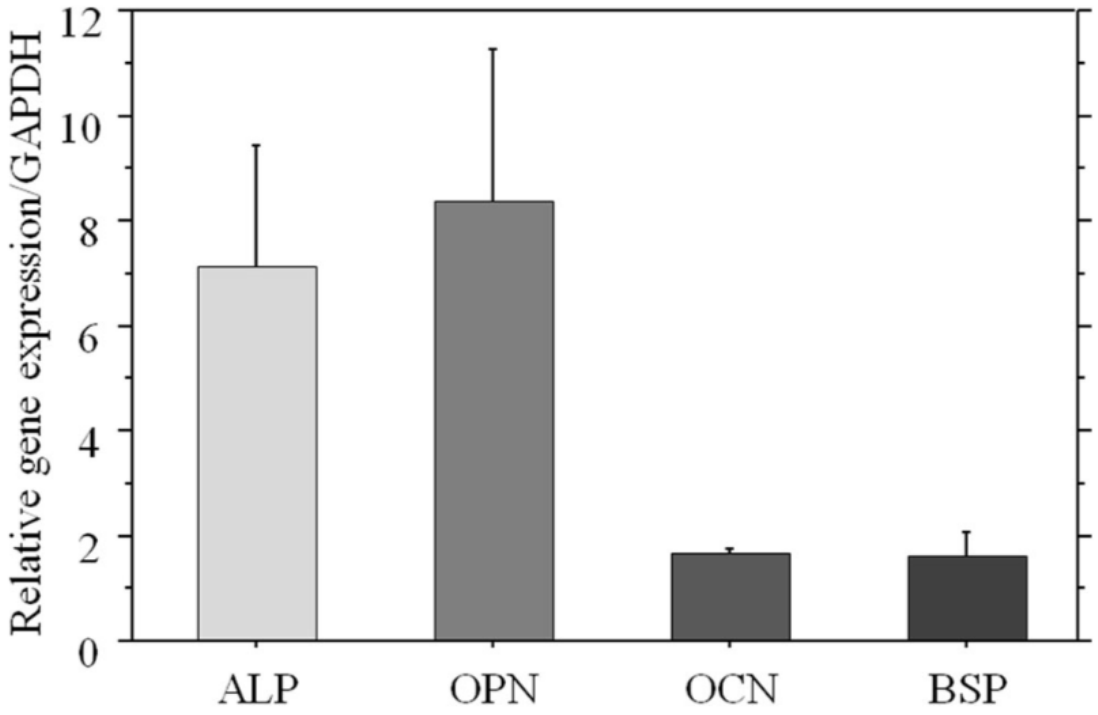

Relative gene expression/GAPDH
12
10
8
6
4
2
0
ALP
OPN
OCN
BSP

Fig. 2

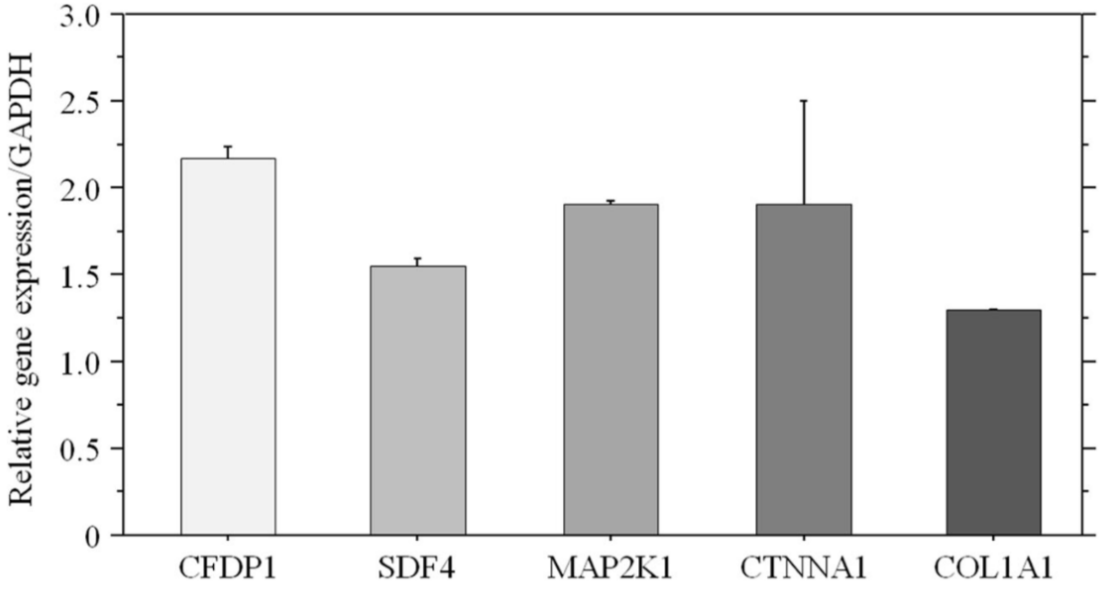

Relative gene expression/GAPDH
3.0
2.5
2.0
1.5
1.0
0.5
0
CFDP1
SDF4
MAP2K1
CTNNA1
COL1A1

Fig. 3

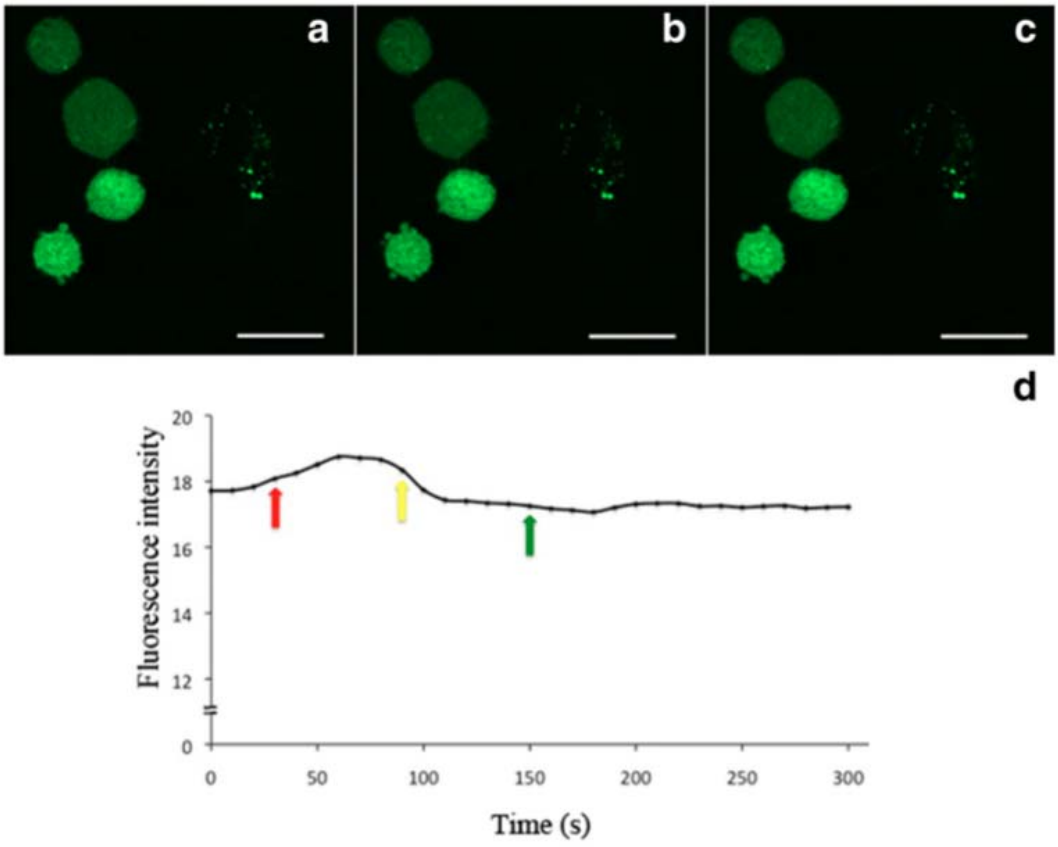

a
b
c
d
Fluorescence intensity
20
18
16
14
12
0
0
50
100
150
200
250
300
Time (s)

Fig. 4

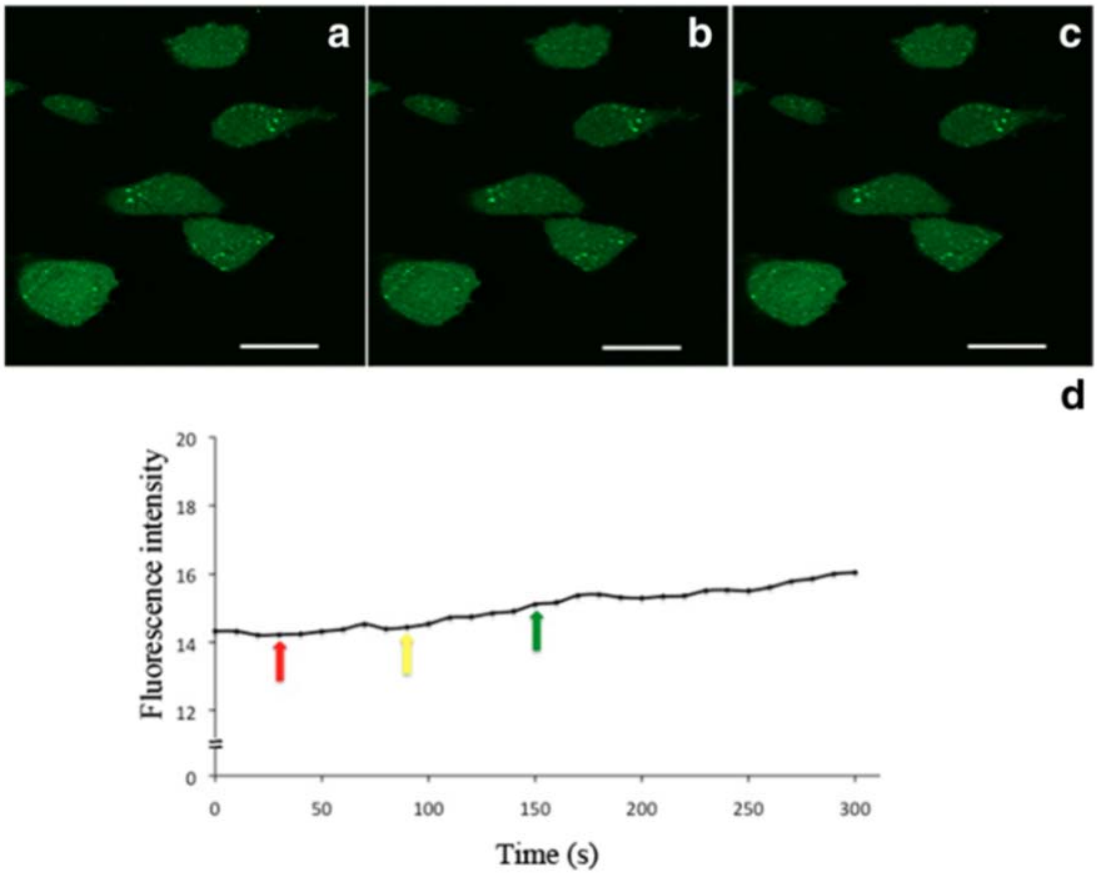

a
b
c
d
Fluorescence intensity
20
18
16
14
12
0
0
50
100
150
200
250
300
Time (s)

Fig. 5

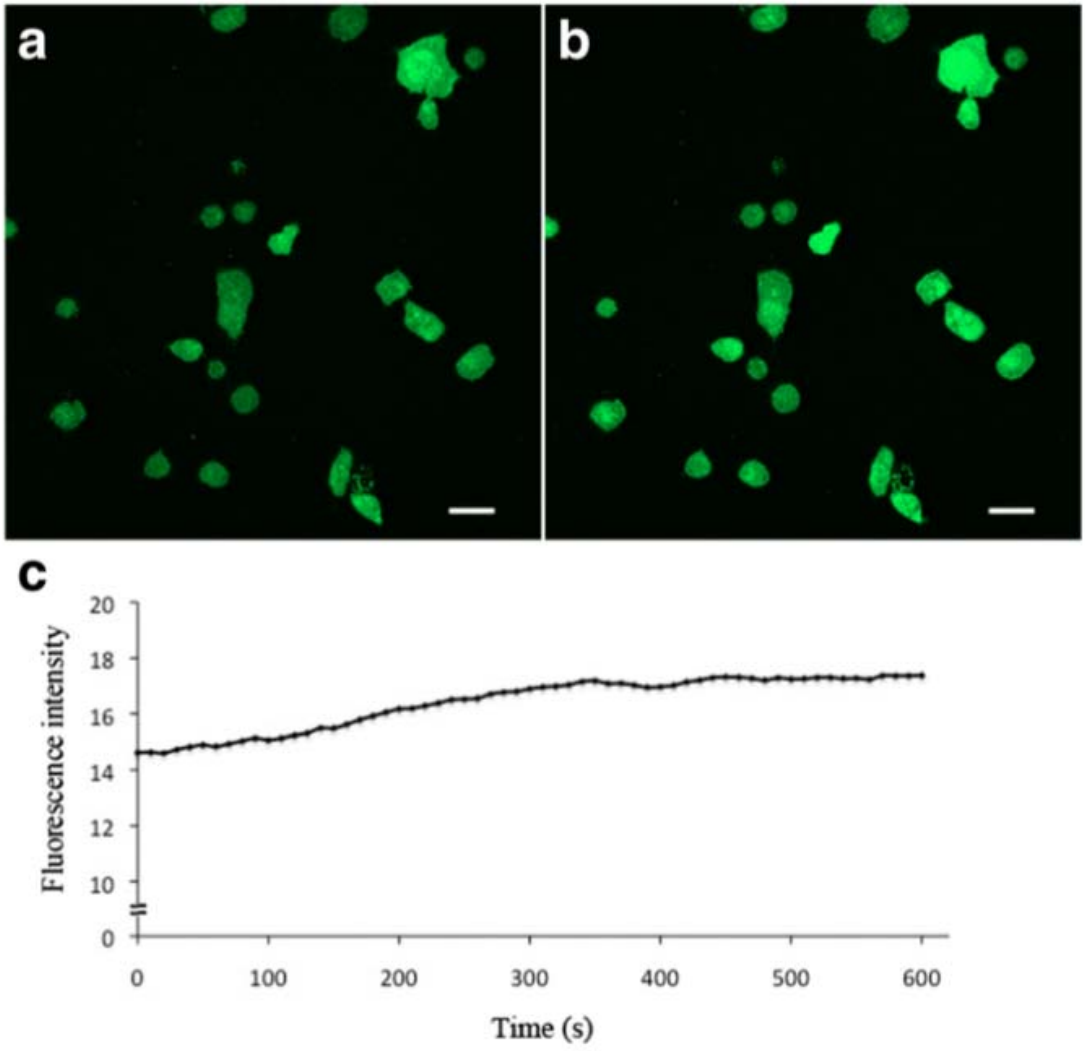
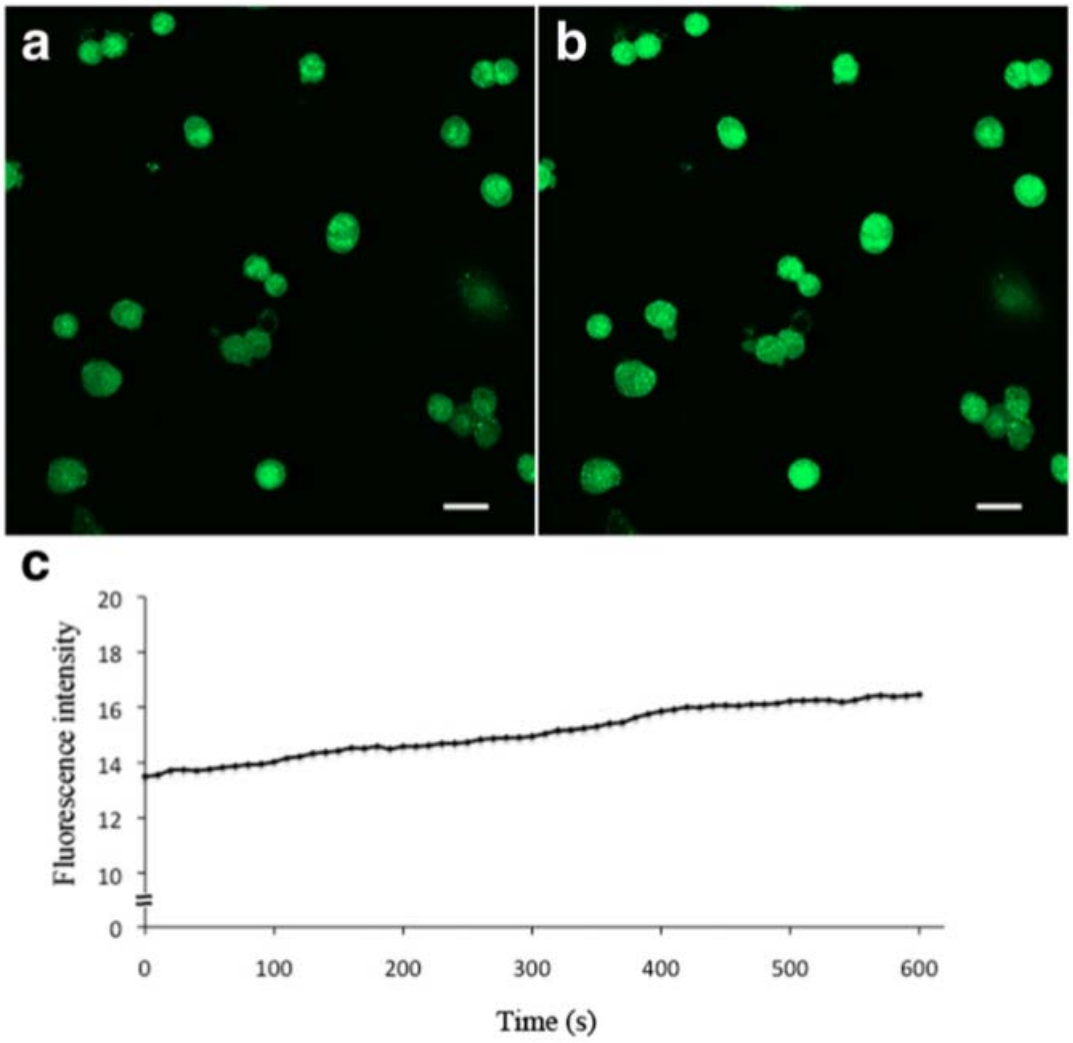

a
b
c
Fluorescence intensity
20
18
16
14
12
10
0
0
100
200
300
400
500
600
Time (s)

Fig. 6


a
b
c
Fluorescence intensity
20
18
16
14
12
10
0
0
100
200
300
400
500
600
Time (s)