Mineral trioxide aggregate induces osteoblastogenesis via Atf6

Toyonobu Maeda a, Atsuko Suzuki a, Satoshi Yuzawa a, Yuh Baba b, Yuichi Kimura c, Yasumas Kato a,⁎

a Department of Oral Function and Molecular Biology, Ohu University School of Dentistry, Koriyama 963-8611, Japan
b Department of General Clinical Medicine, Ohu University School of Dentistry, Koriyama 963-8611, Japan
c Division of Endodontics, Department of Conservative Dentistry, Ohu University School of Dentistry, Koriyama 963-8611, Japan

Abstract

Mineral trioxide aggregate (MTA) has been recommended for various uses in endodontics. To understand the effects of MTA on alveolar bone, we examined whether MTA induces osteoblastic differentiation using MC3T3-E1 cells. MTA enhanced mineralization concomitant with alkaline phosphatase activity in a dose- and time-dependent manner. MTA increased production of collagens (Type I and Type III) and matrix metalloproteinases (MMP-9 and MMP-13), suggesting that MTA affects bone matrix remodeling. MTA also induced Bglap (osteocalcin) but not Bmp2 (bone morphogenetic protein-2) mRNA expression. We observed induction of Atf6 (activating transcription factor 6, an endoplasmic reticulum (ER) stress response transcription factor) mRNA expression and activation of Atf6 by MTA treatment. Forced expression of p50Atf6 (active form of Atf6) markedly enhanced Bglap mRNA expression. Chromatin immunoprecipitation assay was performed to investigate the increase in p50Atf6 binding to the Bglap promoter region by MTA treatment. Furthermore, knockdown of Atf6 gene expression by introduction of Tet-on Atf6 shRNA expression vector abrogated MTA-induced mineralization. These results suggest that MTA induces in vitro osteoblastogenesis through the Atf6–osteocalcin axis as ER stress signaling. Therefore, MTA in endodontic treatment may affect alveolar bone healing in the resorbed region caused by pulpal infection.

© 2015 The Authors. Published by Elsevier Inc. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
the ER chaperone BiP/GRP78 in response to ER stress and is cleaved by Ca\(^{2+}\)-dependent Golgi-associated proteinases (S1P and S2P) to release the transcriptionally active N-terminal fragment (p50Atf6) of 50 kDa including the bZIP sequence (Ye et al., 2000; Chen et al., 2002). Atf6 is thought to be a master regulator of the UPR to reduce ER stress along with two other molecules, i.e., IRE1 (inositol requiring 1) and PERK [PKR (protein kinase R)-like ER kinase] (Kauffman, 1999; Mori, 2000). Involvement ER stress signaling in osteoelastic differentiation has been reported (Jang et al., 2012; Liu et al., 2012; Wang et al., 2014a). In addition, Hino et al. (2010) showed that ER stress marker expression was down-regulated in osteoblasts of osteoporosis patients, suggesting ER stress play an important role for osteoblastic differentiation. Expansion of the ER capacity to prevent ER overload-induced apoptosis by increasing production of ECM proteins, such as Type I collagen (Tohminda et al., 2011).

Although previous studies showed that MTA affected the differentiation of odontoblasts and bone marrow stromal cells with elevated expression of some genes, such as Alp (alkaline phosphatase), Osx (Sp7, osterix), Bglap (osteocalcin), and Coll1a1 (Type I collagen) (Min et al., 2008, 2009; Wang et al., 2014b; Zhao et al., 2012), molecular mechanism of MTA-induced bone healing mechanisms remains unclear. The present study was performed to determine whether MTA induces osteoblastic differentiation and tissue mineralization through the ER stress signaling pathway.

2. Materials & methods

2.1. Reagents

ProROOT MTA was provided by Dentsply (Johnson City, TN). Isogen total RNA extraction kit was purchased from Nippon Gene (Tokyo, Japan). SYBR Premix Ex Taq II and enzyme-linked immunosorbent assay (ELISA) kits for Mouse Gla-Osteocalcin and BMP-2 were from Takara (Tokyo, Japan) and R&D Systems (Minneapolis, MN), respectively. Alpha modifi ed Eagle’s MEM (αMEM) was from ICN Pharmaceuticals (Aurora, OH). Alkaline phosphatase (ALP) determination kit (Lab Assay ALP) were from Wako (Osaka, Japan). Blocking regent N102 was from NOF (Tokyo, Japan). Immobilon-P PVDF membranes and chemiluminescence reagent were from Merck Millipore (Billerica, MA). Avidin-biotin-conjugated horseradish peroxidase (HRP) was from Bio-Rad (Hercules, CA). Xfect Transfection Reagent was from Clontech (Mountain View, CA). Genetin was from Roche (Basel, Switzerland). Doxycycline was from Sigma Aldrich (St-Louis, MO).

2.2. Vectors and transfection

The coding region of p50Atf6 was cloned by RT-PCR and inserted into the p3 × FLAG-CMV-10 vector (Sigma-Aldrich, St-Louis, MO) to intracellularly express N-terminal Flag-tagged p50Atf6 fusion protein. The shRNA for Atf6 mRNA expression vector was constructed using the pSingle-tTS-shRNA vector (Clontech, Mountain View, CA) with an oligonucleotide targeting Atf6. Target sequence to construct an inducible shRNA expression vector for Atf6 using the pSingle-tTS-shRNA vector was as follows: 5′-TCC AGG CTC GTA CAT GAA GCC AGA TAC GAG AAG TCT GCC TTC ATG TCT GAG CTT TTT TAC GCC TTT C-3′; where the target in indicated in bold underlined italic font. The resultant plasmid was designated as pSingle-tTS-Atf6-shRNA. Vectors were transfected with Xfect Transfection Reagent (Clontech).

2.3. Antibodies

Rabbit polyclonal antibodies against Atf6 and Type I collagen were purchased from Abcam (Cambridge, UK). Rabbit polyclonal antibodies against Type III collagen were from Santa Cruz (Dallas, TX). Biotin-conjugated goat anti-rabbit IgG (H + L) was from Jackson ImmunoResearch (West Grove, PA). Anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody was from Gene Tex Inc. (Irvine, CA).

2.4. Cell and cell culture

MC3T3-E1 cells (a clonal pre-osteoblastic cell line derived from newborn mouse calvaria) were grown in growth medium consisting of αMEM supplemented with 10% fetal bovine serum (FBS) as described previously (Maeda et al., 2003). For differentiation of the cells, we used differentiation medium consisting of growth medium supplemented with 50 μg/mL ascorbate 2-phosphate, 10 mM β-glycerophosphate, and 40 mM HEPES (pH 7.4).

2.5. Preparation of MTA solution

The culture medium soluble fraction of MTA was used for this assay according to Hakki et al. (2009) with some modifications. MTA powder (1 g) was suspended in 50 mL of αMEM, swelled, and incubated for 2 weeks at 4 °C with gentle shaking to release biologically active substances from the powder. After centrifugation at 3000 × g for 15 min at 4 °C, the supernatant used as stock solution (20 mg-powder/mL) was diluted 20,000- and 8000-fold with αMEM to final concentrations of 1.0 μg/mL and 2.5 μg/mL, respectively, and used for the experiment as MTA solution. The IC_{50} was 9.5 μg/mL. MTA saturated solution at 2.5 μg/mL contained 2.3 μM calcium and showed negligible levels of mineral deposition without cells. It also did not affect the pH of the culture medium.

2.6. Alizarin Red S (AR-S) staining

Mineralized matrix in the plates was stained with AR-S. Briefly, cells were ﬁ xed in 70% ethanol for 1 h at room temperature, washed with Ca\(^{2+}\)- and Mg\(^{2+}\)-free Dulbecco’s phosphate-buffered saline (PBS (−)) and stained with 2.3 μg/mL AR-S at pH 4.2 for 15 min to eliminate nonspeciﬁ c staining. The stained matrix was photographed using a digital camera. The red stain was quantified using Molecular Imager (BioRad, Hercules, CA).

2.7. Measurement of ALP activity

The membrane fraction of the cells was extracted with 0.1 M Tris–HCl (pH 7.2) containing 0.1% Triton X-100. ALP activity was determined using a Lab Assay ALP kit (Wako, Osaka, Japan).

2.8. Measurement of osteocalcin and BMP-2 in conditioned medium (CM)

Each time point, culture medium was replaced with fresh medium without FBS and cells were cultured for a further 24 h. CM from 24-hour cultures were collected and concentrated by acetone precipitation. Osteocalcin and BMP-2 were measured by enzyme-linked immunosorbent assay (ELISA).

2.9. Inducible knockdown system

For knockdown of the mouse Atf6 gene, we used the Knockout Single Vector Inducible RNAi System. The sequence targeting Atf6 was cloned into the pSingle-tTS-shRNA vector. Stably transfected cells were selected by genetin. Cloned cells showed signiﬁ cant reduction of Atf6 mRNA product in the presence of 1 μg/mL doxycycline (Dox).

2.10. Real-time reverse-transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated with Isogen, and reverse-transcribed to synthesize cDNA with a High-Capacity cDNA Reverse Transcription Kit (Life Technologies, Rockville, MD). Resultant cDNA samples were amplified
by SYBR Premix Ex Taq II with specific primers in a Thermal Cycler Dice Real Time System (TP-870; Takara, Tokyo, Japan). The level of Actb (β-actin) mRNA was used as an internal control. The specific primer sequences used are shown in Table 1.

2.11. Western blotting

Western blotting was performed essentially as described in our previous reports (Kato et al., 2005). Whole-cell lysates (10 μg/lane) were separated by 7.5% or 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto Immobilon-P PVDF membranes. Membranes were blocked with TBS-T (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween 20) containing 20% blocking regent N102, treated with first antibody followed by incubation with biotin-conjugated secondary antibody and avidin-conjugated horseradish peroxidase. Signals were detected with enhanced chemiluminescence reagents. In some cases, antibodies were stripped off from the membrane with stripping buffer consisting of 6 M guanidine hydrochloride, 20 mM Tris–HCl (pH 7.5), 0.2% Nonidet P-40, and 0.1 M β-mercaptoethanol (Yeung and Stanley, 2009). Anti-GAPDH antibody was used as a loading control.

2.12. Gelatin and casein substrate zymography

Zymography was performed as described in our previous reports (Kato et al., 2005). Briefly, gelatinolytic and caseinolytic activity in CM were detected by 7.5% SDS-PAGE containing 0.1% gelatin or casein, respectively. SDS in the gel was removed by Triton X-100 and enzyme reaction was carried out in the presence of 10 mM CaCl₂. The destained bands in the gels, indicating proteolytic activities, were quantified using Molecular Imager (BioRad, Hercules, CA).

2.13. Chromatin immunoprecipitation (ChIP) assay

ChIP was performed as described previously (Agata et al., 2001). Briefly, MC3T3-E1 cells were fixed with 1% formaldehyde and sonicated. Target protein in the sample was precipitated by anti-Atf6 antibody or non-immune IgG as a control. Real-time PCR was performed with the co-precipitated oligonucleotide as the template. Input DNA was also amplified by PCR for normalization. Primer sequences for ChIP assay are shown in Table 1.

Table 1

| Targets     | Sequences                           | Product size (bp) |
|-------------|-------------------------------------|-------------------|
| Actb        | Forward GAT GCC TTG GGA GTC AGA CC  | 162               |
|             | Reverse CCA ACT CCT CAG GAA GCT CCT |                   |
| Bglap       | Forward GCA ACT CCT ACC ACA GCC TCT| 96                |
|             | Reverse CCA TCA CCT ACC ACA GCA TCC TTT GGG T | |
| Bglap promoter| Forward GCG GAT GCT GCC AGG ACT AAT TGG C | 77               |
| Bmp2        | Forward TGA CTG CAT CCT GCC ACC TC | 112               |
|             | Reverse CAG AGT CTG CAC TAT GGC ATG GTA | 68               |
| Bmp4        | Forward ACC CGG CCT AAG ACT GTG AG | 113               |
|             | Reverse TCA CTG GTC CCT GGG ATG TTC |                   |
| Col1a1      | Forward GAC ATG TTC AGT TTT GTG CAC CT | 119              |
|             | Reverse GGG ACC CTT AGG CCA TTG TGT A |                   |
| Col3a1      | Forward AAC GGA CCT GGC CCC AT     | 113               |
|             | Reverse CCA TCA CTG CCC CGA GCA CC |                   |
| Mmp9        | Forward GCC CGG GAA CTC ACA AGA   | 85                |
| Mmp13       | Forward TGC CCT TGG GCA ACA AAG   | 120               |
| Actb        | Forward CAT CGG TAA AGA CCT ATA TGC CCA C | 171              |
|             | Reverse ATG GAG CCC ATC CAC A     |                   |

3. Results

3.1. Promotion of mineralization in MC3T3-E1 cells by MTA

First, we examined whether MTA affects mineralized nodule formation by osteoblastic MC3T3-E1 cells. Alizarin Red-S staining showed that MTA induced nodule formation in a dose-dependent manner (Fig. 1A and B). ALP activity was also induced dose-dependently by MTA, and statistical significance was confirmed from day 8 and later (Fig. 1C).

3.2. MTA increases collagen (Col1a1 and Col3a1) and MMP (Mmp9 and Mmp13) mRNA expression

Type I collagen is a major extracellular matrix protein in bone. On the other hand, Type III collagen, which is a fibril-forming collagen, is an important factor for formation of Type I collagen fibers. MTA induced accumulation of both Col1a1 and Col3a1 at mRNA expression level (Fig. 2A–E). It should be noted that the maximum induction of Col3a1 mRNA expression (day 4) occurred earlier than that of Col1a1 mRNA expression (day 8) (Fig. 2D and E). This was in good agreement with the roles of Type I and Type III collagen in fiber formation.

![Fig. 1. MTA induces mineralization of extracellular matrix in MC3T3-E1 cells.](image-url)

Fig. 1. MTA induces mineralization of extracellular matrix in MC3T3-E1 cells. The cells were cultured for 16 days in the absence or presence of MTA at indicated concentrations. (A) AR-S staining was performed to examine mineralized nodule formation. (B) A quantitative analysis of the intensity for AR-S staining was determined by Molecular Imager FX (Bio-Rad). (C) The time course of stimulation of ALP activity by MTA in MC3T3-E1 cells. ALP samples from whole-cell extracts were assayed using an ALP kit. Data represent the mean ± SEM (n = 4). Statistical significance of differences between MTA treated group and corresponding time-matched vehicle control is indicated by *P < 0.05 and ***P < 0.001.
MMP-13 cleaves Type I and Type III collagens at specific sites generating 3/4 N-terminal and 1/4 C-terminal fragments, as the major fibrous collagen-degrading proteinase in mice. These fragments are rapidly denatured at physiological temperature and are degraded by MMP-2 and MMP-9. MTA significantly induced production of MMP-9 and MMP-13 at both mRNA and protein level, while their active forms were negligible level (Fig. 3). Maximum mRNA levels of both molecules were observed on day 12 (Fig. 3C and F). This elevation was observed at a later phase compared to the collagen expression peaks (see Fig. 2D and E), and it showed good agreement with the osteocalcin expression peak (Fig. 4).

**Fig. 2.** MTA induces collagen synthesis in MC3T3-E1 cells. (A) The cells were cultured for 8 days in the absence or presence of MTA at indicated concentrations. Whole-cell lysates were subjected to western blotting (A). A quantitative analysis of intensity for each immunoreacted band for Type I collagen α1 (Col1a) (B) and Type III collagen α1 (Col3a) (C) was performed by Molecular Imager FX (Bio-Rad). Total RNA was extracted, reverse-transcribed and amplified by real-time PCR with primer sets for Col1a1 and Col3a1. Data are expressed as mean ± SEM (n = 4). Statistical significance of differences between MTA treated group and corresponding time-matched vehicle control is indicated by *P < 0.05 and ***P < 0.001.

**Fig. 3.** Production of MMPs in MTA-treated MC3T3-E1 cells. The cells were cultured in the absence or presence of MTA for 12 days. The medium was changed to serum-free conditioned medium. After 24 h, the conditioned medium was collected and concentrated. (A) MMP-9 was detected by gelatin-zymography as the precursor. (D) MMP-13 was detected by casein-zymography and western blotting as the precursor. A quantitative analysis of the intensity of each band was performed by Molecular Imager FX (Bio-Rad) (B and E). Total RNA was extracted and determined mRNA expression of Mmp9 (C) and Mmp13 (F) by real-time RT-PCR. Data represent the means ± SEM (n = 4). Statistical significance of differences between MTA treated group and corresponding time-matched vehicle control is indicated by *P < 0.05, **P < 0.01 and ***P < 0.001. Zymo, zymography; WB, western blotting.
3.3. Contribution of ER stress signaling to Bglap mRNA expression induced by MTA

As BMP signal through the expression of Runx2 enhances Bglap gene expression (Guo et al., 2012; Liu et al., 2013) and the Bglap gene was also driven by Atf6 (Jang et al., 2012), we examined Bmp-2, Bmp-4, and Atf6 expression in the presence or absence of MTA. MTA treatment affected neither BMP-2 nor BMP-4 expression (Fig. 5A–C). Similarly, there was no significant difference in BMP-2 protein level between MTA treated and untreated control groups.

Interestingly, Atf6 gene expression was significantly upregulated by MTA treatment on day 8 as the maximum induction (Fig. 5D), while mRNA levels of the other ER stress signaling transducer such as Atf4 and OASIS (old astrocyte specifically induced substance) were not changed (data not shown). Western blotting analysis showed a significant increase in Atf6 production by MTA treatment (Fig. 5E). As the activation ratio (p50Atf6 vs. total Atf6; 0.54 in control, 0.47 in 1 μg/mL MTA, 0.45 in 2.5 μg/mL MTA) did not change markedly, it was suggested that the MTA-induced increase in p50Atf6, the active form of Atf6, was due to enhancement of production rather than its activation efficiency (Fig. 5F).

3.4. Atf6 contributes to MTA-stimulated mineralization

To obtain direct evidence regarding the contribution of Atf6 in MTA-stimulated bone formation, we investigated whether p50Atf6 was transcriptionally active by MTA treatment. Because p50Atf6 drives the Bglap gene promoter (Jang et al., 2012), we performed ChIP assay for the promoter region of Bglap gene. Expectedly, MTA treatment led to more significant binding Atf6 to the promoter region of the Bglap gene (Fig. 6).
In addition, we constructed a Flag-tagged p50Atf6 (constitutively active Atf6) expression vector and investigated if forced expression of active Atf6 induces Bglap gene expression. Transient expression of the dominant active Atf6 protein, the level of which was 2.5-fold higher than the mock control, significantly upregulated Bglap mRNA expression (Fig. 7).

Finally, we investigated whether Atf6 contributes to MTA-stimulated mineralization. We constructed a Dox-inducible shRNA expression vector for Atf6 (pSingle-tTS-Atf6-shRNA) according to the Tet-on single-vector inducible RNA interference system. After transfection of pSingle-tTS-Atf6-shRNA vector into cells, stably transfected cells, which were selected by geneticin, were cloned and obtained for mineralization assay. Our data clearly showed that reduction of Atf6 expression by addition of Dox attenuated MTA-stimulated Bglap mRNA expression and also mineralization (Fig. 8) to a level equivalent to that of the control. These data suggested that MTA contributed to bone formation at least in part through Atf6.

4. Discussion

Osteoblast differentiation is regulated by a number of hormones and factors, such as TGF-β (transforming growth factor-β), BMP-2, and bFGF (basic fibroblast growth factor) (Hurley et al., 1994). Among them, BMP-2 is the most effective inducer of proliferation, differentiation, and mineralization in osteoblasts (Higuchi et al., 2002; Hiraki et al., 1991). Previous studies have shown that MTA promoted osteoblastic mineralization concomitant with increased BMP-2 mRNA expression (Ghasemi et al., 2014; Jeong et al., 2014). Here, we also investigated MTA-induced osteoblastic mineralization without BMP-2 mRNA elevation compared to controls. Several recent reports have revealed the contribution of ER stress signaling in osteoblastic differentiation. For example, Atf4 is enriched in osteoblasts and acts as a downstream effector of PERK (pancreatic ER kinase) through elf-2α activation in ER stress signaling to induce Bglap mRNA expression (Saito et al., 2011). In addition, it has been reported that Atf6 upregulates Bglap mRNA level (Jang et al., 2012) and that OASIS (old astrocyte specifically induced substance), which is a member of the CREB/Atf family, is highly expressed in osteoblasts and contributes to bone formation (Murakami et al., 2009). In the present study, we examined the expression of ER stress signaling transducers, such as Atf4, Atf6, and OASIS. Among them, only Atf6 expression was upregulated by MTA treatment. Moreover, p50 the activated form Atf6 was dose-dependently increased by addition of MTA. Because Atf6 plays an important role of differentiation in both odontoblast (Kim et al., 2014) and osteoblast (Jang et al., 2012), we examined whether Atf6 is the candidate “MTA activated transcription factor” to induce osteoblastic differentiation. As expected, MTA induced Bglap mRNA expression in a BMP-2-independent manner. Mineralized nodule formation was enhanced by MTA treatment, but its timing was not changed. Therefore, it was suggested that MTA induced osteoblastic differentiation at a rather late phase.

ER stress signaling plays an important role for expansion of the ER capacity to prevent ER overload-induced apoptosis by increasing production of ECM proteins, such as Type I collagen, and it is also associated with osteoblastic differentiation (Hino et al., 2010; Tohmonda et al., 2011). Here, we showed enhancement of tissue mineralization by
MTA is probably due to not simple induction of collagen production but also activation of collagen remodeling. To accumulate and form collagen fibers, Type III collagen has been shown to trigger Type I collagen fibrillogenesis (Liu et al., 1997). In addition, Type III collagen has growth stimulatory activity for osteoblasts (Maehata et al., 2007). Our data indicated that MTA increased not only both Types I and III collagen synthesis but also MMP-9 and MMP-13 expression. Several studies have shown that induction of osteoblastic differentiation concomitantly increased MMPs. For example, osteoblastic differentiation induced by ascorbate 2-phosphate concomitantly enhanced both MMP-9 and MMP-13 production (Mizutani et al., 2001). The proteolytic cascade by MMP-2/MMP-13/MT1-MMP was shown to be involved in osteoblastic differentiation through mechanical forces (Barthelemi et al., 2012). In addition, osterix, a master regulator of osteoblastic differentiation, induced both MMP-1 (Nakashima et al., 2002) and MMP-13 (Zhang et al., 2012). Our data indicated that maximal induction of Types III and I collagen gene expression by MTA appeared on days 4 and 8, respectively. Mmp-9 and Mmp-13 mRNA expression were seen on day 12, just before mineralization. Taken together, it seemed reasonable that MTA induced MMPs during osteoblastic differentiation through collagen remodeling.

Our preliminary experiment showed strong toxicity for osteoblasts near to the MTA disk. Therefore, we used the medium-soluble fraction of MTA, according to the protocol (Hakki et al., 2009). The medium-soluble fraction, which may contain biologically active substances released from MTA, did not show any obvious toxicity for MC3T3-E1 cells, but stimulated MMP, collagen, and osteocalcin mRNA expression. Although MTA contains calcium silicate, bismuth oxide, and calcium aluminate (Camilleri, 2008), the ratio of substances released from MTA may differ according to the protocol. This may explain the discrepancies regarding BMP-2 expression by MTA. Our data, at least in part, suggested that substances released from MTA in the root were able to diffuse and affect distal osteoblasts in alveolar bone for late-stage differentiation and tissue mineralization in vivo through ER stress signaling.

5. Conclusions

Hashiguchi et al. (2011) investigated that MTA inhibits bone resorption. Taken together, we concluded that MTA in endodontic treatment may affect alveolar bone healing in the resorbed region caused by pulpal infection.

Conflict of interest

The authors confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

Acknowledgments

A part of this work was supported by JSPS KAKENHI Grant (#21791862 and #10382756).
References

Agata, Y., Katakai, T., Ye, S.K., Sugai, M., Gonda, H., Honjo, T., et al., 2001. Histone acetylase
treatment determines the developmentally regulated accessibility for T cell receptor γ gene recombination. J. Exp. Med. 193, 873–880.

Barthelémi, S., Robinet, J., Cardinal, M., Hesse, E., Girard, R., et al., 2012. Mechanical forces-induced human osteoblast differentiation involves MMP-2;F-13/MT1-MMP proteolytic cascade. J. Biochem. 113, 760–772.

Bemba, P.F., Azuma, M.M., Ferreira, L.L., Dezan-Júnior, E., Gomes-Filho, J.E., Cintra, L.T., 2013. Root reconstructed with mineral trioxide aggregate and guided tissue regeneration in apical surgery: a 5-year follow-up. Braz. Dent. J. 24, 428–432.

Camilli, J., 2006. The chemical composition of mineral trioxide aggregate. J. Conserv. Dent. 9, 141–143.

Chen, X., Shen, J., Prywes, R., 2002. The luminal domain of ATF6 senses endoplasmic reticulum (ER) stress and causes translocation of ATF6 from the ER to the Golgi. J. Biol. Chem. 277, 13045–13052.

Ducy, P., Desbois, C., Boyce, B., Pinero, G., Story, B., Dunstan, C., et al., 1996. Increased bone formation in osteocalcin-deficient mice. Nature 382, 448–452.

Ghasemi, N., Rahimi, S., Lotfi, M., 2007. BMP2 protein enhances cell survival, gene expression associated with mineralized tissues, and biomineralization of cementoblasts. J. Endod. 2007, 33, 912–920.

Hiraki, Y., Inoue, H., Shigeno, C., Sanma, Y., Bentz, H., Rosen, D.M., et al., 1991. Bone mor-phogenetic proteins (BMP-2 and BMP-3) promote growth and expression of the differ-entiated phenotype of rabbit chondrocytes and osteoblastic MC3T3-E1 cells. J. Bone Miner. Res. 6, 1373–1385.

Hiraki, Y., Inoue, H., Shigeno, C., Sanma, Y., Bentz, H., Rosen, D.M., et al., 1991. Bone mor-phogenetic proteins (BMP-2 and BMP-3) promote growth and expression of the differ-entiated phenotype of rabbit chondrocytes and osteoblastic MC3T3-E1 cells. J. Bone Miner. Res. 6, 1373–1385.

Hurley, M.M., Abreu, C., Gronowicz, G., Kawaguchi, H., Lorenzo, J., 1994. Expression and regulation of basic fibroblast growth factor mRNA levels in mouse osteoblastic MC3T3-E1 cells. J. Biol. Chem. 269, 9392–9396.

Jang, W.G., Kim, E.J., Kim, D.K., Ryoo, H.M., Lee, K.B., Kim, S.H., et al., 2012. BMP2 protein regulates osteocalcin expression via Runx2-mediated Atf6 gene transcription. J. Biol. Chem. 287, 905–915.

Jeong, Y., Kang, W., Ko, H., Kim, M., 2014. The effects of bone morphogenetic protein-2 and enamel matrix derivative on the bioactivity of mineral trioxide aggregate in MC3T3-E1 cells. Restor. Dent. Endod. 39, 187–194.

Kato, Y., Lambert, C.A., Colige, A.C., Mineur, P., Noël, A., Frankenne, F., et al., 2005. Acidic chondrocyte differentiation and bone formation. J. Biol. Chem. 280, 10938–10944.

Kaufman, R.J., 1999. Stress signaling from the lumen of the endoplasmic reticulum: coordination of gene transcriptional and translational controls. Genes Dev. 13, 1211–1226.

Kim, J.W., Choi, H., Yoon, C.G., Oh, S.H., Hur, S.W., Lee, B.N., et al., 2009. Signaling mediated by the endoplasmic reticulum stress transducer OASIS is involved in bone formation. Nat. Cell. Biol. 11, 1205–1217.

Kobayashi, K., Zhou, X., Kunkel, G., Zhang, Z., Deng, J.M., Behringer, R.R., et al., 2002. The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. Cell 108, 17–29.

Koyama, M., Torabinejad, M., 2010. Mineral trioxide aggregate: a comprehensive literature review – part II: clinical applications, drawbacks, and mechanism of action. J. Endod. 36, 400–413.

Price, P.A., Bao, J.L., 2005. MMP13: a comprehensive literature review – part I: clinical applications, drawbacks, and mechanism of action. J. Endod. 31, 1785–1794.

Qin, H., Cai, J., Fang, J., Xu, H., Gong, Y., 2010. Could MTA be a novel medicine in the cure of traumatic injury? J. Orthop. Trauma 24, 386–390.

Roy, B., Li, W.W., Lee, A.S., 1996. Calcium-sensitive transcriptional activation of the proximal CAAT regulatory element of the grp78/BiP promoter by the human nuclear factor C/EBP. J. Biol. Chem. 271, 28995–29002.

Sato, A., Ochiha, K., Sondoh, S., Tsumagari, K., Murakami, T., Cavener, D.R., et al., 2011. Endoplasmic reticulum stress response mediated by the PEKR-ERK2-AKT4 pathway is involved in osteoblast differentiation induced by BMP2. J. Bone Miner. Res. 26, 4908–4918.

Yeom, J.T., Miyachi, Y., Ghosh, R., Yoda, M., Uchikawa, T., Takito, J., et al., 2011. The IRE1α-XBP1 pathway is essential for osteoblast differentiation through promoting transcription of osterix. EMBO Rep. 12, 451–457.

Wang, X., Schroder, H.C., Feng, Q., Diehl-Seifert, B., Grebenjuk, V.A., Muller, W.E., 2014a. Matrix metalloproteinase 13 (MMP13) is a direct target of vitamin K-dependent bone protein by osteosarcoma cells. J. Biol. Chem. 289, 11660–11665.

Wang, X., Schroder, H.C., Feng, Q., Diehl-Seifert, B., Grebenjuk, V.A., Muller, W.E., 2014b. Matrix metalloproteinase 13 (MMP13) is a direct target of vitamin K-dependent bone protein by osteosarcoma cells. J. Biol. Chem. 289, 11660–11665.

Yeom, J.T., Miyachi, Y., Ghosh, R., Yoda, M., Uchikawa, T., Takito, J., et al., 2011. The IRE1α-XBP1 pathway is essential for osteoblast differentiation through promoting transcription of osterix. EMBO Rep. 12, 451–457.

Wu, B.C., Huang, S.C., Ding, S.J., 2013. Comparative osteogenesis of radiopaque dicalcium silicate cement and white-colored mineral trioxide aggregate in a rabbit femoral model. Materials 2013, 6275–5680.

Yazdizadeh, M., Bouzarjomehri, Z., Khalighinejad, N., Sadri, L., 2013. Evaluation of apical microleakage in open apex teeth using MTA apical plug in different sessions. ISRN Dent. 2013, 959813.

Ye, J., Lawton, R.B., Kornuro, R., Chen, X., Dave, U.P., Prywes, R., et al., 2000. ER stress indu-ces cleavage of membrane-bound ATF6 by the same proteases that process SREBP. Mol. Cell. 5, 1364–1375.

Zhang, C., Tang, W., Li, Y., 2012. Matrix metalloproteinase 13 (MMP13) is a direct target of osteoblast-specific transcription factor osterix (Osterix) in osteoblasts. PLoS One 7, e50252.