Matrix Gla protein negatively regulates calcification of human aortic valve interstitial cells isolated from calcified aortic valves

（石灰化大動脈弁由来のヒト大動脈弁間質細胞はマトリックスGla蛋白によって石灰化が抑制される）

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Abstract

Calcified aortic valve stenosis (CAS) is a common heart valve disease in elderly people, and is mostly accompanied by ectopic valve calcification. We recently demonstrated that tumor necrosis factor-α (TNF-α) induces calcification of human aortic valve interstitial cells (HAVICs) obtained from CAS patients. In this study, we investigated the role of matrix Gla protein (MGP), a known calcification inhibitor that antagonizes bone morphogenetic protein 2 (BMP2) in TNF-α-induced calcification of HAVICs. HAVICs isolated from aortic valves were cultured, and calcification was significantly induced with 30 ng/mL TNF-α. Gene expression of the calcigenic marker, BMP2, was significantly increased in response to TNF-α, while the gene and protein expression of MGP was strongly decreased. To confirm the role of MGP, MGP-knockdown HAVICs and HAVICs overexpressing MGP were generated. In HAVICs, in which MGP expression was inhibited by small interfering RNA, calcification and BMP2 gene expression were induced following long-term culture for 32 days in the absence of TNF-α. In contrast, HAVICs overexpressing MGP had significantly decreased TNF-α-induced calcification. These results suggest that MGP acts as a negative regulator of HAVIC calcification, and as such, may be helpful in the development of new therapies for ectopic calcification of the aortic valve.

Keywords: aortic valve stenosis; human aortic valve interstitial cells; matrix Gla protein; aortic valve calcification

1. Introduction

Aortic valve stenosis (AS) is one of the most common heart valve conditions in elderly people (1). The main etiologies of AS are rheumatic changes, congenital bicuspid valve, and degenerative calcification (2). As the country's population has shifted towards older ages, calcific aortic valve stenosis (CAS) has become the most common cause of AS instead of rheumatic changes (3, 4). Aortic valves from patients with CAS are characterized by extensive fibrotic thickening of the valve leaflets and focal ectopic calcification (5). Aortic valve calcification is irreversible. The most effective treatment is surgical aortic valve replacement; however, this treatment is invasive (6).

Recently, transcatheter aortic valve replacement (TAVR) has become popular as a less invasive treatment for AS patients (7). In combination with TAVR, to delay the progression of CAS, development of a medication-based treatment is important. It is necessary to establish the detailed mechanism underlying aortic valve calcification. Although several groups have focused on identifying this mechanism, it has not yet been established (8-10). CAS is an active process that appears to correlate with several inflammatory factors (11). Inflammation is a prominent feature of aortic valve calcification, and may develop due to endothelial dysfunction fueled by atherosclerotic risk factors (12, 13). Macrophages and T lymphocytes have been identified in calcified aortic valve lesions (14, 15). These immune cells release cytokines, including tumor necrosis factor-α (TNF-α) (16), transforming growth factor-β1 (17), and interleukin-1 (18), all of which contribute to extracellular matrix formation, remodeling, and local calcification.

TNF-α is a pleiotropic cytokine that is mainly secreted by activated macrophages and T lymphocytes in response to many factors including exposure to oxidized low-density lipoprotein (19), damaged extracellular matrix (20), or bacterial infection (21). TNF-α has been detected in the atherosclerotic lesions and calcified aortic valves of both humans and mice, and plays an important role in ectopic calcification of the aortic valve (16, 22). Our recent study reported that TNF-α promotes osteogenic differentiation of human aortic valve interstitial cells (HAVICs) obtained from CAS patients by stimulating the nuclear factor kappa-light-chain- enhancer of activated B cells (NF-κB) signaling pathway. NF-κB activates the gene expression and activity of bone morphogenetic protein 2 (BMP2), which induces the gene expression of SMAD4, Runt-related gene 2 (Runx2), and Distal-less homeodomain 5 (Dlx5) and alkaline phosphatase (ALP) activation (23). However, inhibition of the signaling pathway upstream of BMP2 only partially inhibits TNF-α- induced calcification of HAVICs. Consequently, we hypothesized that another pathway for inducing HAVIC calcification may exist.

Matrix Gla protein (MGP), a γ-carboxyglutamic acid-rich and vitamin K-dependent protein, is a well-known ectopic calcification inhibitor (24). Mice born lacking the MGP gene have extensive calcification of the large elastic and muscular arteries and heart valves, and die within 2 months from arterial rupture and heart failure (25). Recently, Yao et al. reported that MGP inhibits BMP2 and protects against atherosclerosis and vascular calcification (26). These reports suggest that MGP may be a naturally occurring inhibitor of ectopic cardiovascular calcification. However, there have been no reports on the interaction between TNF-α and MGP.

In this study, we investigated and confirmed the role of MGP in TNF-α-induced calcification of HAVICs. Our results showed that TNF-α substantially downregulated MGP gene expression in HAVICs. Investigating the role of MGP using MGP knockdown HAVICS and HAVICs overexpressing MGP, which were obtained from CAS patients, revealed that MGP is a negative regulator of aortic valve calcification.

2. Materials and methods

2.1 Materials

Alpha Modified Eagle's Medium (α-MEM) was obtained from Nacalai Tesque (Kyoto, Japan). Fetal bovine serum (FBS, Biofluids™) and penicillin and streptomycin (Gibco™; Gaithersburg, MD, USA) were obtained from Invitrogen (Carlsbad, CA, USA). TNF-α and other analytical grade reagents were purchased from Wako Pure Chemical Industries (Osaka, Japan). All primers used for quantitative real-time polymerase chain reaction (qPCR) were obtained from Fasmac (Kanagawa, Japan). Power SYBR® Green PCR Master Mix was supplied by TOYOBO (Osaka, Japan). Control small interfering RNA (siRNA) and MGP siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All chemicals used were of the highest purity commercially available. All solutions were freshly made at sufficiently high concentrations that only very small volumes had to be added to the culture medium. Primary mouse antibodies against human MGP and ß-actin, and a secondary goat anti- mouse IgG antibody conjugated to Alexa Fluor 680, were purchased from Invitrogen.

2.2 Isolation and culture of HAVICs

Human aortic valves were obtained from patients with calcific aortic valve stenosis (CAS group; mean age 64.3 ± 5.8 years, n=6) and patients with aortic dissection or aortic regurgitation (Non-CAS group; mean age 66.0 ± 10.0 years, n=4) who underwent aortic valve replacement at Hirosaki University Hospital (Aomori, Japan). There were no statistically significant differences in clinical features associated with CAS between these two groups (data not shown). All patients gave written informed consent, and the study was approved by the Institutional Review Board of the Hirosaki University Hospital. Human aortic valve specimens were gently cut into 2 ± 1 mm long pieces, and HAVICs were isolated by collagenase digestion as previously described (23, 27). The cells were cultured in α-MEM containing 10% FBS, and the fourth passage of cells was used in all experiments. After the HAVICs reached confluency they were further cultured in the presence or absence of 30 ng/mL TNF-α for 7 or 14 days, with the medium changed every 3–4 days.

2.3 Identification of calcification

Cells were seeded in a 12- or 96-well plate and grown for 3 d until confluent. Then they were further cultured with or without 30 ng/mL TNF-α for 7, 12, 14, 20, or 32 day. The degree of calcification was quantified using Alizarin Red S staining (28) visualized with a digital camera (Nikon, Tokyo, Japan). Alizarin Red S dye released from the extracellular matrix following incubation of the cells in 100 mM aqueous

cetyl-pyridinium chloride solution was quantified by spectrophotometry at 550 nm (29).

2.4 Measurement of gene expression

Total RNA was isolated from cells using a QuickGene RNA cultured cell kit S (Fujifilm, Tokyo, Japan). An aliquot of total RNA was reverse transcribed to cDNA using random primers. For qPCR, the cDNA was amplified (ABI PRISM 7000, Life Technologies, Carlsbad, CA, USA) for 40 PCR cycles (95°C for 15 s and 60°C for 1 min) after initial denaturation at 95°C for 1 min. The reaction volume contained 3 µL of a 1:4 dilution of the first-strand reaction product, 0.6 µL of 10 µM specific forward and reverse primers, 0.4 µL of 50× ROX reference dye, 5.4 µL pure water, and 10 µL SYBR qPCR, and was adjusted to a final volume of 20 µL. The primers used for MGP, BMP2, osteocalcin, and glyceraldehyde 3-phosphate dehydrogenase (G3PDH) were designed using NCBI Primer BLAST (The National Center for Biotechnology Information, Bethesda, MD, USA) and their sequences are shown in Table 1. Amplification of the housekeeping gene G3PDH served as the normalization standard. The qPCR data are represented as cycle threshold (Ct) levels and were normalized using the individual G3PDH control Ct values. Relative gene expression was calculated using the 2(-delta delta Ct) method (30).

2.5 Western blot analysis of MGP

Cytoplasmic extracts were obtained by lysing the cells in 20 mM Tris-HCl, pH 7.4, containing 0.05% Triton X-100. After a protein assay using Pierce Quantitative colorimetric peptide assay (Thermo Scientific, Waltham, MA, USA), 0.2 µg of each protein was resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and separated on a Bolt® Bis-Tris Plus 4–12% [MES] SDS-PAGE gel (Thermo Scientific). Proteins were transferred to a polyvinylidene difluoride membrane using the iBlot™ Dry Blotting System (Thermo Scientific). Then membranes were incubated with primary antibody (MGP 1:1000, ß-actin 1:1000), followed by Alexa Fluor 680 goat anti-rabbit secondary antibody using an iBind™ Western System (Thermo Scientific). Proteins were detected using the Odyssey Imaging System (LI-COR Biosciences, Lincoln, NE, USA).

2.6 HAVICs with MGP gene knockdown

HAVICs were seeded at a density of 2×10^4 cells per well into a 96-well plate and cultured overnight. Cells were treated with 100 pM control and MGP siRNA using MultiFectam transfection reagent (Promega, Madison, WI, USA), according to the manufacturer's instructions. The mixture was applied dropwise to the cells, after which they were incubated at 37°C for 4 h. The reaction mixture was changed to normal medium following transfection. For stable reduction of MGP gene expression, the transfection media was replenished at 4 days intervals. qPCR and western blots were used to verify the effect of MGP siRNA transfection. The transfection schema is depicted in Figure 1A.

2.7 HAVICs overexpressing the MGP gene

HAVICs were seeded at a density of 1×10^4 cells per well in complete growth medium in a 96-well plate to obtain 50–70% confluence the following day of transfection. A mixture of 1 μg plasmid DNA (cytomegalovirus [CMV]-LacZ or CMV-MGP-tagged with myc) in Opti-MEM I and Turbofectin 8.0 transfection reagent (Origene Technologies, Rockville, MD, USA) was incubated for 15 min at room temperature. The mixture was added dropwise to the cells, and the cells were incubated at 37°C for 24 h. The reaction mixture was changed to normal medium following transfection. For constitutive MGP overexpression, the transfection medium was replenished at 4 days intervals. The transfection schema is depicted in Figure 1B.

2.8 Statistical analysis

All statistical analyses were performed using KyPlot 5.0 software (Kyenslab, Tokyo, Japan). Group comparisons were performed by analysis of variance using a Student– Newman–Keuls post hoc correction procedure. Comparisons between two independent data sets were assessed by Student's t-test. Values are presented as the mean ± standard error of the mean. P values less than 0.05 were considered statistically significant.

3. Results

3.1 Effects of TNF-α on HAVIC calcification and MGP expression

HAVICs obtained from patients with calcific aortic valve stenosis (CAS group, n=6) and from patients with aortic dissection or aortic regurgitation (Non-CAS group, n=4) were cultured in the presence or absence of 30 ng/mL TNF-α for 14 d after reaching confluency. Computed tomography scans did not detect calcification in aortic valves obtained from the Non-CAS group. HAVICs from the CAS group were positively stained with Alizarin Red S (Fig. 2A), and spectrophotometric quantification showed that calcification was accelerated by TNF-α in the CAS group (Fig. 2B). In both the CAS and Non-CAS groups, MGP gene expression was strongly decreased during 14 days of treatment with TNF-α compared with Control (Fig. 2C). In the CAS group, MGP protein expression was significantly and constitutively decreased at days 2 and 7 (Fig. 2D). BMP2 gene expression, an index of calcification, was significantly increased in the CAS group (Fig. 2E). In a previous study, we confirmed that BMP2 gene expression of HAVICs obtained from the Non-CAS group was not altered in the presence of TNF-α (23). Thus, to determine the pathophysiological role of MGP, we used HAVICs from the CAS group, which has high sensitivity to TNF-α-induced calcification. These data suggest the possibility that decreased MGP gene and protein expression induces increased sensitivity of TNF-α-induced calcification of HAVICs obtained from CAS groups.

3.2 Inhibition of MGP gene expression induces calcification of HAVICs

To confirm whether the inhibition of MGP gene expression induces the HAVICs calcification like TNF-α administration, we transfected MGP siRNA into HAVICs obtained from the CAS group such that MGP expression was stably reduced (Fig. 1A). MGP expression was significantly decreased 2 and 7 days after transfection, in

a similar manner to that observed with TNF-α administration (Fig. 3A). This reduced expression persisted 20 and 32 days after the first transfection such as TNF-α administration (Supplemental Fig. 1A). We confirmed that total RNA level, reflecting cell viability isolated from each cultured cell, was maintained during culture periods between 0 and 32 days (data not shown). MGP knockdown for 32 days (Fig. 3B), but not 14 days (Supplemental Fig. 1B), significantly induced calcification of HAVICs (Fig. 3C). MGP protein expression was also significantly reduced at day 7 (Fig. 3D). We further confirmed that BMP2 gene expression was increased with MGP gene knockdown (Fig. 3E). These results suggest that continuous inhibition of MGP gene expression induces calcification of HAVICs is similar to that observed with TNF-α administration.

3.3 MGP gene overexpression inhibits TNF-α-induced calcification of HAVICs

To confirm whether MGP gene overexpression in HAVICs can inhibit TNF-α-induced calcification, we stably overexpressed the MGP gene in HAVICs obtained from the CAS group (Fig. 1B). MGP gene expression was substantially increased 2 days after transfection, whereas Empty CMV vector negative control did not alter MGP expression levels (Fig. 4A). TNF-α-induced calcification was strongly inhibited in these cells by MGP overexpression (Fig. 4B, C). On the other hand, BMP2 gene expression was not inhibited by MGP overexpression (Fig. 4D). In HAVICs containing Empty vector, BMP2 expression appeared to increase, but an additional increase in expression did not occur with MGP gene overexpression. Together, these results strongly suggest a possibility that elevated MGP effectively inhibits the ectopic calcification of HAVICs by inhibiting BMP2 activity.

4. Discussion

In this study, we demonstrated that TNF-α induces both calcification of HAVICs and inhibition of the expression of MGP, a known antagonist of BMP2. Conversely, long-term inhibition of MGP gene expression by siRNA induced calcification of HAVICs obtained from CAS. We further confirmed that MGP gene overexpression significantly inhibited TNF-α-induced calcification of HAVICs. These findings suggest that MGP plays a critical role as a negative regulator of HAVIC calcification.

Mohler et al. reported that TNF-α elevated BMP2 expression via the NF-□B pathway (31). Venardos et al. reported that, in normal aortic valve interstitial cells, TNF-α administration induces the gene expression of various calcigenic markers (32). Our previous study demonstrated that TNF-α-induced calcification of HAVICs obtained from CAS but not Non-CAS patients without calcified aortic valve proceeded via the BMP2-Dlx5-ALP pathway (23). Thus, we further investigated the role of MGP on the ectopic calcification using HAVICs obtained from CAS patients. In this study, we confirmed that long-term reduction of MGP gene expression by siRNA induced BMP2 gene expression, resulting in calcification of HAVICs. Boström et al. also reported that, in human aortic endothelial cells, MGP siRNA induced BMP2 gene expression in high glucose condition (33). In addition, overexpression of the MGP gene in HAVICs completely inhibited calcification of HAVICs in the presence of TNF-α without inhibition of BMP2 gene expression (Fig. 4D). Because MGP tends to colocalize with BMP2, these results suggest that excess carboxylated MGP, a post-translational product, completely inhibits BMP2 activity

through its antagonizing effects, resulting in the inhibition of HAVICs calcification (34). In contrast, the reduction in MGP gene expression induced BMP2 gene expression. This is particularly the case for HAVICs obtained from the CAS group, in which reduced expression of MGP contributed to the acceleration of TNF-α-induced calcification through the NF-κB-BMP2-ALP pathway (23). These results suggest that TNF-α-induced calcification of HAVICs includes two signaling pathways: NF-κB signaling activation and inactivation of BMP2 inhibition by MGP (Fig. 5). In Western blot analysis, we measured both undercarboxylated and carboxylated MGP. The small but significant decrements of MGP protein expression by TNF-α or MGP siRNA in comparison with MGP gene expression (Figures 2C, 2D, 3A, and 3D) is ascribable the accumulation of post-translationally carboxylated MGP synthesized by γ-glutamyl carboxylase.

Osteocalcin is member of a family of vitamin-K2 dependent, Gla-containing proteins like MGP, and is a known marker of calcification. Both osteocalcin and MGP are members of a family of vitamin K-dependent proteins. They are believed to have diverged from a common ancestor and share some common protein features including similar calcium binding sequences (35). Viegas et al. reported that in the calcified aortic valves obtained from CAS patients, the gene expression of both MGP and osteocalcin is significantly increased (36). To directly confirm these similarities, we investigated whether TNF-α alters osteocalcin gene expression in HAVICs, and found that its expression was unchanged in the presence of TNF-α (Supplemental Fig. 2A). We also found that vitamin K2, an enhancer of the vitamin K cycle which is associated with preparation of Gla proteins, had no effect on TNF-α-induced reduction in MGP gene expression (Supplemental Fig. 2B). These results suggest a

possibility that TNF-α-induced reduction in MGP gene expression in HAVICs obtained from CAS patients is not related to activation of the vitamin K cycle, generally existing in the endoplasmic reticulum of liver cells.

The main limitation of this study is that the molecular mechanism of the TNF-α- induced reduction in the gene expression of MGP, but not osteocalcin, remains unclear. Vitamin D, parathormone, Runx2, and retinoic acid enhance both MGP and osteocalcin genes expression by promoter activation (37-39). We hypothesized that TNF-α selectively interacts with the MGP promoter sequence to inhibit MGP gene transcription. To explain this discrepancy, the detailed mechanism underlying the TNF-α-induced reduction in MGP gene expression then the inhibition of BMP2 gene expression by MGP will need further investigation. Another limitation is that in this study, we investigated with the use of HAVICs but not mesenchymal undifferentiated cells. We recently demonstrated that mesenchymal undifferentiated cells are abundant in the calcified aortic valve, and the cells expressing on their surface CD73, CD90 and CD105, while lacking the expression of CD34, and CD45 surface markers were more sensitive to the calcification induction (40). Future studies should investigate each undifferentiated cell population in HAVICs to determine the pathophysiological roles of MGP in these cells.

In conclusion, we demonstrated that TNF-α substantially reduces MGP gene expression in HAVICs. We also demonstrated that inhibition of MGP gene expression induces calcification of HAVICs obtained from CAS patients and BMP2 gene expression, and that MGP overexpression significantly inhibits TNF-α-induced calcification of HAVICs. Together, these findings suggest that MGP plays a critical

role as the negative regulator of calcification of HAVICs through BMP2 expression. Our results will help to further clarify the mechanism underlying aortic valve calcification and develop new therapies for CAS.

Conflicts of interest

The authors have no financial conflicts of interest to declare.

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Figure Legends

Fig. 1.

Experimental schemata of MGP gene knockdown and overexpression in HAVICs. A: Schema of MGP gene knockdown in HAVICs. HAVICs (2×10^4 cells) were seeded into a 96-well plate and cultured overnight. Cells were treated with control siRNA and MGP siRNA (100 pM) using MultiFectam transfection reagent, according to the manufacturer's instructions. The mixture was added dropwise to the cells, and the cells incubated at 37°C for 4 h. Following transfection, the reaction mixture was replaced with normal medium. The transfection media was replenished at 4 days intervals to maintain constitutive MGP gene knockdown. B: MGP gene overexpression in HAVICs. The day before transfection, HAVICs were seeded at a density of 1×10^4 cells per well in a 96-well plate in complete growth medium, to obtain 50–70% confluence the following day. A mixture of 1 μg plasmid DNA (cytomegalovirus (CMV)-LacZ or CMV-MGP-tagged with myc) in Opti-MEM I and Turbofectin 8.0 transfection reagent was incubated for 15 min at room temperature. The mixture was added dropwise to the cells, and the cells incubated at 37°C for 24 h. Following transfection, the reaction mixture was replaced with normal medium. The transfection medium was replenished at 4 days intervals to maintain constitutive MGP gene overexpression.

Fig. 2.

TNF-α reduces MGP gene and protein expression in HAVICs. HAVICs were cultured in α-MEM containing 10% FBS. After reaching confluence (day 0), HAVICs were cultured for a further 14 days. A: Typical images of Alizarin Red S staining of HAVICs from a CAS (CAS) patient and a control patient with aortic regurgitation (Non-CAS) in the presence or absence of 30 ng/mL TNF-α. B: Calcification of HAVICs from both Non-CAS and CAS groups in the presence or absence of TNF-α was measured and quantified using Alizarin Red S staining. All ratios were calculated against the Non-CAS group at day 0. Gray bars: Non-CAS group, black bars: CAS group at days 0, and 14. Bars represent the mean ± standard error of the mean (SEM; CAS group: n = 4, Non-CAS group: n = 4). A significant difference ($P < 0.05$) between the CAS and Non-CAS groups at day14 is indicated with '#', and a significant difference ($P < 0.05$) when compared against control at day 0 in CAS group is indicated by '*'. C: qPCR analysis of MGP mRNA expression in HAVICs isolated from CAS and Non-CAS groups. After reaching confluence (day 0), HAVICs were further cultured in α-MEM containing 10% FBS for day 7 or day 14 in the absence or presence of TNF-α. Gray and black bars represent the mRNA levels detected in HAVICs isolated from the Non-CAS (n = 4) and CAS (n=5) groups, respectively. Significant differences between the day of treatment and day 0 for each group are indicated with '#' ($P < 0.05$) and '##' ($P < 0.01$). D: Western blot analysis of MGP protein (upper images) in HAVICs obtained from CAS patients. After reaching confluence (day 0), HAVICs were further cultured in the presence of 30 ng/mL TNF-α for days 2 and 7. Band intensity was normalized to the level of ß-actin as an internal standard (lower images). Lane 1, Control (day 2); lane 2, 30 ng/mL TNF-α (day 2); lane 3, Control (day 7); lane 4, 30 ng/mL TNF-α (day 7). Values are the mean ± SEM (CAS group: n =4). Significant difference ($P < 0.05$)

between the TNF-α treatment and Control is indicated with '#'. E: qPCR analysis of BMP2 mRNA expression in HAVICs isolated from the CAS group. After reaching confluence (day 0), HAVICs were further cultured in α-MEM containing 10% FBS for day 7. White and black bars represent the mRNA levels in HAVICs in the absence and presence of TNF-α, respectively. All ratios were calculated against the TNF-α (-) group at day 0. Gene expression levels were normalized using G3PDH gene expression. Bars represent the mean ± SEM (CAS group: n = 4). A significant difference ($P < 0.05$) with the TNF-α (-) group at day 0 is indicated with '#'.

Fig. 3.

MGP gene knockdown induces calcification of HAVICs. HAVICs obtained from the CAS group were cultured in α-MEM containing 10% FBS. Cells (2×10^4 cells) were seeded into a 96-well plate. After culturing overnight (day 0), HAVICs were transfected with MGP siRNA or control siRNA and cultured for a further 32 days, as described as Figure 1A. TNF-α was continuously administrated every 3–4 days as a positive control for calcification of HAVICs. A: qPCR analysis of MGP mRNA expression at day 2 and 7 in HAVICs isolated from the CAS group. All ratios are calculated against the expression level at day 0. Gene expression levels were normalized to G3PDH gene expression levels. Bars represent the mean ± standard error of the mean (SEM; CAS group: n = 6). White bar: Control, black bar: TNF-α (+), blue bar: Control siRNA, and red bar: MGP siRNA. A significant difference when compared against Control is indicated by '*' ($P < 0.01$), and a significant difference when compared against the Control siRNA is indicated by '#' ($P < 0.05$). B: Representative images of Alizarin Red S staining from five different HAVIC

populations isolated from patients with CAS in the presence or absence of 30 ng/mL TNF-α, Control siRNA, or MGP siRNA. C: Calcification of HAVICs was measured and quantified using Alizarin Red S staining. All ratios were calculated against the TNF-α (-) group at day 32. Bars represent the mean ± SEM (CAS group: n = 5). White bar: TNF-α (-), black bar: TNF-α (+), green bar: transfection reagent alone, blue bar: Control siRNA, and red bar: MGP siRNA. A significant difference when compared against TNF-α (-) at day 32 is indicated by '*' ($P < 0.05$), and a significant difference when compared against the Control siRNA at day 32 is indicated by '#' ($P < 0.05$). D: Western blot analysis of MGP protein expression (upper images) at days 2 and 7 in HAVICs isolated from patients with CAS. Band intensity was normalized to the β-actin levels (lower images). Lane 1: Control siRNA (Day 2), lane 2: MGP siRNA (Day 2), lane 3: Control siRNA (Day 7), and lane 4: MGP siRNA (Day 7). Values are the mean ± SEM of four experiments. Significant differences when compared against Control siRNA are indicated by '#' ($P < 0.05$). E: qPCR analysis of BMP2 mRNA expression at day 2 in HAVICs isolated from CAS patients. All ratios were calculated against the TNF-α (-) group at day 0. Gene expression levels were normalized to G3PDH gene expression levels. Bars represent the mean ± SEM (CAS group: n = 5). Black bar: TNF-α administration, blue bar: Control siRNA, and red bar: MGP siRNA. Significant differences when compared against Control siRNA are indicated by '#' ($P < 0.05$).

Fig. 4.

Overexpression of MGP in HAVICs inhibits TNF-α-induced calcification. HAVICs obtained from the CAS group were cultured in α-MEM containing 10% FBS.

HAVICs were seeded at a density of 1×10^4 cells per well in a 96-well plate in complete growth medium. After overnight culturing (day 0), HAVICs were transfected either with CMV expressing the MGP gene or with Empty CMV, and were cultured for a further 12 days in the presence or absence of TNF-α (as described as Fig. 1B). A: qPCR analysis of MGP mRNA expression in HAVICs at day 2. All ratios were calculated against the control group at day 0. Gene expression levels were normalized to G3PDH gene expression levels. Bars represent the mean ± standard error of the mean (SEM; CAS group: n = 4). Black bar: 30 ng/mL TNF-α, light green bar: Transfection reagent alone, green bar: TNF-α + transfection reagent, light blue bar: Empty CMV control, blue bar: TNF-α + empty CMV control, light red bar: CMV overexpressing MGP, red bar: TNF-α + CMV overexpressing MGP. The left axis is for the MGP gene expression of TNF-α alone, transfection reagent alone, empty CMV alone, TNF-α + transfection reagent, and TNF-α + empty CMV control. The right axis is for the MGP gene expression of CMV overexpressing MGP and TNF-α + CMV overexpressing MGP. A significant difference when compared against Empty CMV control is indicated by '#' ($P < 0.01$), and a significant difference when compared against TNF-α + CMV overexpressing MGP is indicated by '*' ($P < 0.05$). B: Representative images of Alizarin Red S staining from four different HAVIC populations isolated from a patient with CAS at day 12 in the presence or absence of 30 ng/mL TNF-α, Empty CMV control, or CMV overexpressing MGP. C: Calcification of HAVICs was measured and quantified using Alizarin Red S staining. All ratios were calculated against TNF-α (-) at day 14. Bars represent the mean ± SEM (CAS group: n = 4). White bar: TNF-α (-), black bar: TNF-α, light green bar: Transfection reagent alone, green bar: TNF-α + transfection reagent, light blue bar: Empty CMV control, blue bar: TNF-α + empty CMV control,

light red bar: CMV overexpressing MGP, red bar: TNF-α + CMV overexpressing MGP. Significant differences when compared against TNF-α (-) are indicated by '#' ($P < 0.05$). D: qPCR analysis of BMP2 mRNA expression in HAVICs isolated from CAS patients at day 2. All ratios were calculated against the TNF-α (-) group at day 0. Gene expression levels were normalized to G3PDH gene expression levels. Bars represent the mean ± SEM (CAS group: $n = 4$). Black bar: TNF-α, light green bar: Transfection reagent alone, green bar: TNF-α + transfection reagent, light blue bar: Empty CMV control, blue bar: TNF-α + empty CMV control, light red bar: CMV overexpressing MGP, red bar: TNF-α + CMV overexpressing MGP. Significant differences when compared against TNF-α are indicated by '#' ($P < 0.05$).

Fig. 5.

Proposed mechanism for TNF-α-induced calcification of HAVICs obtained from the CAS group. Black arrows indicate the pathway obtained by previous study (23). TNF- α accelerates BMP2 gene expression via translocation of NF-□B into the nucleus, which stimulates Dlx5 gene expression. This results in ALP activation, followed by calcification, via Runx2 activation (23). Red arrows indicate the pathway supported by experimental data. In this study, we demonstrated that TNF-α also strongly decreased MGP gene expression. We also demonstrated that inhibition of MGP gene expression induced calcification of HAVICs obtained from CAS patients. We also confirmed that MGP overexpression significantly inhibited TNF-α-induced calcification of HAVICs by inhibiting BMP2 activation. These findings suggest that MGP has a key role primarily in negatively regulating the calcification of HAVICs.

Table 1. Primers used for quantitative real-time PCR

Gene symbol	Genbank Accession no.	Sequences (5′-3′)
BMP2	NM_001200	Forward: cggactgcggtctcctaa
		Reverse: ggaagcagcaacgctagaag
MGP	NM_000900	Forward: tcacatgaaagcatggaatctta
		Reverse: acaggcttagagcgttctcg
Osteocalcin	NM_199173	Forward: tgagagccctcacactcctc
		Reverse: acctttgctggactctgcac
G3PDH	NM_002046	Forward: tgcaccaccaactgcttagc
		Reverse: ggcatggactgtggtcatgag

Figure 1

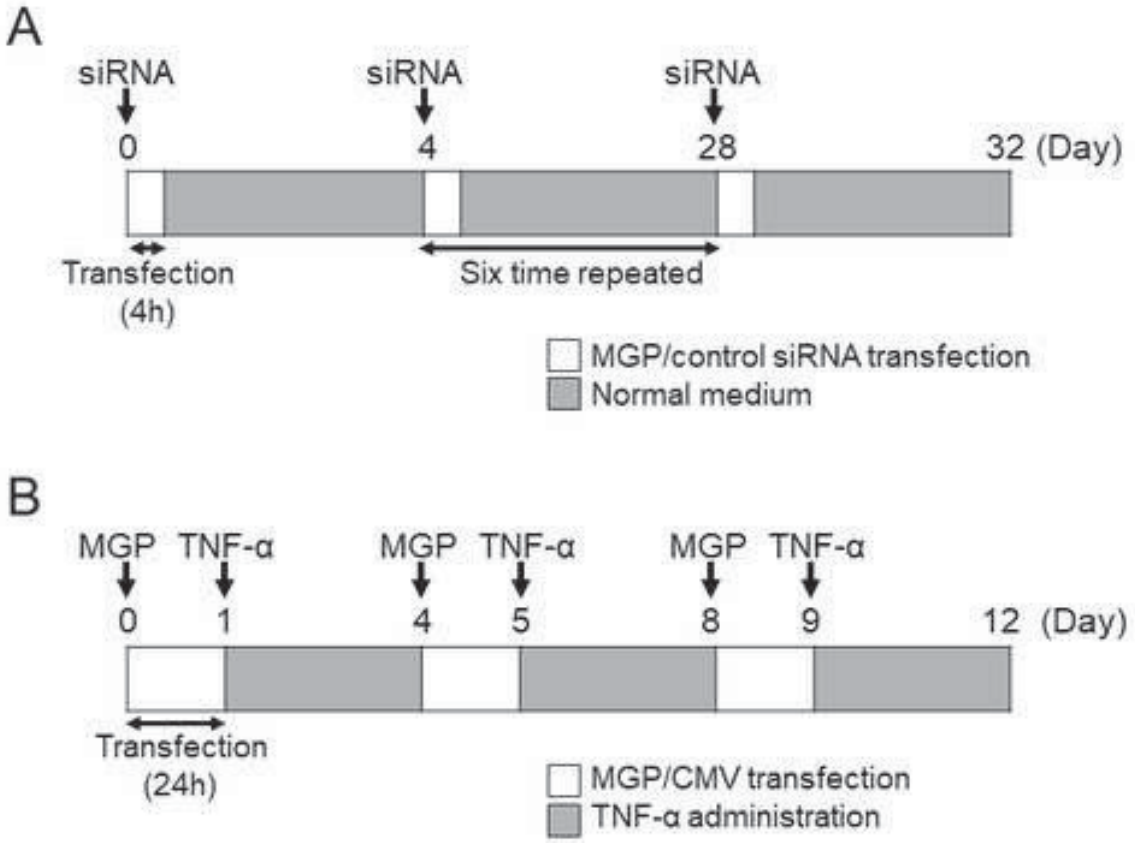
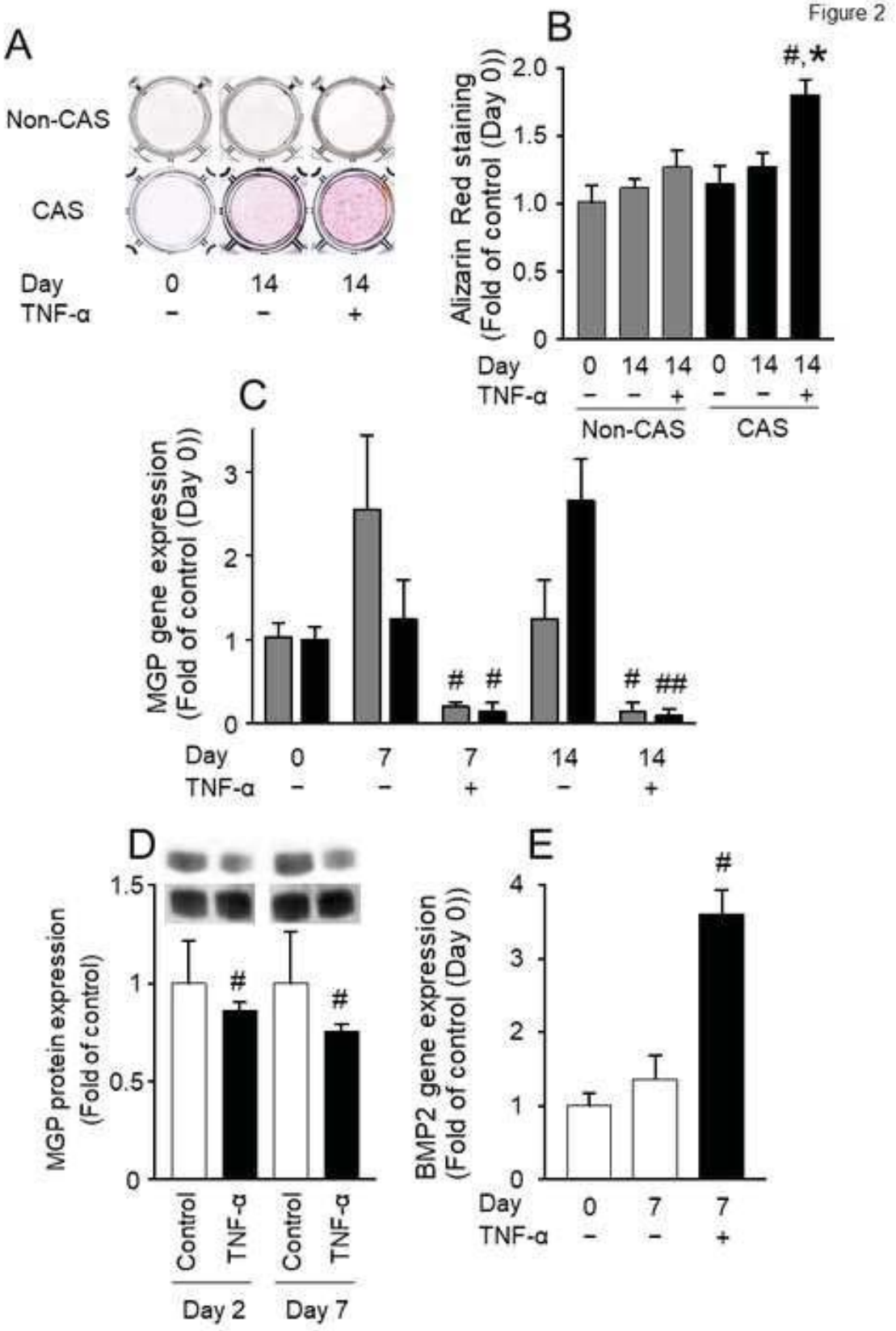

A
siRNA
siRNA
siRNA
0
4
28
32 (Day)
Transfection
(4h)
Six time repeated
MGP/control siRNA transfection
Normal medium
B
MGP
TNF-α
MGP
TNF-α
MGP
TNF-α
0
1
4
5
8
9
12 (Day)
Transfection
(24h)
MGP/CMV transfection
TNF-α administration

Figure 2


A
Non-CAS
CAS
Day 0 14 14
TNF-α - - +
B
Alizarin Red staining (Fold of control (Day 0))
2.0
1.5
1.0
0.5
0
#,*
Day 0 14 14 0 14 14
TNF-α - - + - - +
Non-CAS
CAS
C
MGP gene expression (Fold of control (Day 0))
3
2
1
0
##
Day 0 7 7 14 14
TNF-α - - + - +
D
MGP protein expression (Fold of control)
1.5
1
0.5
0
# #
Control TNF-α Control TNF-α
Day 2 Day 7
E
BMP2 gene expression (Fold of control (Day 0))
4
3
2
1
0
#
Day 0 7 7
TNF-α - - +

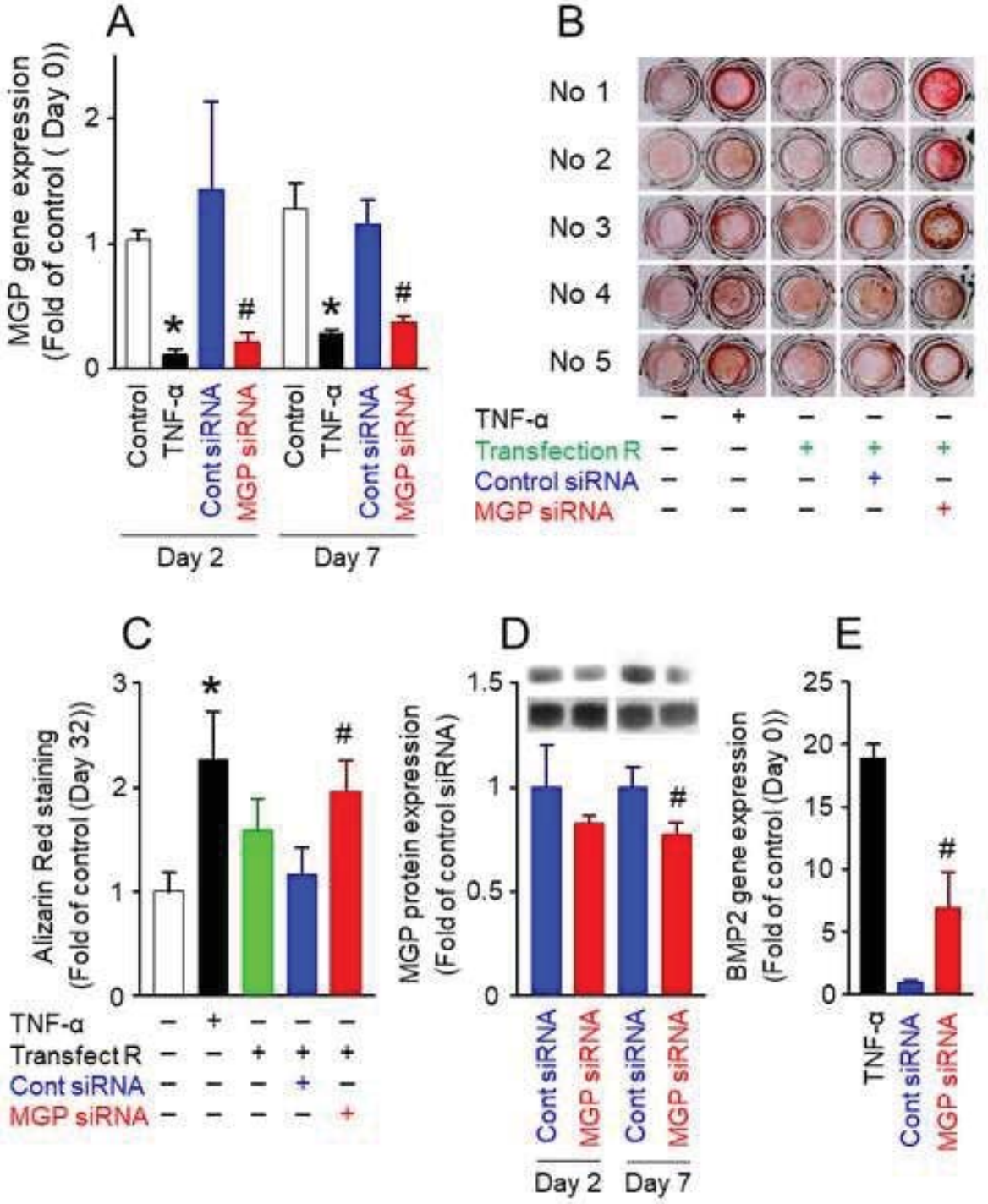

A
MGP gene expression
(Fold of control (Day 0))
Control
TNF-α
Cont siRNA
MGP siRNA
Day 2
Day 7
B
No 1
No 2
No 3
No 4
No 5
TNF-α
Transfection R
Control siRNA
MGP siRNA
C
Alizarin Red staining
(Fold of control (Day 32))
D
MGP protein expression
(Fold of control siRNA)
E
BMP2 gene expression
(Fold of control (Day 0))

Figure 4

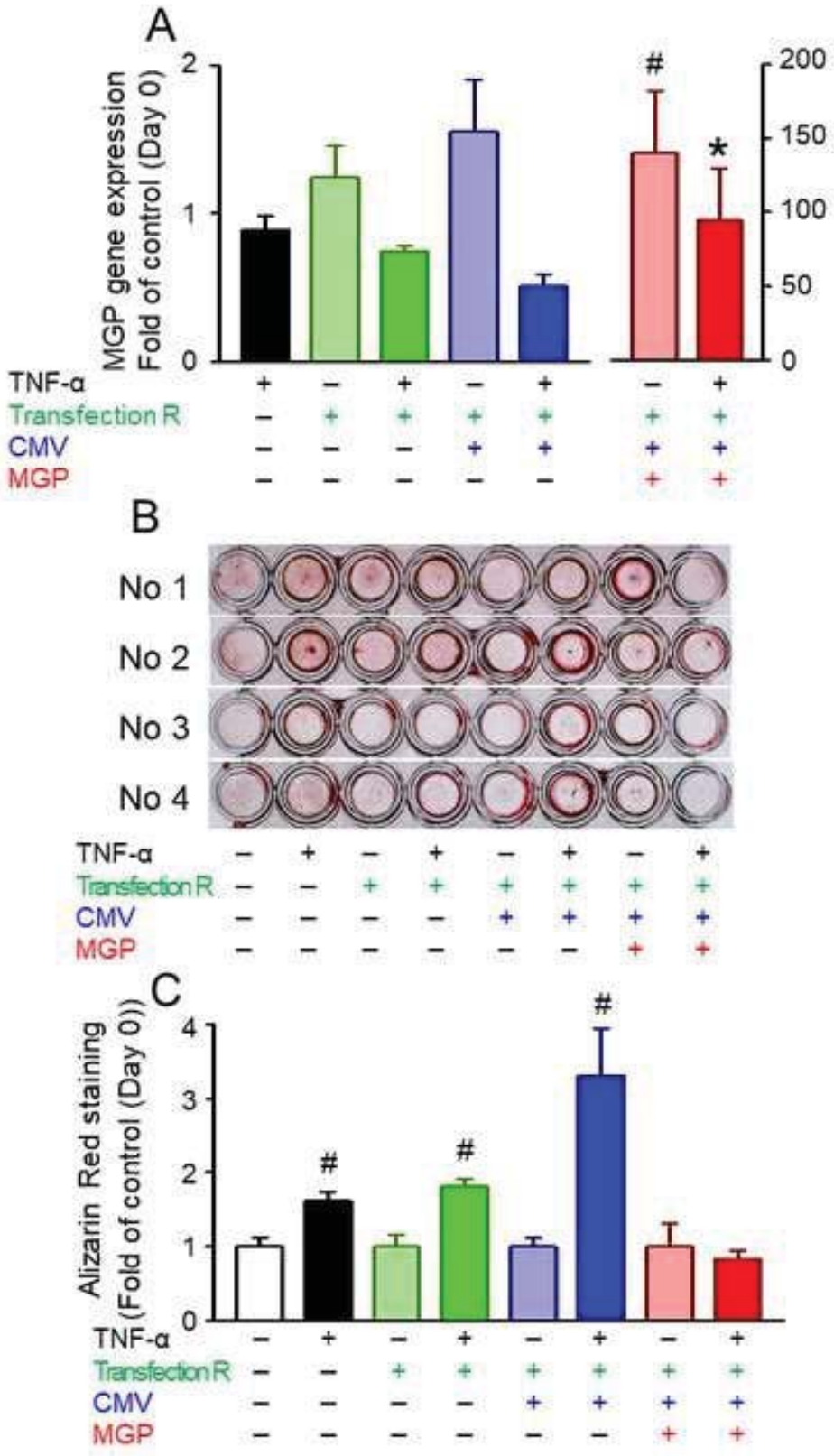

A
MGP gene expression
Fold of control (Day 0)
2
1
0
200
150
100
50
0
#
*
TNF-α + − + − + − +
Transfection R − + + + + + +
CMV − − − + + + +
MGP − − − − − + +
B
No 1
No 2
No 3
No 4
TNF-α − + − + − + − +
Transfection R − − + + + + + +
CMV − − − − + + + +
MGP − − − − − − + +
C
Alizarin Red staining
(Fold of control (Day 0))
4
3
2
1
0
#
#
#
TNF-α − + − + − + − +
Transfection R − − + + + + + +
CMV − − − − + + + +
MGP − − − − − − + +

Figure 4 (continued)

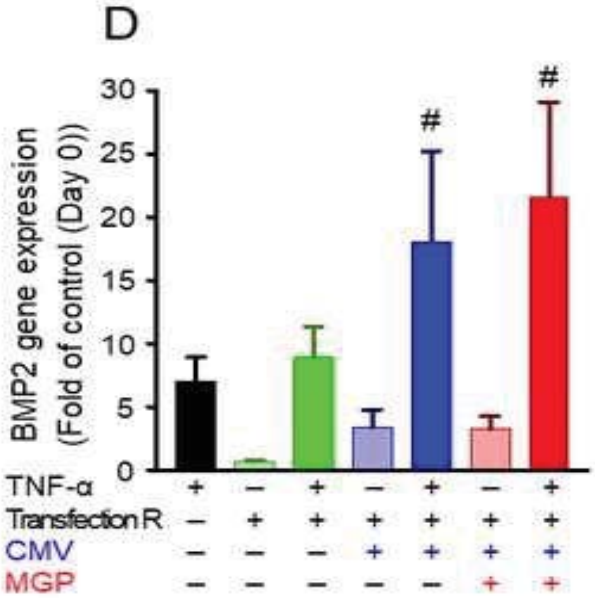
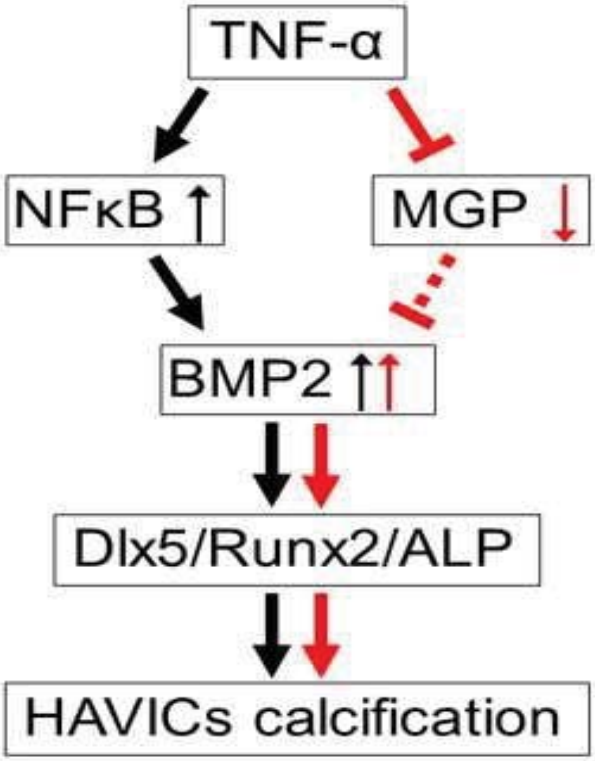
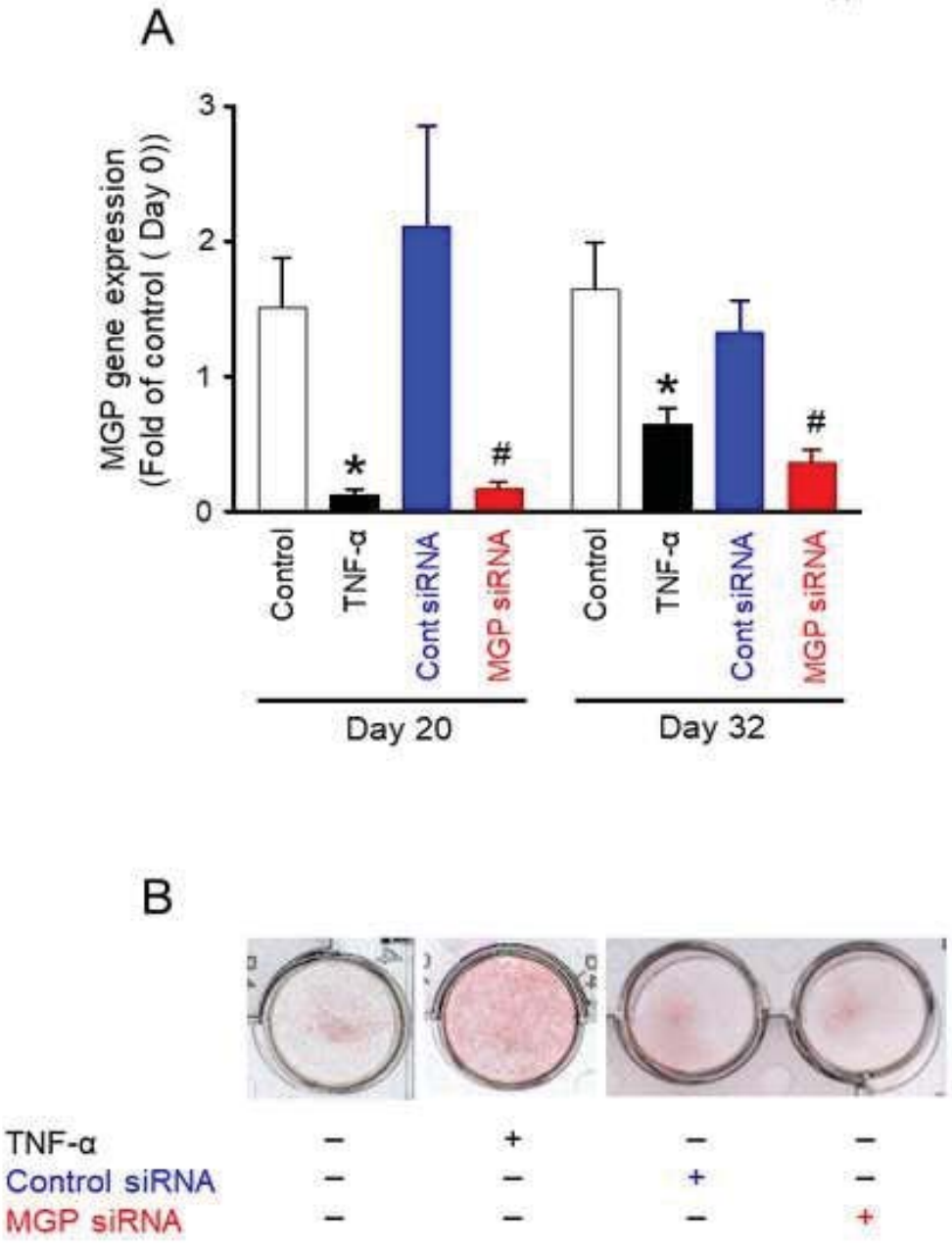
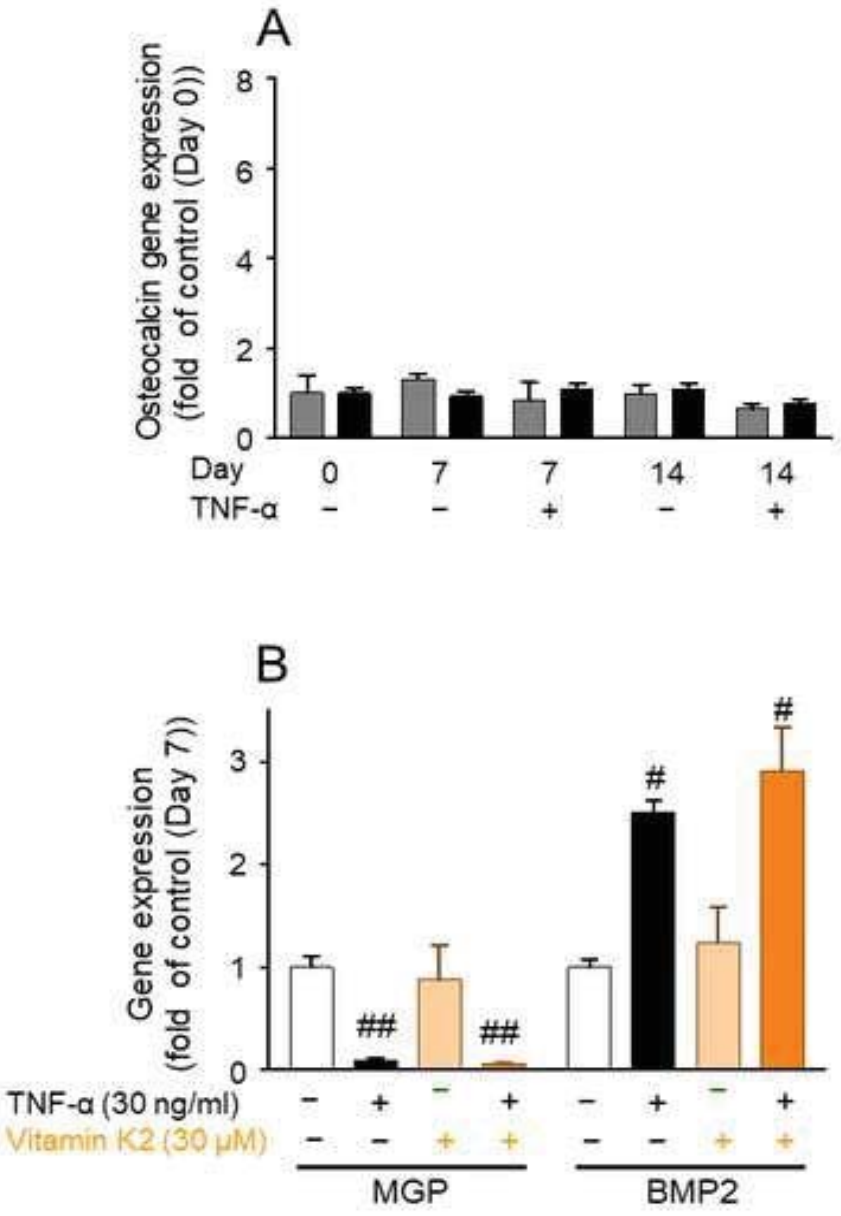


Figure 5



Supplemental figure 1




A
Osteocalcin gene expression
(fold of control (Day 0))
8
6
4
2
0
Day 0 7 7 14 14
TNF-α − − + − +
B
Gene expression
(fold of control (Day 7))
3
2
1
0
##
##
#
#
TNF-α (30 ng/ml) − + − + − + − +
Vitamin K2 (30 μM) − − + + − − + +
MGP
BMP2