博 士 論 文

# Osteocytes as main responders to Low Intensity Pulsed Ultrasound for the treatment of fracture healing

（骨細胞は低出力超音波パルスによる骨折治癒において主に応答する）

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## Introduction

Mechanical loading generally supplied by gravity and exercise is effective to induce bone remodeling and maintain bone mass due to regulate biological function of bone cells such as osteoblasts, osteoclasts and osteocytes [1, 2]. Bone embedded osteocytes are considered to mechano-sensitive cells. Osteocytes compose over 90-95% of the whole bone cells which reside in bone matrix, and form lacunar–canalicular network throughout bone tissue [3]. Osteocytes sense mechanical stimuli by the unique morphologies and generate biological signals that affect osteoblasts and osteoclasts via osteocytic projection reached the bone surfaces, and then organize bone formation and resorption [4]. Mechanical loading can induce several signaling pathways in osteocytes such as Wnt signaling mediated by Insulin-like growth factor 1 (IGF1) and sclerostin (SOST) [5], cAMP/PKA signaling triggered by prostaglandin E2 (PGE2) [6] and calcium signaling [7, 8]. However, it is still unclear whether input certain biological signals by mechanical loading in osteocytes interact with the activation of neighboring bone cells to maintain bone homeostasis.

Mechanical stimuli given by low-intensity pulsed ultrasound (LIPUS) treatment is an established therapy for bone fracture and the U.S. Food and Drug Administration approved the EXOGEN™ (LIPUS system) for the accelerated healing of certain fresh fractures in 1990s [9, 10]. Not only in human but also mice and rats, LIPUS promote healing and regeneration of bone fracture due to direct and indirect effect of osteogenesis [11]. Interestingly both osteoblasts and osteocytes are capable of responding to LIPUS and synergistic action of these cells likely important to the potency of LIPUS treatment [12]. It has been well reported that osteoblasts are sense LIPUS and contribute to fracture healing through bone-forming and inflammatory

regulation [13]. On the other hand, there are not so many reports compared to osteoblasts but osteocytes also respond to LIPUS and have an important role in the biological function of fracture healing. LIPUS stimulation, acoustic radiation force, of bone expand surface wave and induce fluid flow in osteocyte lacunar-canalicular. Fluid flow-induced shear stress increased PGE2 release and cyclooxygenase 2 (Cox2) mRNA expression in murine long bone osteocyte Y4 (MLO-Y4) cells and that PGE2 was involved in up-regulation of connexin 43-based gap junction of MLO-Y4 cells [14]. Furthermore, fluid flow shear stress enhanced the mRNA and/or protein expression of anabolic and metabolic factors such as IGF1, mechano growth factor (MGF), vascular endothelial growth factor (VEGF), and hepatocyte growth factor (HGF) in MLO-Y4 cells [15]. Interestingly, Li *et al*. reported that LIPUS-stimulated osteocytic MLO-Y4 cells secreted PGE2 and NO into culture media and the differentiation of osteoblastic MC3T3-E1 cells cultured in this media was significantly changed [16]. However, there are no report which demonstrated the role and necessity for osteocytes in LIPUS-mediated fracture repair by *in vivo* study model.

Although fracture healing is a complex, well-orchestrated regenerative process by various tissues and cell types. The repair process is mainly divided into four stages such as inflammation, soft callus formation, hard callus formation and remodeling [17]. The injury initiates inflammatory response with white blood cells accompanied by the secretion of inflammatory cytokines, tumor necrosis factor-a (TNF-a), interleukin (IL)-1, IL-6, IL-11 and IL-18. The response induces the hematoma to coagulate around the fracture site and be a template for callus formation. Cartilaginous callus is formed by collagen matrix produced by specific mesenchymal stem cells derived from the surrounding soft tissues and bone marrow. Within the callus, endochondral formation

and endochondral ossification are occurs through the transforming growth factor-beta (TGF-b) and bone morphogenetic protein (BMP)-2, -5 and -6. As callus chondrocytes differentiation progresses, extracellular matrix undergoes calcification and this phase terminates with chondrocyte apoptosis. The primary soft callus is resorbed by osteoclasts and replaced by a hard callus by osteoblasts. Macrophage colony-stimulating factor (M-CSF), receptor activator of nuclear factor kappa B ligand (RANKL), osteoprotegerin (OPG) and TNF-a support recruit both osteoclasts and osteoblasts to make woven bone. Revascularisation and neoangiogenesis at the fracture site achieved by an angiopoietin-dependent pathway and a VEGF-dependent pathway are also important during the nascent bone tissue replace cartilage. Subsequently, the hard callus is resorbed by osteoclasts to remodel into bone's original cortical structure characterized by coupled cycles of osteoblast and osteoclasts activity. The bone remodeling is organized by IL-1, TNF-a, TGF-b and BMPs. During this period, marrow space is re-established and vascular remodeling take place and finally new bone is regenerated. Thus, fracture healing requires the interaction of various tissues and types of cells appropriately in each step [18]. Although the role of osteoblasts and osteoclasts in fracture healing especially in bone remodeling process has been identified, physiological role of osteocytes is still incompletely understood.

Previous paper have shown that LIPUS promote fracture healing at all of stages of the healing process in their experiments utilized in a rat closed femoral fracture model [17]. Experimental study utilized murine model has also been demonstrated that Cox2, as a mechanical-response amplifier in bone cells is essential for LIPUS-mediated fracture healing during the endochondral ossification [19]. Osteocytes are mechano-sensitive and various *in vitro* experiments have been

suggested that osteocytes respond LIPUS stimulation and could give effect on fracture healing [16, 20]. However, it has not been confirmed that whether osteocytes are necessary and valuable for LIPUS-mediated fracture healing by *in vivo* study. It is very difficult to long-term targeted ablation of osteocytes in mammalian model due to the origin of osteocytes. Since osteocytes are differentiated osteoblasts, if osteocytes will be deleted in bone tissue, remained osteoblasts will become newly embedded osteocytes after few months. To solve the problem, we utilized two common laboratory fish species, medaka and zebrafish in the present study. Fish bone is composed of the same component of mammalian bone such as mineral, water, collagen, cells, and other proteins [21]. Bone modelling system is also carried out by osteoblasts and osteoclasts in all of teleost fishes as well as mammalian [22]. However, most advanced fishes called neoteleosts including medaka completely lack bone embedded osteocytes [23]. Although It has been thought that zebrafish equipped with osteocyte-rich bone should be more sensitive to mechanical loading and more effective in bone modelling than medaka, Ofer *et al*. showed that both medaka which have anosteocytic bones and zebrafish which have osteocytic bones can respond to mechanical loading brought by swimming and showed similar new bone formation rate [24]. Because, instead of osteocytes, osteoblasts and chondrocytes modulate local SOST expression to control the bone modelling in anosteocytic medaka skeleton. Interestingly, medaka and zebrafish are utilized for a fracture healing model and bone fractures of caudal bony fin ray of both medaka and zebrafish are repaired through four healing process as inflammation, soft callus formation, hard callus formation and remodeling healing process [25, 26]. These reports suggest that osteoblasts rather than osteocytes are essential for autonomous osteogenic function induced by mechanical loading and

during fracture healing. However, we mammalian have osteocytic bone probably because of a functional advantage for the evolution. Indeed, osteocytes support and control osteogenesis by balancing the function of osteoblasts and osteoclasts [27]. Furthermore, osteocytes involve in the physiological function of not only skeletal tissue but also other organs such as hematopoiesis in bone marrow, phosphate metabolism in kidney and fat metabolism [28-30]. In this report, we showed that osteocyte embedded zebrafish bone is more beneficial to get LIPUS effect on fracture healing. In addition, the global gene expression analysis suggested that LIPUS-stimulated osteocytes may indirectly modify the function of osteogenic cells and inflammatory cells to accelerate fracture healing through the target genes of transcription factors regulated by LIPUS.

## Results

### LIPUS promote fracture healing in zebrafish but not in medaka

To examine whether osteocytes are necessary for the effect of LIPUS on fracture healing, we compared the rate of fracture healing in medaka and zebrafish. Medaka has an acellular bone lacking osteocytes and zebrafish has an osteocyte-containing bone but both bones are extremely similar in structure, mechanical properties and mineral density [21]. Fin rays contain segmented dermal bones and we gave fracture by a sharp glass capillary in 5 places for each fish (Fig. 1A). Alizarin red and Alcian blue staining was performed to observe the process of fracture healing in both medaka and zebrafish (Fig. 1B). Fractured region formed Alcian blue-positive callus and gradually replaced Alizarin red-positive hard callus. Then, Alizarin red-positive callus were remodeled to a smooth surface. Since the surface was not repaired to the normal after few months, this point was considered to be completely healing of bone fracture. The fracture bone of medaka took more days, approximately 7 days, to be completely healed than that of zebrafish (Fig. 1B). Next, to examine LIPUS effect on accelerating bone fracture healing in anosteocytic or osteocytic bone, medaka and zebrafish were stimulated by LIPUS for 20 minutes at 30 mW / $cm^2$ once a day until fracture have been completely recovered. Interestingly, LIPUS treatment accelerated fracture healing in zebrafish (Fig. 1C) but not in medaka. Whereas, the condition factors (K-factors: weight (g)/length $(cm)^3$) X 100) as an indicator of physiological state of the fish depending on adequate management (certain feeding density and climate) were not changed by LIPUS in both species (Fig. 1D). LIPUS provided an advantage in fracture healing probably through direct effect on skeletal tissue rather than systemic change of physiological condition. Because of the effect of

LIPUS on fracture healing is apparent in zebrafish bone contained osteocytes, osteocytes seem to be essential for LIPUS-mediated fracture healing.

**Global transcriptomic analysis in LIPUS stimulated osteocytes**

Osteocytes could respond to LIPUS stimulation and contribute to accelerate fracture healing. In order to understand the molecular events triggered by LIPUS in osteocytes, we performed RNA-sequencing (RNA-seq) analysis in the MLO-Y4 cells [31] exposed to LIPUS or kept in the control situation. Cells were stimulated for 20 minutes by LIPUS and subsequently placed in a $CO_2$ incubator. After 30 minutes incubation, RNA was extracted and determined the transcriptomes. Among 16,476 genes, 179 genes were significantly changed by LIPUS stimulation (Fig. 2A). Of the 179 LIPUS-responsive genes, 93 genes were upregulated and 86 genes were downregulated by LIPUS stimulation (Fig. 2A). As most of bone-related genes were not changed, LIPUS-stimulated osteocytes may participate to fracture healing not through their osteogenic function (Fig. 2B). To clarify the biological mean of these 179 genes, functional annotation analysis was performed by the DAVID, functional annotation tool, and Uniprot keywords was selected as the database. Major annotated terms categories included Immunity, Antiviral defense, Cytoplasm, Secreted, Extracellular matrix and Transcription in top ranks (Fig. 2C). Further, these genes were clustered into 9 and enrichment score > 1.3 (which corresponded to negative log of the P-value < 0.05) were considered significant, resulting 7 clusters were significantly enriched (Table 2). Consistent with annotation categories, Immunity, Transcription and Secreted (bold and underline in Tale 2) were found in top 4 clusters of results (Table 2). It seems to be reasonable that LIPUS modulate these functions in osteocytes because through the

fracture healing, inflammatory immune response are necessary and osteocytes-derived secreted factors are likely to play a role for recruitment of MSC and blood cells to form callus and induce vascularization. Transcriptional factors can be also important to regulate gene expression related in all process of fracture healing containing inflammatory phase and bone remodeling phase. We investigated Individual gene expression contained in three clusters such as immunity, secreted and transcription. Many genes belong to secreted factors were increased by LIPUS, whereas many immune-related genes tend to decrease (Fig. 2D). In transcriptional factors, there is an almost equal amount of up-regulated and down-regulated genes by LIPUS (Fig. 2D). These results demonstrated that osteocytes respond to LIPUS with 179 genes involved in inflammatory response, transcriptional regulation and protein secretion to affect the rate of fracture healing.

**Gene expression change by LIPUS in zebrafish fin ray**

Next, to examine whether genes categorized Immunity, Transcription and Secreted are also modulated by LIPUS *in vivo*, gene expression level assigned to each cluster categories was measured in zebrafish fin. Bone fracture was induced in zebrafish caudal fin and stimulated by LIPUS for 20 minutes once a day. 30 minutes after the stimulation, RNA was isolated from the fin ray at day 1 and 7. To perform qPCR with zebrafish samples, we isolated appropriate genes by below step Immunity in cluster A included 20 genes (Table 2). Among these genes, first we isolated 14 genes which have smallest q value (0.00388), next we eliminated genes which don’t have zebrafish ortholog or not possible to design primer set for qPCR. Finally, 6 genes were left (labeled with an asterisk in Table 2) and measured gene expression change

by qPCR. Similarly, 17 genes were isolated from Transcription in cluster B, and 16 genes were isolated from Secreted in cluster D for qPCR (labeled with an asterisk in Table 2). Most genes categorized in Immunity and Secreted were not changed by LIPUS stimulation in zebrafish fin. In Immunity only 1 out of 6 genes (16.6%), C1s2 (Complement component 1, s subcomponent 2), were increased and in Secreted only 1 out of 16 genes (1.5%), Saa3 (Serum amyloid A-3 protein), were changed (Fig. 3A). However, many genes contained in Transcription, 11 out of 17 genes (64.7%), were significantly changed (Fig. 3B). Reactivity for LIPUS of classified genes as Transcription were quite parallel between MLO-Y4 cells and zebrafish fin ray. Therefore, we focused on transcription genes and performed target gene analysis.

**Target gene analysis of transcription factors modulated by LIPUS**

To know target genes regulated by LIPUS-responding 11 transcriptional factors, we utilized online ChiP-seq database, ChiP-Atras, and obtained binding scores of *Egr1*, *Egr2*, *nuclear receptor 4a1 (Nr4a1)*, *JunB* and *NFATc1* in promoter region of target genes. Unfortunately, information of *Btg2*, *Snai2*, *Cited2*, *FoxQ1* and *Heiz2* was not available in this database (described in N/A in Table 3). Top 10 potential target genes with highest average score were isolated and evaluated the expression level in osteocytic MLO-Y4 cells with RNA-seq data (column 4 in Table 3). 12 genes which abound in MLO-Y4 cells (FPKM value > 10) were selected and researched their function. These genes participate energy metabolism (*Abhd6* and *Adssl1*), cell survival (*Ncapg2*, *Mcl1* and *Brox*), cell-cell contact (*Arhgap17*) and involve several signaling pathway (Cbr1, Dynlt1b, Aida and Htra3) (summarized in Table 3). Especially, TGF-b and Cox2/PGE2 pathways modulated by *Htra3* and *Cbr1* [32, 33] are interesting

because they are induced and secreted in early step of fracture healing (inflammation phase) as the pro-inflammatory molecule [18]. Wnt/b-catenin signaling in osteocytes can stimulate osteoblastic differentiation through the activation of Notch signaling [34] Since Axin is key scaffold protein for the b-catenin destruction complex to inhibit Wnt/b-catenin signaling [35], Axin interacting protein, *Aida*, may partially commit to bone formation phase in fracture healing to regulate both osteoblast and chondrocyte differentiation from osteochondral progenitors. *Cst3* which is most abundant in MLO-Y4 cells, is a secreted factor and promote the differentiation of osteoblasts associated with BMP signaling [36]. Other transcription factors, *Btg2*, *Snai2*, *Cited2*, *FoxQ1* and *Heiz2* were referred by previous reports because their target genes were not available in the ChiP-Atras database. *Btg2* can be transcriptional coactivators and allow interaction with and modulation of various nuclear receptors such as all-trans retinoic acid receptor, estrogen receptor and androgen receptor [37]. It was reported that estrogen receptor in osteocytes was important for bone formation [38]. Snai2 act as transcriptional repressors and regulate cell detachment and attachment from extracellular matrix, cell apoptosis and cell migration [39]. *Cited2* is a CBP/p300-binding transcription co-activator and enhanced TGF-b mediated transcription [40]. Interestingly, previous study utilized fracture model in rat reported that Cited2 was a negative regulator of fracture healing associated with the increase of matrix metalloprotease [41]. LIPUS stimulation decreased *Cited2* expression in fractured fin ray (Fig. 3B) and it was consistent with the positive effect of LIPUS in fracture healing. *FoxQ1* is a member of the forkhead transcription factor family and regulates cell proliferation via TGF-b and Wnt signaling [42]. *HELZ2 (helicase with zinc finger 2, transcriptional coactivator)* is a coregulator of *peroxisome proliferator-activated*

*receptor (PPARγ)* [43]. Since *PPARγ* in osteocytes was important for coupling of bone formation and resorption [44], an increase of *Helz2* by LIPUS (Fig. 3B) may control fracture healing process such as bone remodeling given by osteoblasts and osteoclasts. Taken together, 10 transcription genes shown in Table 3 were also modulated by LIPUS stimulation not only *in vitro* but also *in vivo* and their target genes can be involved in the promotion of fracture healing probably through activation of inflammatory response and osteogenic cell differentiation.

**Measurement of candidate transcription genes for LIPUS effect in medaka fin ray**

In zebrafish bones contained osteocytes, several transcription genes reacted with LIPUS under fracture healing condition. To assess whether osteocytes are valuable for LIPUS action via these transcriptional factors, another *in vivo* experiments with medaka featured lacking osteocytes bone were performed. Medaka fin ray given bone fractures were isolated after LIPUS treatment and RNA was extracted as well as zebrafish samples. Among Isolated 10 transcription genes shown in Table 3, 7 genes exist in medaka and were able to design primer set for qPCR. Interestingly, *Egr1*, *Egr2*, *JunB*, *FoxQ1* and *NFATc1* did not respond for LIPUS in medaka (Fig. 4) and these genes were considered as osteocyte-dependent genes induced by LIPUS treatment. Other genes such as *Snai2* and *Nr4a1* were also changed by LIPUS in medaka similar to zebrafish (Fig. 4), suggesting these genes could be key for both osteocyte- and osteoblast-mediated LIPUS effect. These *in vivo* results showed that osteocyte contribute to LIPUS-promoted fracture repair through transcriptional regulation of *Egr1*, *Egr2*, *JunB*, *FoxQ1* and *NFATc1* which can induce target genes expression mediated

cellular metabolism and survival or triggered various signaling pathway such as TGF-b and Wnt needed for fracture healing event.

## Discussion

In this report, we demonstrated that zebrafish equipped with osteocytic bone is more effective to gain LIPUS-induced promotion of fracture healing than medaka equipped with anosteocytic bone. LIPUS stimulation changed gene expression profile in osteocytes and some transcription genes altered by LIPUS seem to be important for exert the role of osteocytes that modify the function of osteogenic cells and inflammatory cells involved in fracture healing process

In the laboratory, to elucidate the LIPUS effect on fracture healing, numerous *in vitro* and *in vivo* study has been performed. *In vivo* study demonstrated that LIPUS accelerated all stages of the fracture repair process (inflammation, bone formation and bone remodeling) with enhancement of mineralization and inflammatory response [17, 45-47]. *In vivo* studies identified overall biological action, but detailed mechanisms and cellular response were better examined *in vitro* studies. Ample *in vitro* studies showed that osteoblasts and chondrocytes rather than osteocytes responded to LIPUS and directly activated their function and differentiation were important for the LIPUS-dependent acceleration of fracture healing [48, 49]. Indeed, osteoblasts and chondrocytes directly generate new bone and it is proposed that they are exactly key player in endochondral ossification of fracture healing process. Whereas, osteocytes are terminally differentiated cells and do not exert a direct action on osteogenesis, then how osteocytes contribute to the effect of LIPUS on fracture healing is vague in spite of their mechanosensitivity. Besides, *in vivo* animal studies have not been fully clarified that whether osteoblasts are sufficient to exert the effect of LIPUS on fracture healing without osteocytes. Animal study utilized rats demonstrated that LIPUS shortens the fracture healing period by affecting various cellular reactions involved in all phase of

the healing process such as inflammatory reaction, angiogenesis, chondrogenesis, intramembranous ossification, endochondral ossification, and bone remodeling [17]. Naruse *et al*. showed that cyclooxygenase-2 (Cox2) knockout mice failed the LIPUS accelerated fracture healing effect [19], but Cox2 expression was observed several cells such as osteoblasts, osteocytes, chondrocytes and inflammatory cells. To elucidate the role of osteoblasts and osteocytes in the LIPUS effect, *in vivo* experiments with genetic animal model lacking each cell should be useful. However, as osteocytes are derived from osteoblasts, it is very difficult to long-term targeted ablation of each cell in animal model. Thus, *in vivo* studies still could not define the main target cells contributed to the effect of LIPUS.

In this report, we utilized medaka as a natural osteocyte knock-out model to elucidate the potency of osteocytes in LIPUS-induced fracture repair. As a comparative model, zebrafish which have osteocyte-rich bone was utilized. Because size, ecology and swimming mode are similar in both fish species and they have been employed for the comparative study to examine the role of osteocytes in bone modeling so far [24]. Comparing these species partially suited for bone research focused on osteocytes but it should be understood the difference in species due to evolutionary distance. It is estimated that medaka and zebrafish separated from their last common ancestor ∼110 million years ago from genomic comparisons [50, 51]. This evolutionary distance is reflected in their biological function. For example, Regeneration of heart following injury observed in zebrafish is not shared by medaka [52] and the response to the estrogen, 17β-estradiol, was faster in medaka than in zebrafish [53]. Although fracture healing process seems to be similar in medaka and zebrafish, their biological difference could affect the experiments of fracture healing with LIPUS stimulation. For obtaining the

more precise finding by *in vivo* approach utilizing fish, our present study only with medaka and zebrafish could be not sufficient. Not only zebrafish but also goldfish, carp, catfish and salmon also have osteocytes containing bone. Tilapia, fugu and platyfish don't have osteocytes in their skeletal tissue as well as medaka [54, 55]. Cohen *et al*. performed comparative study with the common carp Cyprinus carpio (a fish with cellular bone) and the tilapia Oreochromis aureus (a fish with acellular bone) to know the mechanical properties of two types of bone tissue [56]. Further studies using other fish species are needed in order to reveal the role of osteocytes in LIPUS effect more clear.

Since *Egr1*, *Egr2*, *JunB* and *FoxQ1* were altered in MLO-Y4 cells and osteocytic bone in zebrafish but not in anosteocytic bone in medaka, these were considered to be important transcription genes changed by LIPUS in osteocytes. Whereas, *Nr4a1* and *Snai2* were respond to LIPUS in MLO-Y4 cells and both osteocytic and anosteocytic fish bone, suggesting that these genes likely depend on both osteocytes and osteoblasts. Because MLO-Y4 cells are osteocyte "like" cells and some papers utilized MLO-Y4 cells as late mature osteoblasts in experiments [57]. Accordingly, MLO-Y4 cells not only have osteocytic profile but also keep osteoblastic characters. Yang *et al*. performed Gene Set Enrichment Analysis (GSEA) using osteoblast 2T3 cell model and osteocyte MLO-Y4 cell model. As a result of this study, gene expression pattern of MLO-Y4 is similar to macrophages, mesenchymal fibroblasts, and osteoblasts [58]. As MLO-Y4 cells seem to remain osteoblastic signature partially, altered genes by LIPUS may include both osteocyte- and osteoblast-mediated genes. Indeed, *Nr4a1* and *Snai2* could respond to LIPUS even in

medaka which bone lack osteocytes but contain osteoblasts, suggesting these genes may react LIPUS via not only osteocytes but also osteoblasts.

Finally, we focused on *Egr1*, *Egr2*, *JunB* and *FoxQ1* as osteocyte specific and LIPUS-sensitive genes. *Egr1* and *2*, especially *Egr1*, has been reported as a transcription factor induced by mechanical stimulation in various cells such as endothelial cells [59], tendon cells [60] and myocytes [61]. In osteoblastic cells, 15 minutes gravity loading induced an increase in expression of *Egr1* [62] and substrate stretch (Flexcell) also upregulate *Egr1* expression [63]. In osteocytic cells, *Egr1* was increased by lack of phosphate-regulating gene, PHEX [64] and activation of parathyroid hormone (PTH) signaling [65]. However, any papers which indicate the interaction between mechanical stress including LIPUS treatment and *Egr1* expression in osteocytes were not found. Fibroblast Growth Factor 23 (FGF23), regulatory factor of phosphate metabolism, is mainly produced by osteocytes and *Egr1* was known as a target of FGF/FGFR signaling [66]. Although we speculated that LIPUS affect *Egr1* expression via the alteration of FGF23 in osteocytes, it was not be able to confirm because MLO-Y4 cells does not express FGF23. Previous study demonstrated that MLO-Y4 highly expressed 181 genes compared to osteoblastic cells and very few genes of them are found in osteocytes directly isolate from murine bone. It was considered that many of osteocyte specific genes may require a mineralized matrix that is not provided *in vitro* for MLO-Y4 cells [58]. Since primary osteocytes isolated from murine bone expressed FGF23 [64], further study with primary cells is needed to define LIPUS/FGF23/*Egr1* axis in osteocytes. *FoxQ1* regulate the osteogenic differentiation of mouse bone mesenchymal stem cells [67] however, the role in not only osteocytes but also osteoblasts has been still not known. It was reported that *JunB* expression was

altered by mechanical stretch or loading in murine osteoblastic cells [68] and in rat chondrocytes [69], but not established in osteocytes. In the present study, we found novel candidate transcription genes which are mechano-sensitive and contribute to the promotion of fracture healing caused by LIPUS most likely in osteocyte depending manner.

To predict the functional meaning of *Egrs*, *FoxQ1* and *JunB* regulated by LIPUS in osteocytes, we performed target genes analysis and focused on some molecular signaling which can induce the potency of osteocytes to repair the bone fracture cooperate with neighboring cells. Osteocytes do not form bone directly because they are fully differentiated and nonproliferating cells [70]. Rather, osteocytes indirectly control bone formation and remodeling through the conduction of osteoblast and osteoclast activity [71]. Osteocytes secrete protein SOST, produced by the *SOST* gene, which is a negative regulator of bone mass and osteoblast differentiation via inhibition of Wnt/b-catenin signaling [72]. It has been reported that SOST was induced by mechanical loading through TGF-b dependent mechanism [73] and osteocyte-intrinsic TGF-b elevated the expression of *SOST* genes [74]. Indeed, osteocyte-specific TGF-b receptor II deficient mice demonstrated that osteocyte-intrinsic TGF-b signaling maintains bone quality and fracture resistance through perilacunar/canalicular re-modeling which engage the activity of osteocyte, osteoblast and osteoclast [74]. Osteocytes also released PGE2 and it could stimulate osteoblastic activity through the control of Wnt/b-catenin pathway [75] probably because PGE2 repress SOST expression [76]. Interestingly, SOST and PGE2, that control osteoblastic activity are modulated by mechanical loading in osteocytes and enhance bone formation and remodeling as a consequence of mechanical response of osteocytes [77, 78].

Moreover, TGF-b and Cox2/PGE2 are important molecules for the activation of inflammatory cells to commit early phase of fracture repair [18]. Our present study revealed that mechanical stimuli provided by LIPUS upregulated the expression of transcriptional factor genes such as *Egr1*, *Egr2*, *FoxQ1* and*JunB 1* in osteocytes embedded bone. These transcription factors regulate some target gene transcription which trigger TGF-b, Wnt/b-catenin signaling and PGE2 synthesis. Previous global gene expression analysis using exon array in rat bone following mechanical loading identified loading-induced genes and performed clustering analysis [79]. In this study, forelimb of rat was loaded for 3 minutes per day and global gene expression change was evaluated over a time course of 4 hours to 32 days. Early response genes which was upregulated within 12 hours was contained *JunB* but not *Egrs* and *FoxQ1*. Interestingly, many gene groups known to be essential for bone formation were identified within clusters related in matrix-formation, Wnt/β-catenin signaling, and TGF-β signaling. This result was partially but almost consistent with our RNA-seq analysis with MLO-Y4 cells treated by LIPUS. LIPUS seems to increase “operator” potency of osteocytes which instruct the combination work with not only bone cells but also inflammatory cells in several process of fracture healing.

Despite the effect of “active” bone forming osteoblasts and chondrocytes on LIPUS-induced fracture repair has been well reported, the role of “static” osteocytes which control bone generation indirectly is still not fully understood. In addition, synergistic action of multiple cell types triggered by LIPUS on fracture healing mimicking physiological condition was remain to be elucidated. Present *in vivo* and *in vitro* studies showed that osteocytes were LIPUS-sensitive cells and their assisted reaction caused by early response transcriptional genes on bone forming cells and

inflammatory cells seems to be necessary to promote fracture healing rate. However, further experiments would be required to investigate whether LIPUS-targeted signals and molecules in osteocytes truly affect the function of other collaborating cells, and what kind of molecular and functional changes has caused in other cells to exert biological (physiological) action on fracture repair. Because the molecular response of osteocytes to LIPUS can be altered as the progress of fracture healing for a long term, it is also important to examine the changes of the LIPUS effect on osteocytes to control other cells and harmonize the function of various cells in each phase of fracture healing.

## Methods

### Fish and creating bone fracture model

Adult zebrafish (Danio rerio) and medaka (Oryzias latipes) were obtained from a local aquarium store (Homac, Sapporo, Japan). The fish were maintained at 28℃ under a 14h light/ 10h dark cycle and fed a TetraMin® Tropical Flakes produced by Tetra, automatically two times a day. Zebrafish and medaka were anesthetized with tricaine (Sigma-Aldrich) and the tail bones were fractured with scalpel under a microscope.

### Bone staining

Zebrafish and medaka caudal fin rays were fixed overnight at 4℃ with 4% paraformaldehyde in PBS for Alizarinred and Alcianblue staining. After fixation, fin rays were stained with Alcian blue solution (70% ethanol and 30% acetic acid containing 0.1% Alcian blue) at room temperature overnight. Fins were treated with ethanol and washed in water. Alizarin red solution (4% Alizarin red, 0.5% potassium hydroxide) were added and incubated overnight at room temperature. Stained samples were stored in 80% glycerol for imaging.

### *In vivo* LIPUS stimulation

1 day after the bone fracture, fishes were placed into 6 well plate with water (1 fish per 1 well). LIPUS (Teijin Pharma Ltd.) was generated by an array of 6 PZT-4 (lead-zirconate titanate) transducers (2.5 cm diameter) fixed with a locking device. The plate contained fish was placed on the array and the locking device was immersed in a water tank. The LIPUS signal was set to work at 1.5-MHz, 200-msec burst width sine waves

at 1.0 kHz, and was delivered at an intensity of 30mW/cm$^2$. Fishes were exposed to LIPUS for 20 minutes daily for up to 4 weeks until the fracture has completely healed. Fractured bone sites were observed by optical microscope (Nikon) every day.

**Total RNA extraction from fish fin ray**

Fin rays were homogenized using a BioMasher homogenizer (BioMasher II). The homogenate was lysed in TRizol Reagent (Invitrogen) and total RNA was extracted following instructions for the RNeasy Mini, RNA isolation kit (Qiagen).

**Cell culture and LIPUS stimulation**

MLO-Y4 cells were kindly provided by Dr. Lynda F. Bonewald, Department of Oral Biology, University of Missouri at Kansas City School of Dentistry, Kansas City, MO. Cells were maintained in α-modified essential medium (α-MEM) containing 10% fetal bovine serum (FBS) at 37℃ in a humidified atmosphere of 95% air and 5% $CO_2$. Before the LIPUS stimulation, cells were seeded on collagen-coated 6-well dishes and precultured overnight. Cell culture plate were located on the array of LIPUS system and fixed with locking device. The locking device was immersed in a water tank and then LIPUS stimulation was given (1.5 MHz, pulsed-wave mode intensity of 30 mW/cm$^2$) for 20 minutes. Cells were harvested after LIPUS treatment and total RNA was extracted using the spin column kit (Zymo Research).

**Quantitative RT-PCR**

Total RNA was extracted from fish fin ray and MLO-Y4 cells. RNA (500 ng-1 μg) was reverse transcribed with a high-capacity complementary DNA (cDNA) reverse

transcription kit (Applied Biosystems). qRT-PCR assays were run and quantified in the ABI STEP one real time PCR system using SYBR green PCR Master Mix (Qiagen). Relative mRNA expression was determined by the ΔCt method, and the values were normalized to the expression of b-actin. Primer set for qPCR are shown in Table 1.

**RNA-sequencing and analysis**

Total RNA was isolated from MLO-Y4 cells at 30 minutes after the LIPUS stimulation using the spin column kit (Zymo Research). The integrity and purity of total RNA were assessed using an Aligent 2100 Bioanalyzer (Agilent Technologies). The Illumina TruSeq Stranded mRNA protocol was used for the preparation of RNA-seq libraries and sequenced on a HiSeq 2500 machine (Illumina), as paired-end, 100 base paires reads. Paired-end sequencing reads from HiSeq 2500 were subjected to adaptor-trimming and quality-trimmed with the default parameters to map the reference genome. FPKMs were using Cufflinks (version2.2.1) with a transcriptome reference (Ensembl Mouse Transcript). To identify signature genes for each group, we normalized the read count matrix and performed gene-wise moderated F-tests to test whether a gene is differentially expressed across the cell groups. Signature genes for each group were identified using the following criteria: adjusted q-value < 0.05. Significance difference was defined by q-value. q is FDR-adjusted enrichment p-value and $q < 0.05$ (i.e., -Log 10 (q) > 1.3) is defined as significant. The Functional Annotation Tool of the Database for Annotation, Visualization, and Integrated Discovery (DAVID), v6.8, was used to characterize gene-annotation enrichment analysis.

**Target gene analysis of transcription factor**

To identify the putative target genes of transcription factors, mouse Chip-seq database in ChIP-Atlas (http://chip-atlas.org/) was used. If transcription factor's ChIP-seq peaks settled within the gene's promoter region (5 kb around the transcription start site), this gene was identified as target gene [80].

**Statistics**

All statistics were calculated using Microsoft Excel and GraphPad Prism. All data were presented as mean ± SEM and comparisons were made by Student's t-test. Results were considered significant if *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. All experiments presented in the manuscript were performed in at least two-three independent experiments except the RNA-seq study. The experiments were not randomized and the sample size was not predetermined.

**Data availability**

Data supporting the conclusions are available from the corresponding author upon reasonable request. RNA-seq data will be deposited to GEO prior to the publication of this manuscript.

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**Author Contribution**

T.S. carried out all of experiments and analyzed data. N.F. carried out bioinformatic analysis using RNA-seq data. T.F., M.K., and K.T. helped editing the paper. M.S. directed the research, designed the experiments and wrote the manuscript with M.T.

**Competing interests**

The authors declare no competing interests.

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**Table. 1. Primer sequences for qRT-PCR.**

**Table.2. List of enriched annotation clusters of LIPUS-responsive 179 genes.**

**Table 3. List of genes targeted by transcription factors.**

## Table 1. Primers used in Real-time PCR.

| Gene (Zebrafish) | Primer sequence (Forward) | Primer sequence (Reverse) |
|---|---|---|
| *C1s* | GACCTGTGACGCCAACATCTA | GGATAACCGGACTCCACTGTC |
| *Saa3* | CAAGTATTTCCATGCACGCGG | GCAGCATCTGAATTGCCTCTG |
| *Btg2* | CATTCTGATCTTTGCCGGACG | GGAACCAATGGTGCTGGTAGT |
| *Cited2* | GGAGAGCATACGCTTCTTGTTG | CGACCATGGTTCATTGCCATC |
| *Egr1* | TTCTCAACGCCACAGCACCTGAAGG | GGTCTGATCTGACAGAGGTTTCTCC |
| *Egr2* | GTCTATGGTCTGGATGAGATTCCC | AGATCAAGGTCCCGCTTTTCC |
| *FoxQ1b* | TGGAGGTTTTCTCTGCGAGTC | TAAGGTGGTTTGGGTCTACGC |
| *Helz2a* | GGACTGCCTCGTTTCACTGTA | CCAATAGGCTGTCCTTGGTGT |
| *JunBa* | GACCCTCCGCTCCGAAAT | TGATGCTCCGACCGTACAAATA |
| *JunBb* | TCCCACATACAGCAGAGCCA | GCGTTCCTGCGAGTCCAT |
| *NFATc1* | CAAGCATGAAATCCGCAGAGG | CCGGATGTTTGGAAGTAGCCT |
| *Nr4a1* | CCTCTCTCGTTACTGCCCATATC | CCTGATCACATCCATTGACCCTG |
| *Snai2* | CAGCATGCCTCGTTCATTCCT | CCGGGAGGGCTTTTAAGACATA |
| **Gene (Medaka)** | **Primer sequence (Forward)** | **Primer sequence (Reverse)** |
| *Egr1* | CGTACGACCACCTTACTGGAG | GACCACTGAACAGACCCAAGA |
| *Egr2* | GGCCACTACGACCAACTCAAT | GCTGGATAAGGGGAGTCGATG |
| *FoxQ1b* | GGAAAGGGAAACCCTACACCC | TGAGGGAGAGGTTGTGTCTCA |
| *JunBb* | CAACACTGAACGCCTATTGCC | GGGCTCCTCCTTCAAGGTAAC |
| *NFATc1* | TACAGGCAGGGAACACGTTTG | GAAACGTTCAGACTGTGGGTC |
| *Nr4a1* | CAATGCCTCCTGTCAGCACTA | GACACTTCTGGAAGCGACAGA |
| *Snai2* | CCAAGATGCCACGCTCTTTTC | AGGCTACTGGTAGTCCACACT |

# Table 2. List of significantly enriched annotation clusters

| Annotation Cluster | Name | Gene Count | P_Value | Gene name included in | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Cluster A Enrichment Score 7.44 | Innate immunity | 17 | 7.10E-11 | **Immunity** | | | | | | | | | |
| | Antiviral defense | 12 | 3.50E-10 | *Oas1a* | *Oas2* | *Oas3* | *Oasl2* | *Ddx58* | *Zbp1* | *Adar** | *Bst2** | *C1rb** | *C1s2** |
| | **Immunity** | **20** | 3.60E-10 | *Cfb** | *Gbp2b* | *Hp** | *Irgm1* | *Iigp1* | *Ifit1* | *Ifit3* | *Lgals9* | *Lcn2* | *Pml* |
| | RNA-binding | 8 | 1.90E-01 | | | | | | | | | | |
| Cluster B Enrichment Score 3.88 | Nucleus | 59 | 3.40E-05 | **Transcription** | | | | | | | | | |
| | Transcription regulation | 30 | 1.30E-04 | *Btg2** | *Cited2** | *Irx3** | *Lmcd1** | *Phf11d* | *Smad9** | *Sox9** | *Adar** | *Egr1** | *Egr2** |
| | **Transcription** | **30** | 2.30E-04 | *Egr3** | *Egr4* | *FoxQ1** | *FoxS1* | *Helz2** | *Ifi204* | *JunB** | *Sp100* | *NFATc1** | *Nfkbiz** |
| | DNA-binding | 27 | 2.80E-04 | *Nr4a1** | *Nr4a2* | *Parp14* | *Pml* | *Stat1* | *Stat2* | *Snai2** | *Trim30a* | *Maff* | *Vdr* |
| Cluster C Enrichment Score 3.3 | Metal-binding | 50 | 7.70E-06 | | | | | | | | | | |
| | Zinc | 30 | 1.60E-03 | | | | | | | | | | |
| | Zinc-finger | 22 | 1.00E-02 | | | | | | | | | | |
| Cluster D Enrichment Score 3.21 | **Secreted** | **33** | 2.00E-06 | **Secreted** | | | | | | | | | |
| | Disulfide bond | 44 | 1.00E-04 | *Isg15** | *Sparcl1** | *Cpg* | *Ccl5** | *F13a1** | *Cfb** | *Ctgf** | *Cyr61** | *Dcn** | *Dmp1* |
| | Signal | 49 | 1.20E-02 | *Fmod* | *Fbln5** | *Fbln7** | *Grem1** | *Gbp2b* | *Hp** | *Igfbp5* | *Lgals9* | *Lgals3bp* | *Lcn2* |
| | Glycoprotein | 39 | 5.80E-02 | *Mmp13** | *Mmp3* | *Nbl1* | *Nampt* | *Orm1* | *Pla1a** | *Plat* | *Plau* | *Ptn* | *Saa3** |
| | | | | *Tnc** | *Tnn* | *Tcn2* | | | | | | | |
| Cluster E Enrichment Score 1.48 | ATP-binding | 19 | 1.90E-02 | | | | | | | | | | |
| | Magnesium | 10 | 2.10E-02 | | | | | | | | | | |
| | Transferase | 19 | 9.20E-02 | | | | | | | | | | |
| Cluster F Enrichment Score 1.32 | Lipid biosynthesis | 6 | 8.30E-03 | | | | | | | | | | |
| | Cholesterol biosynthesis | 3 | 1.00E-02 | | | | | | | | | | |
| | Sterol biosynthesis | 3 | 1.70E-02 | | | | | | | | | | |
| | Steroid biosynthesis | 3 | 3.50E-02 | | | | | | | | | | |
| | Fatty acid biosynthesis | 3 | 6.60E-02 | | | | | | | | | | |
| | Cholesterol metabolism | 3 | 6.80E-02 | | | | | | | | | | |
| | Sterol metabolism | 3 | 8.40E-02 | | | | | | | | | | |
| | Lipid metabolism | 7 | 1.10E-01 | | | | | | | | | | |
| | Steroid metabolism | 3 | 1.10E-01 | | | | | | | | | | |
| | Fatty acid metabolism | 3 | 2.50E-01 | | | | | | | | | | |
| Cluster G Enrichment Score 1.31 | Microsome | 6 | 3.70E-03 | | | | | | | | | | |
| | Oxidoreductase | 10 | 6.20E-02 | | | | | | | | | | |
| | Heme | 4 | 1.50E-01 | | | | | | | | | | |
| | Iron | 6 | 1.70E-01 | | | | | | | | | | |

# Table 3. List of genes targeted by transcriptional factors

| Transcription genes | Target genes | Average score | Expression level in MLO-Y4 (FPKM value) | Function | Ref |
|---|---|---|---|---|---|
| *Egr1* | *Greb1* | 1109.833333 | 1.53125 | | |
| | *Olfr806* | 790.5 | n.d. | | |
| | ***Abhd6*** | 774.166667 | **15.7218** | Serine hydrolases, Monoacylglycerol lipase | PMID: 28880480 |
| | *Wwox* | 725.666667 | 6.25733 | | |
| | *Aadacl2fm1* | 613.666667 | n.d. | | |
| | *Nkx2-6* | 581.5 | n.d. | | |
| | *Setd4* | 549.333333 | 0.960806 | | |
| | ***Cbr1*** | 549.333333 | **31.0524** | Carbonyl reductases, Catalyst for the reduction of endogenious prostaglandine and steroids | [33] |
| | *Cldn34d* | 526.666667 | n.d. | | |
| | *Tecpr1* | 434.166667 | 2.83544 | | |
| *Egr2* | ***Dynlt1b*** | 756 | **34.0942** | Activators of G-protein signaling | PMID: 11358340 |
| | *Lcmt1* | 749.909091 | 8.62011 | | |
| | ***Arhgap17*** | 749.909091 | **15.6857** | Rho GTPase activating proteins, Maintenance of tight junctions | PMID: 16678097 |
| | ***Ncapg2*** | 746.181818 | **27.2157** | A component of the chromosome condension II complex, which is critical for mitosis | PMID: 31176678 |
| | *Mtrf1l* | 740.818182 | 3.14548 | | |
| | *Sdhaf3* | 712.454545 | 0.920439 | | |
| | *D10Wsu102e* | 690.090909 | 1.87224 | | |
| | *Cenpu* | 677.818182 | 15.7252 | | |
| | *Cplane1* | 676.272727 | 6.84822 | | |
| | *Cmtm6* | 647.636364 | 7.80277 | | |
| *Nr4a1* | ***Adssl1*** | 811.428571 | **24.6413** | Adenylosuccinate synthetase, which catalyzes AMP synthesis. | PMID: 15786719 |
| | ***Mcl1*** | 809.285714 | **126.123** | Anti-apoptotic protein, pro-survival protein | PMID: 20023629 |
| | *Txk* | 800.285714 | n.d. | | |
| | *Tmem179* | 767.428571 | 1.11521 | | |
| | *Cpm* | 706.142857 | 3.52267 | | |
| | *Greb1* | 679.428571 | 1.53125 | | |
| | *Olfr266* | 655 | n.d. | | |
| | *Rapgef2* | 630.285714 | 5.72616 | | |
| | ***Cst3*** | 607.857143 | **454.443** | Cysteine proteinase inhibitor, An inhibitor of bone resorption | [36] |
| | ***Brox*** | 606.285714 | **15.5085** | Apoptosis-linked gene, Controlling of cell death through the regulation of the endolysosomal system | PMID: 16407552 |
| *JunB* | *Cldn34d* | 895.351351 | n.d. | | |
| | ***Brox*** | 763.783784 | **15.5085** | Apoptosis-linked gene, Controlling of cell death through the regulation of the endolysosomal system | PMID: 16407552 |
| | ***Aida*** | 763.783784 | **67.1444** | Axin-interacting protein, Blocking Axin/JNK signaling | [35] |
| | *Cdc42bpg* | 643.567568 | 5.53688 | | |
| | *Mefv* | 604.567568 | n.d. | | |
| | *Ank1* | 598.972973 | 7.66976 | | |
| | *Prss56* | 598.027027 | n.d. | | |
| | *Chrnd* | 598.027027 | n.d. | | |
| | *Cass4* | 595.810811 | 0.0982373 | | |
| | ***Htra3*** | 574.432432 | **10.6126** | Serine protease, An inhibitor of TGF-b signaling | [32] |
| *Nfatc1* | *Greb1* | 1166 | 1.53125 | | |
| | *Olfr855* | 844.333333 | n.d. | | |
| | *Olfr854* | 844.333333 | n.d. | | |
| | *Abhd6* | 796.333333 | 1.07994 | | |
| | *Nkx2-6* | 765.333333 | n.d. | | |
| | *Wwox* | 748.333333 | 6.25733 | | |
| | *Olfr806* | 723 | n.d. | | |
| | *Acacb* | 657.666667 | 0.787511 | | |
| | *Prl3c1* | 591.666667 | n.d. | | |
| | *Setd4* | 562.333333 | 0.960806 | | |
| *Btg2* | N/A | | | | |
| *Snai2* | | | | | |
| *Cited2* | | | | | |
| *FoxQ1* | | | | | |
| *Heiz2* | | | | | |

**Fig. 1. The effect of LIPUS treatment on fracture healing in medaka and zebrafish.**

(A) Tail bones of zebrafish and medaka were fractured (The red arrows indicate the fracture site) with scalpel under a microscope. Original magnification X2 (Left panel) X10 (Right panel) (B) Stained images of the fracture healing process of medaka and zebrafish in the steady state. The fracture site was stained with Alcian blue and Alizarin red. Original magnification X10 (C) Upper: Body weight and length of medaka and zebrafish were measured every week and condition factor (K-factor; g/cm2 X 103) was calculated at indicated point. Lower: The percent change of condition factor. (n=3-6) (D) Upper: Days required for complete fracture healing in LIPUS-treated and untreated medaka and zebrafish. Lower: Promotion rate of fracture healing caused by LIPUS stimulation in medaka and zebrafish. (n=3-5). Data are represented as mean ± SEM. P values were determined by Student's t-test. * $p < 0.05$, ** $p < 0.01$.

**Fig. 2. RNA-seq analysis of LIPUS-stimulated MLO-Y4 cells.**

(A) Comparison of gene expression levels by scatter plot.179 genes were identified as significantly changed genes, of which 93 genes were up-regulated (red frame) and 86 genes were down-regulated (green frame) by LIPUS. (B) Heat map showing the expression change of osteogenic genes in LIPUS-treated and untreated MLO-Y4 cells. (C) Using 179 genes, annotated terms categories were identified. Log10(P-Value) >1.3 (P-Value < 0.05) was considered to be significantly different. (D) Heat map showing the expression change of genes involving in immunity (left), transcription (center) and secretion (right) in LIPUS-treated and untreated MLO-Y4 cells.

**Fig. 3. Measurement of candidate gene expression in LIPUS-treated zebrafish.**

Zebrafish tail bones were fractured and stimulated by LIPUS for 20 minutes every day. At day 1 and 7 after the fracture, RNA was extracted from the fin ray. (A) Immunity, Secreted and (B) Transcription-related genes were measured. (n=3) The values denote the mean ± SEM and comparisons were made by Student's t-test. *$p < 0.05$; **$p < 0.01$.

**Fig. 4. Measurement of candidate gene expression in LIPUS-treated medaka.** Medaka tail bones were fractured and stimulated by LIPUS for 20 minutes every day. At day 1 and 7 after the fracture, RNA was extracted from the fin ray. Indicated genes were measured by qPCR. (n=3) The values denote the mean ± SEM and comparisons were made by Student's t-test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

**Figure 1**

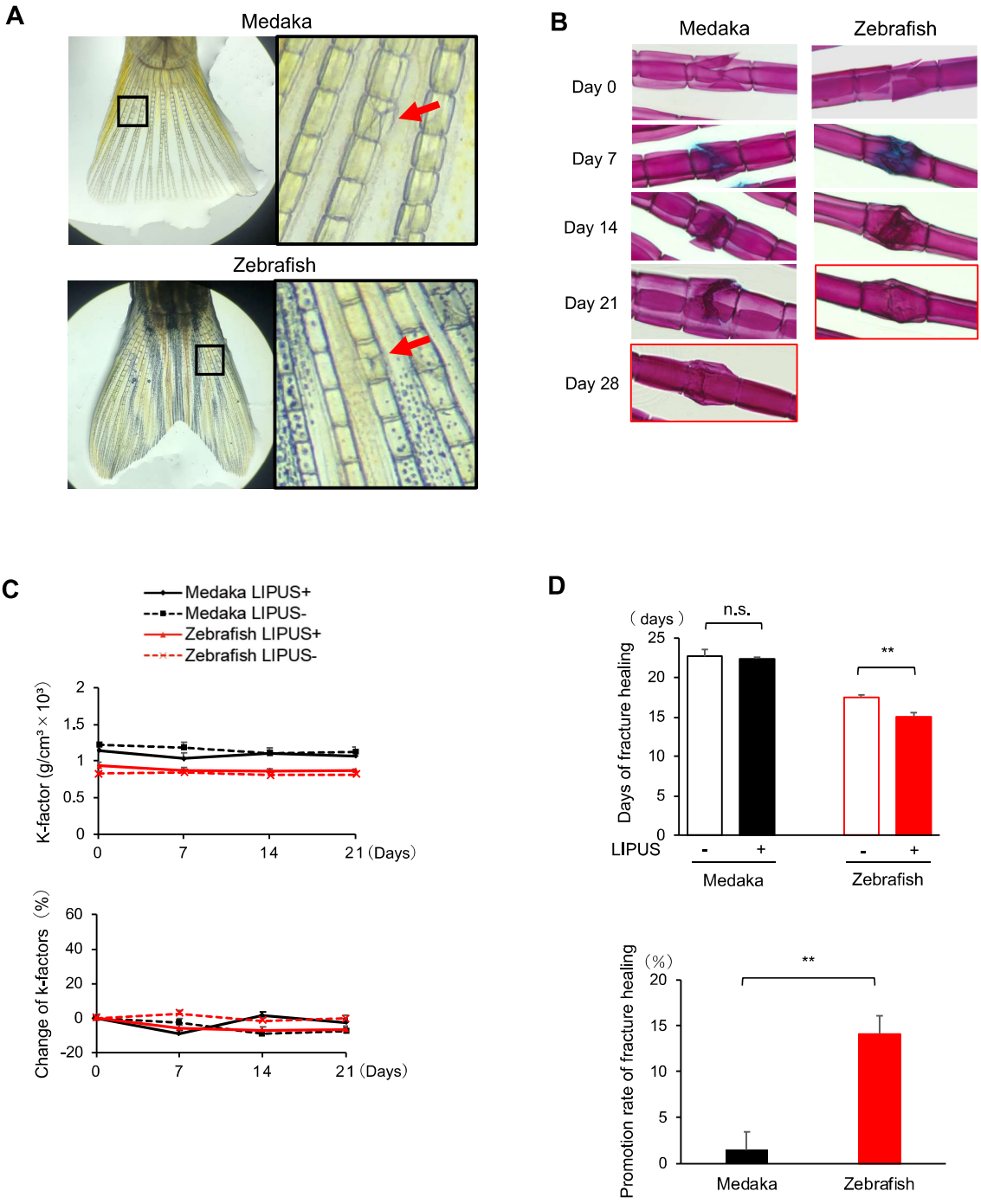

A
Medaka
Zebrafish
B
Medaka
Zebrafish
Day 0
Day 7
Day 14
Day 21
Day 28
C
Medaka LIPUS+
Medaka LIPUS-
Zebrafish LIPUS+
Zebrafish LIPUS-
K-factor (g/cm³ × 10³)
2
1.5
1
0.5
0
0
7
14
21 (Days)
Change of k-factors (%)
60
40
20
0
-20
0
7
14
21 (Days)
D
( days )
n.s.
**
Days of fracture healing
25
20
15
10
5
0
LIPUS
-
+
-
+
Medaka
Zebrafish
(%)
**
Promotion rate of fracture healing
20
15
10
5
0
Medaka
Zebrafish

**Figure 2**

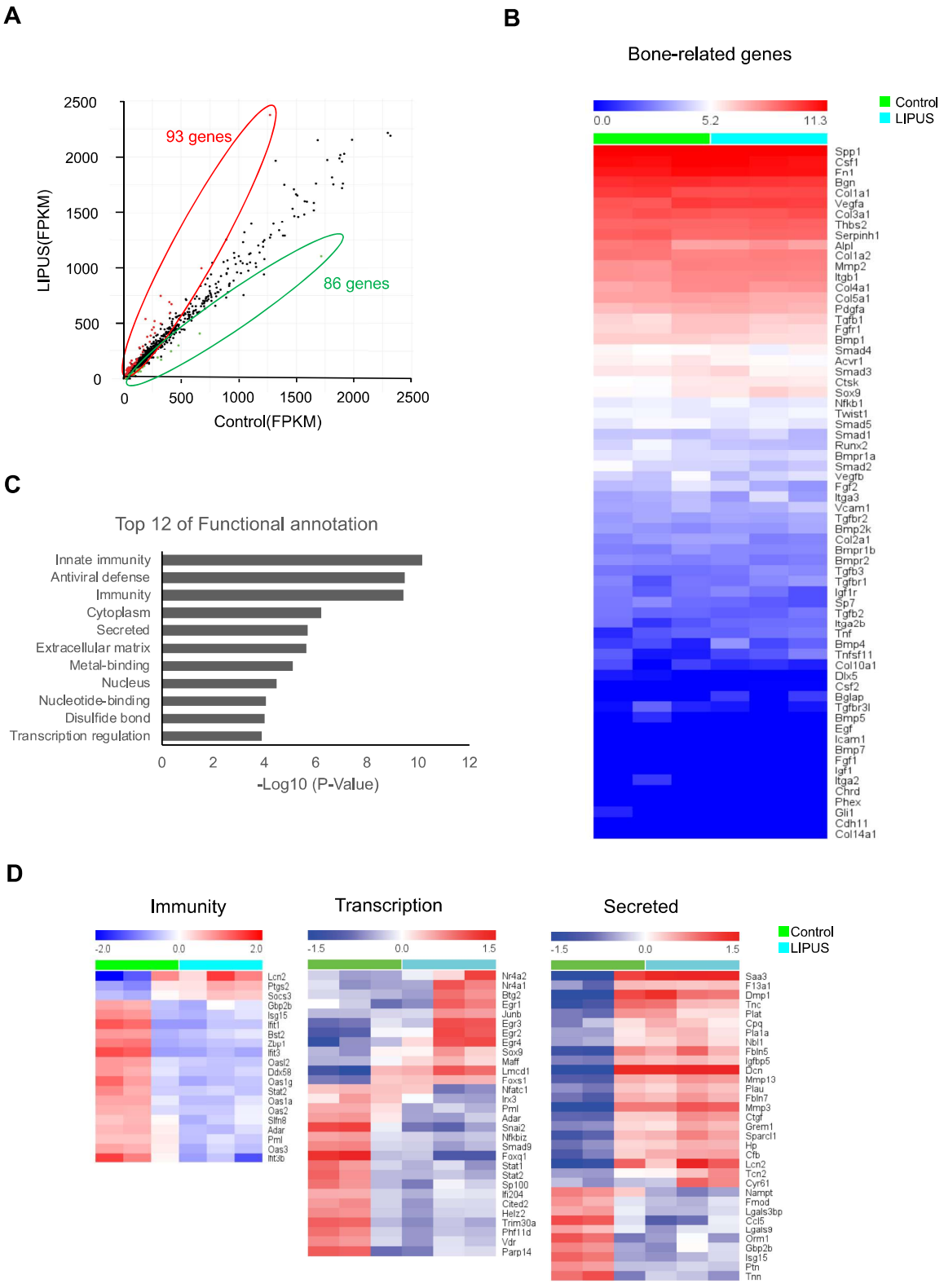

A
93 genes
86 genes
LIPUS(FPKM)
Control(FPKM)
B
Bone-related genes
Control
LIPUS
C
Top 12 of Functional annotation
Innate immunity
Antiviral defense
Immunity
Cytoplasm
Secreted
Extracellular matrix
Metal-binding
Nucleus
Nucleotide-binding
Disulfide bond
Transcription regulation
-Log10 (P-Value)
D
Immunity
Transcription
Secreted
Control
LIPUS

**Figure 3**

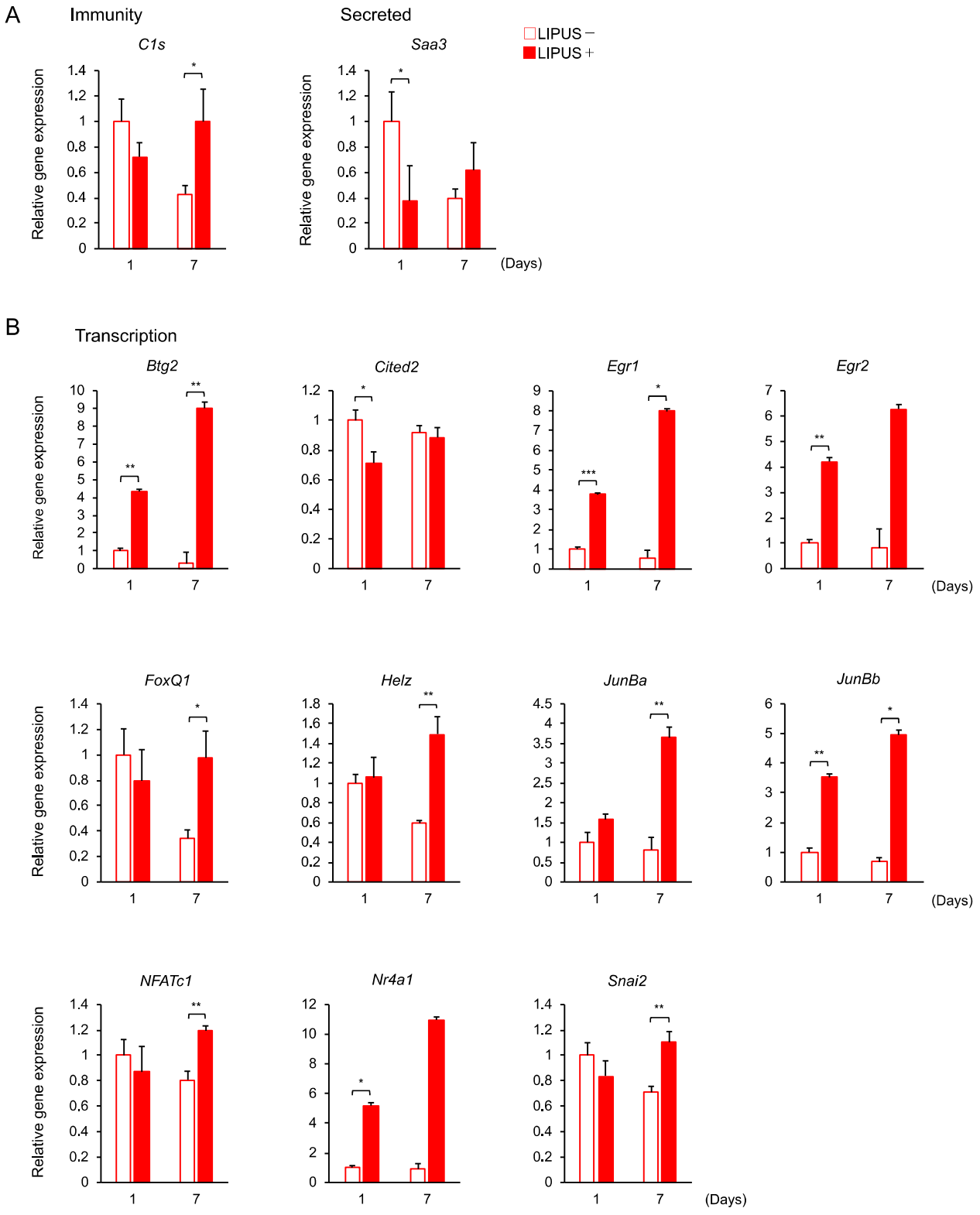
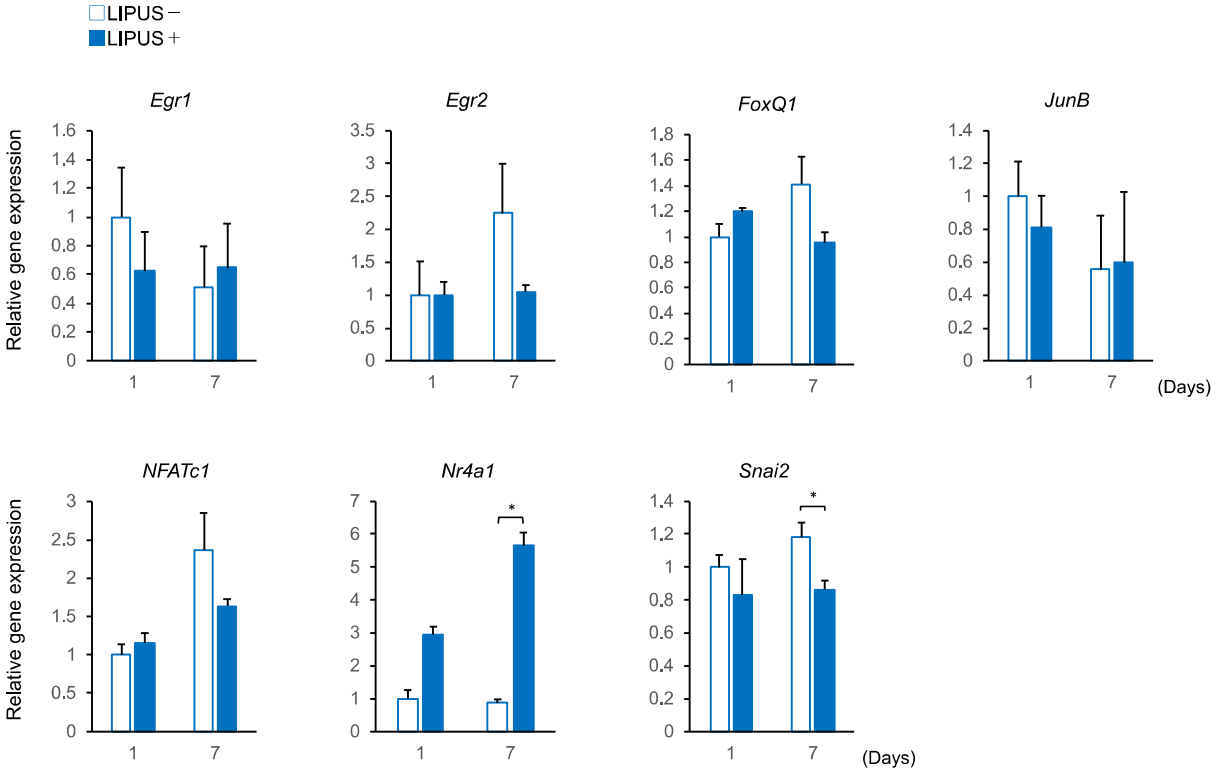

A
Immunity
Secreted
C1s
Saa3
LIPUS −
LIPUS +
Relative gene expression
(Days)
B
Transcription
Btg2
Cited2
Egr1
Egr2
FoxQ1
Helz
JunBa
JunBb
NFATc1
Nr4a1
Snai2

**Figure 4**


LIPUS −
LIPUS +
Egr1
Egr2
FoxQ1
JunB
NFATc1
Nr4a1
Snai2
Relative gene expression
Relative gene expression
(Days)
(Days)
*
*