ARTICLE IN PRESS

BMB Reports - Manuscript Submission

Manuscript Draft

Manuscript Number: BMB-16-170

Title: Extracellular S100A4 negatively regulates osteoblast function by activating the NF-κB pathway

Article Type: Article

Keywords: S100A4; osteoblast; NF-κB; mineralization; inflammation

Corresponding Author: Hong-Hee Kim

Authors: Haemin Kim[1], Yong Deok Lee[1], Min Kyung Kim[1], Jun-Oh Kwon[1], Min-Kyoung Song[1], Zang Hee Lee[1], Hong-Hee Kim[1,*]

Institution: [1]Cell and Developmental Biology, Seoul National University School of Dentistry,

UNCORRECTED PROOF

ARTICLE IN PRESS

Manuscript Type: Article

Title: Extracellular S100A4 negatively regulates osteoblast function by activating the NF-κB pathway

Author's name: Haemin Kim**,** Yong Deok Lee, Min Kyung Kim, Jun-Oh Kwon, Min-Kyoung Song, Zang Hee Lee, and Hong-Hee Kim*

Affiliation: Department of Cell and Developmental Biology, BK21 Program and DRI, School of Dentistry, Seoul National University, Seoul 03080, Republic of Korea

Running Title: The role of extracellular S100A4 in bone formation

Keywords: S100A4, osteoblast, NF-κB, mineralization, inflammation

Corresponding Author's Information: Professor Hong-Hee Kim, Department of Cell and Developmental Biology, School of Dentistry, Seoul National University, 101 Daehak-ro, Jongro-gu, Seoul 03080, Korea. Tel: +82-2-740-8686, FAX: +82-2-765-8656, E-mail: hhbkim@snu.ac.kr

UNCORRECTED PROOF

ARTICLE IN PRESS

ABSTRACT

Patients with inflammatory bone disease or cancer have an increased risk of fractures and delayed healing of bones. S100A4 protein is a member of the calcium-binding S100 protein family, which is abundantly expressed in inflammatory diseases and cancers. In this study, we investigated the effects of extracellular S100A4 on osteoblasts, cells responsible for bone formation. Treatment of mouse primary calvarial osteoblasts with recombinant S100A4 resulted in reductions in matrix mineralization. The expression of osteoblast marker genes including osteocalcin and osterix was also suppressed. Interestingly, S100A4 stimulated the nuclear factor-kappaB (NF-κB) signaling pathway in osteoblasts. More importantly, *ex vivo* organ culture of mouse calvariae with recombinant S100A4 decreased the expression levels of osteocalcin, supporting the results of our *in vitro* experiments. Our findings suggest that extracellular S100A4 plays an important role in the regulation of bone formation by activating the NF-κB signaling pathway in osteoblasts.

UNCORRECTED PROOF

ARTICLE IN PRESS

INTRODUCTION

S100 proteins are calcium-binding proteins comprised of more than 20 members and play important roles in inflammation, cytoskeleton dynamics, enzyme activity, and cell growth and differentiation (1-3). Many studies have revealed the roles of S100 proteins in pathological conditions, especially in tumor progression and arthritis (3). For instance, S100A2, S100A4, S100A6, S100A7, and S100B were found to be differentially expressed in cancer cells, while S100A12 was detected at a high level in synovial tissues of arthritis patients (4). However, each of the S100 proteins is thought to have a specific role or as yet unidentified critical role(s) under certain circumstances. Notably, S100A8 has been shown to activate osteoclasts, cells responsible for bone resorption, by interacting with toll-like receptor 4 (TLR4) (5).

Over the last decade, S100A4 has been linked to rheumatoid arthritis pathogenesis. Klingelhofer and colleagues reported that synovial tissues of rheumatoid arthritis patients express S100A4 (6). In addition, S100A4 was reported to stimulate matrix metalloproteinase (MMP)-13 secretion via receptor for advanced glycation end products (RAGE) in chondrocytes, resulting in cartilage degradation (7). S100A4 also plays important roles in tumor progression by increasing tumor cell migration and invasion and MMP secretion (8). Both intracellular and extracellular functions of S100A4 have been studied. Intracellularly, S100A4 binds to tumor suppressor protein p53, where it aids in tumor survival, and tumor cell migration is regulated through interactions with non-muscle myosin II. Extracellularly, S100A4 binds to cell surface receptors such as RAGE to activate nuclear factor-kappaB (NF-κB) and mitogen-activated protein kinase pathways for tumor cell invasion and survival (9).

UNCORRECTED PROOF

Recently, the NF-κB signaling pathway was reported to be a negative regulator of osteogenesis (10, 11). NF-κB activation by proinflammatory cytokines such as tumor necrosis factor (TNF)-α in osteoblast precursors inhibits osteogenic differentiation (12). RAGE, a receptor of S100A4, was shown to mediate activation of NF-κB in endothelial cells, macrophages, and lymphocytes (13). A study by Ogawa *et al*. reported that osteoblastic cells expressed RAGE, and that the addition of high glucose with AGE, one of the ligands of RAGE, inhibited mineralization (14). Therefore, we postulated that S100A4 might signal through RAGE to activate the NF-κB signaling pathway and suppress osteoblast function.

In this study, we demonstrated that extracellular S100A4 did not affect early osteoblast differentiation. However, it inhibited mineralization activity due to activation of the NF-κB signaling pathway in osteoblasts. Also, *ex vivo* culture of calvariae of neonatal mice with recombinant S100A4 decreased the expression of osteocalcin.

UNCORRECTED PROOF

ARTICLE IN PRESS

RESULTS

S100A4 does not affect early stage of osteoblast differentiation

To test the effect of S100A4 on bone formation, we first investigated its influence on an *in vitro* culture of primary osteoblasts. Preosteoblasts derived from mouse calvarial tissues were treated with recombinant mouse S100A4 protein or control vehicle in osteogenic differentiation medium. S100A4 protein did not affect the viability of calvarial preosteoblasts at concentrations up to 2 μg/ml (Fig. 1A). Cells became positive for ALP, an early stage marker of osteoblast differentiation, within 3 days of culture in osteogenic media (Fig. 1B). S100A4 treatment did not alter the extent of ALP staining compared to the vehicle-treated culture (Fig. 1B). Consistently, we did not observe any differences in ALP activity by S100A4 treatment at day 2 or day 4 of the culture (Fig. 1C). We next assessed mRNA and protein levels of Runx2, the key transcription factor of early osteoblast differentiation. The mRNA expression of Runx2 in S100A4-treated cultures was comparable to that in the vehicle-treated group (Fig. 1D, upper). On the other hand, we observed a slight decrease in Runx2 protein level by S100A4 treatment at day 3 of the culture (Fig. 1D, lower). Taken together, these results indicate that S100A4 has little effect on the early stage of osteoblast differentiation.

S100A4 inhibits mineralization and the expression of late-stage osteoblast markers

We examined the effect of S100A4 on matrix mineralization, a crucial function of

UNCORRECTED PROOF

osteoblast during bone formation. Calvarial preosteoblasts were cultured in osteogenic media for 10 days and the assessment of mineralization was performed by Alizarin-red staining. The cell proliferation and survival was not affected by the presence of S100A4 at concentrations up to 2 μg/ml until day 6 of the culture and only slightly decreased at 2 μg/ml in 9 day cultures (Fig. 2A). Interestingly, the addition of S100A4 at 1 μg/ml in osteogenic media significantly delayed matrix mineralization by calvarial osteoblasts (Figs. 2B and 2C). The expression level of osteocalcin (*Bglap*), a critical component of bone matrix, was also decreased by S100A4 treatment (Fig. 2D). Induction of the protein level of osterix, a transcription factor necessary for mineralization, was also suppressed by S100A4 treatment (Fig. 2E). Collectively, our results show that an excessive amount of extracellular S100A4 impairs the mineralization activity of osteoblasts and delays induction of late osteoblast markers.

S100A4 activates the NF-κB pathway

A number of studies have reported that activation of the NF-κB pathway in osteoblast precursors impaired osteogenic differentiation (10, 11). To investigate the mechanism of mineralization inhibition by S100A4, we tested whether S100A4 activated the NF-κB pathway in calvarial osteoblasts. Stimulation of calvarial osteoblasts with recombinant mouse S100A4 increased the phosphorylation of IKKα/β, IκB, and p65 within 60 minutes (Fig. 3A). We also assessed the protein level of p65 in the nucleus by fractionating the cytosol and nuclear proteins. Increased levels of p65 were observed in the nuclei of S100A4-treated cells (Fig. 3B). Consistent with our Western blotting results,

UNCORRECTED PROOF

immunofluorescence confocal microscopy also demonstrated localization of p65 in the nuclei after S100A4 stimulation (Fig. 3C). Finally, the decreases in mineralization caused by S100A4 were attenuated by treatment with BAY11-7085, a NF-κB inhibitor (Fig. 3D). These data suggest that S100A4 negatively regulates osteogenic differentiation by activating the NF-κB signaling pathway.

S100A4 suppresses osteocalcin expression in *ex vivo* organ culture of calvariae

To obtain further evidence supporting the regulatory role of S100A4 on bone mineralization activity of osteoblasts, we next utilized an *ex vivo* organ culture system. Calvariae from 4-day-old mice were cultured for 9 days in osteogenic media in the presence or absence of S100A4 protein. The osteogenic medium was changed with 2 day intervals and total RNAs were isolated for cDNA synthesis (Fig. 4A). Real-time PCR analyses revealed a strong suppression in *Bglap* (osteocalcin) gene expression by S100A4 treatment (Fig. 4B).

UNCORRECTED PROOF

ARTICLE IN PRESS

DISCUSSION

In this study, we report the role of extracellular S100A4 on the inhibition of bone formation by activating the NF-κB pathway in osteoblasts. Although early differentiation of osteoblast was not affected (Fig. 1), late-stage differentiation of osteoblast and mineralization activity were reduced by S100A4 treatment (Fig. 2). Consistent with the results of our *in vitro* experiments, S100A4 addition to *ex vivo* culture of neonatal calvariae decreased the expression levels of osteocalcin (Fig. 4). Mechanistically, the NF-κB activation in osteoblasts by S100A4 seems to be responsible for the inhibition of bone formation (Fig. 3).

Bone-loss in inflammatory conditions largely depends on enhanced bone resorption by osteoclasts and delayed bone formation by osteoblasts. Among inflammatory cytokines, TNF-α was shown to directly inhibit mineralized nodule formation and osteocalcin secretion from osteoblasts (18). Moreover, the expression of Runx2 was decreased upon TNF-α treatment (19), and TNF-α induced expression of Smad ubiquitination regulatory factor 1/2 (Smurf 1/2), which ubiquitylates Runx2 for proteasomal degradation (20). Yamazaki *et al*. demonstrated that bone morphogenetic protein 2 (BMP2)-mediated activation of Smads, crucial transcription factors of osteoblast differentiation, was abrogated by TNF-α via NF-κB activation (12). Moreover, the activation of NF-κB by TNF-α induced cAMP response element-binding protein H (CREBH), which in turn up-regulated Smurf 1 to inactivate the Smad/Runx2 regulatory system (21). Mice specifically overexpressing the dominant negative form of IKK in osteoblasts displayed increased bone mass (10). These studies are in accordance with our finding of S100A4 mediating inhibition of mineralization through the activation of NF-κB and strongly support a negative correlation between bone anabolism and

UNCORRECTED PROOF

the activation of NF-κB in osteoblasts.

The NF-κB signaling pathway is also critical to the differentiation of osteoclasts by RANKL (receptor activator of NF-κB ligand) (22). Interestingly, mice lacking S100A4 showed a decrease in the number of osteoclasts (23). Therefore, it is reasonable to postulate that S100A4 also mediates the activation of NF-κB in osteoclasts, accelerating bone catabolism. We are currently investigating whether S100A4 directly regulates osteoclastogenesis via the NF-κB pathway and whether S100A4 would be a useful therapeutic target to treat bone metabolic disorders.

It was previously demonstrated that the synovial fluids of rheumatoid arthritis patients had mean concentration of 1.98 μg/ml of S100A4 which was significantly higher than the concentration of 0.247 μg/ml in osteoarthritis patients (6). Since rheumatoid arthritis promotes and maintains a more inflamed condition near the articular joints than osteoarthritis, the effect of extracellular S100A4 on bone cells might be more pronounced in rheumatoid arthritis. In our previous study, we determined that S100A4 was secreted from lipopolysaccharide-stimulated human periodontal ligament cells, supporting the possibility of S100A4 regulation of bone cells in inflammatory diseases (24). The concentrations of recombinant S100A4 used in this study for the *in vitro* tests were 1 or 2 μg/ml, which are similar to the level found in the inflammatory environment in arthritis patients.

Here, we described the effect of extracellular S100A4 on the activation of the NF-κB pathway. Finding receptor(s) that mediate activation in osteoblasts is an interesting subject of further study. RAGE and TLR4 are the two most well characterized receptors for S100A4 in a variety of cell types (25-28). NF-κB signaling in peripheral blood mononuclear cells was

UNCORRECTED PROOF

shown to be activated by S100A4 via TLR4 (25), while a series of studies demonstrated that S100A4 mediates signals via RAGE, especially in cancer cells (26-28). Both receptors are critical components of the NF-κB signaling pathway.

In conclusion, our study demonstrates that extracellular S100A4 interrupts the mineralization function of osteoblasts and causes an imbalance in bone homeostasis by inhibiting new bone formation. Here, we showed for the first time that this phenomenon is mediated through NF-κB activation. Further investigations of S100A4 may open new avenues for developing novel therapeutics for inflammatory bone-destructive conditions.

UNCORRECTED PROOF

ARTICLE IN PRESS

MATERIALS AND METHODS

Animals

All animals were kept in an specific pathogen-free animal facility with consistent temperature (22℃) and humidity (55%) and a 12-hour light/dark cycle. The facility was operated by experienced animal handlers who were responsible for changing bedding material and providing food and sterilized water under the supervision of a veterinarian. One-day-old ICR mice were purchased from OrientBio (Sungnam, Korea) and were used for *in vitro* calvarial osteoblast preparation and *ex vivo* organ culture experiments. Animal experiments were approved by the Committees on the Care and Use of Animals in Research at Seoul National University (SNU-130311-4-3).

Reagents

Recombinant mouse S100A4 was purchased from Prospec (East Brunswick, NJ, USA). Phosphospecific antibodies against IκB kinase (IKK)α/β ($Ser^{176/180}$), IκB (Ser^{32}), p65 (Ser^{536}), IKKα/β, IκB, and p65 were obtained from Cell Signaling Technology (Beverly, MA, USA). Antibodies against osterix, laminB, and p65 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-β-actin (AC-74) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-Runx2 was purchased from MBL International (Woburn, MA, USA). The alkaline phosphatase (ALP) assay kit was purchased from Takara Bio Inc. (Fukui, Japan). β-Glycerophosphate, cetylpyridium chloride, and ascorbic acid were purchased from Sigma-Aldrich. Bay11-7082 was purchased from Alexis Biochemicals

UNCORRECTED PROOF

(Grunberg, Germany). The Cell Counting Kit-8 (CCK-8) was purchased from Dojindo (Kumamoto, Japan).

Cell viability assay

Calvarial osteoblasts were seeded in 96-well plates at the density of $1x10^4$ cells per well. Cells were cultured in the presence of 10 mM β-glycerophosphate and 100 μM ascorbic acid together with vehicle or recombinant mouse S100A4 (0.1~2 μg/ml) for 3~9 days. Cells at day 0, day 3, day 6, and day 9 were subjected to cytotoxicity assay using the CCK-8 assay kit. After incubating cells with the reagents for an hour, the optical density was measured at 450 nm with an iMark Microplate Absorbance Reader (Biorad, Hercules, CA, USA).

Real-time PCR analysis

Quantification of mRNA level by real-time PCR analysis was performed as previously described (15). Primers for real-time PCR analysis were as follows: *Runx2* forward, 5'-CGCACGACAACCGCACCA-3'; *Runx2* reverse, 5'-CAGCACGGAGCACAGGAAGTT-3'; *Bglap* forward, 5'-CCGGGAGCAGTGTGAGCTTA-3'; *Bglap* reverse, 5'-TAGATGCGTTTGTAGGCGGTC-3'; *Hprt* (hypoxanthine-guanine phosphoribosyltransferase) forward, 5'-CCTAAGATGAGCGCAAGTTGAA-3'; *Hprt* reverse, 5'-CCACAGGGACTAGAACACCTGCTAA-3'.

Calvarial preosteoblast preparation and differentiation

Calvarial preosteoblasts were prepared from 1-day-old mice as previously described (16). Prepared calvarial preosteoblasts were seeded onto 48-well or 6-well cell culture plates

UNCORRECTED PROOF

in alpha minimum essential medium (WELGENE, Daegu, Korea) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA). Osteogenic differentiation was induced by supplementing the culture media with 10 mM β-glycerophosphate and 100 μM ascorbic acid. For evaluation of osteoblast differentiation, ALP activity was measured using the ALP assay kit following the manufacturer's protocol. Mineralization was assessed after 10 days of culture by Alizarin-red staining as previously described (17), and the mean intensity was measured using the ImageJ program. Alternatively, Alizarin-red stained wells were incubated with 100 mM cetylpyridium chloride for 2 hours at 37℃ and the optical density of eluates was measured at 415 nm.

Western blotting

Cells were washed with cold PBS and lysed with RIPA buffer (10 mM Tris pH 7.2, 150 mM NaCl, 0.1% sodium dodecyl sulfate, 1% Triton X-100, 1% sodium deoxycholate, 5 mM ethylenediaminetetraacetic acid). Whole cell lysates or nuclear lysates were subjected to Western blot analysis as previously described (16).

***Ex vivo* calvarial organ culture**

Calvariae were dissected from 4-day-old ICR mice and cultured in 60 mm center well culture dishes (Corning, NY, USA). Osteogenic differentiation was induced by supplementing BGJb medium (Invitrogen) with 10 mM β-glycerophosphate and 100 μM ascorbic acid. The culture medium containing mouse recombinant S100A4 (1 or 5 μg/ml) or vehicle was changed every other day for 9 days. For real-time PCR analysis, calvariae were snap-frozen in liquid nitrogen and ground to powder for total RNA isolation using TRIzol (Invitrogen).

UNCORRECTED PROOF

Immunofluorescence microscopy

Calvarial osteoblasts seeded on 12-mm cover glass were fixed with 3.7% formaldehyde and permeabilized with 0.1% Triton X-100 for 15 minutes. After blocking non-specific sites with 1% bovine serum albumin in PBS for 1.5 hours, cells were incubated with anti-p65 (1/200 dilution) and anti-laminB (1/200 dilution) antibodies for 2 hours, followed by a 1-hour incubation with secondary mouse-FITC and goat-Cy3 (1/300 dilution) antibody. Prepared slides were viewed under a Zeiss LSM 700 laser-scanning microscope with the following conditions: Objective lenses; C-Apochromat 40x/1.20 W, Detectors; PMT, Filter model; Green (BP 490-555), Red (560 IF), and Lasers; 488 nm, 555 nm. Pictures were taken and analyzed using the ZEN2010 program (version 6.0.0.320).

Statistics

All experiments, except the mice calvariae organ culture studies, were performed at least three times. All quantitative measurements had 3 or 4 replicates. Two-way ANOVA followed by the Bonferroni test and Student's *t* test were performed to define differences between multiple and 2 groups, respectively. Quantitative data are presented as the mean ± SD. A p value < 0.05 was considered significant.

UNCORRECTED PROOF

ARTICLE IN PRESS

ACKNOWLEDGEMENTS

This work was supported by a grant from the National Research Foundation of Korea (NRF-2014R1A2A1A10050406) to H-HK.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

UNCORRECTED PROOF

REFERENCES

1. Heizmann CW, Fritz G, Schafer BW (2002) S100 proteins: structure, functions and pathology. Front Biosci 7, d1356-1368

2. Roth J, Vogl T, Sorg C, Sunderkotter C (2003) Phagocyte-specific S100 proteins: a novel group of proinflammatory molecules. Trends Immunol 24, 155-158

3. Emberley ED, Murphy LC, Watson PH (2004) S100 proteins and their influence on pro-survival pathways in cancer. Biochem Cell Biol 82, 508-515

4. Foell D, Kane D, Bresnihan B, et al (2003) Expression of the pro-inflammatory protein S100A12 (EN-RAGE) in rheumatoid and psoriatic arthritis. Rheumatology (Oxford) 42, 1383-1389

5. Grevers LC, de Vries TJ, Vogl T, et al (2011) S100A8 enhances osteoclastic bone resorption in vitro through activation of Toll-like receptor 4: implications for bone destruction in murine antigen-induced arthritis. Arthritis Rheum 63, 1365-1375

6. Klingelhofer J, Senolt L, Baslund B, et al (2007) Up-regulation of metastasis-promoting S100A4 (Mts-1) in rheumatoid arthritis: putative involvement in the pathogenesis of rheumatoid arthritis. Arthritis Rheum 56, 779-789

7. Yammani RR, Carlson CS, Bresnick AR, Loeser RF (2006) Increase in production of matrix metalloproteinase 13 by human articular chondrocytes due to stimulation with S100A4: Role of the receptor for advanced glycation end products. Arthritis Rheum 54, 2901-2911

8. Sherbet GV (2009) Metastasis promoter S100A4 is a potentially valuable molecular target for cancer therapy. Cancer Lett 280, 15-30

9. Boye K, Maelandsmo GM (2010) S100A4 and metastasis: a small actor playing many roles. Am J Pathol 176, 528-535

10. Chang J, Wang Z, Tang E, et al (2009) Inhibition of osteoblastic bone formation by nuclear factor-kappaB. Nat Med 15, 682-689

11. Chang J, Liu F, Lee M, et al (2013) NF-kappaB inhibits osteogenic differentiation of mesenchymal stem cells by promoting beta-catenin degradation. Proc Natl Acad Sci U. S. A. 110, 9469-9474

12. Yamazaki M, Fukushima H, Shin M, et al (2009) Tumor necrosis factor alpha represses bone morphogenetic protein (BMP) signaling by interfering with the DNA binding of Smads through the activation of NF-kappaB. J Biol Chem 284, 35987-35995

13. Hofmann MA, Drury S, Fu C, et al (1999) RAGE mediates a novel proinflammatory axis: a central cell surface receptor for S100/calgranulin polypeptides. Cell 97, 889-901

14. Ogawa N, Yamaguchi T, Yano S, Yamauchi M, Yamamoto M, Sugimoto M (2007) The combination of high glucose and advanced glycation end-products (AGEs) inhibits the mineralization of osteoblastic MC3T3-E1 cells through glucose-induced increase in the receptor for AGEs. Horm Metab Res 39, 871-875

UNCORRECTED PROOF

15. Lee Y, Hyung SW, Jung HJ, et al (2008) The ubiquitin-mediated degradation of Jak1 modulates osteoclastogenesis by limiting interferon-beta-induced inhibitory signaling. Blood 111, 885-893
16. Ryu J, Kim HJ, Chang EJ, Huang H, Banno Y, Kim HH (2006) Sphingosine 1-phosphate as a regulator of osteoclast differentiation and osteoclast-osteoblast coupling. EMBO J 25, 5840-5851
17. Oh JE, Kim HJ, Kim WS, et al (2012) PlexinA2 mediates osteoblast differentiation via regulation of Runx2. J Bone Miner Res 27, 552-562
18. Gilbert L, He X, Farmer P, et al (2000) Inhibition of osteoblast differentiation by tumor necrosis factor-alpha. Endocrinology 141, 3956-3964
19. Gilbert L, He X, Farmer P, et al (2002) Expression of the osteoblast differentiation factor RUNX2 (Cbfa1/AML3/Pebp2alpha A) is inhibited by tumor necrosis factor-alpha. J Biol Chem 277, 2695-2701
20. Kaneki H, Guo R, Chen D, et al (2006) Tumor necrosis factor promotes Runx2 degradation through up-regulation of Smurf1 and Smurf2 in osteoblasts. J Biol Chem 281, 4326-4333
21. Jang WG, Jeong BC, Kim EJ, et al (2015) Cyclic AMP Response Element-binding Protein H (CREBH) Mediates the Inhibitory Actions of Tumor Necrosis Factor alpha in Osteoblast Differentiation by Stimulating Smad1 Degradation. J Biol Chem 290, 13556-13566
22. Lee ZH, Kim HH (2003) Signal transduction by receptor activator of nuclear factor kappa B in osteoclasts. Biochem Biophys Res Commun 305, 211-214
23. Erlandsson MC, Svensson MD, Jonsson IM, et al (2013) Expression of metastasin S100A4 is essential for bone resorption and regulates osteoclast function. Biochim Biophys Acta 1833, 2653-2663
24. Mah SJ, Lee J, Kim H, et al (2015) Induction of S100A4 in periodontal ligament cells enhances osteoclast formation. Arch Oral Biol 60, 1215-1221
25. Cerezo LA, Remakova M, Tomcik M, et al (2014) The metastasis-associated protein S100A4 promotes the inflammatory response of mononuclear cells via the TLR4 signalling pathway in rheumatoid arthritis. Rheumatology (Oxford) 53, 1520-1526
26. Herwig N, Belter B, Wolf S, Haase-Kohn C, Pietzsch J (2016) Interaction of extracellular S100A4 with RAGE prompts prometastatic activation of A375 melanoma cells. J Cell Mol Med 20, 825-835
27. Medapati MR, Dahlmann M, Ghavami S, et al (2015) RAGE Mediates the Pro-Migratory Response of Extracellular S100A4 in Human Thyroid Cancer Cells. Thyroid 25, 514-527
28. Siddique HR, Adhami VM, Parray A, et al (2013) The S100A4 Oncoprotein Promotes Prostate Tumorigenesis in a Transgenic Mouse Model: Regulating NFkappaB through the RAGE Receptor. Genes Cancer 4, 224-234

UNCORRECTED PROOF

ARTICLE IN PRESS

FIGURE LEGENDS

Figure 1. Extracellular S100A4 did not influence the early differentiation of osteoblasts. (A) Mouse calvarial preosteoblasts cultured with osteogenic medium containing vehicle (Veh.) or indicated concentration of recombinant mouse S100A4 for the 3 days were subjected to cell viability assay. (B) Mouse calvarial cells cultured with non-osteogenic or osteogenic medium containing vehicle or S100A4 (1 μg/ml) were ALP-stained after 3 days of culture. (C) ALP activities were measured at day 2 and day 4. (D) Calvarial preosteoblasts were cultured with vehicle or S100A4 in osteogenic medium for the indicated days and subjected to real-time PCR for *Runx2* expression. HPRT was used as an internal control (upper). The protein levels of Runx2 were assessed by Western blotting. β-Actin is shown as a loading control (lower). Error bars represent S.D. of mean values. n.s., not significant.

Figure 2. Extracellular S100A4 inhibited matrix mineralization. (A) Mouse calvarial preosteoblasts cultured with osteogenic medium containing vehicle (V) or indicated concentration of recombinant mouse S100A4 for the indicated days were subjected to viability assay. (B) Mouse calvarial preosteoblasts were cultured with vehicle (Veh.) or S100A4 (1 μg/ml) in osteogenic medium for 10 days. Alizarin-red staining was performed, and a representative image is shown. (C) The intensity of the image from (B) was measured using ImageJ program (left). The intensity of Alizarin-red stain solubilized with cetylpyridium chloride was also quantified (right). (D) Mouse calvarial preosteoblasts were cultured with vehicle or S100A4 (1 μg/ml) in osteogenic medium for the indicated days and subjected to real-time PCR for *Bglap* (osteocalcin) expression. HPRT was used as an internal control. (E) Mouse calvarial preosteoblasts were cultured with vehicle or S100A4 (1 μg/ml)

UNCORRECTED PROOF

in osteogenic medium for the indicated days. The protein levels of osterix were assessed by Western blotting. β-Actin was included as a loading control. Error bars represent S.D. of mean values. **, $p < 0.01$ versus Veh.. n.s., not significant.

Figure 3. S100A4 induced NF-κB activation in osteoblasts. (A) Mouse calvarial preosteoblasts were serum-starved for 5 hours, stimulated with either vehicle (Veh.) or recombinant mouse S100A4 (2 μg/ml) for the indicated time, and subjected to Western blotting to detect protein levels of phosphorylated p65 (p-p65), total p65, phosphorylated IκB (p-IκB), total IκB, and phosphorylated IKKαβ. β-Actin is shown as a loading control. (B) Calvarial cells were serum-starved for 5 hours and stimulated with either vehicle or S100A4 (2 μg/ml) for 1 hour. Cytosolic proteins (30 μg) and nuclear proteins (8 μg) were separated and subjected to Western blotting to detect protein levels of p65, laminB, and α-tubulin. (C) Calvarial cells were serum-starved for 5 hours and stimulated with either vehicle or S100A4 (2 μg/ml) for 1 hour. Cells were stained with anti-laminB (red) and anti-p65 (green). LaminB was labeled to locate the nuclear membrane. Cells were subjected to confocal microscopy, and representative images are shown (upper). Green positive (+) nuclei were counted and are depicted as a graph (lower). Primary antibodies were not added for 2nd only samples. (D) Mouse calvarial preosteoblasts were cultured with vehicle or S100A4 (1 or 2 μg/ml) in osteogenic medium for 9 days, together with DMSO or indicated concentrations of BAY11-7085. Cells were subjected to Alizarin-red staining. The intensity of Alizarin-red stain was quantified after solubilization using cetylpyridium chloride. Error bars represent S.D. of mean values. **, $p < 0.01$ versus Veh.. #, $p < 0.01$ versus DMSO.

Figure 4. S100A4 treatment decreased *Bglap* expression in *ex vivo* calvaria culture. (A) A schematic timeline describing the experiment. Calvarie from 4-day-old mice were cultured in

UNCORRECTED PROOF

organ culture dishes with the osteogenic medium containing either mouse recombinant S100A4 (1 or 5 μg/ml) or vehicle for 9 days. The medium was changed every other day. At the end of the culture, calvariae were frozen and ground for total RNA isolation (B) After, reverse transcription, the mRNA levels of *Bglap* were determined by real-time PCR analyses. n=3 to 4 per group. **, $p < 0.01$ between the indicated groups.

UNCORRECTED PROOF

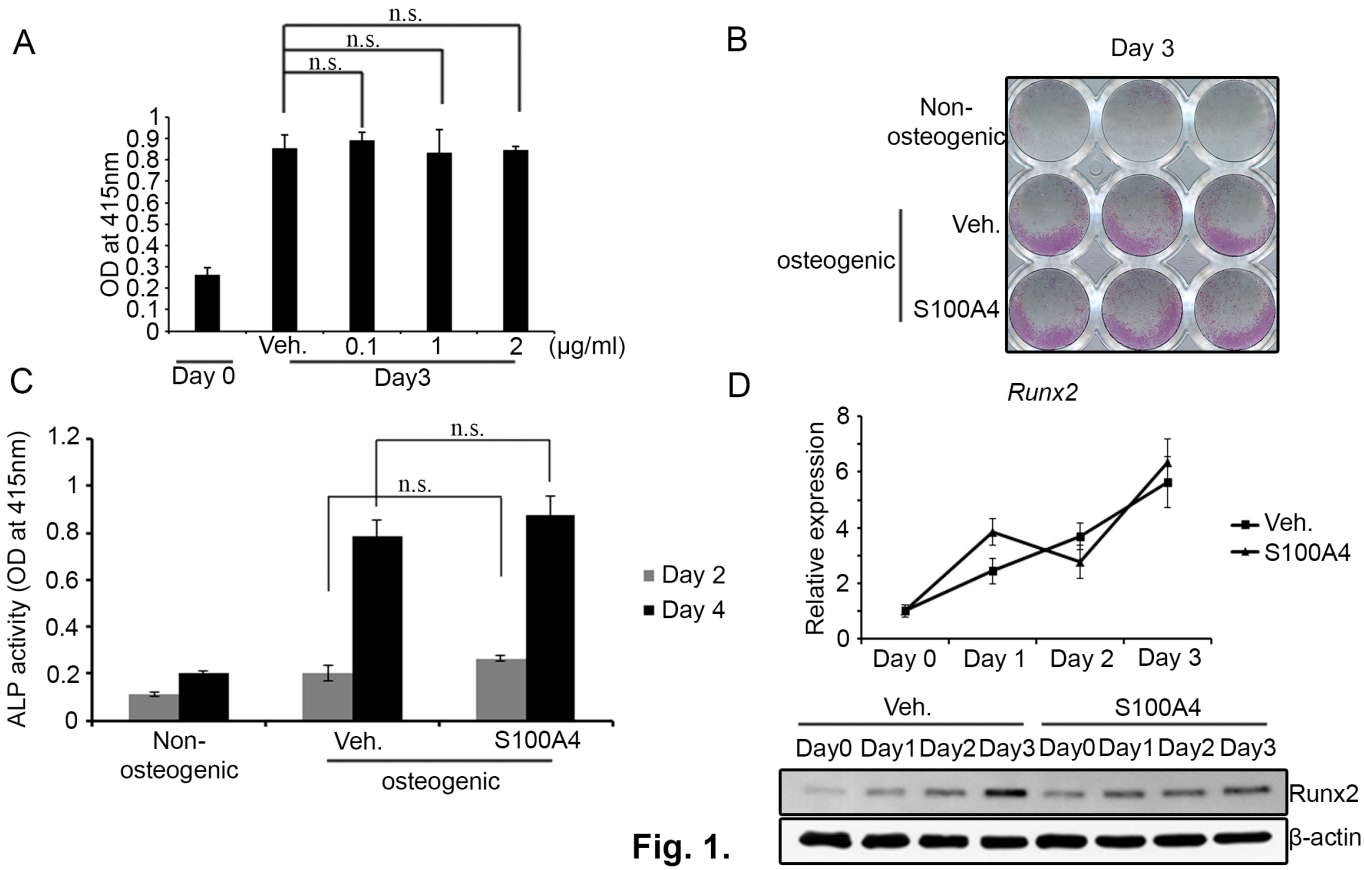

Fig. 1.

Fig. 1 Fig 1

UNCORRECTED

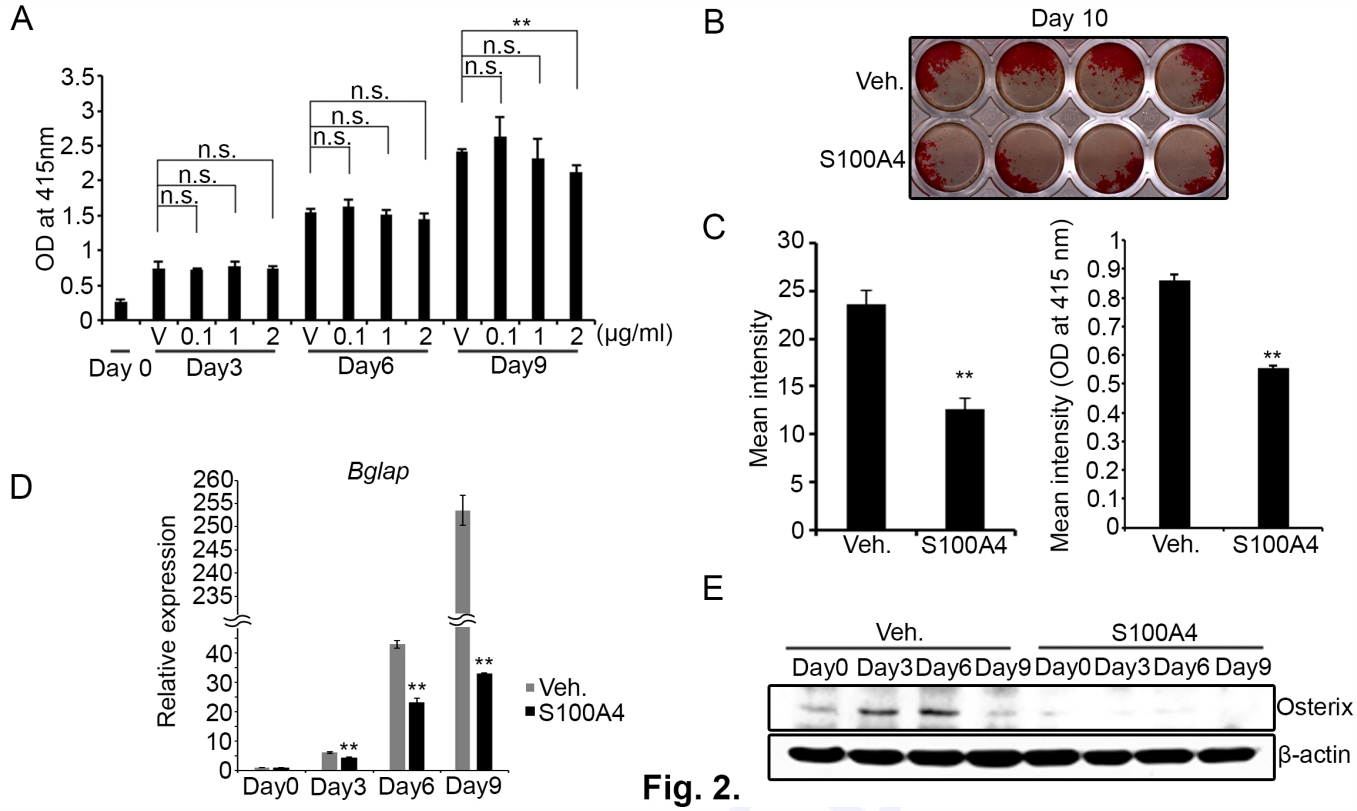

Fig. 2.

Fig. 2 Fig 2

UNCORRECTED

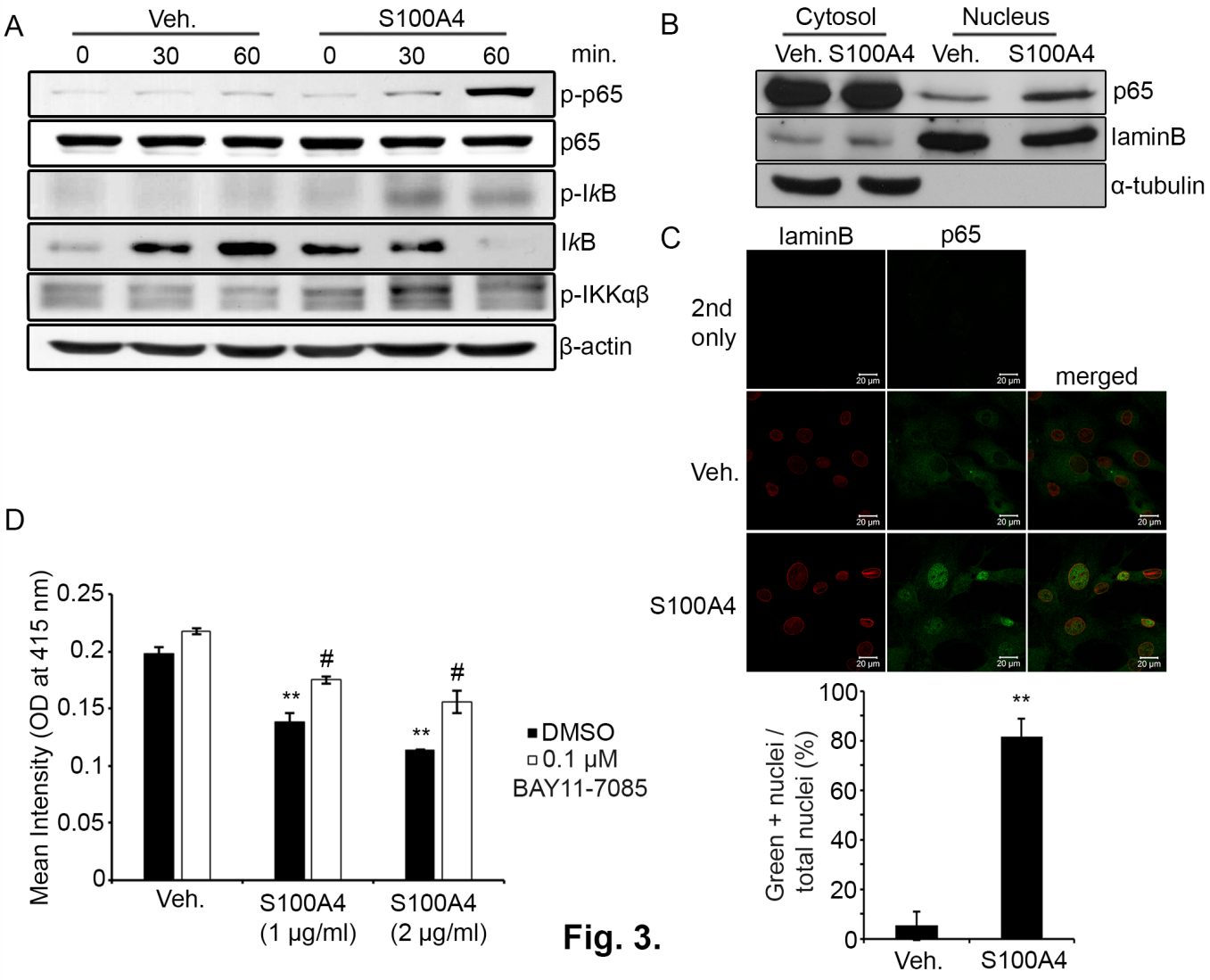

Fig. 3.

Fig. 3 Fig 3

UNCORRECTED

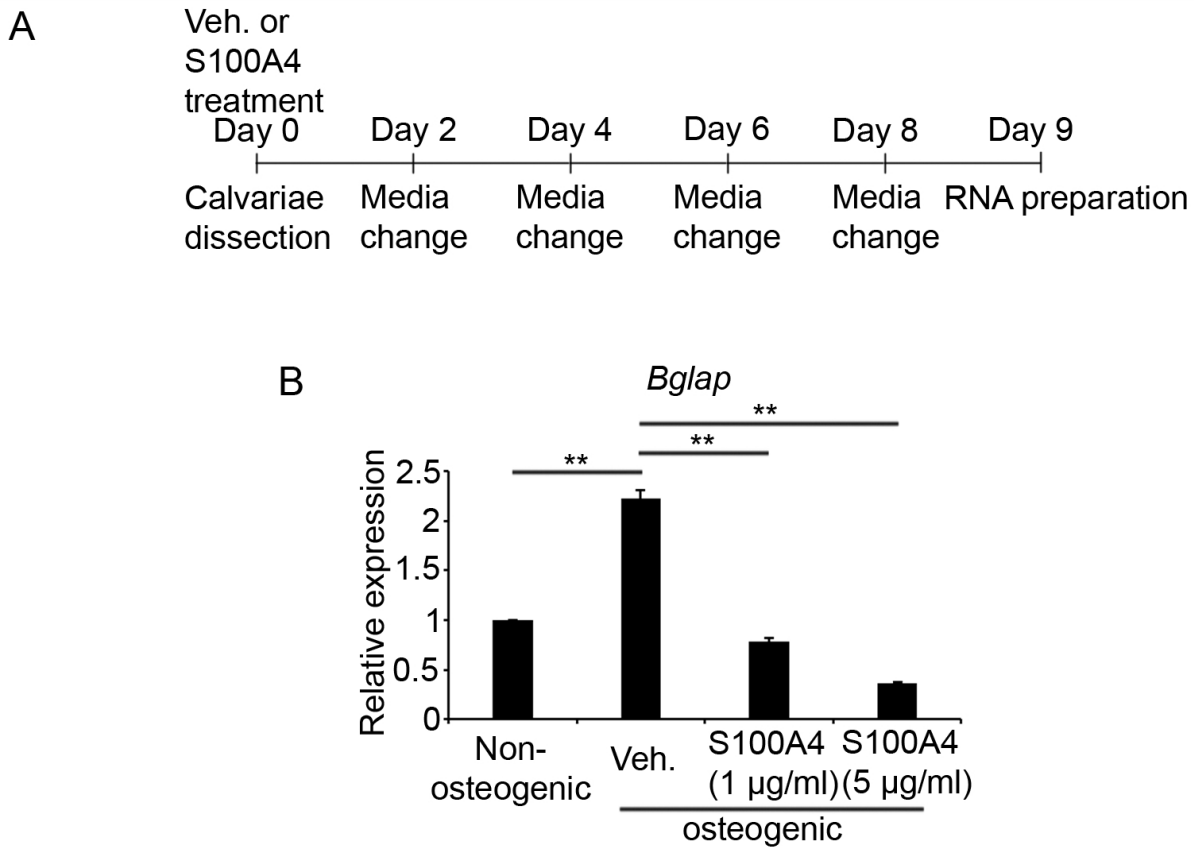

Fig. 4.

Fig. 4 Fig 4

UNCORRECTED