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One-day Treatment of Small Molecule 8-Bromo-cyclic AMP Analogue Induces Cell-based VEGF production for *In Vitro* Angiogenesis and Osteoblastic Differentiation

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Abstract

Small molecule based regenerative engineering is emerging as a promising strategy for regenerating bone tissue. Small molecule cAMP analogues have been proposed as novel biofactors for bone repair and regeneration, and while promising, the effect that these small molecules have on angiogenesis, a critical requirement for successful bone regeneration, is still unclear. Our previous research demonstrated that the small molecule cAMP analogue 8-bromoadenosine-3',5'-cyclic monophosphate (8-Br-cAMP) was able to promote initial osteoblast adhesion on a polymeric scaffold via cAMP signaling cascades. Here, we report that 8-Br-cAMP is capable of inducing *in vitro* cell-based VEGF production for angiogenesis promotion. We first demonstrated that treating osteoblast-like MC3T3-E1 cells with 8-Br-cAMP for one day significantly increased VEGF production and secretion. We then demonstrated that 8-Br-cAMP induced cell-secreted VEGF is biologically active and may promote angiogenesis as evidenced by increased endothelial cells (HUVECs) migration and tubule formation. In addition, treatment of MC3T3-E1 cells with 8-Br-cAMP for as short as a single day resulted in enhanced ALP activity as well as matrix mineralization, demonstrating *in vitro* osteoblastic differentiation. A short term 8-Br-cAMP treatment also addresses the concern of non-specific cytotoxicity, as our data indicate that a one-

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day 8-Br-cAMP treatment scheme supports cellular proliferation of MC3T3-E1 cells as well as HUVECs. While the major concern associated with small molecule drugs is the risk of non-specific cytotoxicity, the short exposure treatment outlined in this paper provides a very promising strategy to mitigate the risk associated with small molecules.

Keywords

angiogenesis; osteoblastic differentiation; regenerative engineering; bone regeneration; small molecules; cAMP; morphogenesis

Introduction

Regenerative engineering technologies using bioactive molecules, stem cells, and advanced biomaterials have been proposed as promising approaches for bone repair and regeneration (Laurencin and Khan, 2012; Ozdemir et al., 2013). Foremost, the delivery and evaluation of a variety of bioactive molecules have been widely researched (Cui et al., 2013). One such bioactive molecule, bone morphogenetic proteins (BMPs), have shown great potential for use in bone regeneration and repair (Bessa et al., 2008; Lo et al., 2012d). While these proteinaceous growth factors have been shown to facilitate bone formation within tissue engineered scaffolds, the resulting neotissue is often found only at the surface of the scaffold and not within the scaffold interior due to poor vascularization and limited oxygen supply (Kim et al., 2012; Tischer et al., 2010). To enhance vascularization, recombinant angiogenic factors such as vascular endothelial growth factor (VEGF) have been extensively researched as cofactors to be delivered in scaffolds (Chiu et al., 2011; Cui et al., 2013; Ennett et al., 2006). It should be noted that while the identification and production of recombinant protein growth factors that play key roles in bone repair and regeneration have generated much enthusiasm and numerous clinical trials, the outcomes of many of these clinical trials have been disappointing (Lee et al., 2010; Lo et al., 2012d). In general, the ability to regenerate functional tissues from recombinant protein growth factors have been hindered by a number of limitations, including high manufacturing cost, contamination, and unwanted immune responses (Lo et al., 2012a; Lo et al., 2011a). Therefore, an alternative form of biofactors is needed to diminish these drawbacks. Small molecules that carry the capacity to induce bone regeneration have recently gained more spotlight in the field because they possess intrinsic physical properties (e.g. non-immuogenic, chemically stable) that allow them to overcome the issues observed with recombinant protein growth factors (Doorn et al., 2013; Egusa et al., 2010; Lo et al., 2012a; Lo et al., 2011a; Masuya and Teno, 2010; Sefcik et al., 2008; Segar et al., 2013). We have previously demonstrated that the small molecule 6-Bnz-cAMP, a target-specific cAMP-analog that interacts via the protein kinase A (PKA) signaling pathway, was able to induce initial cell adhesion, differentiation, and mineralization of early passage osteoblast-like MC3T3-E1 cells on biodegradable polymeric scaffolds (Lo et al., 2012b; Lo et al., 2011b). Similarly, it has been shown that other cAMP analogues have effects on regulating osteoblastic differentiation and mineralization *in vitro* and *in vivo* (Doorn et al., 2012a; Siddappa et al., 2008). cAMP is found ubiquitously in mammalian cells and acts as a common secondary messenger controlling diverse cellular processes including cell differentiation and morphogenesis (Beavo and Brunton, 2002). For instance,

in mouse and chick limb buds, a transient increase in intracellular cAMP levels during the onset of chondrogenesis has been observed (Ho et al., 1982; Solursh et al., 1979), suggesting that cAMP plays an important role in regulating this process. Similarly, dibutyryl cAMP analogue has been shown to enhance cartilage differentiation in the limb-bud mesoderm in both cell and organ cultures (Kosher and Savage, 1980), suggesting that cAMP plays a crucial role in complex tissue regeneration.

Although the effect of cAMP analogues on angiogenesis is unclear, cAMP mediated signaling pathways have been implicated in angiogenesis regulation through increased VEGF expression (Namkoong et al., 2009). This observation has prompted us to test whether various cAMP analogues can induce *in vitro* angiogenesis by increasing VEGF production. In this report, we investigated the effects of various cAMP analogues (8-Br-cAMP, 6-Bnz-cAMP, and 8-CPT-2Me-cAMP) on VEGF production using MC3T3-E1 osteoblast-like cells. As illustrated in figure 1A, 8-Br-cAMP is an activator of PKA and the exchange protein activated by cyclic AMP (Epac) whereas 6-Bnz-cAMP and 8-CPT-2Me-cAMP exclusively target PKA and Epac, respectively. These cAMP analogues have been well established as tools for studying various cAMP mediated signal transduction in a wide range of cellular processes (Christensen et al., 2003; Lo et al., 2011b; Schwede et al., 2000). The hypothesis of our investigation was that the 8-Br-cAMP small molecule plays a highly important role in bone regeneration for bone regenerative engineering.

Methods

Reagents

N^6-benzoyladenosine-3’,5’-cyclic monophosphate (6-Bnz-cAMP), 8-bromoadenosine-3’,5’-cyclic monophosphate (8-Br-cAMP), and 8-(4-Chlorophenylthio)-2’-O-methyladenosine-3’, 5’-cyclic monophosphate (8-CPT-2Me-cAMP) were purchased from Alexis Biochemicals (San Diego, CA); Fibronectin was purchased from Gibco (Grand Island, NY).

Cell Culture

All cells were maintained at 37°C in a 5% CO_2-humidified incubator. MC3T3-E1 osteoblast-like cells (American Type Culture Collection, Manassas, VA) (passage number 21 to 30) were used to study VEGF synthesis in response to cAMP treatment and *in vitro* osteoblastic differentiation and proliferation. The cells were maintained in regular growth medium containing alpha minimal essential medium (α–MEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin G, and 100 mg/mL streptomycin. Cells cultured in osteogenic medium (alpha minimal essential medium supplemented with 10% FBS, 1% of antibiotic, 3 mM β-glycerolphosphate, and 10 μg/ml ascorbic acid) served as a positive control for osteoblastic differentiation. HUVECs, purchased from Lonza (Basel, Switzerland) (passage number 3 to 8) were used for *in vitro* angiogenesis studies. Cells were grown in endothelial cell growth medium 2 (EGM2) (Lonza, Basel, Switzerland) supplemented with 10% FBS and 1% of antibiotic (100U/ml penicillin G and 100mg/ml streptomycin).

Cell Proliferation Assay

Cell proliferation studies were performed using a non-radioactive cell proliferation assay kit (MTS) (Promega, Madison, WI) which was performed according to the manufacturer's instructions. Cells were collected at days 1, 3, and 7 for the proliferation assays. For one-day treatment, 100 μM of 8-Br-cAMP was added to the medium at the time of cell seeding. 100 μM of 8-Br-cAMP was found to be an optimal concentration to induce MC3T3-E1 cell adhesion (Lo et al., 2011b). About 24 h post-seeding, the medium was replaced with fresh medium without 8-Br-cAMP supplementation. For continuous 8-Br-cAMP treatment, 100 μM of 8-Br-cAMP was added to the medium at the time of cell seeding. The medium supplemented with 8-Br-cAMP was replaced every 3 to 4 days.

Cell Viability Assay

Viabilities of MC3T3-E1 and HUVEC cells on day 1, 3 and 7 with or without one-day 8Br-cAMP treatment were measured by the live/dead viability kit (Life Technologies, Grand Island, NY). Cells were stained with calcein AM for live cells (green) and ethidium homodimer for dead cells (red). The stained cells were then counted using confocal microscopy (Zeiss, USA). Experiments were performed in triplicate. Three regions were randomly selected and analyzed for each experiment.

Enzyme-Linked Immunosorbent Assay (ELISA)

A mouse Quantikine intact VEGF ELISA kit (R&D Systems, Minneapolis, MN) was used to quantify cell-secreted VEGF according to the manufacturer's instructions. The assay product solution was visualized using a Biotek Synergy™ HT microplate reader (Winooski, VT) at a wavelength of 450 nm. The absorbance was converted to VEGF concentration with the use of a kit provided mouse VEGF standard (0 – 500 pg/mL).

Conditioned media preparation

Conditioned media were prepared because we wanted to investigate the trophic effect of the cAMP treated cells. Briefly, osteoblast-like MC3T3-E1 cells (1 million) were plated in 10-cm tissue culture plates in regular growth medium with or without 100 μM 8-Br-cAMP supplement. After 1 day of incubation, media were collected and directly applied to HUVECs for *in vitro* angiogenesis analysis. Medium collected from the 8-Br-cAMP treated cells was designated as "*8-Br-cAMP conditioned medium*" and medium collected from the untreated control cells was designated as "*Control conditioned medium*".

Real-time reverse transcription PCR analysis

HUVEC were plated and grown to 90% confluence in 10 cm^2 plate. They were then treated with corresponding conditioned media for 3 hours. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was used to assess the angiogenesis markers Vegfa, Icam1, and Vcam1 gene expressions. Total RNA was isolated using the RNeasy Mini System (Qiagen, Hilden, Germany) according to the manufacturer's instructions. 1μg total RNA was used as a template for RNA to cDNAEcoDry Premix System (Clontech) to synthesis complementary DNA (cDNA). A Light cycler instrument (Bio-Rad iCycler iQ system) was used to perform real time PCR using SuperMix Premix (Biorad) and Taqman

Gene Expression Assays (Applied Biosystems, Guilford, CT). ΔΔCt method is used to calculate the relative fold expression of the genes of interest by normalized to a housekeeping gene, GAPDH, and untreated control.

In vitro capillary network formation assay

Matrigel (270 μL) (BD Biosciences, San Jose, CA) was added to each well of a 24-well culture plate according to the manufacturer's instruction. After the gel solidified, 40,000 HUVECs in 1 ml of corresponding medium were added to the well. The cells were incubated at 37 °C and 5% CO_2 for 10-12 h (He et al., 2013). The formation of the capillary/tube-like networks were examined using a light microscope (Olympus, USA). Five random fields for each sample were taken at 4X magnification. ImageJ software (NIH) was used to quantify the total length of capillary tubes. A tubule was defined as a capillary structure with a length at least 4 times the width of individual well-separated cells (Chen et al., 2004; Wieghaus et al., 2006). Assays were performed in triplicate.

Endothelial cell transwell migration assay

The migration activity of HUVEC was assayed using a Boyden Chamber assay method. 50,000 HUVEC cells in serum-free starvation EBM-2 medium were placed into 24-well sized Transwell (Corning, New York, NY) which were pre-coated with 10 μg/ml fibronectin. Corresponding conditioned media were added to the lower chamber. The plate was incubated at 37 °C with 5% CO_2 for 10-12 h. The cells that remained on the upper surface of the filter membrane were rinsed with PBS and removed by cotton swab. The migrated cells that located on the lower surface of the filter membrane were fixed in 100% cold methanol for 15 min. The membranes were then cut off, stained with 50 μg/ml DAPI (Sigma-Aldrich, St Louis, MO) for 10 min, and mounted to cover slides. The migrated cells were quantified under a confocal microscope (Zeiss, USA). Assays were performed in triplicate.

Alkaline phosphatase activity (ALP) assay

To investigate the osteogenic effect of one-day treatment of 8-Br-cAMP on osteoblastic differentiation, we first measured the ALP activity since this is one of the most important early markers for osteoblastic differentiation (Katagiri et al., 1994). MC3T3-E1 Cells were cultured with regular growth medium with either short or continuous 8-Br-cAMP treatments at a concentration of 100 μM. ALP activity was measured at 7 days in culture. ALP activity was measured using an alkaline phosphatase substrate assay kit according to the manufacturers' instructions (Bio-Rad, Hercules, CA). For one-day 8-Br-cAMP treatment, 100 μM 8-Br-cAMP was added to the medium at the time of cell seeding. About 24 h after incubation, the medium was replaced with fresh medium without 8-Br-cAMP supplement. For continuous 8-Br-cAMP treatment, 100 μM 8-Br-cAMP was added to the medium at the time of cell seeding. The medium supplemented with 8-Br-cAMP was replaced every 3 to 4 days. At day 7, media were removed and cells were washed 3-4 times with PBS. The washed cells were lysed with 1% Triton X-100 at room temperature for 5 min. ALP substrate solution was added to the cell lysate at 37 °C for 1-2 h. The assay product solution was visualized using a Biotek Synergy™ HT microplate reader (Winooski, VT) at a wavelength of 405 nm. The absorbances were normalized to cellular DNA. Cellular DNA

measurements were performed using PicoGreen dsDNA assay kit (Molecular Probes, Eugene, OR) according to the manufacturers' procedures.

Matrix mineralization assay

After day 21 of culture in osteogenic medium, the mineralized matrix of the cultured osteoblasts was quantified by colorimetric determination of total calcium deposited in the matrix (Calcium Liquicolor, Stanbio Laboratory, Boerne, TX). This procedure was performed as previously described (Lo et al., 2012c).

Statistical Analysis

Statistical analysis was performed on samples using the Student's t-test (Microsoft Excel). The experiments were performed in at least triplicate and the level of significance was set at $p < 0.05$. An asterisk (*) in the figures denotes significance between groups.

Results

Effect of various cAMP analogues on VEGF synthesis in osteoblast-like MC3T3-E1 cells

To investigate the role of cAMP analogues on VEGF production in MC3T3-E1 cells, we employed several different cAMP analogues (figure 1A). As shown in figure 1B, the ELISA data revealed that 100 μM 8-Br-cAMP significantly promoted VEGF secretion by MC3T3-E1 cells after one day of incubation when compared to untreated control cells. There were no statistically significant differences in 6-Bnz-cAMP and 8-CPT-2Me-cAMP treated cells compared to the untreated control cells.

Effect of conditioned media on the angiogenic behavior of endothelial cells in vitro

Since VEGF has been demonstrated to have angiogenic effects on endothelial cells, the angiogenic activities of the secreted VEGF in conditioned media was firstly investigated by examining its influence on various angiogenic markers expression in endothelial cells. We examined the effects of conditioned media on Vascular Endothelial Growth Factor A (VEGF-A), Intercellular Adhesion Molecule 1 (ICAM-1), and Vascular Cell Adhesion Protein 1 (VCAM-1) mRNA levels in HUVECs that had been cultured in conditioned media for 3h. Figure 1C reveals that HUVECs cultured in 8-Br-cAMP conditioned medium resulted in a significant increase in VEGF-A, ICAM-1, and VCAM-1 mRNA levels.

During angiogenesis, endothelial cell migration plays an essential role in vascular budding (Lamalice et al., 2007; Osusky et al., 2004). To test the effects of the conditioned media on endothelial cell migration, conditioned media from treated or untreated MC3T3-E1 cells was directly added to HUVECs in Boyden Chambers and endothelial cell migration activity was analyzed. Figure 2A revealed that increased cell migration was observed when HUVECs were supplemented with 8-Br-cAMP exposed MC3T3-E1 cell conditioned medium. In contrast, conditioned medium from control untreated cells lacked this activity (Figure 2B). The numbers of migrated cells were quantitated in each experimental group (Figure 2C).

It is generally believed that the ability of endothelial cells to form capillary tube network is an important prerequisite for subsequent angiogenesis (Folkman and Haudenschild, 1980;

Osusky et al., 2004). To test the ability of secreted VEGF to induce capillary tube formation, conditioned media from osteoblast-like MC3T3-E1 cells was directly added to HUVECs and capillary network formation was determined. An endothelial tube was defined as a multicellular structure with a length at least 4 times its width (Sun et al., 2004; Wieghaus et al., 2006). HUVECs were cultured in Matrigel with conditioned media for 10-12 h. Figure 3A revealed that HUVECs supplemented with 8-Br-cAMP conditioned medium displayed more capillary tube like structures than the control sample as shown in Figure 3B. These observations were quantified using ImageJ software (NIH) to determine the length of the tube-like structures (Figure 3C). Taken together, the observations from Figures 2 and 3 indicated that the 8-Br-cAMP conditioned medium significantly enhanced migration and capillary formation *in vitro*.

Effect of one-day treatment of 8-Br-cAMP on osteoblastic differentiation

We next tested the effect of the one-day 8-Br-cAMP treatment on osteogenic differentiation of MC3T3-E1 cells. As shown in Figure 4A, one-day 8-Br-cAMP treatment showed significant increases in ALP activities when compared to the untreated control group at day 7. These observations indicated that treatment of cells with 8-Br-cAMP for one day was able to induce osteoblastic differentiation *in vitro*. Matrix mineralization is a late stage marker of osteoblastic differentiation. As shown in figure 4B, MC3T3-E1 cells exposed to one-day 8-Br-cAMP treatment scheme exhibited a significant increase in calcium level as compared to cells cultured in osteogenic medium alone.

Effect of small molecule 8-Br-cAMP on cellular proliferation and cell viability

The effects of 8-Br-cAMP treatment on MC3T3-E1 and HUVEC proliferation were assessed next. Both MC3T3-E1 and HUVECs were cultured in regular growth medium with either one-day or continuous 8-Br-cAMP treatments at a concentration of 100 μM. Cellular proliferation was evaluated over a period of 7 days of culture using a cell proliferation assay kit (MTS assay kit). The MTS assay is a routinely used method for accessing *in vitro* cytotoxicity of a chemical (Malich et al., 1997). Untreated cells were used as a control. The absorbance in the figure serves as a surrogate for the number of living cells in culture because a linear correlation between the absorbance (490 nm) and cell number was observed (data not shown). As shown in Figures 5A and 5B, there were no significant differences in cellular proliferation at day 1 among cells treated with 8-Br-cAMP when compared to the untreated control group for both MC3T3-E1 cells and HUVECs. These observations indicated that cell viability was maintained in 8-Br-cAMP treated cells during the early proliferative phase of both cell types. This data also suggested that one-day 8-Br-cAMP treatment does not induce direct cytotoxicity to the MC3T3-E1 cells and HUVECs at the concentration used in this study. At day 3, one-day 8-Br-cAMP did not significantly change the proliferation in MC3T3-E1 cells and HUVECs. However, continuous 8-Br-cAMP treatment significantly inhibited the proliferation of MC3T3-E1 cells when compared to the untreated control cells. At day 7, there was a significant decrease in cell proliferation for both cell types when treated with 8-Br-cAMP continuously. These results were consistent with the literature that has shown the anti-proliferative effects of treating cells with 8-Br-cAMP (Doorn et al., 2012b; Lamb and Steinberg, 2002; Southgate and Newby, 1990; Starzec et al., 1994). Interestingly, there were no statistically significant differences in

cellular proliferation between one-day 8-Br-cAMP treated cells and untreated control cells during the entire period of assay. This data indicates that one-day 8-Br-cAMP treatment is much less cytotoxic than continuous 8-Br-cAMP treatment. To confirm that the low cell cytotoxicity for the one-day 8Br-cAMP treatment scheme, we performed cell viability assay to access the viability of cells for the one-day 8-Br-cAMP treatment. Figure 5C and D demonstrate that both 8-Br-cAMP treated and untreated control groups maintained >80% of the cell viability throughout the entire study period. In addition, there were no significant differences in cell viability among the cells treated with 8-Br-cAMP for 1 day when compared to the untreated control group at any time point. Taken together, these observations demonstrated that one-day 8-Br-cAMP treatment does not induce cell cytotoxicity in either MC3T3-E1 or HUVEC cells.

Discussion

Small molecules biofactors are low molecular weight organic compounds with medicinal properties. Due to characteristics inherent to small molecules, small molecule mediated-bone regeneration is emerging as a promising strategy since it minimizes the limitations observed in protein-based therapeutics (Egusa et al., 2010; Lo et al., 2012a; Lo et al., 2011a; Sefcik et al., 2008). However, the major concern associated with small molecule therapeutics is their non-specific side effects (Lo et al., 2012a; Lo et al., 2011a). Due to their small molecular size, small molecules can easily penetrate through the cell membrane in non-target cells and perturb a number of metabolic and signaling pathways, eliciting unwanted physiological responses, i.e. side effects (Brouwers et al., 2011). Minimizing side effects from small molecules by reducing the frequency of their administration is a promising alternative strategy (Lo et al., 2013).

A number of small molecules have been shown to induce bone regeneration *in vitro* and *in vivo* (Egusa et al., 2010; Lo et al., 2012a), therefore, we chose to focus our study on the small molecule 8-Br-cAMP analogue because our present study demonstrates that medium collected from one-day 8-Br-cAMP treatment enhanced HUVECs' migration and capillary formation, both of which are relevant events for *in vitro* angiogenesis. In addition, treatment of osteoblast-like MC3T3-E1 cells with 8-Br-cAMP for as short as one-day resulted in significant osteoblastic differentiation, shown by the enhanced ALP activity and matrix mineralization. To our knowledge, this is the first observation that a single dose cAMP analogue exposure for one day can lead to enhanced endothelial cell migration and capillary formation as well as osteoblastic differentiation. More importantly, the cell proliferation data indicated that a relatively short oneday 8-Br-cAMP treatment did not interfere with proliferation, whereas continuous 8-Br-cAMP treatment (3 or 7 days) significantly inhibited cellular proliferation (Figure 4). These findings may also provide a broader strategy for how to mitigate cytotoxicity risks associated with small molecules. One methodology for implementing transient small molecular exposure for tissue engineering would be to utilize relatively short *ex vivo* treatments of co-cultured osteoprogenitor cells and endothelial cells seeded on an osteoinductive scaffold with 8-Br-cAMP, followed by surgical implantation. While the results are promising, the activity of 8-Br-cAMP reported here was studied in the *in vitro* mouse cell model. Prior to preclinical animal studies, further *in vitro* cell study is

needed to investigate the differing in physiological effect of 8-Br-cAMP on mice and humans in terms of angiogenic activity, osteogenic activity, dosages, and treatment schemes.

The data presented provides a clear connection between cAMP signaling and VEGF expression in osteoblast-like MC3T3-E1 cells. Given that we and others have previously shown that 8-Br-cAMP triggers both PKA- and Epac-signaling cascades (Lo et al., 2011b; Rangarajan et al., 2003), our results also suggest that both PKA- and Epac-signaling cascades are associated with the extracellular production of VEGF in osteoblast-like MC3T3-E1 cells. This hypothesis was further supported by the fact that neither Epac specific activation (8-CPT-2Me-cAMP) nor PKA specific activation (6-Bnz-cAMP) significantly enhanced VEGF expression (Figure 1B). Consistent with our observations, Namkoong et al. demonstrated that forskolin, a cAMP rising agent, induced VEGF expression via the coordinated cross-talk between Epac- and PKA-mediated signaling in endothelial cells (Namkoong et al., 2009). The data also indicates that short-term treatment of MC3T3-E1 cells with 8-Br-cAMP triggered osteoblastic differentiation as demonstrated by enhanced ALP activity as well as matrix mineralization. It is worth noting that integrin-dependent cell adhesion is responsible for osteoblast function since various integrin subunits have been shown to trigger different signaling which result in activation of distinct downstream signaling pathways (Giancotti and Ruoslahti, 1999; Lo et al., 2012c). Consistent with these observations, we have previously demonstrated that the small molecule 8-Br-cAMP can promote osteoblast cell adhesion, which is mediated by integrin subunits (Lo et al., 2011b). Taken together, these observations imply that the osteoblastic differentiation of MC3T3-E1 cells observed in this study may be partially due to the increased integrin-dependent cell adhesion induced by 8-Br-cAMP. Nevertheless, future experiments are needed to investigate the detailed molecular mechanisms underlying the osteogenic effects from one-day treatment of 8-Br-cAMP.

In this study, it was shown that (1) one-day 8-Br-cAMP treatment does not induce cell cytotoxicity, (2) one-day treatment of 8-Br-cAMP is adequate to induce differentiation of osteoblast-like MC3T3-E1 cells, and (3) medium collected from the one-day 8-Br-cAMP treated cells is adequate to promote migration and capillary formation of HUVECs due to the presence of VEGF. The data presented here provide a necessary prerequisite for the use of the small molecule 8-Br-cAMP for future *in vitro* and *in vivo* bone regenerative engineering studies.

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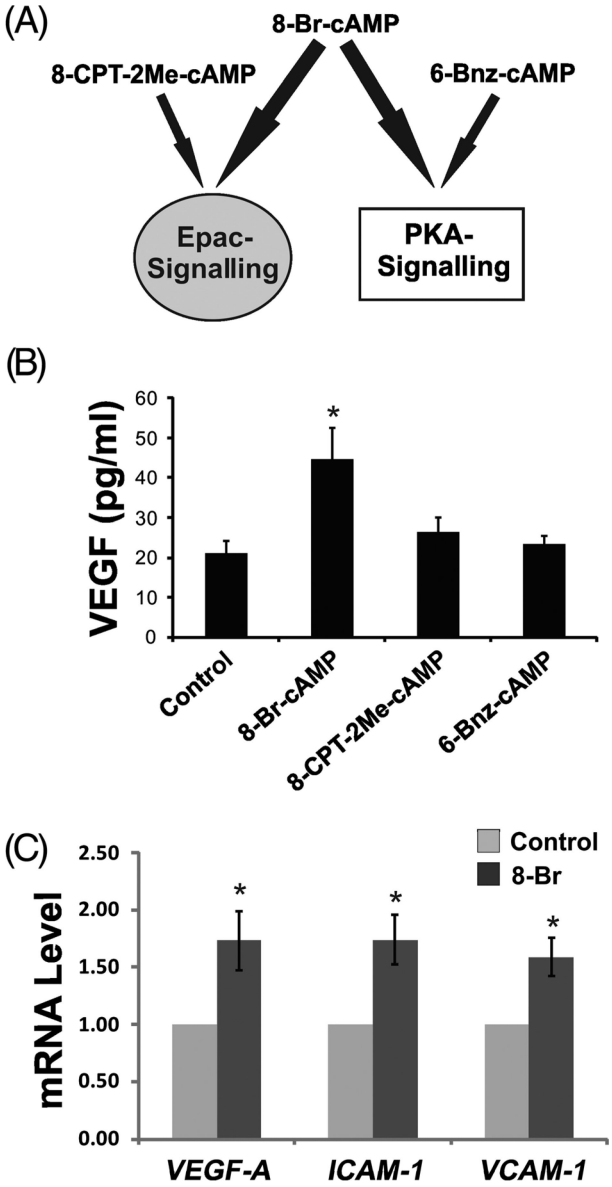

Figure 1.

Effect of various cAMP analogues on extracellular VEGF production in osteoblast-like MC3T3-E1 cells. (A) Schematic representation of various cAMP analogues (8-Br-cAMP, 8-CPT-2Me-cAMP, and 6-Bnz-cAMP) and their signaling pathways targets. (B) The cultured cells were stimulated by 100 μM 8-Br-cAMP, 100 μM 6-Bnz-cAMP, or 100 μM 8-CPT-2Me-cAMP for 24 hours. Untreated cells were used as a control. Media were collected and analyzed by ELISA. Note that only 8-Br-cAMP treatment significantly enhanced secretion of VEGF in osteoblast-like MC3T3-E1 cells. Error bars represent means ± SD (n= 4). (C) VEGF-A, ICAM-1, and VCAM-1 mRNA levels in HUVECs were determined after the cells were cultures in the conditioned media for 3h. Note: Control: control conditioned medium; 8-Br: 8-Br-cAMP conditioned medium. To facilitate the comparison of different experimental settings, cells cultured in “8-Br” condition were normalized to the cells treated with the control conditioned medium. Error bars represent means ± SD (n= 3).

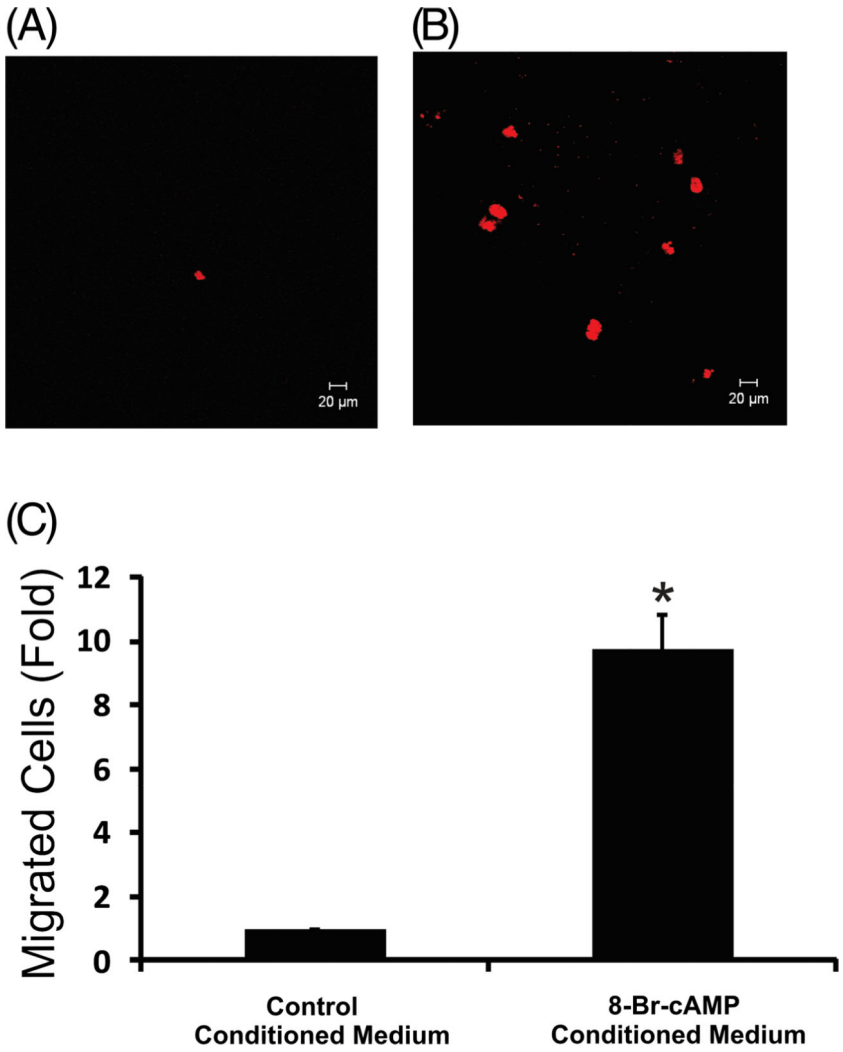

Figure 2.

Effect of conditioned media on endothelial cell migration in the Boyden Chamber assay. HUVECs cultured in fibronectin-coated Boyden Chamber with (A) control conditioned medium or (B) 8-Br-cAMP conditioned medium. (C) Quantitation of HUVECs migration in each experimental group. These results indicate that the 8-Br-cAMP conditioned medium increases HUVECs migration. To facilitate the comparison of different experimental settings, migrated cells were normalized to the cells treated with the control conditioned medium. Error bars represent means ± SD (n= 4).

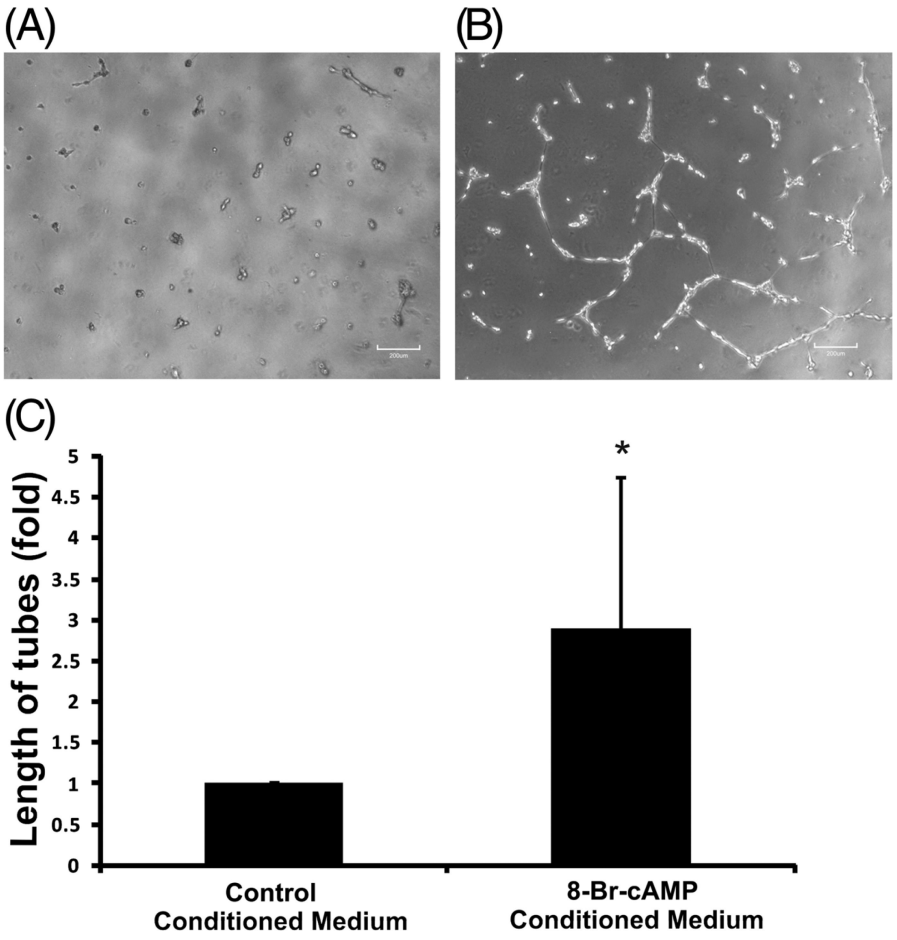

Figure 3.

Effect of conditioned media on capillary network formation *in vitro*. Light micrographs of HUVECs cultured in matrigel with (A) control conditioned medium or (B) 8-Br-cAMP conditioned medium. Scale bars are 200 μM. (C) Quantization of capillary network formation in each sample. These results indicate that the 8-Br-cAMP conditioned medium enhances capillary network formation. The range of the length of tube structures formed in the martigel assay is from ~60 μM to ~722 μM. To facilitate the comparison of different experimental settings, length of tubes were normalized to the length of tubes of the cells treated with the control conditioned medium. Error bars represent means ± SD (n= 6).

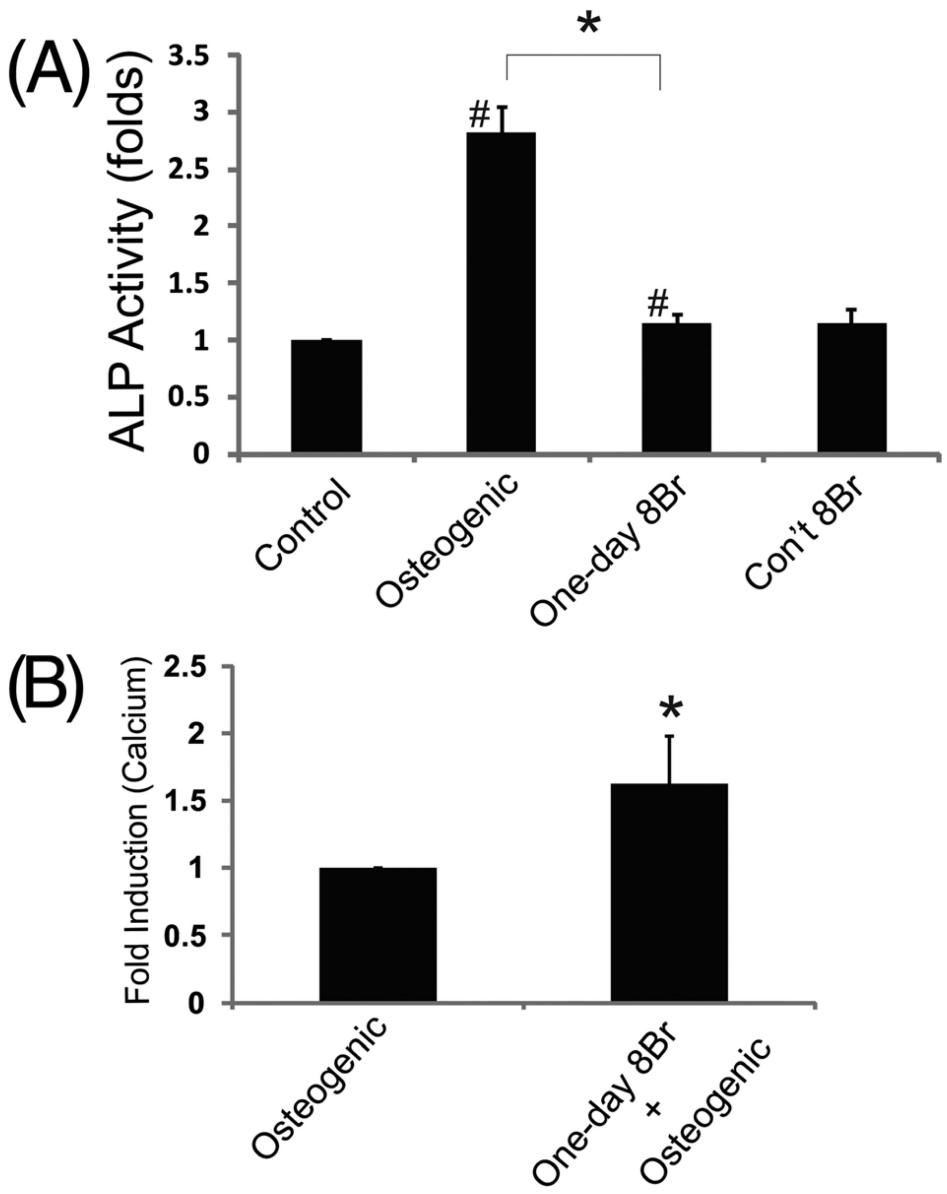

Figure 4.

Effect of various 8-Br-cAMP treatments on osteoblastic differentiation of osteoblast-like MC3T3-E1 cells. (A) ALP activities at day 7 were assessed by a commercial ALP activity substrate assay kit. Absorbance values were normalized to cellular DNA. To facilitate the comparison of different experimental settings, ALP activities were further normalized to the ALP activity of the cells exposed to the untreated control. Cell exposed to osteogenic medium served as a positive control experiment. #$p<0.05$ compared to "Control", i.e. cells cultured in regular growth medium. (B) Effect of one-day 8-Br-cAMP treatment on matrix mineralization of MC3T3-E1 cells. Note that calcium level was significantly increased in cells when they were cultured in osteogenic medium with one-day 8-Br-cAMP treatment. To facilitate the comparison of different experimental settings, the value was normalized to cells exposed to the osteogenic medium. Error bars represent means ± SD (n= 3).

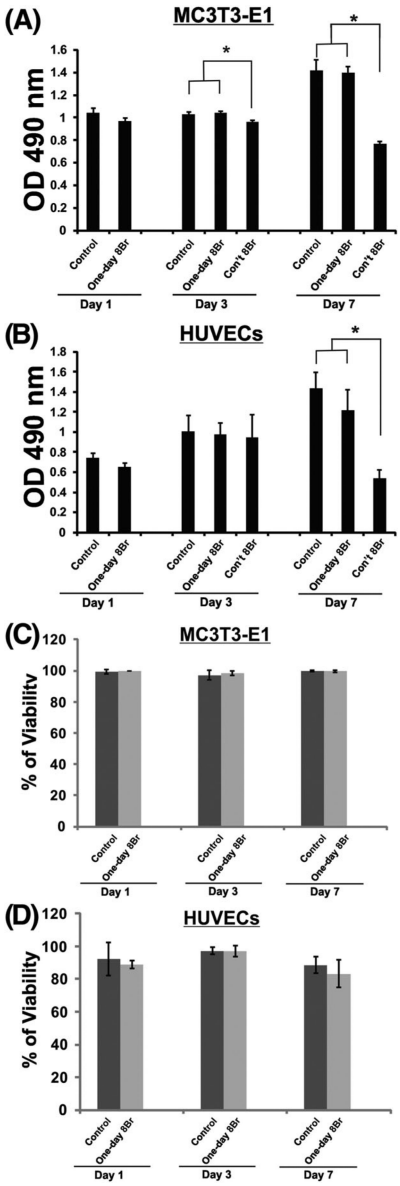

Figure 5.

Effects of various 8-Br-cAMP treatment schemes (one-day treatment versus continuous treatment) on cellular proliferations and viability in osteoblast-like MC3T3-E1 cells and HUVECs. Cellular proliferation of (A) MC3T3-E1 cells and (B) HUVECs in the presence of the small molecule 8-Br-cAMP (100 μM) was measured at days 1, 3, and 7 using a commercial cell proliferation assay kit (MTS). Cell viability was assessed in order to confirm the low cell cytotoxicity of one day 8-Br-cAMP treatment. Cell viability of (C) MC3T3-E1 cells and (D) and HUVECs in the presence of the small molecule 8-Br-cAMP (100 μM) for 1 day was measured at days 1, 3, and 7. Note: "*One-day 8Br*" for one-day 8-Br-cAMP treatment and "*Con't 8Br*" for continuous 8-Br-cAMP treatment. Error bars represent means ± SD (n= 3).