Heterodimeric BMP-2/7 Antagonizes the Inhibition of All-Trans Retinoic Acid and Promotes the Osteoblastogenesis

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Abstract

Objectives: Hypervitaminosis A and alcoholism can result in a low mineral density and compromised regenerative capacity of bone, thus delaying implant osteointegration. The inhibitory effect of all-trans retinoic acid on osteoblastogenesis is considered to be one of the mechanisms. We hypothesized that heterodimeric bone morphogenetic protein-2/7 could antagonize all-trans retinoic acid and enhance osteoblastogenesis, with an aim to accelerate and enhance bone regeneration and implant osteointegration.

Materials and Methods: We applied 5 ng/ml or 50 ng/ml bone morphogenetic protein-2/7 to restore the osteoblastogenesis of pre-osteoblasts (MC3T3-E1 cell line) that was inhibited by 1 μM all-trans retinoic acid. We evaluated the efficacy by assessing cell numbers (proliferation), alkaline phosphatase activity (a marker for early differentiation), osteocalcin (a marker for late differentiation), calcium deposition (a marker for final mineralization) and the expression of osteoblastogenic genes (such as Runx2, Collagen Ia, alkaline phosphatase and osteocalcin) at different time points.

Results: All-trans retinoic acid significantly inhibited the expression of all the tested osteoblastogenic genes and proteins except alkaline phosphatase activity. In the presence of ATRA, 50 ng/ml bone morphogenetic protein-2/7 not only completely restored but also significantly enhanced all the osteoblastogenic genes and proteins. On the 28th day, mineralization was completely inhibited by all-trans retinoic acid. In contrast, 50 ng/ml BMP-2/7 could antagonize ATRA and significantly enhance the mineralization about 2.5 folds in comparison with the control treatment (no ATRA, no BMP2/7).

Conclusions: Heterodimeric bone morphogenetic protein-2/7 bears a promising application potential to significantly promote bone regeneration and implant osteointegration for the patients with hypervitaminosis A and alcoholism.

Introduction

Sufficient bone volume and adequate bone quality are of paramount importance to achieve a rapid establishment of implants’ functions in dentistry and orthopedics. However, various adverse bone conditions such as low density and compromised self-healing capacity of bone can significantly compromise new bone regeneration and implant osteointegration, and thus delay the loading of implants. Such adverse bone conditions can be resulted from some harmful lifestyles, such as the alcoholism and hypervitaminosis A. Alcoholism exhibits very high prevalence: it affects more than 14 million people in the United States. A large body of evidence suggests that a correlation between low bone mass and chronic alcohol abuse [1] with increased fracture risk [2]. Animal studies showed that a alcohol consumption could decrease the new bone formation [3] and delay implant osteointegration [4] possibly by reducing the number and activity of osteoblasts [5]. The detrimental effect of alcohol was even more harmful in comparison with nicotine [6]. On the other hand, researchers have found that hypervitaminosis A caused accelerated bone resorption, bone fragility, and spontaneous fractures [7,8]. The chronic and significant enhancement of all-trans retinoic acid (ATRA), a metabolite of vitamin A, can be one of the mechanisms for such phenomena. The significantly increased ATRA in serum may also account for the detrimental effects of alcoholism [9].

All-trans retinoic acid (ATRA) can induce bone resorption and osteoporosis [10]. The suppression of ATRA on bone metabolism can be mediated by both increasing the osteoclastic bone

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resorption [11] and decreasing the osteoblastic bone formation [12]. It has been established that ATRA can suppress the proliferation of pre-osteoblasts and inhibit the formation of a mineralized matrix. This effect may be due to the induction and maintenance of a partially differentiated non-proliferating state of osteoblasts by ATRA [13].

As robust osteoinductive agents, bone morphogenetic proteins (BMPs) are a group of growth factors under the superfamily of transforming growth factor-β (TGF-β). BMPs, particularly BMP-2 and BMP-7, can significantly enhance all the cellular events during osteoblastogenesis, such as migration, proliferation, differentiation and mineralization [14]. Consequently, BMPs have been extensively investigated to accelerate and enhance bone regeneration and implant osteointegration [15]. However, the effective doses of homodimeric BMPs in clinic to promote bone formation are extremely high (e.g. up to milligrams) [16,17], which results in not only a substantial economic burden to patients but also a series of potential side effects, such as the overstimulation of osteoclastic activity and an ectopic bone formation in an unintended area [18,19]. One alternative approach to solve the problem is to adopt more potent forms of BMPs. We have shown that heterodimeric BMP-2/7 induced osteoblastogenesis with significantly lower optimal concentrations but similar maximum effects [14]. Our in-vivo experiment also proved that low-dose heterodimeric BMP-2/7 facilitated more rapid bone regeneration in a better quality in peri-implant bone defects than the homodimeric BMPs [20].

In this study, we hypothesized that heterodimeric BMP-2/7 could antagonize the inhibitory effect of ATRA and enhance the osteoblastogenesis of pre-osteoblasts. We adopted a murine calvaria-derived cell line (MC3T3-E1), which is the precursor of functionalized osteoblast and is often used to examine the potency of osteoinductive agents like BMPs [21].

Materials and Methods

Study Design

In this study, we tested our hypothesis that heterodimeric BMP-2/7 could antagonize the inhibitory effect of ATRA and enhance the osteoblastogenesis of pre-osteoblasts. We treated the pre-osteoblasts with the following treatments: 1) no ATRA, no BMP-2/7; 2) 1 μM ATRA, no BMP-2/7; 3) 1 μM ATRA, 5 ng/ml BMP-2/7; 4) 1 μM ATRA, 50 ng/ml BMP-2/7; 5) no ATRA, 5 ng/ml BMP-2/7; 6) no ATRA, 50 ng/ml BMP-2/7. The concentrations of BMP2/7 and ATRA were determined basing on the previous studies. In our previous studies, 5 ng/ml was the low-threshold concentration and 50 ng/ml was the optimal concentration for BMP-2/7 to induce the ALP activity and OCN expression [14]. In addition, 1 μM ATRA has repeatedly been adopted to study the in-vitro effects of ATRA on osteogenic differentiation [11,22,23]. We evaluated their effects on the osteoblastogenesis by assessing cell numbers (as a proliferation index), alkaline phosphatase (ALP) activity (a marker for early differentiation), osteocalcin (OCN) (a marker for late differentiation), calcium deposition (a marker for final mineralization) and the expression of various osteoblastogenic genes.

Cell Culture

MC3T3-E1 cells (ATCC; Chinese Academy of science, Shanghai, China) were cultured in a α-Minimum essential medium (α-MEM) containing 10% fetal bovine serum (FBS) (Gibco®, Invitrogen, Grand Island, NY, USA). The medium was changed every 3 days. Exponentially growing cells were plated at a final concentration of 1×10⁵ cells/well in 24-well plates for the cell proliferation assay, at a final concentration of 2×10⁵ cells/well in 6-well plates for the ALP activity assay, OCN detection and PCR analysis, or at a final concentration of 3×10⁵ cells/well in 48-well plates for alizarin red staining. After incubation for 24h, the cells were subjected to a low-serum medium (9% FBS) for another 24 h. Thereafter, the cells were treated with the different combinations of heterodimeric BMP-2/7 (R&D Systems, Inc., Minneapolis, MN, USA) and/or ATRA (Sigma-Aldrich, St. Louis, MO, USA). Triplicates per group were performed for each parameter per time point.

Cell Viability and Proliferation Assay

To investigate the cell viability and proliferation of MC3T3-E1 cells, the cell numbers of each group was determined by the alamar Blue cell viability reagent (Invitrogen Corporation, Carlsbad, CA, USA) after the treatment for 1 day and 4 days. The fluorescent intensity was measured using a fluorescence spectrometer (SpectraMax M5 Molecular Devices, Sunnyvale, CA, USA) at EX 540 nm/EM 590 nm.

ALP Activity Assay

To assess the early differentiation of pre-osteoblasts, the ALP activity and total protein content were measured after the treatment for 4 day and 7 days. The ALP activity in the cell lysate (Sigma-Aldrich, St. Louis, MO) was determined using LabAssay™ ALP colorimetric assay kit (Wako Pure Chemicals, Osaka, Japan). The total protein content was measured at 570 nm using a commercial BCA Protein Assay kit (Beyotime, China). The values were expressed as nmol p-NP/ug total protein/hour to present the ALP activity.

OCN Expression Assay

To assess the terminal differentiation of pre-osteoblasts, the OCN secreted into the cell culture medium was determined. The cell supernatants were collected on the 4th day and the 7th day and centrifuged (10,000 rpm, 4°C, 5 min) before detection. The OCN concentrations in the supernatants were determined by ELISA using a mouse OCN EIA kit (Biomedical Technologies, Stoughton, MA, USA) [24].

Alizarin Red Staining

We compared the mineralization possibility of MC3T3-E1 cells stimulated by BMP-2/7 and ATRA. For this purpose, cells were treated with mineralizing medium (10% FBS, 50 μM L-ascorbic acid, and 10 mM β-glycerophosphate; Sigma-Aldrich, St. Louis, MO, USA) [25] containing BMP-2/7 and ATRA. The medium was replaced every 3 days. On the 21st and 28th day, mineralized nodules were determined by the staining of alizarin red (Sigma-Aldrich, St. Louis, MO, USA) as previously described [25,26]. Culture plates were photographed by NIS-Elements F2.20 (Nikon Eclipse 80i, Tokyo, Japan), and the calcified areas were then quantified using a software of Image-Pro Plus 6.0.

Isolation of Total RNA and Real-time Fluorescence Quantitative Polymerase Chain Reaction (RT-qPCR) Analysis

On the 1st, the 4th and the 7th day, the total RNA was extracted from the cells using RNAeasy Mini Kit and purified with RNase-Free DNase Set reagent (Qiagen, Germany) following the manufacturer’s instructions. Total RNA was reverse transcribed to cDNA using a kit of PrimerScript® RT Master Mix (Perfect Real Time, Takara, Japanese). RT-qPCR was performed using a PrimeScript® RT reagent Kit (Perfect Real Time, Takara, Japan). The fluorescent intensity was measured using a fluorescence spectrometer (SpectraMax M5 Molecular Devices, Sunnyvale, CA, USA) at EX 540 nm/EM 590 nm.
Japanese) according to the manufacturer’s instruction. Specific
primers used for detecting mRNA transcripts of the Runx2, 
Collagen I, ALP, OCN, and β-actin gene are as shown in Table 1.
Transcripts were normalized to the β-actin transcript levels.
Calculate the n-fold upregulation for each gene of interest over the
internal control gene (β-actin gene) according to the delta-delta-Ct
method using the formula: 2^{-ΔΔCt (gene of interest-CT internal
control)sample-CT internal controlcontrol} [27].

Statistical Analysis
Statistical comparisons among the results were made by one-
way analysis of variance (ANOVA). Post Hoc comparisons were
made using Bonferroni corrections. The level of significance was
set at p<0.05. SPSS software (version 20) for a Windows computer
system was employed for the statistical analysis.

Results
On the 1st day, a significant increase in the cell numbers was
detected only under the treatment of no ATRA, 50 ng/ml BMP-
2/7 (Figure 1). Neither significant enhancement nor inhibition in
cell proliferation was detected in the other groups. On the 4th day,
ATRA alone significantly decreased the cell numbers about 50%,
which could be completely restored by 5 ng/ml or 50 ng/ml BMP-2/7. In contrast, 5 ng/ml or 50 ng/ml BMP-2/7 alone
alone could result in a significantly higher cell number than the other
treatments.

In contrast to the inhibitory effect of ATRA on cell
proliferation, ATRA alone didn’t significantly influence the ALP
activity on the 4th day and the 7th day (Figure 2). BMP-2/7 significantly increased the ALP activity in a dose-dependent
manner irrespective of ATRA at both time points. On the 7th day,
5 ng/ml and 50 ng/ml BMP-2/7 (with 1 μM ATRA) significantly
increased the ALP activity 4.5 folds and 16.7 folds respectively in
comparison with the treatment of no BMP-2/7, 1 μM ATRA.

In the absence of BMP-2/7 and ATRA, OCN expression in
pre-osteoblasts significantly increased from the 4th day to the 7th
day (Figure 3). Similar to its effects on cell proliferation, ATRA
alone could significantly inhibit the OCN expression about 55% on
the 4th day and 66% on the 7th day. On the 4th day, 5 ng/ml
BMP-2/7 antagonized the inhibitory effect of ATRA and
completely restored the OCN expression. However, on the 7th
day, 5 ng/ml BMP-2/7 only restored about 70% OCN expression.
In contrast, despite of the presence of ATRA, 50 ng/ml BMP-2/7 significantly enhanced the expression of OCN 1.5 times on
the 4th day and 1.23 times on the 7th day respectively in
comparison with the control treatment (no ATRA, no BMP-2/7).
Albeit so, the BMP-2/7-induced OCN expression was significantly
inhibited by ATRA at both time points.

On the 21st day, the mineralization in cell matrix was found
neither in the two groups without BMP-2/7 nor in the group of
1 μM ATRA, 5 ng/ml BMP-2/7 (Figure 4). 50 ng/ml BMP-2/7 alone
alone resulted in the highest mineralization on the 21st day. In the
presence of ATRA, only 50 ng/ml BMP-2/7 was associated with
significant mineralization. On the 29th day, significant mineraliza-
ation was also found in the control group (no ATRA, no BMP-2/7),
which could be completely inhibited by ATRA (1 μM ATRA,
no BMP-2/7). The mineralization area in the group of 1 μM
ATRA, 5 ng/ml BMP-2/7 was still significantly lower than that in
the control group (no ATRA, no BMP-2/7). In contrast, 50 ng/ml
BMP-2/7 could antagonize ATRA and significantly enhance the
mineralization about 2.5 folds in comparison with the control
group (no ATRA, no BMP-2/7). 50 ng/ml BMP-2/7 alone resulted
in the highest mineralization on the 29th day.

For all the treatment groups, the expression of Runx2 gene
significantly increased from day 1 to day 4, while slightly decreased
from day 4 to day 7. On the 1st day, Runx2 expression was
significantly suppressed by ATRA with or without BMP-2/7 (Figure 5A). The expression of Runx2 gene was, whereas,
significantly enhanced by ATRA alone on the 4th day and the
7th day. BMP-2/7 significantly increased the expression of Runx2
gene in a dose-dependent manner. On the 4th day and the 7th day,
ATRA didn’t significantly influence the expression of Runx2 that
was induced by 5 or 50 ng/ml BMP-2/7. The expression of Collagen Ia gene was significantly suppressed by ATRA alone at
the three time points (Figure 5B). 5 ng/ml BMP-2/7 could
completely restore the expression of Collagen Ia gene that was
inhibited by ATRA at all time points. 50 ng/ml BMP-2/7 could
also restore the expression of Collagen Ia gene on the 1st day and
the 7th day, and significantly enhance it on the 4th day. ATRA
could significantly decrease the gene expression of Collagen Ia that
was induced by 5 or 50 ng/ml BMP-2/7 at all the time points.
Different from the ALP activity, the expression of ALP gene was
significantly suppressed by ATRA alone at all the three time
points. In the presence of ATRA, 5 ng/ml and 50 ng/ml could
significantly enhance ALP gene expression (Figure 5C) in
comparison with the two groups without BMP-2/7. On the 4th
day, the highest expression of ALP gene was detected in the group
of 1 μM ATRA, 50 ng/ml BMP-2/7. On the 7th day, the highest
expression of ALP gene was detected in the group of no ATRA,
50 ng/ml BMP-2/7. The expression of OCN gene was also
significantly suppressed by ATRA alone at all the three time
points. In the presence of ATRA, 5 ng/ml BMP-2/7 completely
restored the expression of OCN gene, and 50 ng/ml could further
significantly enhance the expression of OCN gene (Figure 5D).
BMP-2/7 could significantly enhance the gene expression of OCN
in a time-course and dose-dependent manner irrespective of
ATRA. At both time points, ATRA significantly inhibited the
expression of OCN gene that was induced by 5 or 50 ng/ml BMP-
2/7.

Discussion
Rapid restoration of bone defects and early loading of implants
have been pursued in the field of orthopedics and dentistry.

Table 1. Primer sequences for real-time quantitative
polymerase chain reaction analysis of the expression of Runx2,
collagen I, alkaline phosphatase (ALP) and osteocalcin (OCN)
genes.

| Gene       | Accession No. | Primers (F = forward; R = reverse) |
|------------|---------------|-------------------------------------|
| Akp2 (ALP) | NM_007431     | F: 5'-TGCCCTACCTTGTGGCCTGGA-3'  |
|            | R: 5'-TCACCCGGAGGTGTAGTCAAAATG-3' |
| Osteocalcin| NM_007541     | F: 5'-AGCCAGCTGCGCAGCACTA-3'   |
| Collagen I | NM_007742     | F: 5'-ATGCCGGACACTCAAGATG-3'  |
|            | R: 5'-TGGAGCAGCAGCAGCTGAGTA-3' |
| Runx2      | NM_009820     | F: 5'-CATCGGCGTGCGCAGCAGA-3'  |
|            | R: 5'-TTCTTAACACGCGAGGAGCTCT-3' |
| β-actin    | NM_007393     | F: 5'-AGGAGCAATGTTGGTATGCT-3'  |
|            | R: 5'-TGCCAAACAGTGCTGCTT-3'   |

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BMP-2/7 Antagonizes ATRA and Promotes Osteogenesis
However, the goal becomes very difficult to achieve in the patients with low bone density and compromised self-healing capacity caused by the dietary-accumulated ATRA. In this study, we found that heterodimeric BMP-2/7, a potent osteoinductive cytokine, could antagonize the inhibitory effect of ATRA and enhance the osteoblastogenesis of pre-osteoblasts. Consequently, heterodimeric BMP-2/7 is very promising to accelerate and enhance bone regeneration and implant osteointegration for these patients.

ATRA, an active metabolite of vitamin A, is involved in bone formation [28]. ATRA regulates the gene expression via its receptors. There are two families of retinoic acid receptors, RARs and RXRs, each of which has three isoforms (α, β, γ). In consistency with the previous studies [29,30], ATRA significantly inhibited cell proliferation (Figure 1), OCN expression (Figure 3) and mineralization (Figure 4), but did not influence the ALP activities (Figure 2). Coordinate gene expression patterns showed that ATRA treatment slowed the proliferation of MC3T3-E1 cells and inhibited the formation of a mineralized matrix [13]. This effect may be due to the induction and maintenance of a partially differentiated non-proliferating state. Until now, the molecular mechanisms for the effects of ATRA on the osteoblastogenesis of pre-osteoblasts remain largely unknown. Interestingly, Runx2, a
key modulator for osteogenic differentiation, was significantly decreased only on the 1st day, whereas significantly increased on the 4th day and the 7th day by ATRA (Figure 5A). Runx2 controls osteoblast proliferation and promotes a transition from a proliferative to a post-proliferative stage prior to osteoblast differentiation [31,32]. It remains to be elucidated whether the significant up-regulation of Runx2 by ATRA could partially account for the significant down-regulation of cell proliferation. Besides, RA can induce cell growth arrest through the enhanced and prolonged MAPK signaling with BLR1 as a critical component in a positive feedback [33].

On the other hand, although the expression of Runx2 gene was significantly enhanced by ATRA alone, all the osteoblastic genes e.g. Col., ALP and OCN were significantly suppressed. In contrast, heterodimeric BMP2/7 alone could significantly enhance the ALP activity, OCN expression, mineralization and the all the selected osteogenic genes. ATRA could also suppress the BMP2/7-induced osteoblastogenesis of pre-osteoblasts. The mechanisms accounting for these phenomena remain ambiguous. It has been well established that homodimeric BMP5s bind to transmembrane serine/threonine kinase receptors on the cell surface, triggering specific intracellular pathways that activate and influence gene transcription [34]. Activated BMP type I receptors phosphorylate Smad1, Smad5, and Smad8 (R-Smad), which then assemble into heteromeric complexes with Smad4 (Co-Smad) and translocate into the nucleus to regulate transcription of target genes, such as Runx2 [35]. In addition, the activated BMP receptors can also initiate Smad-independent signaling pathways, resulting in the activation of ERK, p38 and JNK [36,37,38]. Similarly, we also showed that heterodimeric BMP2/7 could significantly enhance the Id1 and Id2 (inhibitor of DNA binding 1 and 2, the canonical targets of BMP signaling) on the 4th day (Figure S1). It was proposed that ATRA could down-regulate Id1 through promoting the degradation of phosphorylated Smad1 [23]. However, this mechanism seems not true in the pre-osteoblasts since ATRA could further promote the BMP2/7-induced Id1 and Id2. In consistency with Id1 and Id2, ATRA didn't suppress the BMP2/7-induced Runx2. Consequently, ATRA might not promote the degradation of phosphorylated Smad1 in pre-osteoblasts. It might be plausible that ATRA interferes with the activation and functions of Runx2 possibly through inhibiting the phosphorylation and translocation to nuclei of Runx2. It is also important to note that the ALP activity remained unchanged under the treatment of ATRA alone. It has been shown that the induction of ALP activity was mediated through the activation of a Smad-independent signaling pathway p38 MAPK induced by BMP ligands [39]. Taken together, ATRA might inhibit the functions of Runx2 but enhance MAPK signaling, thereby facilitating the induction and maintenance of a partially differentiated non-proliferating state. The molecular mechanisms still need to be elucidated. The modulation of ATRA on the BMP signaling-related genes (such as Bambi, Mecom and Smurf1, figure S1) may provide some clues for further investigations.

In comparison with the homodimeric BMP-2 and BMP-7, heterodimeric BMP-2/7 was associated with significantly lower threshold and optimal concentrations, significantly earlier response [14]. Consequently, heterodimeric BMP-2/7 is very promising to efficiently accelerate and enhance bone regeneration and implant osteointegration. In this study, 5 and 50 ng/ml BMP-2/7 could antagonize the inhibitory effect of ATRA and restore the cell proliferation that was inhibited by ATRA (Figure 1). In the presence of ATRA, 5 ng/ml BMP-2/7 significantly enhanced the ALP activity and 50 ng/ml BMP-2/7 resulted in the highest ALP activity (Figure 2). Furthermore, 5 ng/ml BMP-2/7 can only partially rescue OCN that was inhibited by ATRA. In contrast, 50 ng/ml BMP-2/7 could further significantly enhance OCN 1.2 folds in comparison with the control treatment (no ATRA, no BMP-2/7). These results were compliant with our previous findings that BMP-2/7 induced the optimal ALP activity and OCN expression at the concentration of 50 ng/ml [14]. The findings from gene of ALP and OCN confirmed that BMP-2/7 could not only restore but also significantly enhance ALP and OCN that were inhibited by ATRA.
On the 21st day, the mineralization in cell matrix was found neither in the two groups without BMP-2/7 nor in the group of 1 μM ATRA, 5 ng/ml BMP-2/7 (Figure 4). In the presence of ATRA, only 50 ng/ml BMP-2/7 was associated with detectable mineralization. These results indicated that 50 ng/ml heterodimeric BMP-2/7 could significantly accelerate mineralization even in the presence of ATRA. On the 28th day, mineralization also occurred to the control group (no ATRA, no BMP-2/7), while such mineralization could be completely inhibited by ATRA. This result indicated that the up-regulation of Runx2 and maintenance of ALP by ATRA was insufficient for the final mineralization. 5 ng/ml BMP-2/7 only restored 8.5% mineralization, which suggested that the limited up-regulation of cell proliferation and ALP were neither sufficient to rescue ATRA-inhibited osteoblastogenesis. In contrast, 50 ng/ml BMP-2/7, that significantly enhanced all the osteoblastogenic genes and proteins (Figure 2, 3).
and 5), not only restored but also significantly enhanced mineralization 2.5 folds (Figure 4). These results suggested that BMP-induced signaling could antagonize the inhibition of ATRA and significantly enhance osteoblastogenesis. Consequently, heterodimeric BMP-2/7 could not only significantly enhance but also accelerate the osteoblastogenesis, thereby bearing a promising application potential to facilitate bone regeneration and implant osteointegration for the patients with hypervitaminosis A and excessive alcohol consumption.

One of the limitations in this study was that we used a murine-derived pre-osteoblast cell line. It might be more instructive for clinic to investigate the effects of heterodimeric BMP-2/7 and ATRA on human primary cells. In addition, the effects of ATRA exhibited a large diversity on different cell types. Consequently, caution should be taken to extrapolate the results in other cell types.

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Supporting Information
Figure S1 The microarray analysis of 27 BMP signaling-related genes in MC3T3-E1 cells that were treated with 1) no ATRA, no BMP2/7, 2) 1 μM ATRA, no BMP2/7, 3) no ATRA, 5 ng/ml BMP2/7 and 4) 1 μM ATRA, 50 ng/ml BMP2/7 for 4 days. (A) Heat map and (B) table of fold changes (compared to the treatment of no ATRA, no BMP2/7). (DOCX)
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